CYCLIC AMP, CYCLIC AMP-DEPENDENT PROTEIN KINASE AND ITS INHIBITOR PROTEIN IN SALIVARY GLANDS OF UNFED AND FEEDING FEMALE LONE STAR TICKS, *AMBLYOMMA AMERICANUM* (L.)

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TABLE OF CONTENTS

Page

PART III

LIST OF TABLES

LIST OF FIGURES

Figure — Page

PART I

PART II

Figure Page

PART III

INTRODUCTION

Each part of this thesis is a separate and complete manuscript to be submitted for publication. Part I has been published in Comparative Biochemisrtry and Physiology. Part II is being submitted to Comparative Biochemistry and Physiology. Part III is being submitted to Journal Parasitology. Each part is presented in the thesis in the format of the journal to which it is submitted.

PART I

ADENOSINE-3',5'-MONOPHOSPHATE IN SALIVARY GLANDS OF UNFED AND FEEDING FEMALE LONE STAR TICKS, *AMBLYOMMA AMERICANUM* (L.)

ABSTRACT

Basal levels of cAMP in salivary glands of female lone star ticks, Amblyomma americanum (L.), were found to be about 5 pmole/mg protein during all stages of tick feeding. The amount in glands from unfed ticks was 13 pmole/mg protein. Glands from rapidly feeding ticks (>200 mg) that were stimulated by 10^{-5} M dopamine had significantly higher amounts of cAMP (ca 34 pmole cAMP/mg protein). Glands from slowly feeding ticks stimulated by 10^{-5} M dopamine had ca 14 pmole cAMP/mg protein. Glands from rapidly feeding ticks stimulated by 10^{-5} M dopamine and 10^{-2} M theophllyne had ca 120 pmole cAMP/mg protein. Cyclic AMP did not increase in glands of unfed ticks when stimulated by 10^{-7} M dopamine and 10^{-5} M dopamine and 10^{-2} M theophylline. Glands from both slowly and rapidly feeding ticks that were stimulated by 10^{-7} M dopamine and 10^{-2} M theophylline had similar, but significantly higher amounts of cAMP than basal (ca 12 pmole cAMP/mg protein). After stimulation by 10^{-5} M dopamine was removed, cAMP decreased faster in glands from slowly feeding ticks than in glands of rapidly feeding ticks.

Key Word Index: Amblyomma americanum, ticks, salivary gland, secretion, cyclic AMP, dopamine.

INTRODUCTION

The salivary glands of feeding ixodid ticks are important organs of osmoregulation. While a bloodmeal is being ingested, excess fluid is extracted from the gut and returned to the host via the salivary glands (Kaufman and Sauer, 1976). Salivary fluid secretion is controlled by nerves and the neurotransmitter at the neuroeffector junction appears to be dopamine (Kaufman, 1976). A dopamine-sensitive adenylate cyclase is present in the salivary glands and its activity and fluid secretion by glands in vitro are activated half-maximally by the same concentration of dopamine (Sauer et al, 1979; Schmidt, et al, 1982). Exogenous cyclic AMP (cAMP) is also able to stimulate fluid secretion by glands, in vitro (Sauer et al, 1979). Cyclic AMP functions as a secondary messenger of various stimuli in many cells (Berridge, 1975; Weller, 1979). Cyclic AMP is synthesized through activity of adenylate cyclase and hydrolyzed by phosphodiesterases to form inactive adenosine-5'-monophosphate (Sutherland and Rall, 1958; Butcher and Sutherland, 1962). Phosphodiesterase activity in salivary glands of A. americanum changes during the 9-15 day feeding cycle of adult females (McMullen et al, 1978). Dopamine sensitive adenylate cyclase activity also changes during the feeding of A. americanum adult females (Schramke et al, 1984). The observed decrease in phosphodiesterase and increase in

adenylate cyclase activities corresponds with an increase in the rate of salivary gland secretion as the tick progresses from the slow to rapid stage of feeding (Sauer et al, 1979).

The present experiments were designed to measure levels of cAMP in salivary glands of feeding adult female ticks and to ascertain whether a known agonist of salivary gland adenylate cyclase (dopamine), and an antagonist (theophylline) of cAMP phosphodiesterase differentially affect levels of cAMP in glands of slowly and rapidly feeding ticks.

MATERIALS AND METHODS

Experimental Protocol and Tissue Extraction of Cyclic AMP

Female Amblyomma americanum (L.) were reared on rabbits during larval development and on three to four year old ewes for nymphal and adult feeding. The paired glands (i.e., gland pairs from one tick each) were excised and maintained in ice-cold modified TC-199 (Needham and Sauer, 1979) before use. Cyclic AMP was extracted from salivary glands of unfed and feeding female ticks weighing 4 - 1000 mg following stimulation with 10^{-7} M dopamine (DA) and 10^{-2} M theophylline (Th). Cyclic AMP was also measured in salivary glands from slowly feeding female ticks (weighing 40 to 60 mg) and rapidly feeding females (400 - 700 mg) after stimulation with 10^{-5} M DA or 10^{-5} M DA and 10^{-2} M Th in TC-199 at room temperature for 5 min. Other glands were

stimulated with 10^{-5} M DA or 10^{-5} M DA and 10^{-2} Th, washed and then incubated for 1 and 5 min in TC-199 without DA or Th. In the latter experiments, control glands of each pair were treated in the same manner as stimulated glands but without DA and Th. Glands were immersed in liquid nitrogen for 10 sec. in all experiments prior to cAMP extraction. Each gland was immediately transferred to a Dounce ground glass homogenizer containing 1 ml ice-cold 5% (w/v) trichloroacetic acid (TCA) with trace amounts of ^{3}H-cAMP (10 nCi/ml, New England Nuclear) to monitor percent recovery of cAMP during extraction. Homogenized glands were centrifuged for 25 min (4000 x g) and the pellet resuspended in 200 ul 1 N NaOH and used for protein determination (Lowry, et al, 1951). The supernatant (SN) was acidified with 100 ul 1 N HCl. Trichloroacetic acid was removed from the SN by extracting 5 times with 3 ml 98% aqueous ethyl ether (v/v) (Gilman, 1970). Residual ethyl ether was removed by evaporation at 80°C in a water bath.

Assay of Cyclic AMP

The extracted supernatant was frozen, lyophylized overnight and dissolved in 120 ul 50 mM sodium acetate buffer, pH 6.2. Duplicate aliquots (10 ul) of the reconstituted samples were placed in scintillation bags (Nalgene Corp.) containing 5 ml Biocount scintillation fluid (Research Products International) and counted on an LS-3133T liquid scintillation system (Beckman Instruments, Inc.) to monitor percent recovery of extracted cAMP. The

remaining 100 ul was diluted with 1 ml 4.1 mM sodium acetate buffer, pH 6.2. Aliquots of 20, 60 and 100 ul of the prepared samples were acetylated and sample cAMP was assayed by radioimmunoassay (Collaborative Research, Inc.). The assay used is a modification of the the methods developed by Steiner (1974) and Brooker, et al. (1979).

Abbreviations: cAMP and Cyclic AMP, cyclic 3',5'-adenosine monophosphate; DA, dopamine; Th, theophylline; PDE, cyclic AMP-phosphodiesterase; TCA, trichloroacetic acid; SN, supernatant.

RESULTS

Total cAMP in glands of feeding ticks increased as the weight of the tick increased (Fig. 1) in both control glands and those stimulated by 10^{-7} M DA and 10^{-2} M Th. However, the protein content of the salivary glands also increased with increasing tick weight; therefore, cAMP/mg protein in both control and stimulated glands was constant over the weight range of the feeding tick (Fig. 1).

Glands from unfed and recently attached (<5 mg) ticks did not have significantly higher cAMP after being stimulated by 10^{-7} M DA and 10^{-2} M Th (Table 1). The average cAMP/mg protein in glands (n = 5) from slowly feeding ticks (<200 mg) stimulated by 10^{-7} M DA and 10^{-2} M Th for 5 min was 10.3 +/- 2.7 pmole cAMP/mg protein (X +/- SEM). Control glands (n = 5) from the same weight ticks had 3.7 +/- 0.6

pmole cAMP/mg protein (Fig. 2). In rapidly feeding ticks (>200 mg) the average cAMP/mg protein was 9.2 +/- 1.5 in glands (n = 10) stimulated by DA/Th and 6.6 +/- 2.7 in control glands (n = 10). Control glands (n = 4) from other slowly feeding ticks (43.7 - 66.3 mg) contained 3.0 +/- 0.1 pmole cAMP/mg protein while paired glands stimulated by 10^{-5} M DA contained 13.6 +/- 3.8 pmole cAMP/mg protein. Glands (n = 4) from rapidly feeding ticks (372 - 683 mg) stimulated by 10^{-5} M DA had 34.1 +/- 12.6 pmole cAMP/mg protein and control glands (n = 4) 4.1 +/- 0.6 pmole cAMP/mg protein. Other glands from slowly and rapidly feeding ticks were treated for 5 min with 10^{-5} M DA/10^{-2} M Th (Fig. 2). Control glands (n = 4) from slowly feeding ticks in these experiments had 1.9 +/- 0.2 pmole cAMP/mg protein and those stimulated by DA/Th, 77.0 +/- 15.4 pmole cAMP/mg protein (n = 4). Control glands (n = 4) from rapidly feeding ticks had 5.1 +/- 1.3 pmole cAMP/mg protein and stimulated glands (n = 4) 119.3 +/- 20.0 pmole cAMP/mg protein.

Other glands were stimulated with 10^{-5} M DA or 10^{-5} M DA and 10^{-2} M Th and then incubated in TC-199 for 1 or 5 min. without drugs to measure rate of hydrolysis of cAMP. Paired control glands were incubated in the same way, but without prior stimulation with dopamine and theophylline. Levels of cAMP did not change in control glands, but cAMP decline in glands of slowly feeding ticks and more slowly in glands obtained from rapidly feeding ticks (Fig. 3A and B).

DISCUSSION

Levels of cyclic AMP increased in salivary glands with increased tick feeding but cAMP/mg protein remained constant. Basal levels of cAMP/mg protein were slightly higher in glands from unfed ticks. This difference may be due to rapid increase in non-enzyme related protein in salivary glands after tick attachment (McSwain et al, 1982). Salivary glands of unfed females did not increase their cAMP when incubated with 10^{-7} M DA and 10^{-2} M Th (Table 1). This result agrees with the findings of Schramke et al, (1984) who did not detect dopamine-sensitive adenylate cyclase activity in salivary glands of unfed females.

Previous studies demonstrated changes in dopamine sensitive adenylate cyclase and cAMP-PDE activities during tick feeding (McMullen et al, 1983; Schramke, et al, 1984). As the tick progresses from the slow to the rapid phase of feeding, total cAMP-PDE activity declines at a tick weight of ca 200 mg (McMullen and Sauer, 1978). This decline is due entirely to a sharp drop in the soluble (cytosolic) activity; the particulate activity increases slightly. At the same time, dopamine-sensitive adenylate cyclase specific activity increases and reaches a peak in glands of ticks weighing ca 200 mg. Activity then declines slightly as ticks increase in weight to repletion (Schramke et al, 1984). Enzyme differences in glands from slowly and rapidly

feeding ticks apparently affect levels of cAMP during gland stimulation. Stimulation of salivary glands by dopamine (with or without theophylline) increased gland levels of cAMP in a dose-dependent manner. Dopamine at 10^{-5} M caused a much larger (9-fold) increase in cAMP in glands of rapidly feeding ticks than glands of slowly feeding ticks. Inclusion of 10^{-2} M Th along with 10^{-5} M DA caused a 32-fold increase in cAMP in glands from slowly feeding ticks and a 23-fold increase in glands of rapidly feeding ticks. The level of cAMP/mg protein in glands of slowly feeding ticks stimulated by 10^{-5} M DA and 10^{-2} Th was higher than that of glands of rapidly feeding ticks stimulated by 10^{-5} M DA alone illustrating the relatively greater ability of glands of slowly feeding ticks to hydrolyze cAMP. Hydrolysis of newly formed cAMP must occur rapidly because of the marked effect of Th in elevating gland cAMP when incubated with DA. Other cAMP must be hydrolyzed more slowly as indicated by slow hydrolysis of increased cAMP when glands were left in stimulant free medium for up to 5 min. Hydrolysis in the latter experiments occurred more rapidly in glands from slowly feeding ticks. The glands contain a high Km PDE in the cytosol and a low Km PDE bound to membranes (McMullen et al, 1983). The high Km cytosolic PDE may be important for modulation of cAMP when the cellular cAMP concentration is raised above basal. The major role for the low Km, particulate (membrane-bound) cAMP-PDE may be to modulate basal levels of cAMP when the gland is not being stimulated

to complete a biological response.

Thus, the regulation of overall gland cAMP is complex. Increases in cAMP may be localized or compartmentalized to effect a specific function (Hayes and Brunton, 1982). The glands are multicellular and multifunc- tional and undergo substantial morphological and physiological changes in the cAMP mediated events or its enzymes concerned with its synthesis and hydrolysis within individual cells would be useful in determining how cAMP modulates the secretory process.

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Figure 1. Cyclic AMP (pmole/gland) in salivary glands from female lone star ticks incubated 5 min. in TC-199 as a function of gland protein in control and dopamine (10^{-7} M), theophylline (10^{-2} M) stimulated glands.

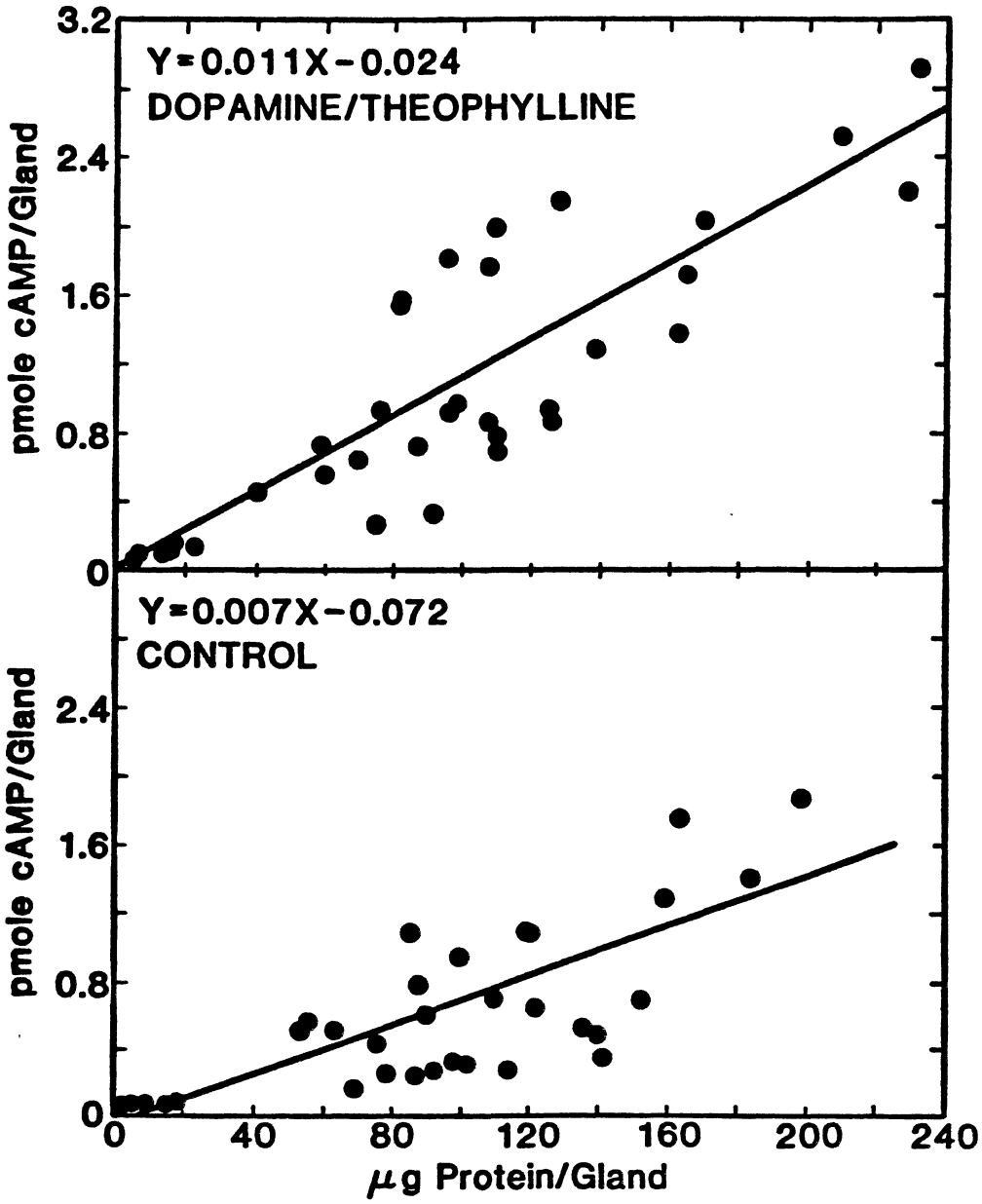

Y=0.011X-0.024
DOPAMINE/THEOPHYLLINE
Y=0.007X-0.072
CONTROL
pmole cAMP/Gland
pmole cAMP/Gland
3.2
2.4
1.6
0.8
0
0
40
80
120
160
200
240
μg Protein/Gland

Table 1. Cyclic AMP in salivary glands obtained from unfed and recently attached female A. americanum ticks stimulated by dopamine (10^{-7}M) and theophylline (10^{-2}M)

Tick	n	Wt(mg)±SEM	cAMP pmole/mg protein ±SEM)	
			Control	Stimulated
Unfed	3	4.07±.33	14.25±2.99	13.43±2.45
Recently attached	3	4.60±.44	6.58±2.03	8.07±0.713

Figure 2. Comparison of cAMP levels (pmole/mg protein +/- S.E.M.) in slowly and rapidly feeding ticks stimulated with 10^{-7} M or 10^{-5} M dopamine with and without 10^{-2} M theophylline. Incubations were performed as indicated in Figure 1.

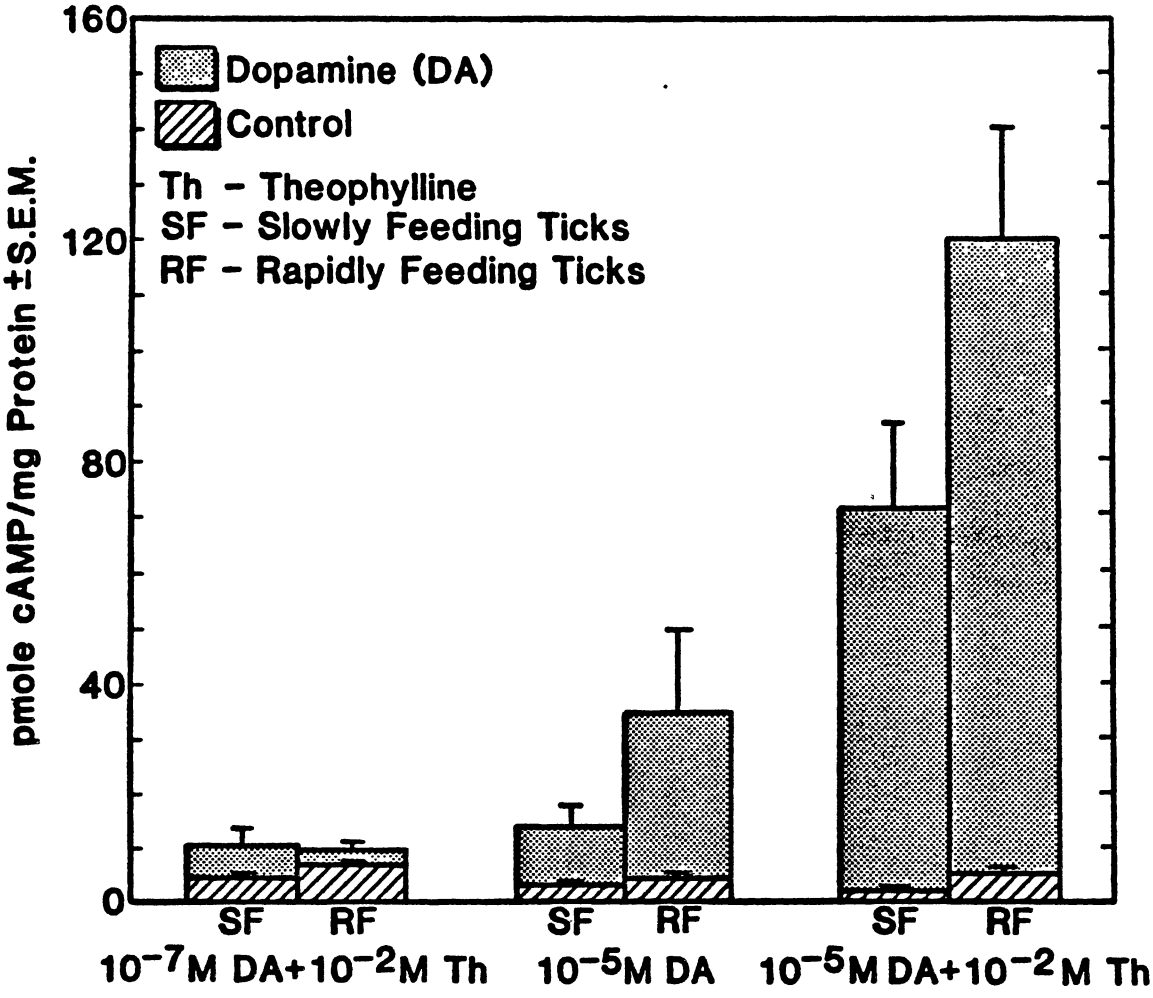
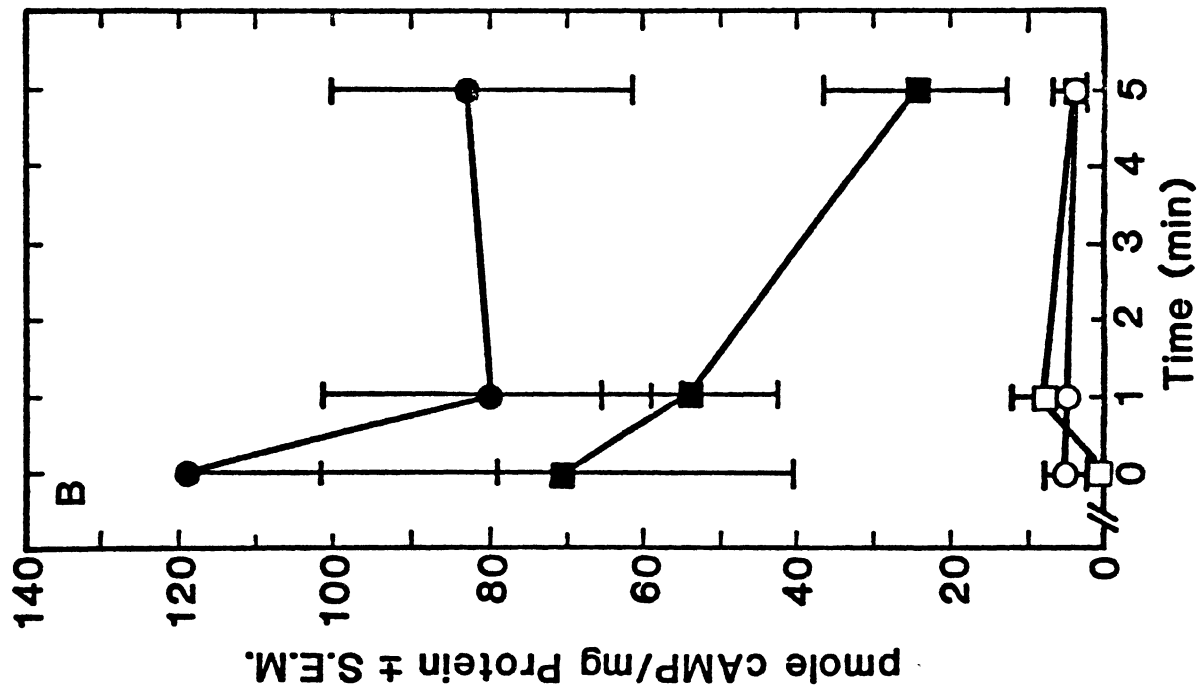
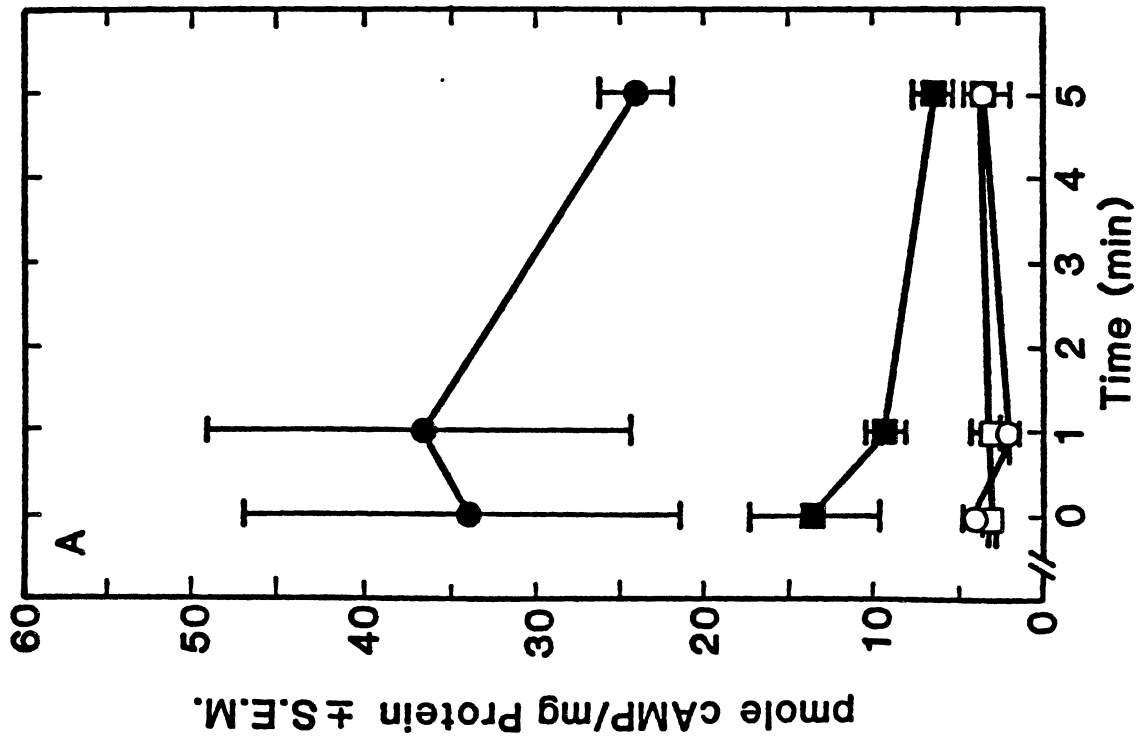

160
120
80
40
0
pmole cAMP/mg Protein ±S.E.M.
Dopamine (DA)
Control
Th - Theophylline
SF - Slowly Feeding Ticks
RF - Rapidly Feeding Ticks
SF
RF
SF
RF
SF
RF
10⁻⁷M DA+10⁻²M Th
10⁻⁵M DA
10⁻⁵M DA+10⁻²M Th

Figure 3. Cyclic AMP (pmole/mg protein +/- S.E.M.) in salivary glands from slowly (□ , ■) and rapidly (○ , ●) feeding adult female ticks following 5 min. stimulation with 10^{-5} M dopamine (A) or 10^{-5} M dopamine and 10^{-2} M theophylline (B). After stimulation, dopamine was removed and glands were incubated in its absence. Open symbols indicate unstimulated glands.


B
pmole cAMP/mg Protein ± S.E.M.
140
120
100
80
60
40
20
0
0
1
2
3
4
5
Time (min)


A
pmole cAMP/mg Protein ± S.E.M.
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (min)

PART II

CYCLIC AMP-DEPENDENT PROTEIN KINASE AND PROTEIN KINASE INHIBITOR IN THE SALIVARY GLANDS OF *AMBLYOMMA AMERICANUM* FEMALES

ABSTRACT

Cyclic AMP-dependent protein kinase activity in salivary glands of feeding female Amblyomma americanum rises to a maximum at 14.6 mg of tick weight and declines thereafter. Most activity (79%) was found in the supernatant of glands from ticks at all stages of feeding. A potent 60,000 - 70,000 MW, heat- and acid-stable cAMP-dependent protein kinase inhibitor protein is present in the glands. Its activity parallels that of cAMP-dependent protein kinase activity.

INTRODUCTION

Ixodid ticks secrete excess fluids back into the host via their salivary glands during feeding. Salivary gland fluid secretion can be stimulated by dopamine and 3',5'-adenosine monophosphate (Sauer et al, 1979; Needham and Sauer, 1979) in the lone star tick, Amblyomma americanum. Gland function appears to be under catecholaminergic neural control (Kaufman and Phillips, 1973; Kaufman, 1978). Schmidt et al, (1981) discovered a D_1-dopamine receptor linked to adenylate cyclase in plasma membranes of tick salivary glands. Dopamine activates the adenylate cyclase to synthesize 3',5'-adenosine monophosphate (cAMP) from adenosine triphosphate (Schmidt et al, 1982; Schramke et al, 1984). Salivary glands in rapidly

feeding ticks (>200 mg) produce higher levels of cAMP in response to dopamine than glands from slowly feeding ticks (<200 mg; Hume et al, 1984). This correlates with higher dopamine-sensitive adenylate cyclase (Schramke et al, 1984) and lower cAMP phosphodiesterase activities (McMullen and Sauer, 1978) in glands of rapidly feeding as compared to slowly feeding ticks.

The only known mode of action of cAMP in eukaryotes is to activate protein kinases that catalyze the phosphorylation of endogenous proteins (Walsh et al, 1968). The phos- phorylated proteins then affect, in some way, the biological response of cells to the neurotrans- mitter. Cyclic AMP-dependent protein kinases of most animals are of two types, I and II. Each of the two enzymes is a tetramer consisting of two copies each of regulatory (R) and catalytic (C) subunits. The enzymes differ in size of cAMP binding regulatory subunit (Erlichman et al, 1974).

In earlier studies we have found similarities but also important differences between those properties reported for cAMP-dependent protein kinases (PK) in most other animals (Mane et al, 1985). Tick salivary gland PK's are similar in that thay appear to consist of at least two isozymes with receptor cAMP binding proteins and catalytic subunits. The receptor subunits have molecular weights near those of Type I and II subunits in other animals (McSwain et al, 1985; Mane et al, 1985). Differences include absence of phosphorylation of high molecular weight subunit but apparent phosphorylation of the low molecular weight

subunit receptor protein. Also, a 10-fold higher concentration of cAMP is required for maximal stimulation of the enzyme as compared to that found in other tissues. Preliminary evidence has also indicated the presence of a heat stable (95°C) and trypsin-sensitive inhibitor in the glands.

The purpose of the present experiments was to determine cAMP-dependent protein kinase and inhibitor activities in salivary glands from ticks at various stages of feeding. We were also interested in further characterizing the heat stable inhibitor protein for comparison to inhibitors in other animals and in measuring its activity during feeding.

MATERIALS AND METHODS

TICK SALIVARY GLAND PROTEIN KINASE ACTIVITY

Cyclic AMP-dependent protein kinase assay

Salivary gland pairs were excised from unfed, feeding, and replete female *A. americanum*. Ticks were fed in surgical stockenettes secured to the sides of 3-4 year old ewes. Dissected glands were held in ice-cold 0.1 M PIPES buffer (piperazine N-N'-bis(2-ethane sulfonic acid)), pH 6.8. Each gland pair was homogenized in a Kontes Duall Glass Homogenizer 20 in 200 ul ice-cold buffer at medium speed with 40 strokes of a Virtis 23 homogenizer. Homogenates were held at 4°C until used. Protein was

measured by the method of Lowry et al, (1951).

Cyclic AMP-dependent protein kinase activity was assayed by the methods of Reimann et al,. (1971) and Gill and Walton (1979). Salivary gland homogenates were incubated in a total volume of 50 ul with 15 ul tissue homogenate protein, 100 ug histone H-1, 10 mM $MgCl_2$, 10^{-5} M cAMP, 0.5 mM ^{32}P-ATP and 0.1 M PIPES, pH 6.8. ^{32}P-ATP was synthesized by the method of Walseth and Johnson (1979) and histone H-1 was prepared according to Jones (1964). Incubations without cAMP and others without histone were used to control for cAMP-independent activity and phosphorylation of endogenous substrates, respectively. Cyclic AMP-dependent PK activity was calculated by subtracting control activities in the presence of cAMP, but without histone from that measured in the presence of both cAMP and histone in the assay medium. Assays were incubated 30 min at 37°C and stopped by placing 30 ul on 2 X 2 cm squares of cellulose phosphate paper (Whatman). Free radioactivity was removed by washing three times with 20 ml doubly distilled, deionized water per square and once with acetone. Dried squares were placed in 5 ml BioCount scintillation fluid (Research Products International) and radioactivity was measured with a Beckman LS-3133T liquid scintillation counter. Cyclic AMP-dependent phospho-transferase activity/tissue protein was calculated as described by Gill and Walton (1979).

1 unit = 1 pmole ^{32}P incorporated min^{-1}. Specific Activity = 1 unit mg $protein^{-1}$.

Subcellular Fractionation of cAMP-dependent Protein Kinase

Excised glands from unfed, feeding and replete A. americanum females were placed in ice-cold PIPES buffer before use. Pairs of glands were homogenized in 0.3 M sucrose as described above. Homogenates were then centrifuged at 105,000 x g for 3 min with a Beckman Airfuge. Supernatants were collected for each gland pair and pellets were resuspended in 100 ul sucrose and recentrifuged. Homogenate supernatants and the respective supernatants from each pellet wash were pooled and each pellet was resuspended in 200 ul sucrose. Aliquots of supernatant and pellet were assayed for protein and the remainder used in the protein kinase assay.

Possible Differences In Inhibitor Or Activator Activities In Salivary Glands Of Slowly And Rapidly Feeding Ticks

Salivary glands from two slowly (29.8 +/- 20.0 mg) and two rapidly (686.5 +/- 146.9 mg) feeding female ticks, each, were homogenized in 400 ul PIPES buffer. Aliquots of 100 ul from slowly and rapidly feeding tick salivary gland homogenates were mixed and enzyme activities compared with unmixed fractions to determine the presence of possible inhibitors or activators.

Cyclic AMP-dependent Protein Kinase Inhibitor Protein

Salivary gland protein kinase inhibitor (PKI) was prepared by the methods of Walsh et at. (1971). Salivary glands (2 glands/5 ul) from feeding female ticks were sonicated with a Fisher Sonic Dismembrator Model 300 at the setting 30% of the maximum frequency output of 20 kilocycles sec^{-1} for 30 sec. The sonicate was heated to 95°C in a water bath and cooled to 4°C. The preparation was centrifuged at 15,000 x g for 15 min at 4°C, in an Eppendorf 5412 Centrifuge. Aliquots of either 70, 140, 210, 280, 350 ug protein - 70 ug homogenate protein was the equivalent of 6 salivary glands - of heat-treated supernanant were added to a cAMP-dependent protein kinase assay along with bovine heart cAMP-dependent protein kinase (Sigma; 2 ug/5 ul). Assay volumes were adjusted to 55 ul with 0.1 M PIPES buffer, pH 6.8 and incubated for 30 min at 37°C. The reaction was started by addition of the enzyme.

Trypsin Treated Cyclic AMP-Dependent Protein Kinase Inhibitor

Trypsin (Sigma) was added to the PKI preparation (1:1, w/w) and incubated 60 min at 30°C. Following trypsinization the solution was heated at 95°C for 10 - 20 min. Trypsinized PKI and PKI, respectively, were added to assay mixtures containing bovine heart cAMP-dependent protein kinase. Tubes contained 10^{-5} M cAMP, 0.5 mM ^{32}P-ATP, 8 ug bovine heart cAMP-dependent protein kinase,

20 mM pH 6.8 MOPS (3-(N-morpholino) propane sulfonic acid), 60 ul PKI in MOPS to give a volume of 120 ul. To this was added 400 ug histone H-1 and 10 mM $MgCl_2$ to start the reaction in a final volume of 160 ul. Reactions were allowed to proceed 30 min. Aliquots were removed at 5 min intervals to determine ^{32}P incorporation as described above.

Protein Kinase Inhibitor Activity During Tick Feeding

Eight ticks per category from the unfed stage to fully engorged-replete ticks were investigated. Salivary glands were taken from unfed and feeding ticks of the following weight categories: unfed, approximately 5 mg; recently attached 10 to 20 mg; partially fed, 20 to 100 mg in 20 mg ranges (e.g. 20 - 40 mg); partially fed ticks 100 to 950 mg in 150 mg ranges (e.g. 100 - 250 mg); and engorged-replete ticks. The 8 pairs of glands in each category were sonicated in 40 ul 20 mM MOPS buffer, pH 6.8 for 30 sec. The sonicates were heated at 95°C for 20 min, cooled to 4°C for 20 min and centrifuged at 15,000 x g for 15 min at 4°C. Fifteen microliters of each PKI preparation was added to assay tubes to a final volume of 50 ul. Percent PKI inhibition of total cAMP-dependent protein kinase activity was determined as above.

Molecular Weight Determination Of PKI

Gland PKI was chromatographed on 1 X 25 cm G-75 Sephadex (Pharmacia) molecular sieve column equilibrated

with 20 mM MOPS, pH 6.8. Fractions (1 ml) were concentrated with Centricon - 10 microconcentrator tubes (Amicon) centrifuged at 9000 x g for 25 min (Sorvall RC2-B) and brought to 100 ul with buffer. PKI assays were conducted using 50 ul of concentrate.

RESULTS

Cyclic AMP-dependent Protein Kinase Over The Feeding Cycle

The levels of cAMP-dependent PK in salivary glands changes as feeding A. americanum females develop to repletion (Fig. 1). Unfed ticks averaging 5.75 mg had a mean PK level of 16.7 units. Females which fed for = < 6 days with a mean weight of 10.4 mg had PK levels of 68.9 units or a 3.7-fold increase. Protein kinase activity reached a significant ($P < 0.05$, by method of finding the lowest sums of square error with a program developed by C-. H. Sung) peak at 14.6 mg of tick weight. This high level of activity decreased slightly, but remained high as the ticks fed to repletion. Engorged-replete ticks had a mean PK level of 89.7 units. Salivary gland homogenates were centrifuged at 105,000 x g to separate soluable and particulate PK. After centrifugation 79% of PK activity was found in the supernatant fraction (Fig. 2). The remaining 21% activity was found in the particulate fraction. Subcellular distribution of PK activity did not change over the the feeding cycle.

Homogenates from slowly and rapidly feeding ticks

were mixed to determine whether there were factors in glands which would enhance or inhibit PK activities. PK specific activity in the mixture was intermediate to activity obtained with homogenates from slowly and rapidly feeding ticks (Table 1). The specific activity of slowly feeding tick homogenate was 769.4 +/- 80.4, rapidly feeding homogenate had specific activity of 152.1 +/- 31.1 and the mixtures had an intermediate of 412.0 +/- 31.2 pmole ^{32}P incorporated min^{-1} mg $protein^{-1}$.

Salivary Gland Protein Kinase Inhibitor

A boiled extract of tick salivary glands inhibited PK in a dose-dependent manner (Fig. 3). PK activity was inhibited 50% with the addition of 6 gland equivalents of crude PKI protein (210.8 ug). PKI was demonstrated to be protein by the significant ($P < 0.05$) reduction in PKI activity after treatment of PKI with trypsin (Fig. 4). Total gland PKI activity increased from undetectable activity in unfed ticks to a significant ($P < 0.05$) peak of inhibition at 24.4 mg (Fig. 5). The high level of inhibition was maintained through to repletion ticks with an average of 42% decrease in total ^{32}P incorporated for ticks greater than 24 mg. PKI eluted from a molecular sieve column at a volume corresponding to an apparent molecular weight of 60,000 and 70,000.

00032

DISCUSSION

Total tick salivary gland cAMP-dependent protein kinase (PK) specific activities change during tick feeding. The ability to secrete fluid also changes during feeding; however, maximum secretion occurs late in feeding when the tick weighs about 300 mg (Sauer et al, 1979). At most times of the year, female Amblyomma americanum ticks require about 14 days to complete feeding while feeding on restrained ovine host in the laboratory (Hume et al, unpublished). Slow feeding occurs during the first 9 - 10 days when the tick increases in weight to approximately 20 mg from an unfed weight of 4 - 5 mg. The female then begins feeding much more rapidly for 3 - 4 days attaining an engorged weight of 500 - 1000 mg. Total tick gland PK activity and specific activity were highest at 15 mg of tick weight corresponding to 7 - 9 days of feeding. Salivary gland protein is maximal at 80 - 100 mg of tick weight (McSwain et al, 1982; Schramke et al, 1984), or after 10 - 11 days of feeding. Therefore, both cAMP-dependent PK activity and total protein precede rapid feeding and high rate of fluid secretion. Female ticks remained at relative low weights for 9 - 11 days during the slow feeding stage (Hume et al, unpublished). This extended time of attachment and slow feeding represents only a small segment of the PK activity profile as presented in Fig. 1. At day 11 of a feeding experiment, feeding ticks (n = 21)

had a mean weight of 118.7 mg. Therefore, the rate of PK activity and or synthesis is greatest in the lower weight, slowly feeding ticks. These results suggest a more complex role for the enzyme in phosphorylating proteins, some of which may not be directly involved in controlling fluid secretion. Secretion can be stimulated by dopamine and cAMP (Sauer et al, 1979) and dopamine stimulates synthesis of cAMP by activating an adenylate cyclase (Schmidt et al, 1982). At least twelve proteins are phosphorylated in response to gland stimulation by dopamine and cAMP (McSwain et al, 1985).

The majority of PK activity (79%) was isolated in the cytosol after ultracentrifugation correlating with the findings of Mane et al, (1985) who found 57% of salivary gland PK activity in the cytosol, 21% in the 100,000 x g pellet (microsomal fraction) and 19% in the 12,000 x g pellet.

The presence of a PK inhibitor protein indicates another possible level of control. Inhibitor activity was highest at a tick weight of 24 mg and near the weight of females demonstrating peak PK activity. Changes in PKI activity paralleled changes in PK activity. The absence of proportionately more activator or inhibitor activity in glands of slowly or rapidly feeding ticks was further demonstrated in mixing experiments.

Tick PKI appears to be a more potent inhibitor of tick PK than of mammalian PK (Mane, personal communication). Ninty-three percent of tick enzyme activity can be

inhibited as compared to only 50% on mammalian PK activity with the same amount of tick inhibitor protein. A low concentration of inhibitor (precipated with treated with 5% trichloroacetic acid) protein with kemptide as a PK substrate resulted in a slight increase in kemtide phosphorylation (Hume et al, unpublished). However, higher levels of inhibitor did decrease PK activity with kemptide. This may indicate a substrate dependent of inhibitor to decrease PK activity. Alternately, denatured proteins of crude inhibitor may be serve as substrates in the presence of kemptide. The 60,000 - 70,000 MW size of tick PKI also indicates a difference with mammalian PKI which has a MW of 22,000 (Whitehouse et al, 1981). Therefore, tick inhibitor appears to be a unique PK inhibitor.

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Figure 1. Cyclic AMP-dependent protein kinase activity over the weight range of *A. americanum* females. Each point represents PK activity in one pair of salivary glands. Gland kinase activity peaked at 14.6 mg. Observations enclosed in the hash-mark rectangle are from unfed ticks.

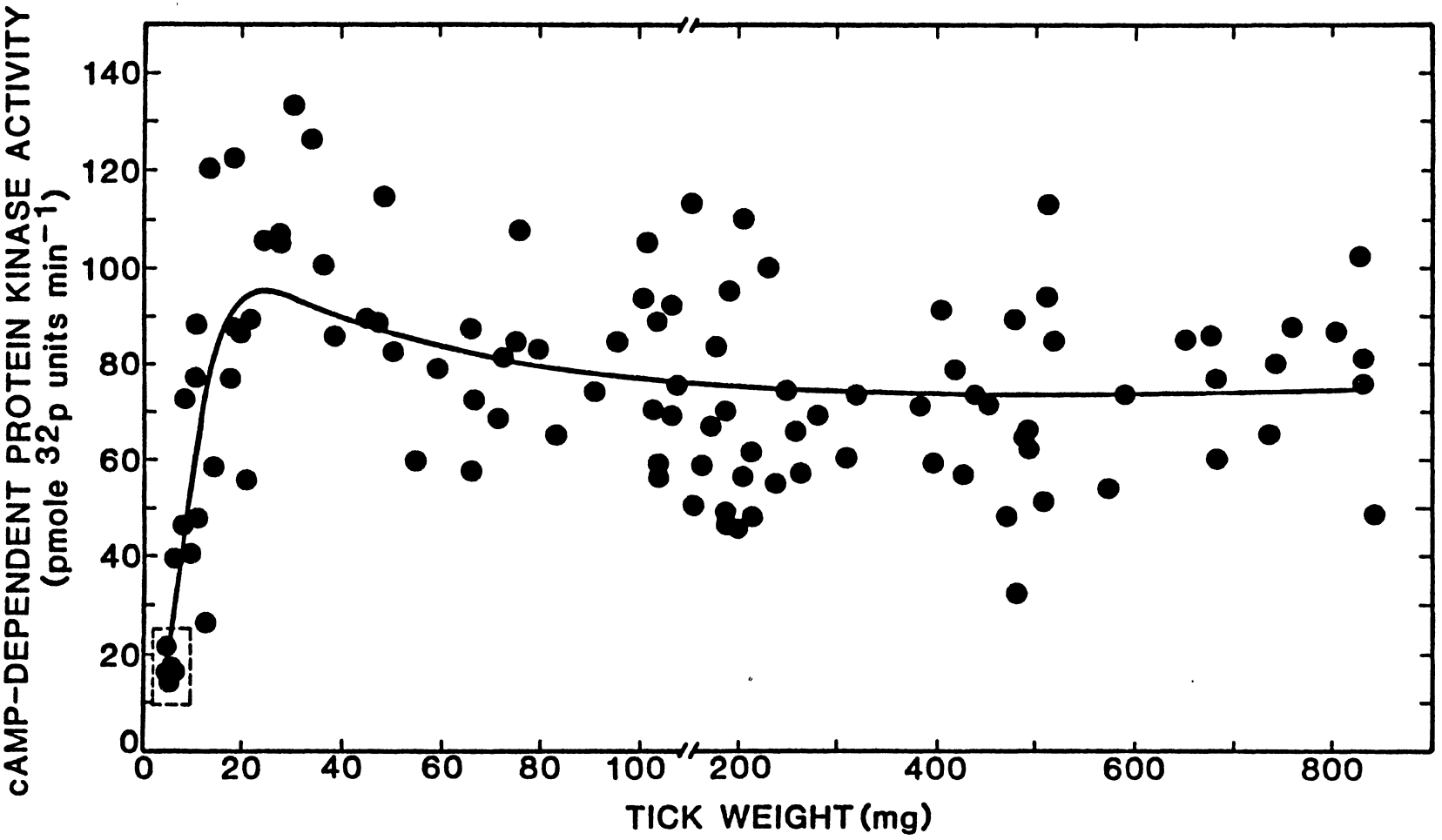

cAMP-DEPENDENT PROTEIN KINASE ACTIVITY
(pmole 32p units min-1)
0
20
40
60
80
100
120
140
0
20
40
60
80
100
200
400
600
800
TICK WEIGHT (mg)

Figure 2. Cyclic AMP-dependent protein kinase activity in supernatant (105,000 x g) of tick glands (1 gland pair/observation) homogenates. Little change in distribution of kinase activity was observed in salivary glands during tick feeding.

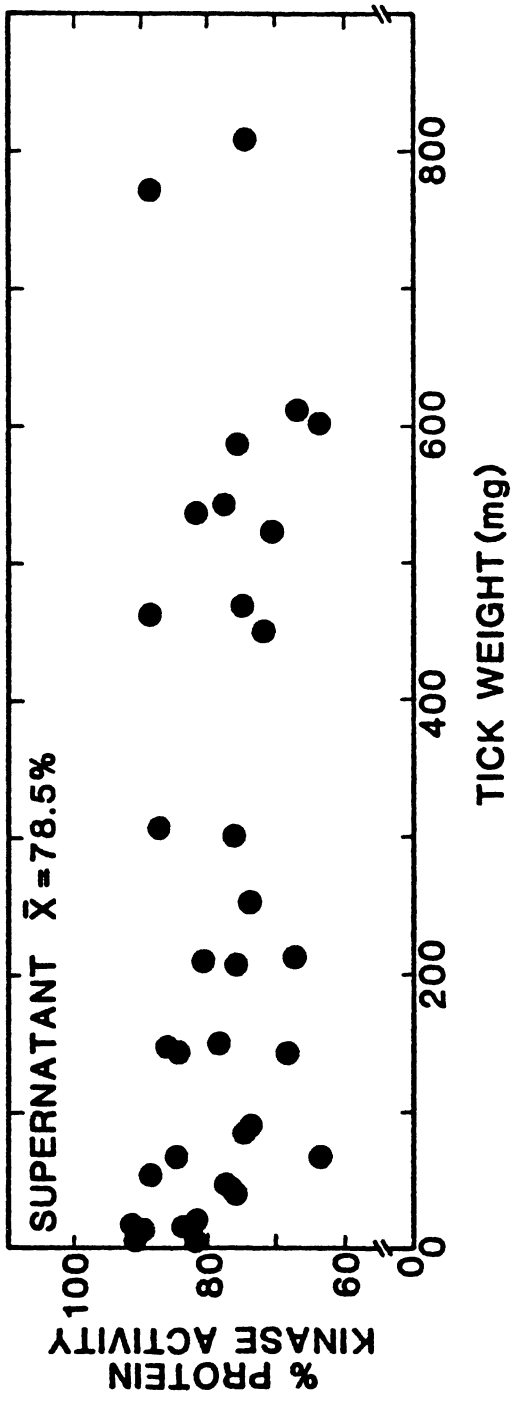

% PROTEIN KINASE ACTIVITY
100
80
60
0
200
400
600
800
TICK WEIGHT (mg)
SUPERNATANT $\bar{X}$=78.5%

Table 1. PK activity of homogenates from slowly and rapidly feeding ticks and of a mixture. Averages are from 3 experiments with 2 gland pairs per feeding stage in each experiment. Numerals indicate means ± S.D.

Feeding Stage	Tick Wt. (mg)	mg Protein	PK S.A.
Slow	29.8+/-20.0	0.37+/-0.3	769.4+/-80.4
Rapid	686.5+/-146.9	0.85+/-0.1	152.1+/-31.1
	MIXTURE	0.27+/-0.1	412.0+/-31.2

Figure 3. Salivary gland protein kinase inhibitor activity as a function of increasing gland protein. Fifty percent inhibition of control activity (i.e. without inhibitor) was observed with 6 gland equivalents of protein (210 ug) added to bovine heart cAMP-dependent protein kinase. Each point is the average of 3 independent experiments with 2 gland equivalents of heat-treated supernatant protein per 5 ul.

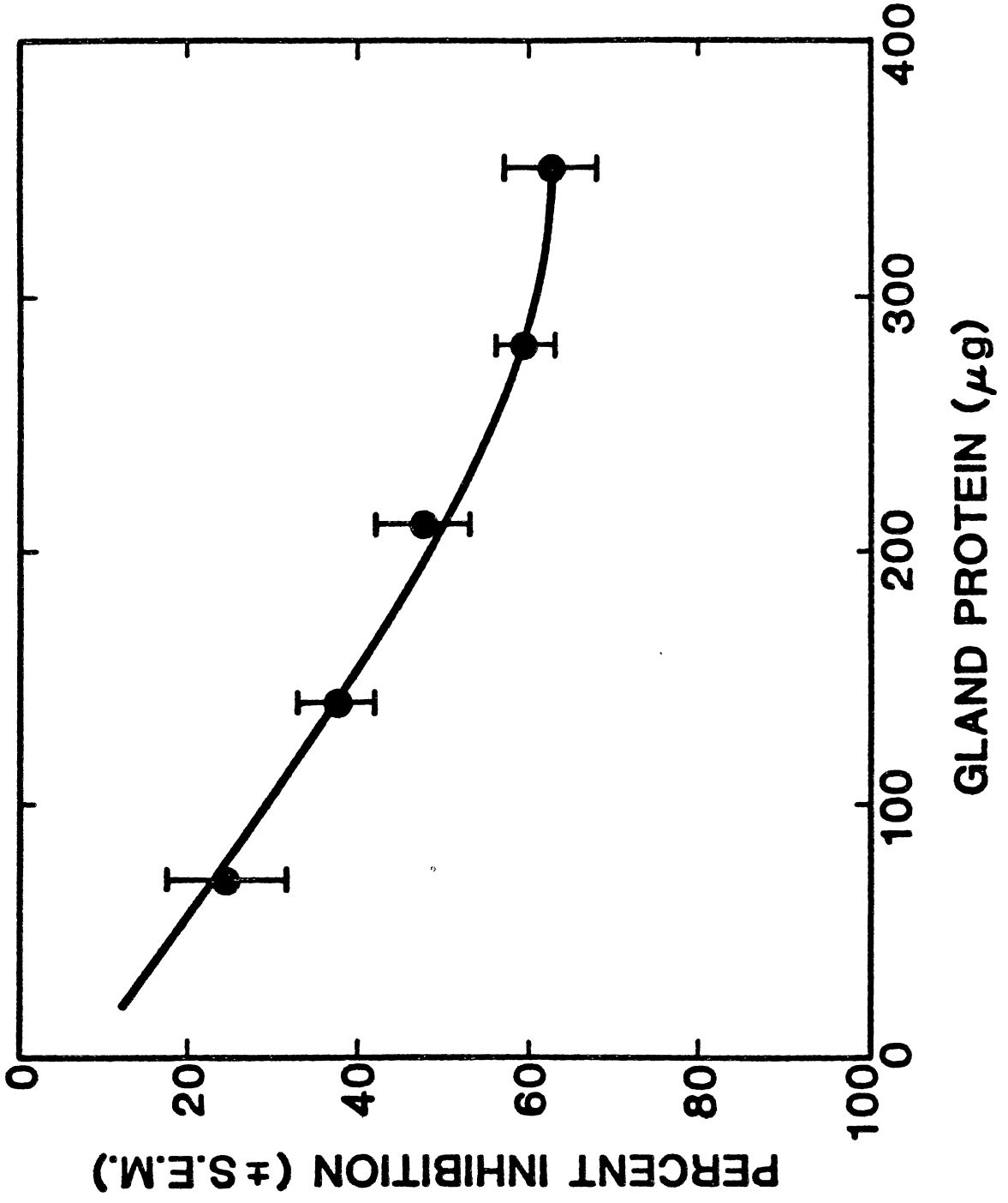

PERCENT INHIBITION (± S.E.M.)
0
20
40
60
80
100
0
100
200
300
400
GLAND PROTEIN (μg)

Figure 4. The effect of trypsin on salivary gland cAMP-dependent protein kinase inhibitor protein. ^{32}P incorporation was measured at 5, 10, 15, 20, 25 min. after intiation of the reaction (○ - PK activity without PKI present, ● - PK activity in the presence of autolysed and heat (95°) denatured trypsin, □ - PK activity in the presence of trypsin lysed inhibitor, ■ - PK activity in the presence of non-denatured inhibitor).

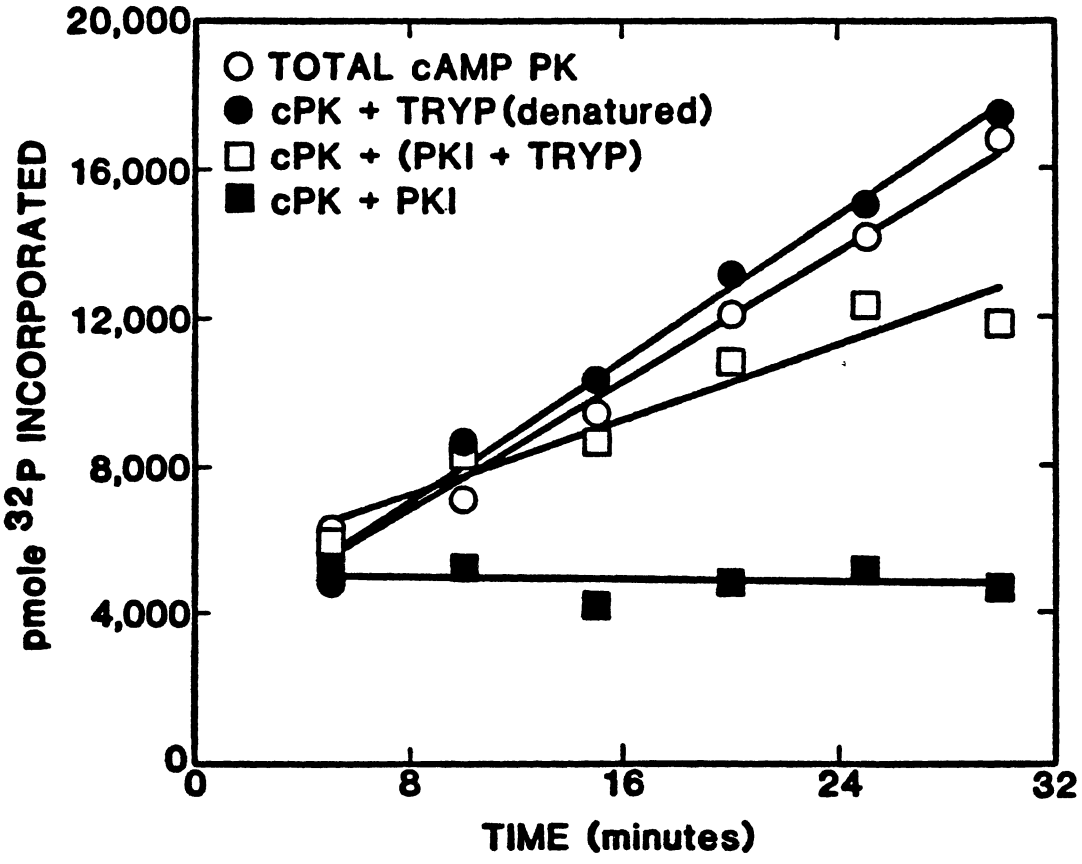
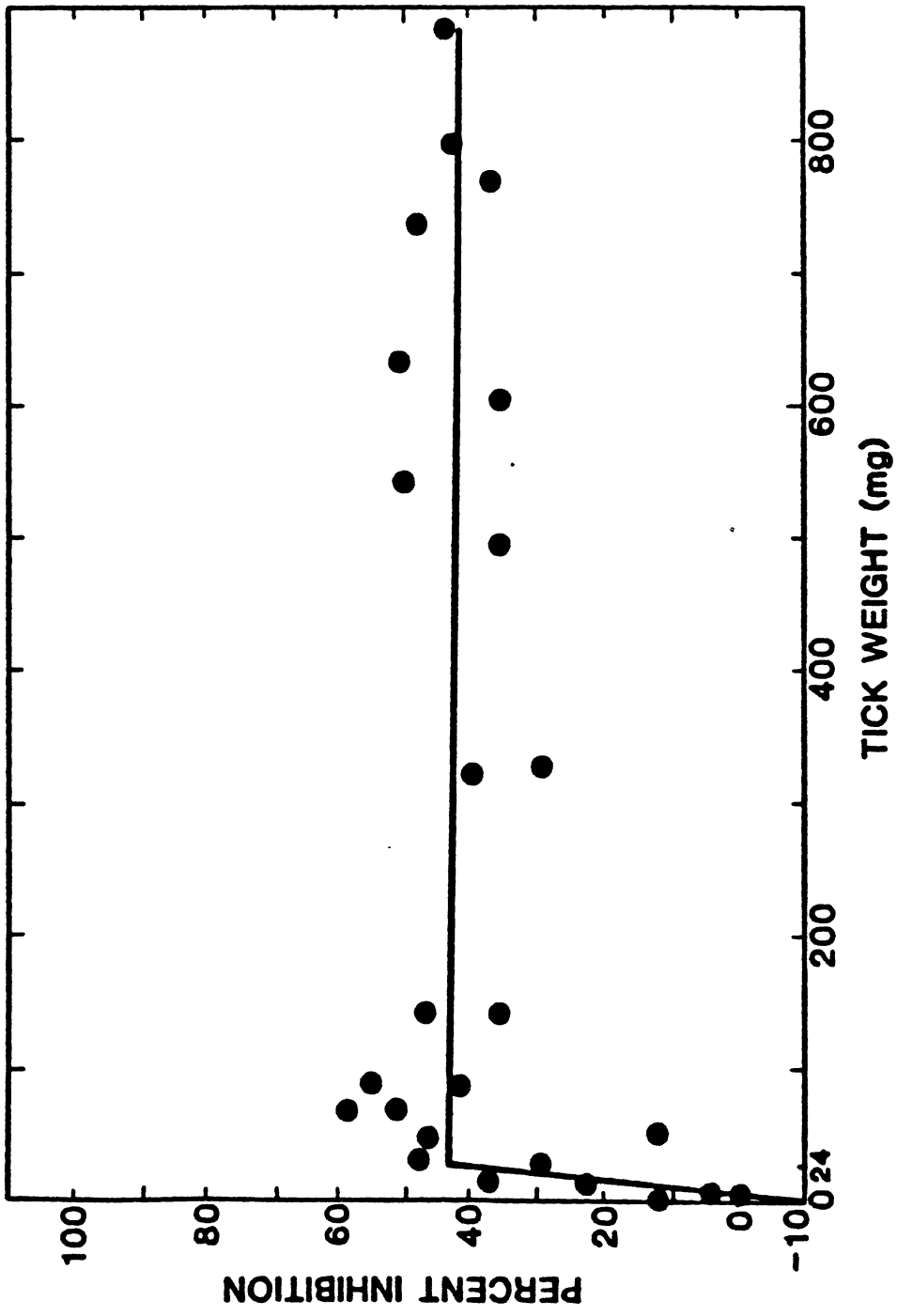

20,000
16,000
12,000
8,000
4,000
0
pmole ^{32}P INCORPORATED
O TOTAL cAMP PK
● cPK + TRYP(denatured)
□ cPK + (PKI + TRYP)
■ cPK + PKI
0
8
16
24
32
TIME (minutes)

Figure 5. Percent inhibition of mammalian cAMP-dependent protein kinase activity by inhibitor protein from tick salivary glands as a function of female weight (here weight is an average of 8 ticks). Each observation was made with 6 gland equivalents of homogenate.


PERCENT INHIBITION
100
80
60
40
20
0
-10
0
200
400
600
800
TICK WEIGHT (mg)

PART III

WEIGHT CHANGES IN INDIVIDUAL, ATTACHED AND FEEDING *AMBLYOMMA AMERICANUM* FEMALE TICKS

ABSTRACT

A reliable method is described for estimating weights of feeding Amblyomma americanum female ticks without removing the tick from the host. Feeding and weight changes were measured over the feeding cycle. Three distinct periods of growth and two to five periods of weight loss were noted. Periods of weight loss during feeding may indicate that, at times, rates of salivation exceed rates of bloodmeal ingestion.

RESULTS

The time and magnitude of tick feeding are critical factors affecting the ability of ixodid ticks to vector pathogens (Burgdorfer, 1975, Journal of Medical Entomology, **12**:269-278). Furthermore, salivary antigens that cause host allergic responses depend upon the duration of tick feeding (Brown and Askenase, 1983, Federation Proceedings, **42**: 1744-1749). Limited information is known about how or when these factors are transmitted to the host. Because of variations in time required for completion of engorgement and final engorgement weights of females, it has been difficult to measure the progress of feeding by a single attached tick. Gothe and Budlemann (1980, Zentralblatt Fuer Veterinaermedizin B. **27**:524-543) reported a method for

estimating weight of feeding Rhipicephalus evertsi evertsi from measurements of tick volume. Here we describe a similar method, in more detail, for estimating the progress of feeding by individual Amblyomma americanum females.

A. americanum females (12) were marked on the scutum with bright paint (Testors enamel) for identification. Females and 12 unmarked males were placed in a stockinette cell fastened with adhesive to the side of the host (3-4 year old ewe previously exposed to tick feeding and restrained in stanchions) and allowed to feed to repletion. The volume of each tick was determined prior to attachment and at various times of feeding thereafter. Volumes were determined by measuring with Mitutoyo calipers (+/- 0.02 mm), tick length (L) along the anterior-posterior medial line, tick width (W) between spiracles and the tick depth (D) dorso-ventrally.

To verify the precision of these linear measurements for estimating volume and then weigh 200 unfed ticks, partially fed ticks (forcibly detached), and replete female ticks were weighed with a Mettler H20 balance (sensitive to 0.01 mg) after determining tick volume as described. Tick weights and estimated volumes (V = L x W x D) were linearly related with little variation (r = 0.99; Fig. 1A). This remarkable correlation indicated that it was feasible to accurately estimate weights of individual attached ticks rapidly with simple linear measurements.

Subsequently, two sets of marked females were placed

on host animals and their volumes determined over the feeding cycle. The average time to repletion was 13.8 +/- 1.3 (mean +/- standard deviation) days and the mean final weight was 642.7 +/- 144.0 mg. Balashov (1972, A Translation of Bloodsucking Ticks - Ixodidea - Vectors of Diseases of Man and Animals) discussed three growth stages in feeding female ixodid ticks. Three stages were observed over the feeding cycle of A. americanum. During the first stage, which lasted 9 - 10 days post-attachment, ticks increased in weight slowly from about 5 to 11 mg. During the second stage they increased rapidly from 10.9 +/- 8.2 mg at day 10 to to 118.7 +/- 154.9 on day 12. The final and even more intense stage to repletion lasted approximately 2 days. During the course of these studies we observed a seasonal effect on the time required, but not the magnitude, of female feeding. From mid-April to mid-May, feeding was greatly accelerated to an average of 9 days or less as compared to 14 days at other times of the year. The reason or cause of this difference is unknown, but it is interesting that intense host-seeking behavior of adult A. americanum in the field is observed at this time of year in Oklahoma (Semtner and Hair, 1973, Annals of the Entomological Society of America, **66**:1264-1268).

Interestingly, individual ticks, while experiencing a large overall increase in weight (4 to about 650 mg), exhibited periods of weight loss as compared to its previous day's weight (Fig. 1B). Ticks were examined daily

and every tick experienced at least 2 - 5 periods of weight loss during the feeding cycle. Because of daily variations when decreases in individual ticks occured, averages of many ticks would erroneously suggest a steady increase in tick weight with increasing time of attachment and feeding. It is likely that weight losses represent periods of greater excretion as compared to ingestion of the blood-meal. Since most excess fluid is eliminated by the salivary glands back to the host (Kaufman and Phillips, 1973, Journal of Experimental Biology, **58**:523-556), it is possible that weight losses represent periods of increased salivation. Alternately, the losses could represent periods of attenuated feeding without reduced salivation. It is likely that the ratio of ingestion to excretion (mostly salivation in A. americanum) is not constant during the feeding cycle. Fluid may accumulate in the hemocoel during tick feeding up to a "threshold" volume that "triggers" a more intense burst of salivation. Recall that Kaufman (1979, Rec. Adv. Acarol., Vol 1:357-363) suggested at least two neural pathways bringing sensory information to the salivary gland to control fluid secretion and elimination of excess fluid following concentration of the bloodmeal. He suggested that likely responses are to stretch receptors in the abdominal muscle or increased hydrostatic pressure in the hemocoel.

This report demonstrates that estimation of weight based upon measurements of tick size is a reliable method

for studying the course of feeding in a single attached ixodid tick. This method may enhance detailed studies of tick feeding, pathogen transmission to hosts and determing when ticks secrete factors that cause allergic or toxic type reactions in host animals.

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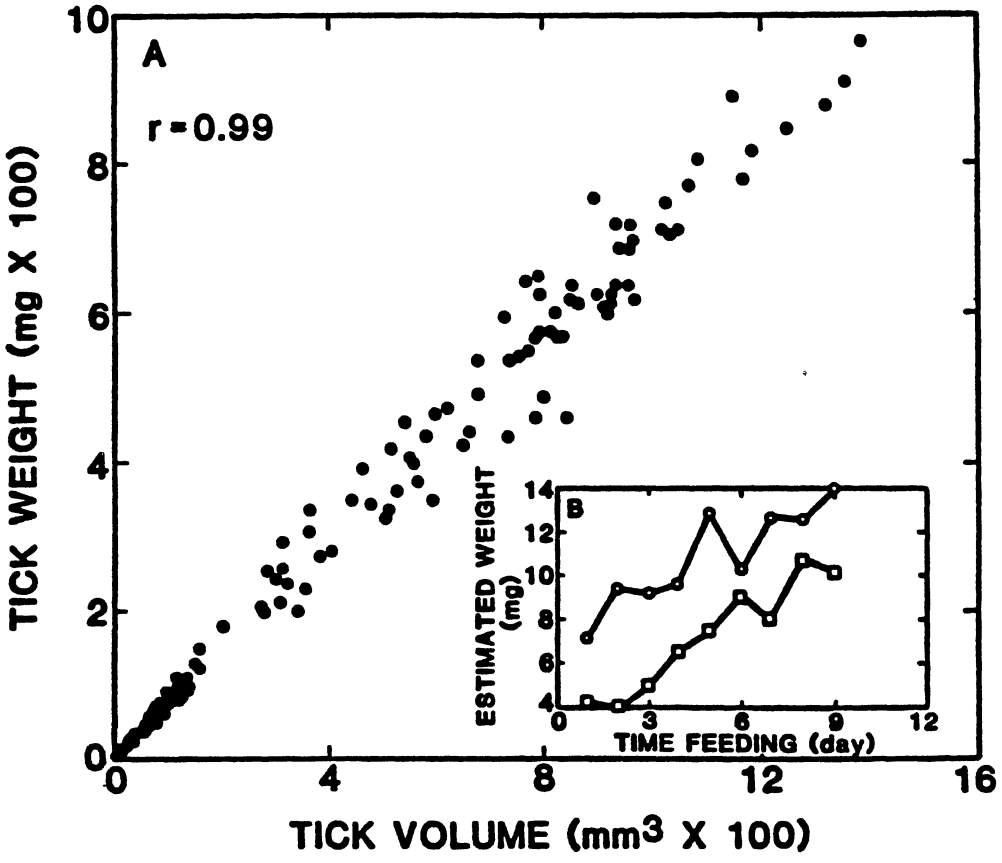
Figure 1. A. The relation between weights of unfed, partially fed and replete females and measured volumes (Volume = length x width x depth). A second degree linear equation was used for weight estimation (Weight Estimated = $0.075X - 5.05 \times 10^{-5}X^2 + 1.75$). B. Typical weight changes of individual female ticks during early stages of the feeding cycle. Decreases were observed at days 3, 6, 8 (○) and 2, 7, 9 (□), respectively, of two representative ticks.


A
r=0.99
TICK WEIGHT (mg X 100)
TICK VOLUME (mm3 X 100)
0
2
4
6
8
10
0
4
8
12
16
B
ESTIMATED WEIGHT (mg)
TIME FEEDING (day)
4
6
8
10
12
14
0
3
6
9
12

VITA

Michael Edwin Hume

Candidate for the Degree of

Doctor of Philosophy

Thesis: CYCLIC AMP, CYCLIC AMP-DEPENDENT PROTEIN KINASE AND ITS INHIBITOR PROTEIN IN SALIVARY GLANDS OF FEMALE LONE STAR TICKS, AMBLYOMMA AMERICANUM (L.)

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