# ROLE OF CYCLIC AMP MEDIATED PROTEIN PHOSPHORYLATION AND ITS RELATIONSHIP TO FLUID SECRETION IN SALIVARY GLANDS OF *AMBLYOMMA AMERICANUM* (L.)

By

JANIS LEE ADAMS McSWAIN

Bachelor of Science
Central State University
Edmond, Oklahoma
1972

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1976

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1983

ROLE OF CYCLIC AMP MEDIATED PROTEIN PHOSPHORYLATION AND ITS RELATIONSHIP TO FLUID SECRETION IN SALIVARY GLANDS OF AMBLYOMMA AMERICANUM (L.)

Thesis Approved:

John R. Sauer
Thesis Adviser

Richard C Essenberg

Ronald W. McNew

R. W. Barker

Norman N. Durham
Dean of Graduate College

# PREFACE

The author would like to express her gratitude to Dr. Richard Essenberg, Professor, Department of Biochemistry, for his valuable advise, teaching, and patience during this study. I greatly appreciate Dr. Bob Barker, Associate Professor, Department of Entomology, for his support, understanding, and interest in this project. Appreciation is also extended to Dr. Ronald McNew, Professor, Department of Statistics, for his guidance on the statistical aspects of this study. I appreciated the help Dr. Don Peters, Department Head, Department of Entomology, has given and would like to express my gratitude to him. Dr. Kermit Carroway, Professor of Biochemistry, was very helpful and was missed when he left Oklahoma State University. All these individuals served on the author's graduate committee.

I would also like to express a special "thank you" to Dr. John Sauer, Professor, Department of Entomology, for his unfailing guidance and support. I much admire his patience, endurance and dedication.

To Debbie Barker, John Krolak, Harold McMullen, Glen Needham, Mary Schramke, Steve Schmidt, Mike Hume, John Williams, Roy Darville, and Suresh Mane, a special "thank you". Working with people like you was a privilege.

I will especially not forget the friendship of Marie Will, Billie Will, Mary Rymer, and La Donna Vernon. I would like to thank them for their continued friendship and concern. To Charlie, my best friend, I can only say that you were always there and I truly appreciate that.

Finally to my family, Rick, Kim, and David, I extend my deepest appreciation for their love and support.

This research was supported in part by Grants PCM 79-21717 and AI-3535 from the National Science Foundation and National Institutes of Health, respectively.

TABLE OF CONTENTS

LIST OF TABLES

Table | Page

PART IV

LIST OF FIGURES

Figure Page

PART III



PART IV

# INTRODUCTION

Each part of this thesis is a separate and complete manuscript to be submitted for publication. Part I has been published in the Journal of Parisitology. Parts II, III, and IV are being submitted to Biochimica Et Biophysica Acta.

Each part appears in this thesis in the format of the journal in which it has been published or to which it is being submitted.

# PART I

# PROTEIN CHANGES IN THE SALIVARY GLANDS OF THE FEMALE LONE STAR TICK, *AMBLYOMMA AMERICANUM*, DURING FEEDING

## ABSTRACT

Several new proteins, as determined by SDS-polyacrylamide gel electrophoresis, appeared in the salivary glands of female Amblyomma americanum soon after attachment of the tick to a host. Other proteins, present in unfed ticks, increased in quantity during tick feeding. The newly synthesized proteins remained undiminished through the remainder of tick feeding, suggesting incorporation, as structural components, into enlarging alveoli Types II and III. All but one of these kinds of protein were found in the salivary glands of unmated, partially fed females attached to a host for equal periods of time; however, total gland protein was much less than that observed in glands of mated females. Another group of proteins, present in the salivary glands of unfed females, were secreted or converted to other substances by the salivary glands of females if mating did not occur. One protein, present in unfed ticks, increased in quantity during early phases of feeding, but during later phases of feeding it was secreted or converted to other substances. This protein remained in the glands of females if mating did not take place. One other protein found in the glands of unfed ticks decreased during early phases of feeding but increased during later phases of feeding. This protein was also seen in the glands of partially fed and unmated females. Results suggested that following attachment, ixodid females were stimulated to feed and synthesize new kinds of salivary gland protein. Mating stimulated additional female feeding and an increase in the amounts but not the number of salivary gland proteins. Following mating, salivary glands were also stimulated to secrete considerable protein.

# INTRODUCTION

Salivary glands are vital to the physiology of ectoparasitic ixodid ticks (Sauer, 1977). During feeding the meal is concentrated by movement of water and ions from the gut of the tick to the hemocoel and back to the host via the salivary glands (Tatchell, 1967; Kaufman and Phillips, 1973a,b,c; Meredith and Kaufman, 1973; Hsu and Sauer, 1975). The glands of most ixodid ticks also secrete cement that is dispersed around the mouthparts of the tick to help anchor the mouthparts to the host during feeding (Balashov, 1972; Chinery, 1973). It is widely believed that many disease-causing organisms are inoculated into the host body by direct introduction of the agent with tick saliva (Sauer, 1977).

The glands in female ticks undergo substantial growth, differentiation and development during feeding, and sequential accumulation and depletion of materials in specific cells of the multialveolar, multicellular tissue is seen via microscopy (Binnington, 1978; Megaw and Beadle, 1979). Development of "water-cells" in Type II and III alveoli is likely a key factor in enhancing the tick's ability to secrete fluid (Meredith and Kaufman, 1973; Megaw and Beadle, 1979).

Various enzymes and other factors have been found in artificially induced saliva from partially fed ixodid ticks (Geczy et al., 1971; Tatchell, 1971; Dickinson et al., 1976; Higgs et al., 1976) but little is known about when or if these materials are secreted naturally. The purpose of the present experiments was to investigate changes in salivary gland proteins during various phases of female feeding. SDS-slab-gel electrophoresis provided a sensitive method for simultaneously comparing specific proteins of salivary glands obtained

from unfed ticks and ticks in different stages of feeding.

## MATERIALS AND METHODS

Male and female lone star ticks Amblyomma americanum (L.) were raised by the methods of Patrick and Hair (1975). Females served as the source of salivary glands and were removed from sheep after feeding for various periods of time. Unfed adults were taken from the existing colony at the Medical Entomology Laboratory at Oklahoma State University.

Salivary glands were dissected at 4° C in a medium of modified oxygenated TC-199 (Difco) at pH 7.0 with penicillin and streptomycin sulfate added in amounts as described by Needham and Sauer (1979). Following dissection, glands were maintained at 4° C and homogenized in small, glass, tissue homogenizers containing 0.3 ml of 10 mM sodium phosphate buffer at pH 7.2. Total protein of tissue homogenates was determined by the method of Lowry et al. (1951).

### Polyacrylamide SDS-gel Electrophoresis of Salivary Gland Protein

Glands were excised from unfed ticks; ticks attached for less than 48 hr (48 hr after infesting ticks on sheep) and weighing about 5 to 6 mg; ticks attached longer and weighing 100 to 250 mg; ticks attached still longer and weighing 300 to 450 mg; and ticks near repletion weighing more than 500 mg; and proteins were compared. Glands were also removed from unmated females attached to the host for 12 to 14 days. The weight of these female ticks never exceeded 35 mg.

An aliquot containing 100 µg of salivary gland protein was placed

in a 2X volume of sample buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.0725 M Tris-HCl buffer (pH 6.8), and 0.01% bromophenol blue. Glands from 10 to 20 unfed ticks and ticks attached to the host for < 48 hrs or from 4 unmated females were required to obtain sufficient protein. The sample buffer containing the tissue homogenate was incubated in boiling water for 2 minutes. An aliquot containing approximately 30 µg of protein of this preparation was subjected to acrylamide gel electrophoresis for 12 to 14 hrs or until the bromophenol blue tracking dye reached the bottom of the gel (17.5 cm by 14 cm by 0.075 cm) at 5 to 10 mA on a vertical plate-gel containing 7.5% acrylamide separating gel and 1.0 cm of 3.5% acrylamide stacking gel. Gels and electrode buffer (pH 8.3) were prepared following the method of King and Laemmli (1971). The molecular weights of the protein bands on the SDS gels were determined by comparing the mobilities of the proteins with proteins of known molecular weight following the method of Weber and Osborn (1969). The following proteins were used as standards: ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (94,000), β-galactosidase (116,500), and myosin (200,000).

Following electrophoresis, the gels were fixed in 25% isopropanol and 10% acetic acid for 30 minutes. Protein bands were stained with a solution of 25% methanol, 10% acetic acid, and 0.025% Coomassie blue R-250 for several hours, and later destained by shaking in 10% acetic acid. After destaining, the gels were placed on several layers of filter paper and slowly dried under vacuum. Chemicals used in all electrophoretic procedures were obtained from Bio-Rad and were of the highest grade commercially available.

# RESULTS

After attachment to sheep, female lone star ticks A. americanum undergo a slow phase of feeding lasting 8 to 14 days during which their weight increases from 4 to 300 mg followed by a rapid phase lasting 12 to 24 hr during which the weight increases from approximately 300 to 800 mg. The average engorged weight is approximately 750 mg (Guenther et al., 1980).

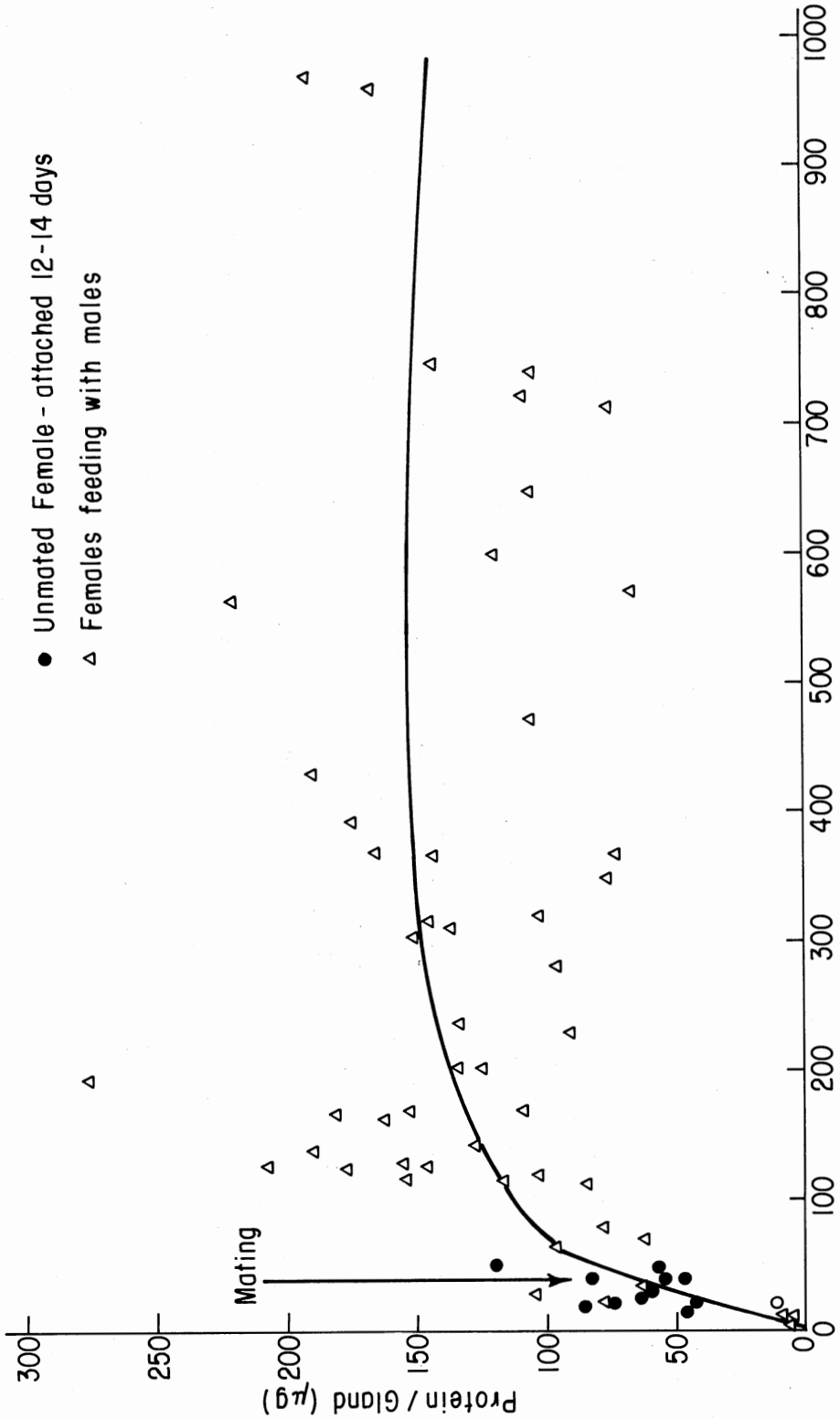
When total female tick salivary gland protein was plotted as a function of tick weight (feeding) (Fig. 1), there was an increase in tissue protein from 4.6 ± 2.0 µg/gland (mean ± SD) in the unfed female to an average of 134.5 ± 49.5 µg/gland (ticks weighing greater than 150 mg).

Gland protein increased rapidly following tick attachment. The mean protein concentration of salivary glands from ticks attached to a host for about 24 hr was 8.0 ± 3.0 µg/gland while the average weight of the whole tick incresed from 3.68 mg to 5.58 mg. Mating is required for the female to engorge fully although attachment and some feeding will occur in the absence of mating.

When female ticks were placed in isolated cells on the host without males, they attached and fed but attained a weight of only 27.1 ± 12.8 mg after 12 to 14 days. Most females of A. americanum, when allowed to feed and mate, achieve their engorged weight by the 14th day postinfestation on sheep. The salivary gland protein in 14-day unmated females was 62.7 ± 27.1 µg/gland. This value did not increase further if mating did not take place.

When salivary gland proteins (obtained from females allowed to mate) were separated by electrophoresis, at least 31 major bands were

Figure 1. Protein measured in salivary glands of ticks of different weights. The maximum weight of females at the time of mating is indicated by the arrow; unmated females never exceeded 35 mg.


● Unmated Female - attached 12-14 days
△ Females feeding with males
Mating
Protein / Gland (μg)
Tick Weight (mg)
0
50
100
150
200
250
300
0
100
200
300
400
500
600
700
800
900
1000

observed to change as female weight (feeding) increased. These proteins and their estimated molecular weights are depicted in Figure 2 representing the changes noted in four separate experiments. Based upon analysis of the gels, the proteins were classified into eight groups A-H (Table 1). Group A proteins (Nos. 12, 13, 14, 19) were not present in unfed ticks but were evident shortly (48 hr) after tick attachment and thereafter. Group B proteins (Nos. 1, 2, 3, 15, 17) were not present in unfed ticks and ticks attached to the host for less than 48 hr but were evident in ticks weighing 100 mg and more. Group C proteins (Nos. 9, 10, 16, 18, 21, 22, 30) were evident in unfed ticks but appeared to increase in quantity as tick feeding progressed. Group D proteins (Nos. 11, 20, 28) were present in the unfed tick but could not be detected shortly after the tick attached to the host. The single member of Group E protein (No. 8) was present in unfed ticks and those attached for 48 hr or less and disappeared as tick feeding progressed. Group F proteins (Nos. 4, 5, 6, 7, 23, 24, 25, 27, 31) were present in unfed ticks and were seen to diminish but not completely disappear as tick feeding progressed. One protein (No. 29), and the only member of Group G, was present in unfed ticks, increased in quantity during early phases of feeding but completely disappeared during the later stages of feeding. Another protein and the only member of Group H (No. 26) was present in the glands of unfed ticks, decreased in glands of ticks in early phases of feeding, and increased during later phases of feeding.

Proteins in glands obtained from unmated females are depicted in Figure 3. Of the thirteen proteins secreted or changed in electrophoretic mobility in mated females (Groups D, E, and F), all but two (Nos. 11 and 20) were still present in the glands of unmated

Figure 2. Representative SDS-polyacrylamide gel electrophoretic pattern showing differences in salivary gland proteins from ticks of varying weights (stage of feeding): (a) unfed female, (b) female attached to the host < 48 hr, (c) 100-mg female, and (d) 500-mg female. Thirty µg of salivary gland protein provided the protein for each panel. The slab gel was stained with Coomassie blue. Thirty-one major protein bands were seen to change during the feeding cycle.

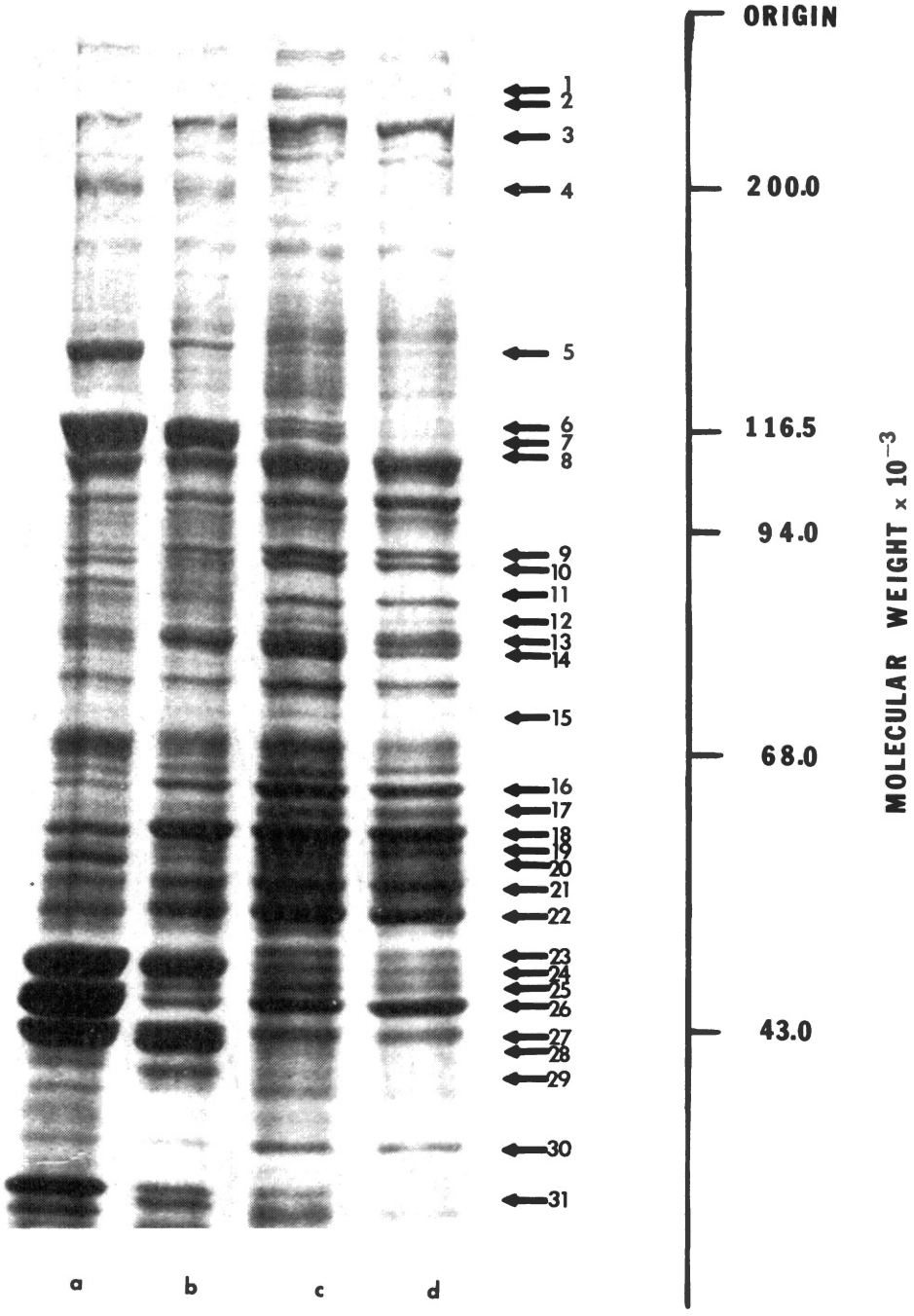

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT x $10^{-3}$
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
a
b
c
d

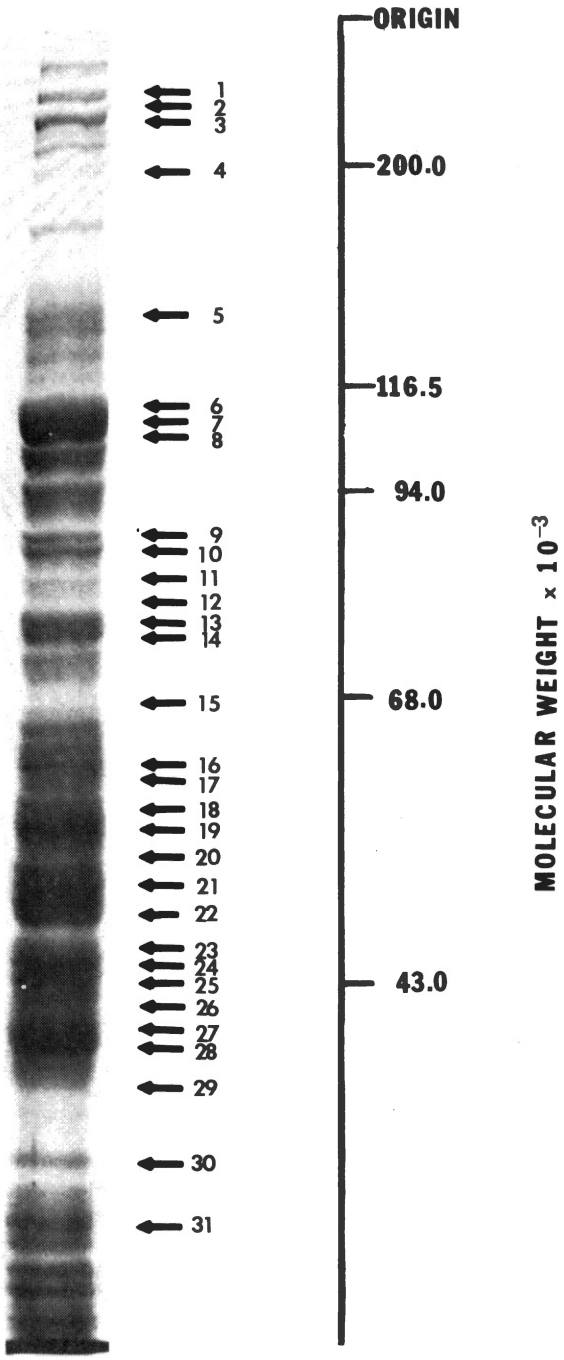
Table 1. Major protein changes in salivary glands of Amblyomma americanum (L.) females during attachment and feeding.†

| Protein | Est. M.W. | Group* | Degree of Coomassie blue staining per extent of feeding as determined from time postattachment or weight | | | |
|---|---|---|---|---|---|---|
| | | | Prefed | 48 hr (10 mg) | 100 mg | 500 mg |
| 1 | 218,000 | B | - | - | ++ | ++ |
| 2 | 216,000 | B | - | - | ++ | ++ |
| 3 | 205,000 | B | - | - | ++ | ++ |
| 4 | 180,000 | F | ++ | ++ | + | + |
| 5 | 135,000 | F | ++ | ++ | + | + |
| 6 | 117,000 | F | ++ | ++ | + | + |
| 7 | 116,000 | F | ++ | ++ | + | + |
| 8 | 114,000 | E | ++ | ++ | + | - |
| 9 | 94,000 | C | + | + | ++ | ++ |
| 10 | 93,000 | C | + | + | ++ | ++ |
| 11 | 88,000 | D | ++ | + | - | - |
| 12 | 84,000 | A | - | + | ++ | ++ |
| 13 | 80,000 | A | - | + | ++ | ++ |
| 14 | 79,000 | A | - | + | ++ | ++ |
| 15 | 72,000 | B | - | - | ++ | ++ |
| 16 | 61,000 | C | + | ++ | ++ | ++ |
| 17 | 60,000 | B | - | - | ++ | ++ |
| 18 | 58,000 | C | + | ++ | ++ | ++ |
| 19 | 55,000 | A | - | + | + | + |
| 20 | 54,000 | D | ++ | + | - | - |
| 21 | 52,000 | C | + | + | ++ | ++ |
| 22 | 48,000 | C | + | + | ++ | ++ |
| 23 | 46,000 | F | ++ | ++ | + | + |
| 24 | 45,000 | F | ++ | ++ | + | + |
| 25 | 43,000 | F | ++ | + | + | + |
| 26 | 41,000 | H | ++ | + | ++ | ++ |
| 27 | 39,000 | F | ++ | ++ | + | + |
| 28 | 38,000 | D | ++ | ++ | - | - |
| 29 | 37,000 | G | + | ++ | - | - |
| 30 | 32,000 | C | + | + | ++ | ++ |
| 31 | 30,000 | F | ++ | + | + | + |

*See text for definitions of groups.

† - = protein not visible in Coomassie blue-stained gels. + or ++ = relative amounts of protein visible in Coomassie blue-stained gels.

Figure 3. SDS-polyacrylamide gel electrophoresis of salivary gland protein from unmated females attached to sheep for 12 to 14 days. The gel was stained with Coomassie blue. The thirty-one major proteins identified in Figure 2 are indicated here for purposes of comparison.


ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT x 10⁻³
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

females. Also, all but one (No. 15) of Groups A, B, and C (growth and development proteins) were present in the glands of unmated femles as was protein No. 29, the single member of Group G, and No. 26, the single member of Group H.

Hemolymph from ticks of various weights was electrophoresed on SDS gels and the proteins were compared to proteins in the salivary glands. The protein bands observed in the hemolymph were unlike those seen in the salivary glands, indicating that hemolymph contamination did not account for the changes observed in salivary gland tissue.

## DISCUSSION

Binnington (1978) and Megaw and Beadle (1979) described sequential changes in salivary gland structure during attachment and feeding of female Boophilus microplus. The glands consist of three types of multicellular alveoli, two of which contain extensive granular material and the third agranular. The same alveolar types have been identified in the salivary glands of female A. americanum (Krolak et al., 1982). The agranular alveoli (Type I) do not change during feeding except that glycogen-like material (Megaw and Beadle, 1979) in pyramidal cells of unfed ticks decreases as feeding progresses. Binnington (1978) and Megaw and Beadle (1979) agree that certain cells in Type II and III alveoli lose their products during early phases of feeding while other cells enlarge and appear to synthesize and secrete their products throughout feeding. Furthermore, Type II and III alveoli enlarge greatly following attachment of the tick to a host. Epithelial cells located between granular cells (Binnington, 1978; Megaw and Beadle, 1979) and called "water cells" by Meredith and Kaufman (1973), are

thought to enlarge greatly. Based upon these anatomical observations, it is likely that the major changes in protein observed occur in alveolar types II and III.

Of the major protein changes seen, Groups A, B, and C appear to be synthesized as a result of tick attachment and feeding. Groups A and C proteins were synthesized soon after attachment and Group B somewhat later. It appears that most or all of the Group A, B, and C proteins are related to growth and development of alveolar types II and III because of continued presence in glands of ticks near repletion. It seems possible that many of these are associated with "water cells" believed to be involved in the process of fluid secretion. Mating itself did not stimulate the synthesis of many new species of protein because all but one of the above were present in the salivary glands of unmated females; however, mating and increased feeding caused total gland protein to increase greatly.

Groups D through F proteins present in the glands of unfed ticks were absent or diminished in glands during late stages of feeding and, therefore, may be secreted or converted to other substances during the feeding process. Only two of these were absent from the glands of unmated females.

The combined results suggest that attachment and limited feeding were the primary stimuli for the synthesis of major new kinds of salivary gland protein and that mating or increased feeding was the primary stimulus for protein secretion. Only a few major proteins were secreted before mating and it may be that these are associated with secretion of cement material. Unmated females are firmly attached to the host and Moorhouse and Tatchell (1966) found that the cement

secretion of B. microplus consists of a cortex of carbohydrate and protein and an internum of lipoprotein.

The results do not draw attention to more subtle changes that may be taking place in the glands during feeding. Less conspicuous proteins, other than those emphasized here, may also change and be of physiological significance to the tick. We have considered only the most prominent proteins in the Coomassie blue-stained electrophoretograms.

Other important but unanswered questions center on possible involvement of endocrine or other factors controlling changes in glandular protein. Ixodid female salivary glands, because of the numerous changes that occur during attachment and feeding, may be an excellent system for studying basic questions associated with control of tissue growth, differentiation, and secretion in acarines.

# LITERATURE CITED

Balashov, Y. S. 1972. A translation of: Bloodsucking ticks (Ixodoidea), Vectors of diseases of man and animals. Misc. Publ. Entomol. Soc. Am. 8:161-376.

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

Chinery, W. A. 1973. The nature and origin of the "cement" substance at the site of attachment and feeding of the adult Haemaphysalis spinigera (Ixodidae). J. Med. Entomol. 10:355-362.

Dickinson, R. G., J. E. O'Hagan, M. Schotz, K. C. Binnington, and M. P. Hegarty. 1976. Prostaglandins in the saliva of the cattle tick, Boophilus microplus. Aust. J. Exp. Biol. Med. Sci. 54:475-486.

Geczy, A. F., M. A. Naughton, J. B. Clegg, and R. W. Hewetson. 1971. Esterases and a carbohydrate splitting enzyme in the saliva of the cattle tick, Boophilus microplus. J. Parasitol. 57:437-438.

Guenther, P. E., D. M. Barker, and J. R. Sauer. 1980. Sodium chloride and water balance in feeding Gulf Coast ticks, Amblyomma maculatum (Koch). Ann. Entomol. Soc. Amer. 73:485-488.

Higgs, G. A., J. R. Vane, R. J. Hart, C. Potter, and R. G. Wilson. 1976. Prostaglandins in the saliva of the cattle tick, Boophilus microplus (Canestrini) (Acrina, Ixodidae). Bull. Entomol. Res. 66:665-670.

Hsu, M. H., and J. R. Sauer. 1975. Ion and water balance in the feeding lone star tick. Comp. Biochem. Physiol. 52A:269-276.

Kaufman, W. R., and J. E. Phillips. 1973a. Ion and water balance in the ixodid tick Dermacentor andersoni. I. Routes of ion and water excretion. J. Exp. Biol. 58:523-536.

_____, and _____. 1973b. Ion and water balance in the ixodid tick Dermacentor andersoni. II. Mechanisms and control of salivary secretion. J. Exp. Biol. 58:537-547.

_____, and _____. 1973c. Ion and water balance in the ixodid tick Dermacentor andersoni. III. Influence of monovalent ions and osmotic pressure on salivary secretion. J. Exp. Biol. 58:549-564.

King, J., and U. K. Laemmli. 1971. Polypeptides of the tail fibers of Bacteriophage T4. J. Mol. Biol. 62:465-477.

Krolak, J. M., C. L. Ownby, and J. R. Sauer. 1982. Alveolar structure of the salivary glands of the lone star tick Amblyomma americanum (L.): Unfed females. J. Parasitol. 68:61-62.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Megaw, M. J. W., and D. J. Beadle. 1979. Structure and function of the salivary glands of the tick, Boophilus microplus Canestrini (Acarina: Ixodidae). Int. J. Insect. Morphol. & Embryol. 8:67-83.

Meredith, J., and W. R. Kaufman. 1973. A proposed site of fluid secretion in the salivary gland of the ixodid tick Dermacentor andersoni. Parasitology 67:205-217.

Moorhouse, D. E., and R. J. Tatchell. 1966. The feeding processes of the cattle tick, Boophilus microplus (Canestrini): A study in host-parasite relations. Parasitology 56:623-632.

Needham, G. R., and J. R. Sauer. 1979. Involvement of calcium and cyclic AMP in controlling ixodid tick salivary fluid secretion. J. Parasitol. 65:531-542.

Patrick, C. D., and J. A. Hair. 1975. Laboratory rearing procedures and equipment for multihost (Acarina: Ixodidae). J. Med. Entomol. 12:389-390.

Sauer, J. R. 1977. Acarine salivary glands--physiological relationships. J. Med. Entomol. 14:1-9.

Tatchell, R. J. 1967. Salivary secretion in the cattle tick as a means of water elimination. Nature, London 213:940-941.

_____. 1971. Electrophoretic studies on the proteins of the haemolymph, saliva, and eggs of the cattle tick, Boophilus microplus. Insect Biochem. I:47-55.

Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by sodium-dodecylsulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

# PART II

# CYCLIC AMP MEDIATED PROTEIN PHOSPHORYLATION OF ENDOGENOUS PROTEINS IN THE SALIVARY GLANDS OF THE LONE STAR TICK, *AMBLYOMMA AMERICANUM* (L.).

## I. DOPAMINE AND CYLCIC AMP INDUCED PROTEIN PHOSPHORYLATION IN WHOLE GLANDS

## SUMMARY

Incubating tick salivary glands *in vitro* with dopamine and cyclic AMP increased the amount of phosphate incorporated into at least 12 endogenous proteins of tick salivary glands. Eleven were shown to lose phosphate after stimulation with dopamine was terminated with dopaminergic antagonists, thioridazine and d-butaclamol. Of the 12 proteins which incorporated additional phosphate, these with molecular weights corresponding to 62,000, 47,000, and 45,000 were the most prominent. The 62,000 dalton phosphoprotein was not present or did not take up phosphate in glands of unfed ticks. The present data and earlier studies are compatible with the hypothesis that cyclic AMP stimulated phosphorylation of specific endogenous proteins is involved in controlling the fluid secretory process in glands of feeding ticks.


Key Words: Cyclic AMP, protein phosphorylation, tick salivary glands.

---

## INTRODUCTION

Covalent modification of specific intracellular proteins has been rocognized as an important means for metabolic and physiological control of cellular processes [1-4]. The effects of many neurotransmitters and hormones are mediated by cyclic adenosine 3',5'-monophosphate (cyclic AMP) [5-6]. Intracellular cyclic AMP is increased after a membrane-bound adenylate cyclase is activated by hormones or neurotransmitters [7]. The effects of cyclic AMP are mediated through activation of cyclic AMP-dependent protein kinases [8] which phosphorylate endogenous proteins. Phosphorylated proteins then affect

in some way the biological response of the hormone or neuotransmitter.

The salivary glands are the primary organs of fluid secretion in feeding ixodid ticks [9]. Fluid secretion is controlled by nerves and the neurotransmitter at the neuroeffector junction is likely dopamine [10]. Exogenous cyclic AMP mimics dopamine in stimulating fluid secretion by isolated glands [11] and intracellular levels of cyclic AMP increase in salivary glands following in vitro stimulation with dopamine [12]. A dopamine sensitive adenylate cyclase is present in tick salivary glands [13].

We have examined the effects of dopamine and cyclic AMP on the phosphorylation of endogenous proteins in tick salivary glands. Phosphorylation was measured in whole salivary glands after incubating glands with substances (dopamine, cyclic AMP) known to stimulate glands to secret fluid. Several proteins were found to incorporate more phosphate when whole glands were incubated with dopamine or cAMP. Results are consistent with the hypothesis that changes in the level of phosphate in specific proteins following gland stimulation by dopamine are necessary steps leading to the secretion of fluid by the glands.

## MATERIALS AND METHODS

Materials. [$^{32}$P] orthophosphate (carrier free) was obtained from New England Nuclear, Boston, MA. Dopamine, chlorpromazine, cyclic AMP, and ATP were obtained from Sigma, St. Louis, MO. Thioridazine was a generous gift from Sandoz; d-butaclamol was from Ayerst. Protein standards and electrophoresis reagents were obtained from Bio-Rad, Richmond, CA.

Tissue preparation. Lone star ticks, Amblyomma americanum (L.),

were raised by the methods of Patrick and Hair [14]. Adult female ticks were used in all experiments and were removed from the host (sheep) after feeding for various periods of time. Salivary glands were dissected at 4°C in a medium of modified, oxygenated TC-199 (Difco) at pH 7.0 containing penicillin and streptomycin sulfate and buffered as described by Needham and Sauer [15].

Phosphorylation in whole glands. Intact pairs of salivary glands were collected and one gland of each was used as control. The phosphorylation of endogenous proteins followed modified procedures of Rudolph and Krueger [16]. Each gland was preincubated in small glass tubes for one hour at 37°C in 300 µl buffered, oxygenated TC-199 (pH 7.0) containing 100 µCi/ml $^{32}P_i$. Following preincubation, glands were placed in 300 µl TC-199 containing dopamine or cyclic AMP/theophylline (concentrations were varied; see figure legends). The stimulation phase of the experiments was performed with or without $^{32}P_i$ in the medium. Similar results were obtained with either. Control glands were placed directly into a stop solution after preincubation. In experiments designed to study dephosphorylation of phosphoproteins both experimental and control glands were preincubated with $^{32}P_i$ and stimulated with dopamine. The control gland was kept in this medium while the experimental gland was transferred to a buffer solution containing dopamine and a dopaminergic antagonist, either thioridazine or d-butaclamol [17]. These drugs are poorly soluble in water and thus were initially dissolved in 95% ethanol. The final of concentration ethanol in the reaction mixture was 1%. Control experiments indicated that this amount of ethanol did not affect phosphate incorporation into proteins. Reactions were terminated in all

experiments by placing the salivary glands directly into 200 µl of a solution containing 0.0625 M-Tris HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. The latter served as marker dye during electrophoresis. Glands were immediately homogenized in this solution, then transferred to a boiling $H_2O$ bath for 2 minutes.

Control glands were not used in experiments designed to show phosphorylation of proteins in salivary glands from ticks of various weight (feeding stage). Glands from 20 unfed ticks were required to obtain sufficient protein. Because glands change in size during tick feeding, glands from 10 ticks were required when studying glands from ticks attached to a host for 48 hrs while glands from only one tick in other stages of feeding contained sufficient protein. Glands were pre-incubated for one hour at 37°C in 300 µl buffered, oxygenated TC-199 (pH 7.0) containing 100 µC/ml $^{32}P_i$. Glands were then stimulated with $10^{-5}$M dopamine for five minutes and prepared for electrophoresis.

Gel electrophoresis and autoradiography. An aliquot containing 70-80 µg of protein from homogenized glands was subjected to SDS-polyacrylamide gel electrophoresis for 12-14 hours (or until the bromophenol blue tracking dye reached the bottom of the gel) at 5-10 mA on a vertical slab (17.5 cm by 14 cm by .075 cm) containing a 7.5% acrylamide separating gel and 1.0 cm of a 3.5% acrylamide stacking gel both containing 0.1% SDS. Gels and electrode buffers (pH 8.3) were prepared following the methods of King and Laemmli [18]. The molecular weights of the protein bands on the SDS gels were determined by comparing the mobilities of the proteins with proteins of known molecular weight. Averages from six gels were used to determine the indicated molecular weights. The following proteins were used as

standards: ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (94,000), β-galactosidase (116,500), and myosin (200,000).

Following electrophoresis, the gels were fixed in 25% isopropanol and 10% acetic acid for 30 minutes. Protein bands were stained with a solution of 25% methanol, 10% acetic acid and 0.025% Coomassie blue R-250 for several hours and destained by shaking in 10% acetic acid. After destaining, the gels were placed on several layers of filter paper and dried on a Bio Rad Gel Slab Dryer (model 224). The dried gels were placed on Kadak film NS-2T and placed in an X-ray exposure holder and exposed for 5-10 days. Developed autoradiograms were scanned densitometrically with a Gilford 252 photometer and model 2520 gel scanner.

## RESULTS

Dopamine stimulation of phosphorylation of endogenous proteins. In initial studies, whole tick salivary glands from feeding ticks (> 200 mg) were used (one gland serving as a control for each pair). Figure 1 shows the distribution of $^{32}P$ incorporated into salivary gland proteins in the presence and absence of dopamine. Densitometric analysis of $^{32}P_i$ incorporation showed consistent significant increases in at least 12 protein bands following stimulation of whole glands with $10^{-5}M$ dopamine (Table I, Figure 2). The most significantly phosphorylated proteins had molecular weights of 47,000, 45,000, and 62,000. Proteins, other than the 12 mentioned, showed an increase in phosphate content in response to dopamine but variations in individual experiments was such that their density was not significantly different

from control ($P > 0.05$). In some experiments an overall darkening of an entire column occurred following gland stimulation. Stimulation times of 30 seconds to 15 minutes gave equivalent results (data not shown).

Dephosphorylation of endogenous proteins. According to the criteria of Krebs [19], if a protein is phosphorylated by a cyclic AMP-dependent protein kinase, a phosphoprotein phosphatase must exist to reverse the process. Figures 3 and 4 illustrate the effect on $^{32}P$ incorporation into salivary gland proteins of terminating dopamine stimulation with thioridazine or d-butaclamol, which are known to inhibit dopamine-stimulated adenylate cyclase in tick salivary glands [17]. Even though stimulation times (1 min.) and dopamine concentrations (4 μM) were less than optimum to stimulate maximum fluid secretion, $^{32}P$ incorporation was rapid in control glands. High concentrations of thioridazine (800 μM) were required to inhibit phosphorylation, possibly because of low permeability of antagonist. Eleven of the proteins whose phosphorylation increased in response to dopamine lost a significant fraction of phosphate after thioridazine addition (Table II). d-Butaclamol gave similar results but was effective at lower concentrations (175 μM) than was thioridazine (Table III).

Cyclic AMP stimulation of phosphorylation. Figures 5 and 6 show the effects of cyclic AMP and theophylline on phosphate incorporation. High concentrations were used to assure permeation as was shown necessary in fluid secretion experiments [11]. The same proteins phosphorylated in response to dopamine showed increased $^{32}P$ incorporation in response to cAMP.

Phosphorylation of endogenous proteins from salivary glands of ticks of various developmental stages. Tick salivary glands in females

undergo substantial growth, differentiation, and development during feeding, and sequential accumulation and depletion of materials in specific cells of the multialveolar, multi-cellular tissue are seen via microscopy [20-21]. Major changes occur in proteins present in salivary glands from different developmental stages [22].

Figure 7 illustrates the dopamine stimulated incorporation of $^{32}P_i$ into proteins of salivary glands obtained from ticks of various weights ranging from unfed ticks to ticks weighing 150 mg. Significantly, glands from unfed ticks showed little or no incorporation of $^{32}P_i$ in the 62,000 dalton protein. A protein of 62,000 daltons, which is hard to detect in unfed ticks [22], appears within 24 hours after attachment of the tick to the host and initiation of feeding. An 88,000 and a 110,000 dalton protein also appear in feeding ticks shortly after attachment. Incorporation of $^{32}P_i$ is apparent in proteins of these sizes within hours after attachment and feeding and these proteins continued to be phosphorylated thereafter. $^{32}P_i$ incorporation into the 47,000 and 45,000 dalton proteins was apparent throughout the feeding cycle. Proteins with molecular weights of 55,000 and 29,000 daltons incorporated significantly more $^{32}P_i$ in glands of unfed ticks than in glands from ticks in later stages of development, as did a protein of 82,000 daltons. In glands from ticks weighing more than 150 mg, the pattern of phosphorylation remained unchanged (data not shown).

## DISCUSSION

The present study establishes that at least 12 proteins in tick salivary glands are phosphorylated in response to dopamine and cyclic AMP/theophylline under conditions and concentrations known to stimulate

fluid secretion [12]. Forty-seven thousand and 45,000 dalton proteins incorporated phosphate to a greater degree than any other proteins. These proteins have molecular weights that closely approximate the regulatory subunit of Type I cyclic AMP dependent protein kinase in other animals [23]. We believe that these proteins may be regulatory subunits of Type I cAMP kinase because two proteins that label heavily with the photo affinity analogue (8-azido [$^{32}$P] cAMP [24] co-migrate on SDS-slab gels. It appears that in this system, the Type I regulatory subunit may be autophosphorylated or that it serves as a substrate for a different phosphotransferase reaction such as cGMP-dependent protein kinase [25]. The regulatory subunit of the Type I protein kinase in S49 mouse lymphoma cells has been shown to be phosphorylated in intact cells [26]. In many systems, the regulatory subunit of Type II protein kinase (55,000 MW) undergoes autophosphorylation [27]. A 55,000 dalton protein was phosphorylated in response to dopamine in this tissue, but it was not a major protein (Table I). A 55,000 dalton protein is more heavily labelled in unfed ticks than in feeding ticks.

The physiological response of cells to neurotransmitters can be attenuated by specific receptor antagonists [17]. If the neurotransmitter receptor is linked to an adenylate cyclase, enzyme activity will be reduced and cellular cyclic AMP and cyclic AMP-dependent protein kinase activity reduced by the antagonist. Under these conditions dephosphorylation of proteins phosphorylated by cyclic AMP-dependent protein kinases should be favored. Several dopamine receptor antagonists including thioridazine and d-butaclamol have been shown to prevent stimulation of tick salivary gland adenylate cyclase [17]. When these drugs were added to whole glands following dopamine

stimulation, a significant dephosphorylation of proteins whose phosphorylation was stimulated by dopamine was observed. The necessity of having to use high concentrations of thioridazine to inhibit phosphorylation may indicate its low permeability in tissue. It is probable that receptor sites are deeply embedded in the multicellular tissue [20]. Blockage of stimulation leads to dephosphorylation of 11 of these proteins phosphorylated after stimulating glands with dopamine. We believe that these results support the hypothesis that phosphorylation of certain endogenous proteins in response to gland stimulation by dopamine is an integral part of the fluid secretory process in tick salivary glands. Future research should be directed to showing where in the glands these phosphoproteins are located and how they help mediate fluid secretion.

When comparing phosphoproteins in unfed ticks to those of feeding ticks, it is interesting to note that proteins having molecular weights of 110,000, 88,000, 62,000 daltons (most notabaly the 62,000 dalton protein) were absent in glands of the unfed ticks. Proteins with molecular weights corresponding to 47,000, and 45,000 daltons were present throughout the feeding cycle and proteins with molecular weights of 55,000, 29,000 and 82,000 daltons incorporated significantly more phosphate in glands of unfed ticks. The significance of these differences is unclear.

Freedman and Jamison [28] recently reported a marked phosphorylation of several endogenous proteins with apparent molecular weights of 62,000, 55,000, 52,000, 49,000, 34,000 and 29,000 in rat pancreas and parotid cells in response to various secretagogues. The 29,000 dalton protein is the S6 ribosomal protein in this tissue.

Although the present study focused on cyclic AMP mediated protein phosphorylation as it relates to fluid secretion it is possible that phosphoproteins are involved in other functional events in the glands. The glands are multicellular and multifunctional [9] and undergo remarkable tissue differentiation during tick attachment and feeding on the host [20, 21]. Thus it is interesting that similar proteins as the above (62,000, 55,000, 29,000) had levels of phosphate increased during gland stimulation by dopamine. It is quite evident that much more work is required to determine the functional significance of the various phosphoproteins in tick salivary glands.

# LITERATURE CITED


1. Krebs, E. G. and Beavo, J. A. (1979) Ann. Rev. Biochem., 48, 923-959.
2. Rudolph, S. A. and Greengard, P. (1974) J. of Biol. Chem. 249, 5684-5687.
3. Baum, B. J., Frieberg, J. M., Ito, H., Roth, G. S. and Filburn, C. R. (1981) J. Biol. Chem. 256, 9731-9736.
4. Whittemore, S. R., Lenox, R. H., Hendley, E. D. and Ehrlich, Y. H. (1981) Neurochemical Research. 6, 775-785.
5. Sutherland, E. W. and Rall, T. W. (1958) J. Biol. Chem. 332, 1077-1091.
6. Bloom, F. E. (1975) Rev. Physiol. Biochem. Pharmacology. 74, 1-103.
7. Rodbell, J. (1980) Nature 284, 17-22.
8. Ruben, C. S. and Rosen, O. M. (1975) Ann. Rev. Biochem. 44, 831-887.
9. Sauer, J. R. (1977) J. Med. Entomology. 14, 1-9.
10. Kaufman, W. R. and Phillips, J. E. (1973b) J. Exp.Biol. 58, 537-547.
11. Needham, G. R. and Sauer, J. R. (1975) J. Insect Physiol. 21, 1893-1898.
12. Sauer, J. R., Mincolla, P. M. and Needham, G. R. (1976) Comp. Biochem. Physiol. 53C, 63-66.
13. Schmidt, S. P., Essenberg, R. C. and Sauer, J. R. (1982) Comp. Biochem. Physiol. 72C 9-14.
14. Patrick, C. D. and Hair, J. A. (1975) J. Med. Entomol. 12, 389-390.

15. Needham, G. R. and Sauer, J. R. (1979) J. Parasitol. 65, 531-542.

16. Rudolph, S. A. and Krueger, B. K. (1979) Adv. Cyclic Necleotide Research 10, 107-133.

17. Schmidt, S. P., Essenberg, R. C. and Sauer, J. R. (1981) Jour. Cyclic Necleotide Research. 7(6):375-384.

18. King, J. and Laemmli, U. K. (1971) J. Mol. Biol. 62, 465-477.

19. Krebs, E. G. (1973) Proc. IV. Int. Congr. Endocrinol. 17-29. Ameraterdam:Excerpta Med.

20. Binnington, K. C. (1978) Int. J. Parasitol. 8, 97-115.

21. Megaw, M. J. W. and Beadle, D. J. (1979) Int. J. Insect Morphol. and Embryol. 8, 67-83.

22. McSwain, J. L., Essenberg, R. C. and Sauer, J. R. (1982) J. Parasitol. 86(1), 100-106.

23. Hofmann, F., Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1975) J. Biol. Chem. 250, 7795-7801.

24. McSwain, J. L., Essenberg, R. C., and Sauer, J. R. (1983) Biophys. Act.

25. Geahlen, R. L. and Krebs, E. G. (1980) J. Biol. Chem. 255, 1164-1169.

26. Steinberg, R. A., O'Ferrell, P. H., Friedrich, U. and Coffino, P. (1977) Cell 101, 381-391.

27. Erlichman, J., Rosenfeld, R. and Rosen, O. M. (1974) J. Biol Chem. 248, 5000-5003.

28. Freedman, S. D. and Jamieson, J. D. (1982) J. Cell. Biol. 95, 903-908.

TABLE I

RELATIVE INCORPORATION OF $^{32}P$ INTO INDIVIDUAL PROTEIN BANDS

| Mr | Peak Height in Cm | | Mean % Change |
|---|---|---|---|
| | Control (n=15) | Dopamine (n=15) | |
| 201000 | 0.85±0.15 | 1.24±0.20 | + 13 |
| 153000 | 4.12±0.53 | 5.07±0.72 | + 15 |
| 148000 | 3.24±0.49 | 4.26±0.65 | + 23 |
| 128000 | 1.91±0.25 | 2.50±0.34 | + 23 |
| 102000 | 3.17±0.60 | 3.75±0.63 | + 17 |
| 97000 | 1.82±0.30 | 2.18±0.41 | + 11 |
| 62000 | 2.80±0.40 | 3.74±0.54 | + 24 |
| 58000 | 1.53±0.26 | 2.01±0.28 | + 30 |
| 55000 | 1.20±0.28 | 1.85±0.33 | + 27 |
| 47000 | 16.14±3.44 | 24.54±3.59 | + 41 |
| 45000 | 17.36±3.59 | 26.04±3.80 | + 40 |
| 37000 | 2.02±0.34 | 2.27±0.28 | + 16 |

Relative incorporation of $^{32}P$ into individual protein bands of salivary glands of Amblyomma americanum (L.). Peak heights of densitometric scans of 15 experiments were used to determine the relative amount of $^{32}P$ incorporation into each protein (mean ± s.e.m.). A paired sample student's t-test was used to determine significant differences. Phosphate increase was significant at the .05 level for the proteins listed.

TABLE II

RELATIVE INCORPORATION OF $^{32}P$ INTO INDIVIDUAL PROTEIN BANDS FOLLOWING INCUBATION IN THIORIDAZINE

| Mr | Peak Height in Cm | | Mean % Change |
|---|---|---|---|
| | Control (n=2) | Thioridazine (n=2) | |
| 201000 | 1.15 | 1.10 | + 5 |
| 153000 | 5.60 | 3.60 | - 35 |
| 148000 | 5.35 | 3.20 | - 41 |
| 128000 | 2.50 | 2.20 | - 17 |
| 102000 | 3.40 | 2.60 | - 24 |
| 97000 | 2.00 | 1.30 | - 29 |
| 62000 | 4.15 | 0.85 | - 79 |
| 58000 | 0.45 | 0.30 | - 35 |
| 55000 | 0.50 | 0.30 | - 40 |
| 47000 | 12.80 | 8.45 | - 34 |
| 45000 | 12.80 | 8.45 | - 34 |
| 37000 | 3.95 | 2.55 | - 38 |

Relative incorporation of $^{32}P$ into individual protein bands of whole salivary glands after blockage of dopamine stimulation. Peak heights of densitometric scans were used to determine the relative amount of $^{32}P$ incorporation into each protein. Numbers in table represent averages of two experiments.

TABLE III

RELATIVE INCORPORATION OF $^{32}P$ INTO INDIVIDUAL PROTEIN BANDS FOLLOWING INCUBATION IN d-BUTACLAMOL

| Mr | Peak Height in Cm | | Mean % Change |
|---|---|---|---|
| | Control | d-butaclamol | |
| 201000 | - | - | - |
| 153000 | 1.9 | 1.4 | - 26 |
| 148000 | 1.3 | 0.7 | - 46 |
| 128000 | 0.7 | 0.4 | - 42 |
| 102000 | 1.9 | 0.4 | - 80 |
| 97000 | 0.5 | 0.2 | - 60 |
| 62000 | 1.8 | 0.3 | - 83 |
| 58000 | 0.8 | 0.3 | - 58 |
| 55000 | 0.4 | 0.3 | - 25 |
| 47000 | 14.6 | 6.6 | - 54 |
| 45000 | 14.6 | 6.6 | - 54 |
| 37000 | 0.6 | 0.3 | - 50 |

Relative incorporation of $^{32}P$ into individual protein bands of salivary glands of Amblyomma americanum (L.). After blockage of dopamine stimulation with d-butaclamol peak height measurements of densitometric scans were used to determine the relative amount of $^{32}P$ incorporation into each protein.

Figure 1. Phosphorylation of proteins of whole salivary glands in response to dopamine. Phosphate incorporation was studied following incubation of whole glands with (+) or without (-) 10 μM dopamine for 1 min. Proteins whose states of phosphorylation were affected by dopamine are indicated by arrows.

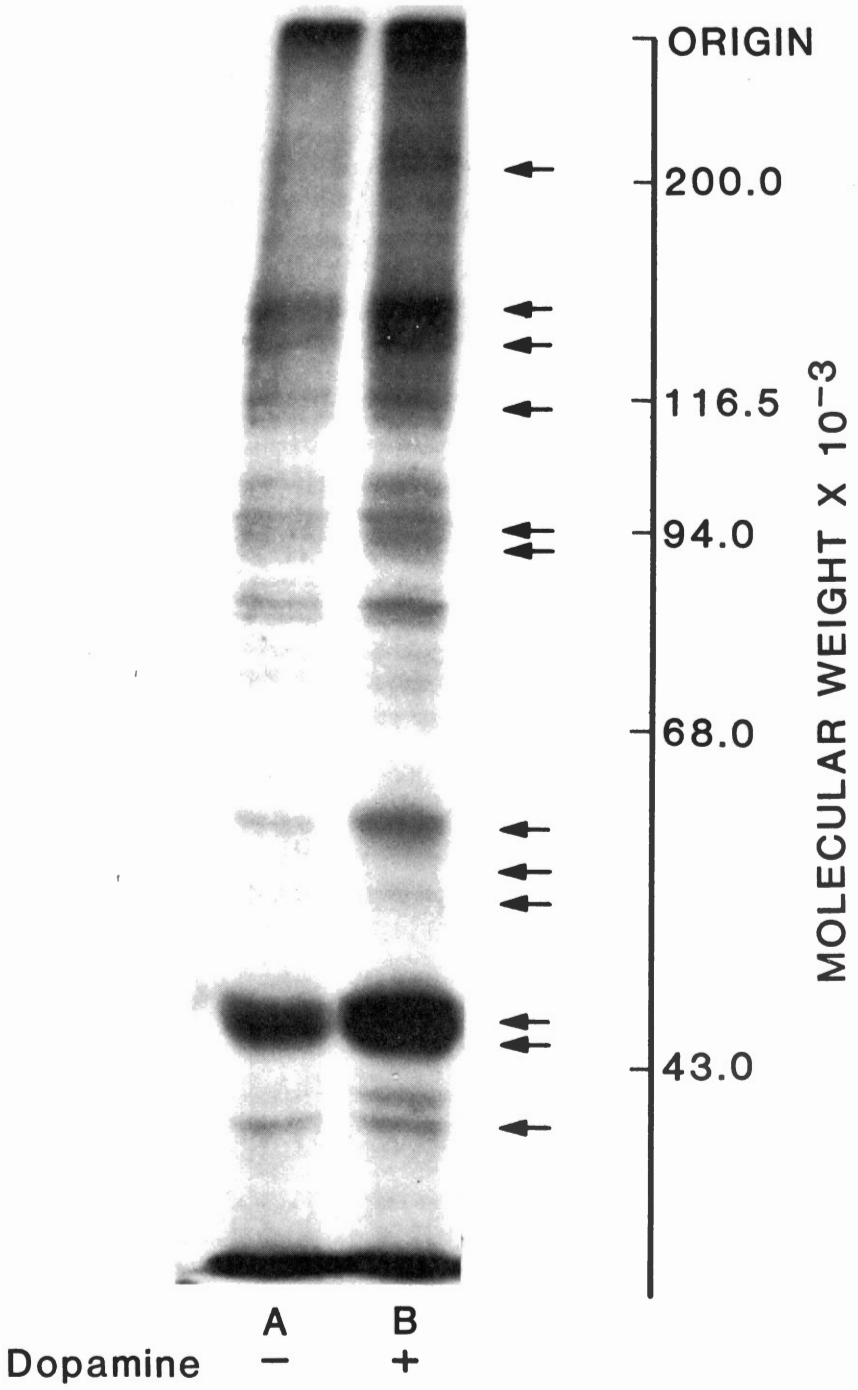

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
Dopamine
–
+

Figure 2. Densitometric scan of the autoradiograph of Figure 1 showing $^{32}P$ incorporation in paired glands _____, dopamine; _ _ _, control. Although other proteins than those labeled appear to incorporate increased $^{32}P$ with dopamine stimulation, they were inconsistent and the increase was not significant over 15 experiments.

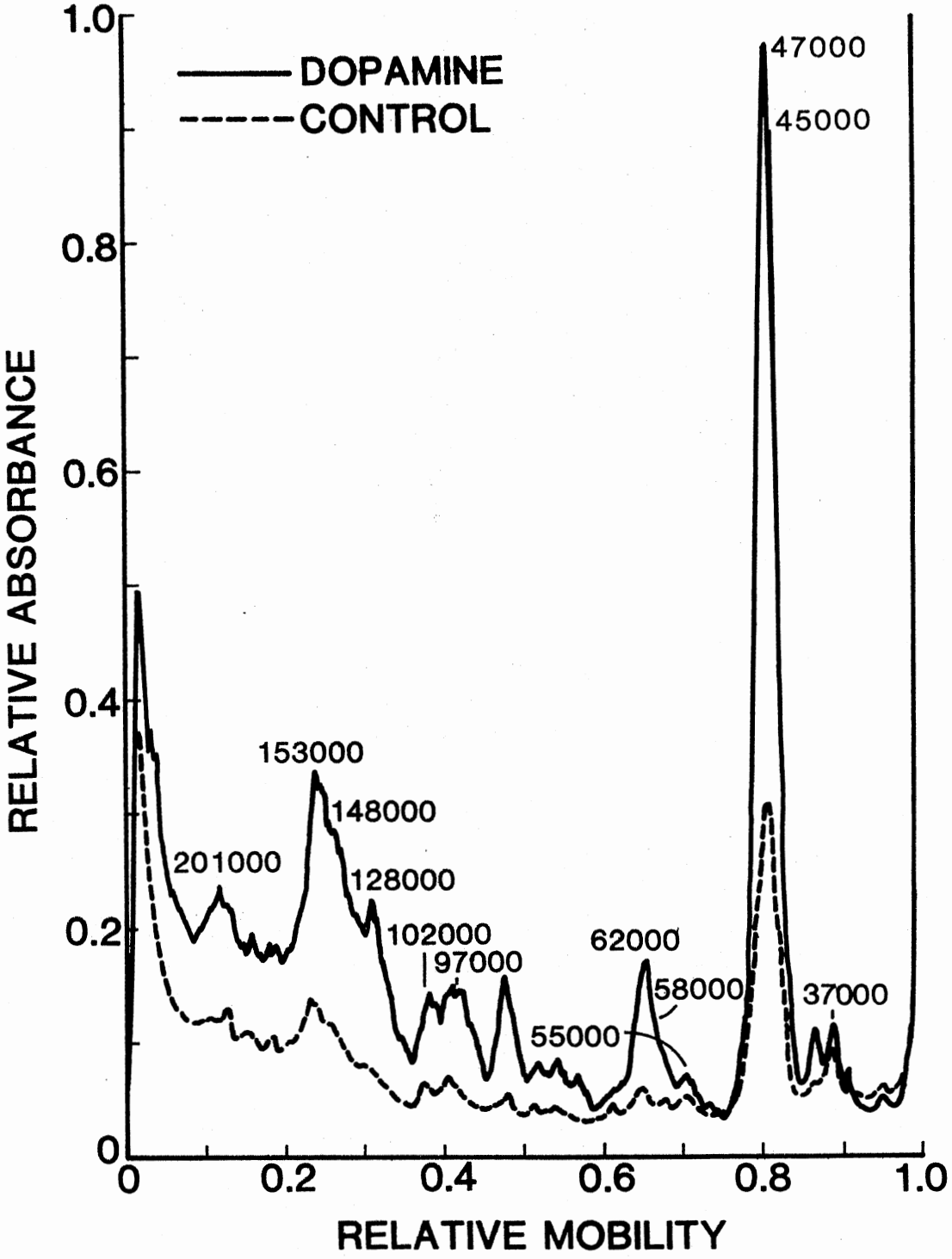

DOPAMINE
CONTROL
RELATIVE ABSORBANCE
RELATIVE MOBILITY
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
47000
45000
153000
148000
201000
128000
102000
97000
62000
58000
55000
37000

Figure 3. Dephosphorylation of proteins of whole salivary glands in response to thioridazine. Phosphate incorporation was measured following incubation of whole glands with 2 μm dopamine for 1 min and then with (+) or without (-) 800 μm thioridazine for 20 min. Control glands had an amount of ethanol added equivalent to that of thioridazine treated. Proteins whose states of phosphorylation was significantly affected by these treatments are indicated by arrows.

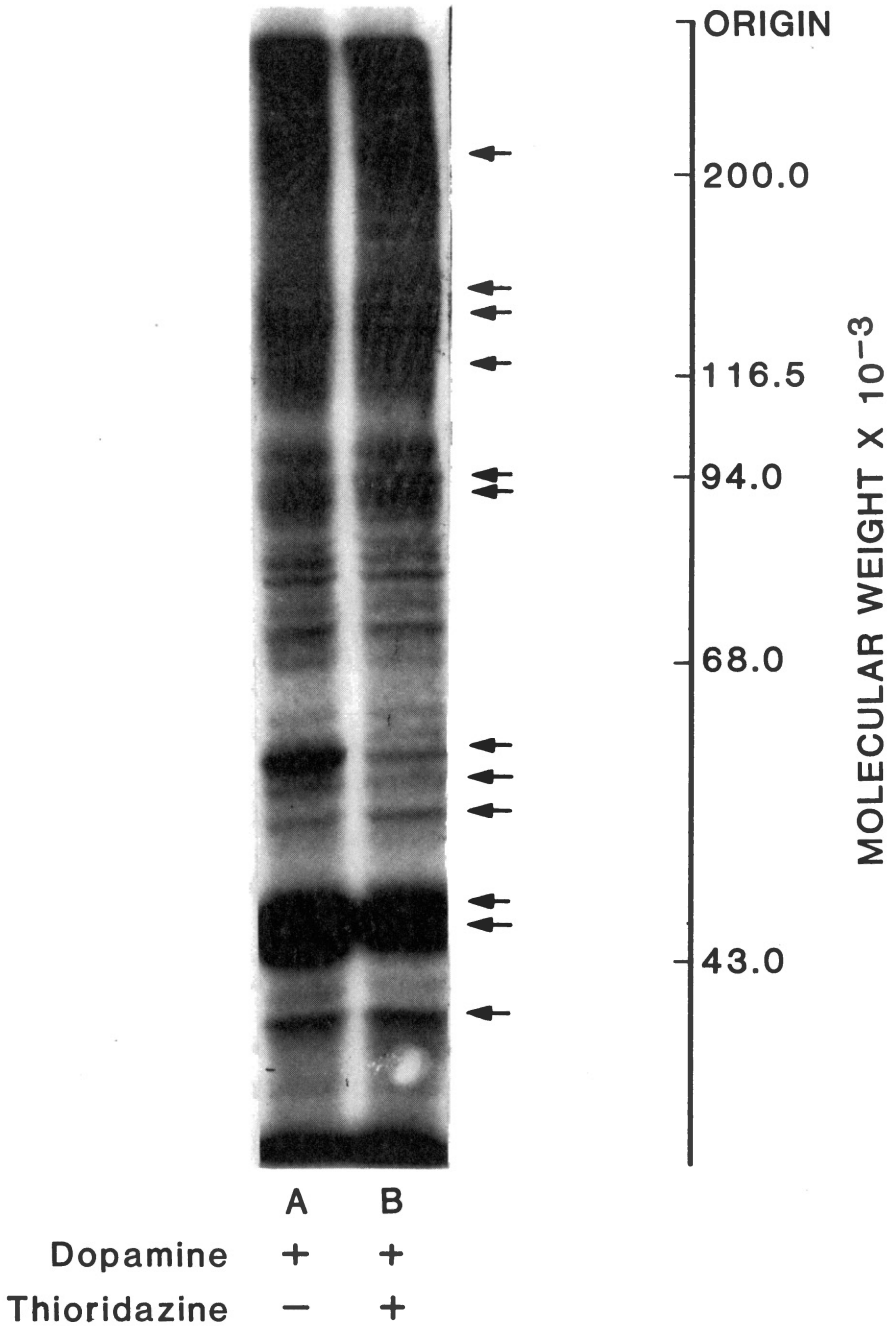

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
Dopamine + +
Thioridazine − +

Figure 4. Dephosphorylation of proteins of whole salivary glands in response to d-butaclamol. Phosphate incorporation was studied following incubation of whole glands with 2 μm dopamine for 1 min and then with (+) or without (-) 175 μm d-butaclamol for 20 min. Proteins whose state of phosphorylation was affected by these treatments are indicated by arrows.

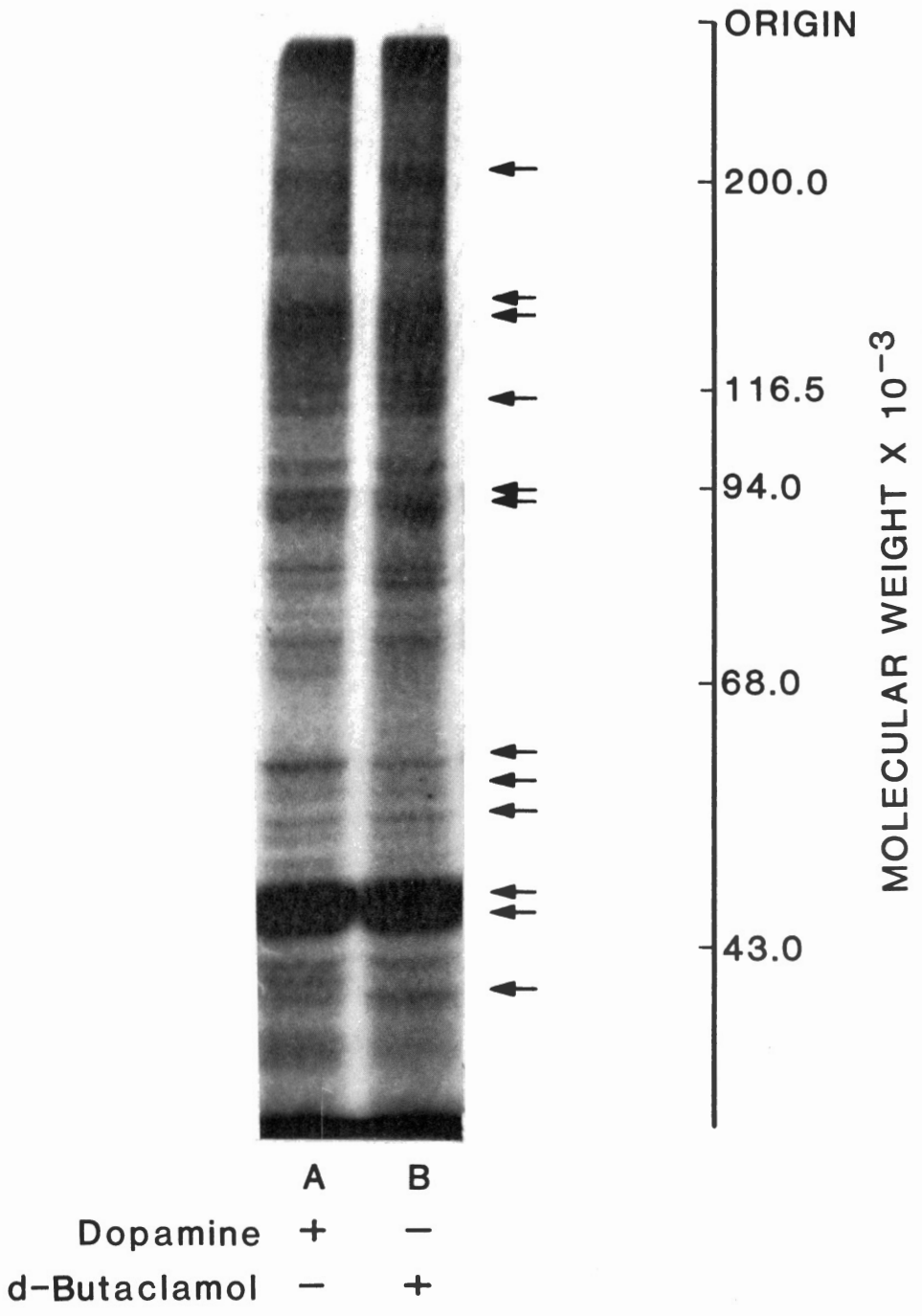

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
Dopamine + −
d-Butaclamol − +

Figure 5. Phosphorylation of proteins of whole salivary gland in response to cAMP. Incorporation of phosphate was measured following incubation with (+) and without (-) $10^{-2}$ M cyclic AMP and $10^{-2}$ M theophylline for 5 min. Proteins whose state of phosphorylation increased in response to cyclic AMP and theophylline are indicated by arrows.

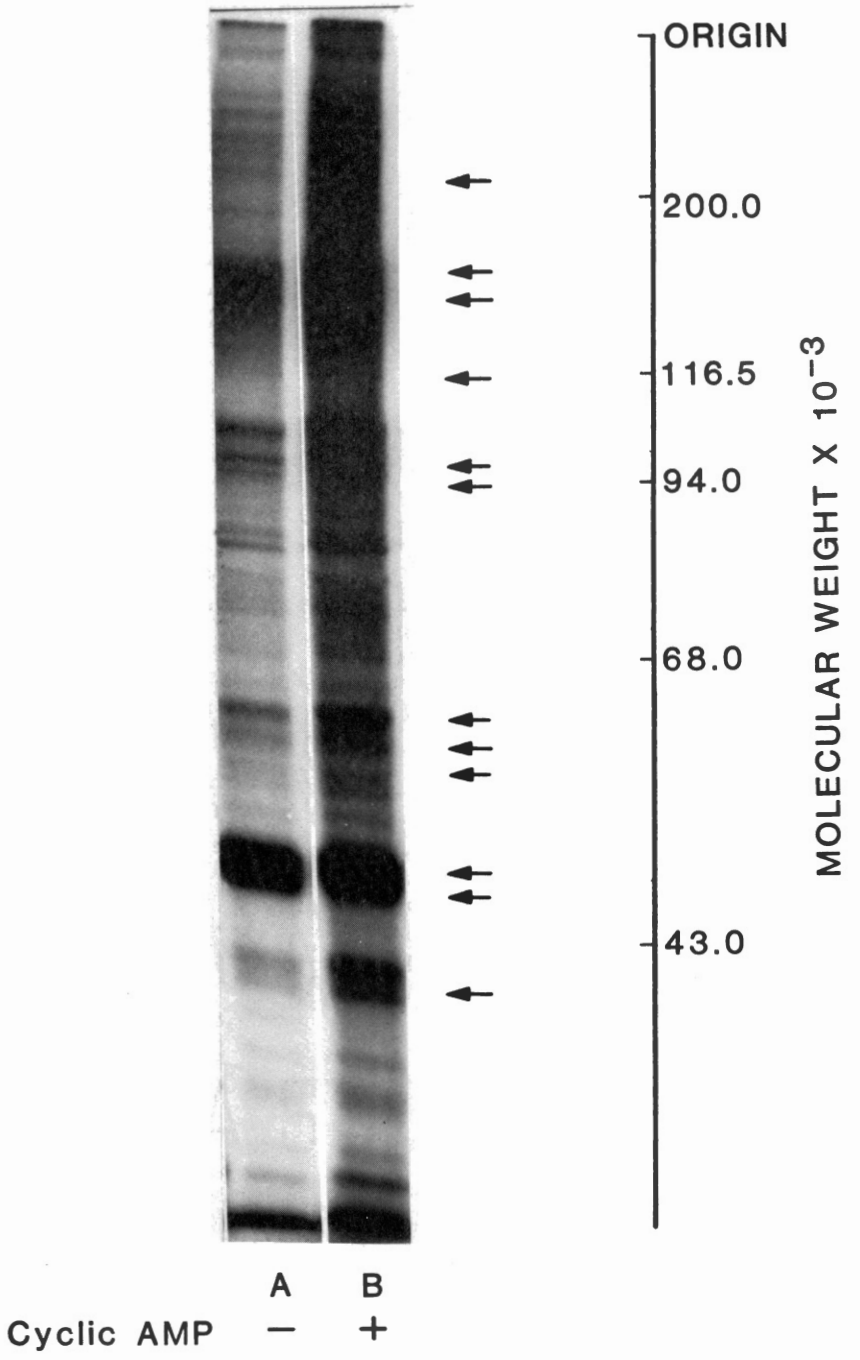

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
Cyclic AMP
—
+

Figure 6. Densitometric scan of the autoradiograph of Figure 5. Proteins whose states of phosphorylation increased in response to cyclic AMP and theophylline are labeled.

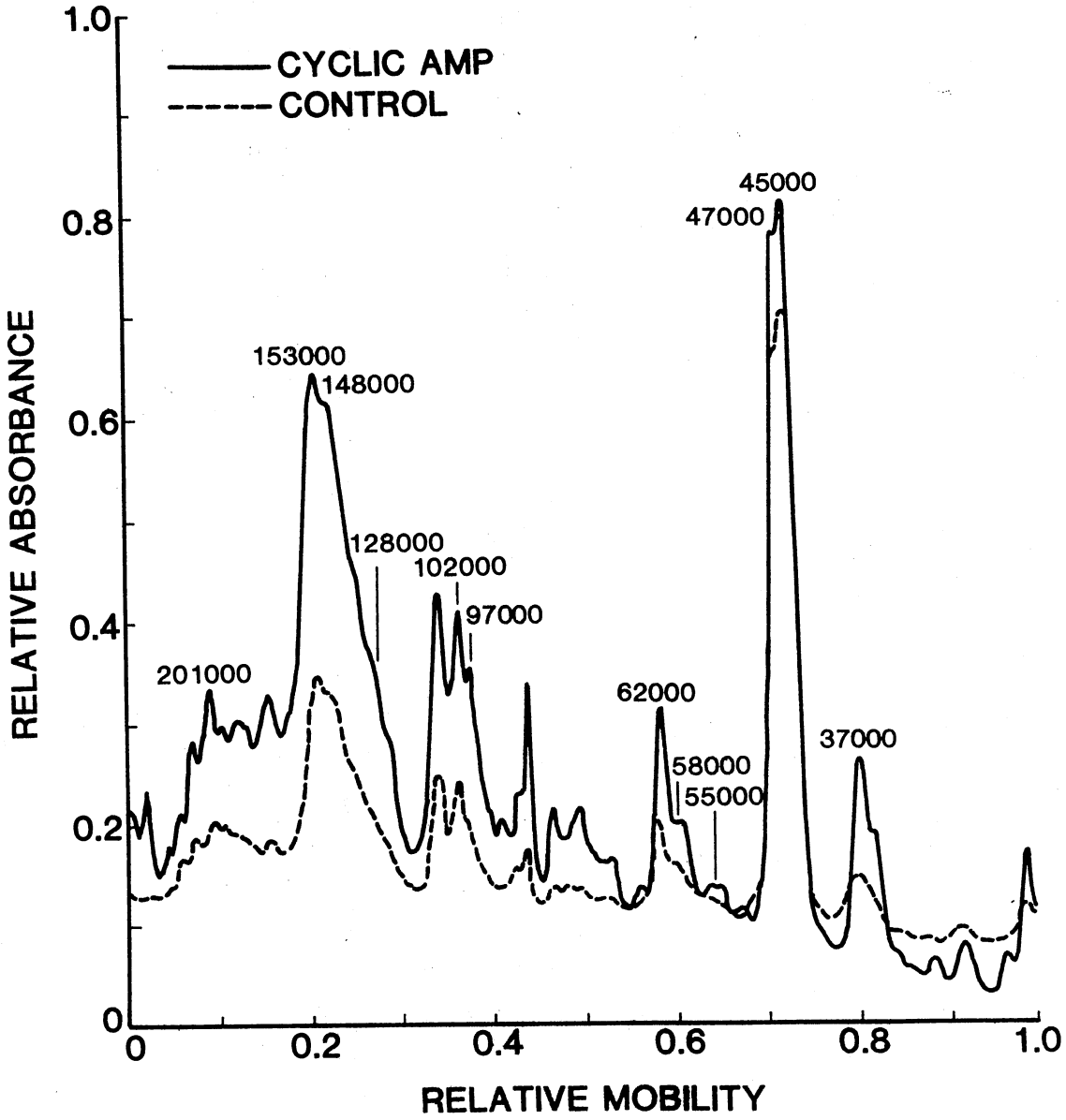
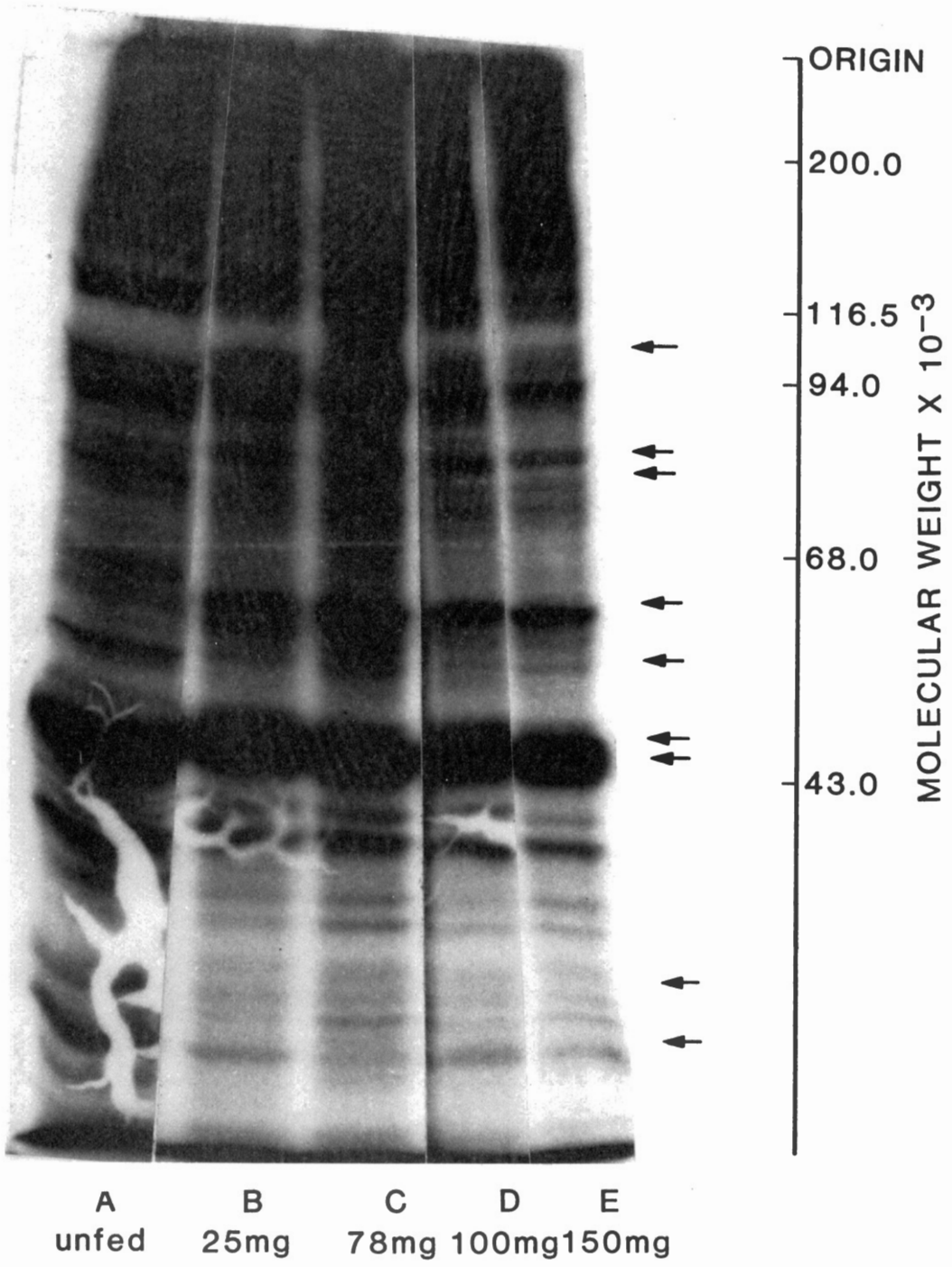

CYCLIC AMP
CONTROL
RELATIVE ABSORBANCE
RELATIVE MOBILITY
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
45000
47000
153000
148000
128000
102000
97000
201000
62000
58000
55000
37000

Figure 7. Phosphorylation of proteins of whole salivary glands from ticks in different stages of feeding. Phosphate incorporation was measured following incubation of whole glands in the presence of 10 μm dopamine for 5 min.


ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
unfed
B
25mg
C
78mg
D
100mg
E
150mg

# PART III

# CYCLIC AMP MEDIATED PROTEIN PHOSPHORYLATION OF ENDOGENOUS PROTEINS IN SALIVARY GLANDS OF THE LONE STAR TICK, *AMBLYOMMA AMERICANUM* (L.).

## II. CYCLIC AMP INDUCED PHOSPHORYLATION IN BROKEN CELLS

## SUMMARY

Cyclic AMP stimulated the phosphorylation of at least 14 endogenous proteins in broken cell preparations of tick salivary glands. Of these, nine were proteins with molecular weights similar to those phosphorylated in response to dopamine and cyclic AMP/theophylline in whole gland experiments. The most prominent phosphoproteins, as in whole gland experiments, had molecular weights of 62,000, 47,000 and 45,000. Endogenous phosphatase activity was demonstrated when the endogenous phosphoproteins were incubated with unlabled ATP or protein kinase inhibitor. Proteins, likely subtypes of the Type I cAMP protein kinase and having molecular weight of 47,000 and 45,000, bound the photoaffinity analogue of cyclic AMP, 8-$N_3$-[$^{32}$P]cAMP, and co-migrated with phosphoproteins of the same molecular weights. A protein with a molecular weight of 55,000 and likely the receptor subunit of Type II cAMP protein kinase also bound the photoaffinity analogue of cAMP.


Key Words: Cyclic AMP, protein kinase, protein phosphorylation, tick salivary glands.

---

## INTRODUCTION

Cyclic AMP functions as a "second messenger" for many homones and neurostransmitters. The best known mode of action of cyclic AMP in eukaryotes is to activate protein kinases that catalyze the phosphorylation of endogenous proteins. Phosphorylated proteins then effect, in some way, the biological response of cells to the hormone or neurotransmitter [1]. Cyclic AMP dependent protein kinases of most animals are of two types (I and II). The enzymes differ in size of

cyclic AMP binding regulatory subunit [2] and ability, in some animals, to phosphorylate their own regulatory subunit [3]. Regulatory subunits of protein kinases have been located on autoradiograms of electrophoretgrams with 8-azidoadenosine 3':5'-monophosphate ($8\text{-}N_3\text{-}[^{32}P]$cAMP), a photoaffinity analog of cAMP that binds to cAMP-binding sites [4].

In the accompanying study [5] we demonstrated that substances (dopamine and cyclic AMP) which stimulate fluid secretion by intact tick salivary glands causes the phosphorylation of several endogenous proteins when added exogenously to whole glands. In this study we demonstrate that the same proteins are phosphorylated in response to cAMP in broken cell preparations of similar glands. We also demonstrate the existence of cAMP binding proteins with molecular weights similar to regulatory subunits of cAMP-dependent protein kinases found in other vertebrate and invertebrate tissue.

## MATERIALS AND METHODS

$[\gamma\text{-}^{32}P]$ ATP was obtained from New England Nuclear, Boston, Massachusetts; ($8\text{-}N_3\text{-}[^{32}P]$cAMP) from ICN, Irvine, California. Cyclic AMP and protein kinase inhibitor were obtained from Sigma, St. Louis, Missouri. Protein standards and electrophoresis reagents were obtained from BioRad, Richmond, California.

Tissue preparation. Male and female lone star ticks Amblyomma americanum (L.) were raised by the methods of Patrick and Hair [6]. Salivary glands were dissected from adult female ticks weighing 200 mg or more in a medium of modified, oxygenated TC-199 (Difco) at pH 7.0 and 4° C containing penicillin and streptomycin sulfate and buffered as

described by Needham and Sauer [7].

Endogenous protein phosphorylation. Six pairs of salivary glands were homogenized in 1 ml of 50 mM Hepes[1] buffer (pH 7.0). The phosphorylation reactions followed the procedures described by Rudolph and Krueger [8]. One hundred µl of tissue homogenate was added to a disposable glass test tube (on ice) containing 80 µl with 50 mM Hepes[1] buffer (pH 7.0), 10 mM $MgCl_2$ solution and 10 mM cyclic AMP where indicated. The phosphorylation reaction was initiated by adding 20 µl of 4.8 µM [$\gamma$-$^{32}$P] ATP (specific activity of 10-20 Ci/mmole). The tubes were agitated briefly and transferred to a shaking water bath for various periods of time. The reaction was terminated by adding 100 µl of a stop solution containing 0.625 M-Tris HCl (pH 6.8), 2% SDS[2], 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. The latter served as a marker dye during electrophoresis. The tubes were then transferred to a boiling $H_2O$ bath for 2 minutes.

Dephosphorylation of endogenous proteins. After stimulating an increase in phosphorylation of endogenous proteins with cyclic AMP, 20 µl of $10^{-3}$ M ATP or 4 µg protein kinase inhibitor per assay tube was added for varying periods of time. Samples were stopped at various times and prepared for electrophoresis as above.

Determination of cyclic AMP binding proteins with 8-$N_3$-[$^{32}$P]cAMP. Photoaffinity labeling experiments were performed as described by Lohmann et al. [9]. The reaction mixture contained 100 µl of 50 mM Mes[3] (pH 6.2), 10 mM $MgCl_2$, 1 mM isobutylmethylxanthine, 1 mM ATP, 0.5 mM

---

[1]4-(2-hydroxyethyl)-1-Piperazine ethanesulfonic acid

[2]Sodium dodecyl sulfate

[3]2(N-morpholino)-ethanesulfonic acid

2-mercaptoethanol, 0.2 μM 8-$N_3$-[$^{32}$P] cAMP (100 Ci/mmol) and 120 μg of protein in the absence or presence of 20 μM cAMP. The reaction medium was incubated for 60 min in the dark at 4°C and then irradiated for 10 min at 254 nm with a Chromato-Vue® ultraviolet light source at 8 cm. Fifty μl of the SDS stop solution was then added to the samples and they were transferred to a boiling water bath for 2 min. A parallel experiment was performed in the same solution except that labelled ATP was used in the place of the photoaffinity label to observe the phosphorylation of protein. The phosphorylation reaction was initiated by adding 20 μl 4.8 μM [$\gamma$-$^{32}$P] ATP (20-40 Ci/mM). After 5 min, the reaction was terminated by adding 50 μl of the SDS stop solution and described above. The solution was then transferred to a boiling water bath for 2 min.

Gel electrophoresis and autoradiography. An aliquot containing 70-80 μg of protein was subjected to SDS-polyacrylamide slab gel electrophoresis for 12-14 hours (or until the bromophenol blue tracking dye reached the bottom of the gel) (17.5 cm by 14 cm by .075 cm) at 50-10 mA on a vertical slab containing a 7.5% acrylamide separating gel and 1.0 cm of a 3.5% acrylamide stacking gel, both containing 0.1% SDS. Gels and electrode buffers (pH 8.3) were prepared following the method of King and Laemli [10].

## RESULTS

Phosphorylation and dephosphorylation of endogenous proteins. When tissue homogenates were incubated with cyclic AMP for 0.5 or 1 min, a significant increase in incorporation of phosphate was observed in at least 14 proteins (Table I, Figure 1). All 14 may be physiologically

important, but proteins with molecular weights of 145,000, 124,000, 103,000, 93,500, 62,000, 55,000, 47,000, 45,000, and 37,000 closely approximate molecular weights of proteins that incorporated increased $^{32}P$ when whole glands were stimulated exogenously by dopamine or cyclic AMP [5]. Although the molecular weights of the corresponding phosphoproteins appeared to be slightly different in the two experiments (whole gland, broken cell), the relative positions of the proteins to one another on the electrophoretograms were identical [5] (Figure 2) indicating that the proteins are homologous. Differences in methods of preparing tissue for electrophoresis (i.e., kind of buffers) may have caused differences in migration patterns in tissue proteins with respect to standard proteins (or vice versa). Some differences in calculated molecular weights have been observed when using different buffers (Mes, Hepes) in preparing tissue for electrophoresis. For consistency, the molecular weights assigned to phosphoproteins in the previous [5] whole gland experiments will be used to designate homologous proteins in present experiments.

Autoradiographical distribution of phosphoproteins stimulated with cyclic AMP (10 μM) for 5 min and then chased with $10^{-3}$ M unlabeled ATP for 30 min is indicated in Figure 3. Longer incubation times with cAMP were used to insure maximum phosphorylation of proteins. Densitometric measurements of $^{32}P$ incorporation (Table II, Figure 4) showed a decrease in phosphate only in phosphoproteins which exhibited increased phosphate when tissue was stimulated with cyclic AMP. When protein kinase inhibitor (4 μg/200 μl assay volume) was used instead of unlabeled ATP, similar results were obtained (Figure 5). Densitometric measurements (Table III, Figure 6) showed a decrease in phosphate in all band except

124,000, 113,000, 103,000, and 93,500 dalton proteins. In both procedures, a decrease in 145,000, 62,000, 47,000, 45,000, and 37,000 dalton proteins was observed. The degree of phosphorylation of the 55,000 dalton protein was only slightly affected by both procedures.

Cyclic AMP binding proteins. Proteins with molecular weights identical to those of three prominent and two lesser phosphoproteins were shown to bind 8-$N_3$-[$^{32}$P] cyclic AMP (Table IV, Figure 7). These proteins have molecular weights of approximately 55,000, 47,000, 45,000, 33,000 and 29,000. Other proteins incorporated 8-$N_3$-[$^{32}$P] cyclic AMP to a slight extent (Figure 7). A 55,000 dalton protein heavily labeled with the photoaffinity analog of cAMP and it co-migrated with a moderately phosphorylated protein whose level of phosphate was enhanced somewhat by cyclic AMP. This protein's molecular weight is similar to the regulatory subunit of Type II cyclic AMP protein kinase found in cells of most eukaryotes [4]. Forty-seven thousand and 45,000 dalton labelled proteins also co-migrated with heavily phosphorylated proteins whose level of phosphate was substantially stimulated by cyclic AMP. The regulatory subunit of Type I cyclic AMP dependent protein Kinase has a molecular weight of 47,000 in cells of most eukaryotes [4].

## DISCUSSION

These experiments show that cyclic AMP stimulates phosphorylation of proteins in broken cell preparations of tick salivary glands. Previous studies have demonstrated that gland levels of cyclic AMP increase when glands are stimulated with dopamine [11] and that exogenous cyclic AMP, theophylline and dopamine all stimulate fluid secretion by isolated glands [12]. Importantly, the proteins

phosphorylated in response to dopamine during whole gland experiments [5] were also phosphorylated in response to cyclic AMP in broken cell preparations in the present experiments.

When intact gland preparations were pre-incubated with $^{32}P$ and then stimulated by dopamine in a manner that also stimulates fluid secretion, 11 phosphoproteins incorporated additional $^{32}P$. Stimulation by exogenous cyclic AMP and theophylline also increased $^{32}P$ into the same proteins. Phosphate was lost from those proteins when stimulation was terminated by addition of the dopaminergic receptor antagonists, thioridazine and d-butaclamol, suggesting that phosphatases to dephosphorylate proteins are present. Phosphoproteins with molecular weights of 62,000, 55,000, 47,000 45,000 and 37,000 incorporated increased $^{32}P$ in the presence of cyclic AMP in broken cells (present experiments). Phosphate incorporation decreased during a chase with unlabeled ATP and after adding protein kinase inhibitor to the medium. These results further support the hypothesis that phosphatases are present to dephosphorylate proteins phosphorylated in response to cyclic AMP. Forty-seven thousand and 45,000 dalton bands were especially prominent in whole gland and broken cell experiments. A 62,000 dalton protein is also prominent and of interest because it is not present or does not incorporate $^{32}P$ in glands of unfed ticks. The salivary glands are the primary organ of osmoregulation. When the tick attaches to a host and takes a bloodmeal, excess fluid and ions are returned into the host via the salivary glands. The 62,000 dalton phosphoprotein appears within 24 hours after tick attachment and remains throughout the remainder of tick feeding. This protein is phosphorylated in both whole gland and broken cell preparations.

Five proteins bound the photoaffinity analogue of cyclic AMP. Of these, 55,000, 47,000 and 45,000 dalton proteins co-migrated with phosphoproteins whose level of phosphate incorporation increased during whole gland and broken cell experiments. Cyclic AMP binds to regulatory subunits of Type I and Type II protein kinases having molecular weights closely approximating 55,00 and 47,000 in other tissues [4]. Co-migration of 8-$N_3$-[$^{32}$P] cyclic AMP labelled 47,000 and 45,000 dalton proteins with phosphoproteins in tick salivary glands is unique because if they are regulatory subunits of a cAMP dependent protein kinase they are two distinct proteins and possibly autophosphorylated or phosphorylated by other factors such as cyclic AMP protein kinase [13]. Walter et al. [14] and Prashard et al. [15] demonstrated that the regulatory subunit of Type I protein kinase has two subtypes differing in isoelectric points but not in molecular weights in mammalian liver cells respectively. It may be that two subtypes of Type I regulatory subunits exist in tick salivary glands that differ slightly in molecular weights and/or electrophoretic mobility.

Walter and Greengard [14] concluded that proteins that bind 8-$N_3$-[$^{32}$P] cAMP and having molecular weights less than 47,000 dltons are likely proteolytic by-products of the Type I and II subunits. Proteins with molecular weight greater than 54,000 daltons occasionally bind slight amounts of 8-$N_3$[$^{32}$P] cAMP non-specifically.

The upper band (47,000 M.W.) of the phosphorylated doublet does not incorporate $^{32}$P as readily as does the lower band (45,000). Also, the 47,000 dalton protein binds less radioactive cyclic AMP analog in the presence of high concentrations of unlabeled cyclic AMP. The significance of this observation is unclear.

The results of these experiments and the previous study with intact glands [5] indicate that factors (i.e., dopamine and cyclic AMP) that affect fluid secretion in tick salivary glands affect the level of phosphorylation of specific endogenous proteins. Other work has shown that the glands are controlled by nerves, that the neurotransmitter is dopamine and that dopamine activates a specific dopamine sensitive adenylate cyclase to increase gland levels of cyclic AMP. Cyclic AMP binding proteins having molecular weights similar to the regulatory subunits of Type I and II cyclic AMP-dependent protein kinases are present. Thus we believe that both Type I and Type II cyclic AMP dependent protein kinases are present in tick salivary glands that phosphorylate at least 12 proteins of possible importance in the fluid secretory process. The receptor subunit of the Type I kinase appears to be unique in that it consists of two phosphorylated subtype proteins. The significance of this to overall gland function is unclear.

Future research should be directed toward determining subcellular location of phosphorylated proteins and demonstration of how they affect the functional ability of the glands. The task is formidable because of the multicellular, multifunctional nature of the glands.

## LITERATURE CITED


1. Walsh, D. A., Perkins, J. P., Krebs, E. G. (1968) J. Biol. Chem. 243, 3763-3765.
2. Hofmann, F., Beavo, J. A., Boethel, P. J., and Krebs, E. G. (1975) J. Biol. Chem. 250, 7795-7801.
3. Erlichman, J., Rosenfeld, R. and Rosen, O. M. (1974) J. Biol. Chem. 249, 5000-5003.
4. Walter, U., Uno, I., Liu, A. Y. C. and Greengard, P. (1977) J. Biol. Chem. 252, 5000-5003.
5. McSwain, J. L., Essenberg, R. C. and Sauer, J. R. (1983) Biochem. Biophys. Acta. In preparation.
6. Patrick, C. D. and Hair, J. A. (1975) J. Med. Entomol. 12, 389-390.
7. Needham, G. R. and Sauer, J. R. (1979) J. Parasitol. 65, 531-542.
8. Rudolph, S. R. and Krueger, B. K. (1979) Adv. Cyclic Nucleotide Research 10, 107-133.
9. Lohmann, S., Walter, U. and Greengard, P. (1980) J. Biol. Chem. 255, 9985-9992.
10. King, J. and Laemmli, U. K. (1971) J. Mol. Biol. 62, 465-477.
11. Sauer, J. R., Mincolla, P. M. and Needham, G. R. (1976) Comp. Biochem. Physiol. 53C, 63-66.
12. Needham, G. R. and Sauer, J. R. (1975) J. Insect Physiol. 21, 1893-1898.
13. Geahlen, R. L. and Krebs, E. G. (1980) J. Biol. Chem. 255, 1164-1169.

14. Walter, U. and Greengard, P. (1982) in Handbook of Experimental Pharmacology, Vol. 58/1. Cyclic Nucleotides I. (Nathanson, J. A. and Kebabian, J. W., eds.) pp. 479-505, Springer-Verlag, New York.

15. Prashad, N., Rosenberg, R. N., Wischmeyer, B., Ulrich, C. and Sparkman, D. (1979) Biochemistry 18, 2717-2725.

TABLE I

RELATIVE INCORPORATION OF $^{32}P$ INTO INDIVIDUAL PROTEIN BANDS IN RESPONSE TO CYCLIC AMP

| Mr | Control (-cAMP) n=2 | Peak height in cm cAMP 10 µm (n=2) | Mean % Change |
|---|---|---|---|
| 145000 | 0.90 | 1.20 | + 25 |
| 124000 | 0.60 | 0.75 | + 20 |
| 113000 | 0.50 | 0.60 | + 16 |
| 103000 | 0.75 | 0.85 | + 12 |
| 93500 | 0.55 | 1.00 | + 45 |
| 74000 | 1.45 | 2.10 | + 30 |
| 62000 | 3.35 | 5.20 | + 35 |
| 55000 | 3.25 | 3.80 | + 14 |
| 49000 | 2.20 | 2.30 | + 4 |
| 47000 | 12.10 | 23.80 | + 48 |
| 45000 | 13.45 | 27.75 | + 55 |
| 37000 | 1.45 | 2.40 | + 40 |
| 34000 | 0.70 | 2.40 | + 70 |
| 29000 | 0.55 | 1.60 | + 65 |

Relative incorporation of $^{32}P$ into individual protein bands of salivary glands of Amblyomma americanum (L.). Peak heights of densitometric scans were used to determine the relative amount of $^{32}P$ incorporation into each protein. Numbers in table represent averages of two experiments.

TABLE II

RELATIVE INCORPORATION OF $^{32}P$ INTO INDIVIDUAL PROTEIN BANDS FOLLOWING DEPHOSPHORYLATION IN RESPONSE TO "COLD CHASE"

| Mr | Peak height in cm | | Mean % Change |
|---|---|---|---|
| | Control (+cAMP 10 μm) n=2 | ATP $10^{-3}$ M n=2 | |
| 145000 | 1.85 | 1.35 | - 27 |
| 124000 | 1.15 | 1.10 | - 4 |
| 113000 | 0.85 | 0.70 | - 18 |
| 103000 | 1.35 | 0.95 | - 27 |
| 93500 | 3.85 | 2.90 | - 25 |
| 74000 | 8.50 | 6.80 | - 20 |
| 62000 | 5.40 | 5.10 | - 6 |
| 55000 | 5.35 | 3.75 | - 29 |
| 49000 | 2.70 | 2.55 | - 6 |
| 47000 | 15.05 | 23.60 | - 29 |
| 45000 | 14.45 | 10.35 | - 29 |
| 37000 | 3.95 | 2.60 | - 34 |
| 34000 | 5.95 | 4.20 | - 29 |
| 29000 | 1.80 | 0.65 | - 30 |

Relative incorporation of $^{32}P$ into individual protein bands of tick salivary gland homogenates of Ambyomma americanum (L.). Peak height measurements of densitometric scans of autoradiographs of electrophorsed proteins were used to determine the relative amount of $^{32}P$ incorporation into each protein.

TABLE III

RELATIVE INCORPORATION OF $^{32}P$ INTO INDIVIDUAL PROTEIN BANDS FOLLOWING DEPHOPHORYLATION IN RESPONSE TO PROTEIN KINASE INHIBITOR

| Mr | Peak height in cm | | % Change |
|---|---|---|---|
| | Control (+cAMP 10 μm) | Protein kinase inhibitor | |
| 145000 | 1.8 | 0.6 | - 67 |
| 124000 | 1.1 | 1.2 | + 08 |
| 113000 | 0.9 | 1.0 | + 10 |
| 103000 | 1.0 | 1.3 | + 23 |
| 93500 | 2.5 | 2.4 | + 04 |
| 74000 | 7.2 | 5.7 | - 20 |
| 62000 | 6.4 | 4.2 | - 34 |
| 55000 | 5.5 | 5.7 | - 3 |
| 49000 | 3.3 | 2.4 | - 27 |
| 47000 | 2.9 | 1.5 | - 48 |
| 45000 | 13.0 | 8.2 | - 37 |
| 37000 | 3.4 | 2.5 | - 27 |
| 34000 | 5.8 | 3.2 | - 49 |
| 29000 | 1.0 | 0.2 | - 80 |

Relative incorporation of $^{32}P$ individual protein bands of tick salivary gland homogenates of Amblyomma americanum (L.). Peak height measurements of densitometric scans of autoradiograph of electrophoresed proteins were used to determine the relative amounts of $^{32}P$ incorporation into each protein.

TABLE IV

INCORPORATION OF 8-$N_3$-[$^{32}$P] cAMP INTO PROTEIN BANDS

| Band | Mr | Co-migration with Phosphorylated protein |
|---|---|---|
| 1 | 170000 | No |
| 2 | 159000 | No |
| 3 | 118000 | No |
| 4 | 109000 | No |
| 5 | 70000 | No |
| 6 | 55000 | Yes |
| 7 | 47000 | Yes |
| 8 | 45000 | Yes |
| 9 | 33000 | No |
| 10 | 29000 | No |

Relative incorporation of 8-$N_3$-[$^{32}$P] cAMP into individual protein bands of salivary gland homogenates of Amblyomma americanum (L.).

Figure 1. Autoradiograph of electrophoresed proteins showing incorporation of $^{32}P$ into endogenous proteins of a broken cell preparation of tick salivary glands. Phosphate incorporation was measured following incubation with (+) or without (-) 10 μM cyclic AMP for 1 min. Proteins whose states of phosphorylation were affected by cyclic AMP are indicated by arrows.

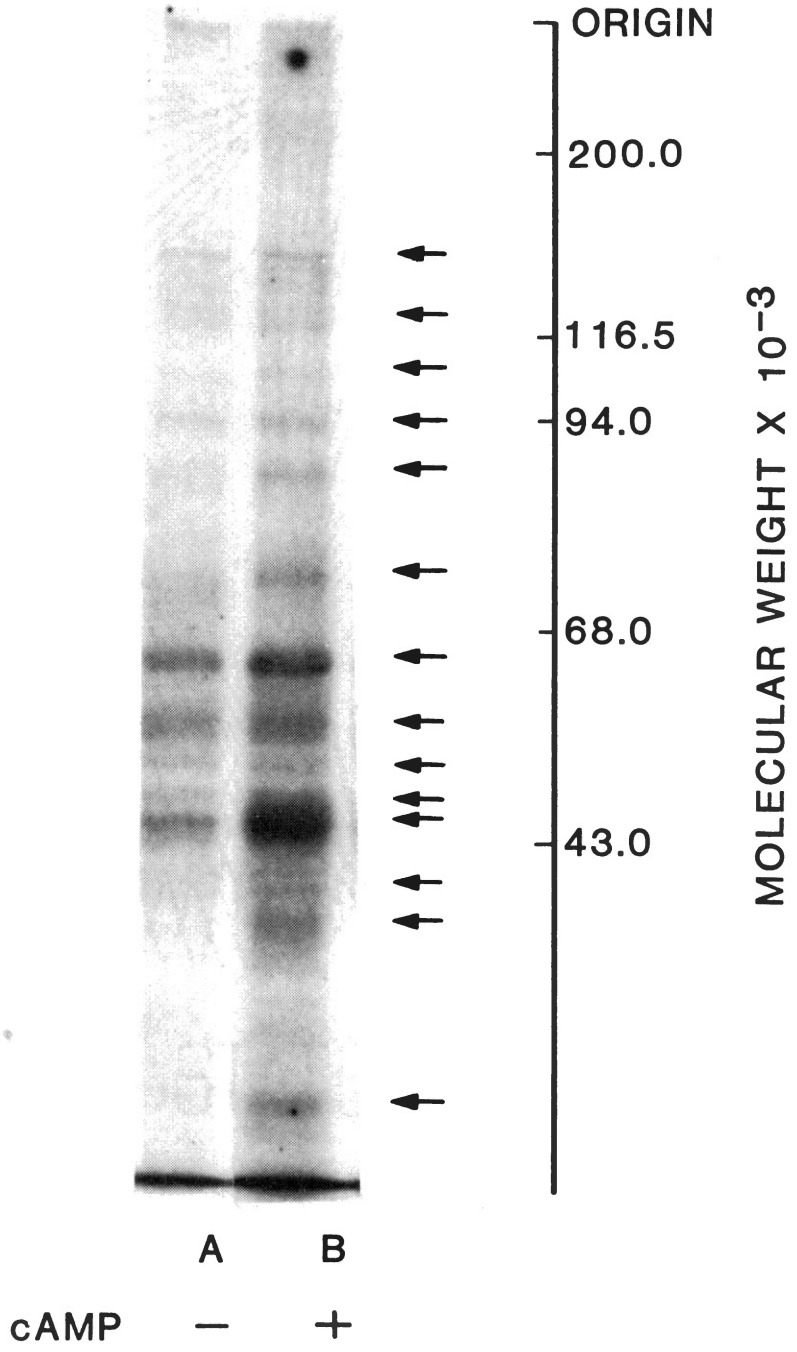

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
cAMP
—
+

Figure 2. Densitometric scan of the autoradiograph in Figure 1. _____, cyclic AMP; _ _ _ _, control.

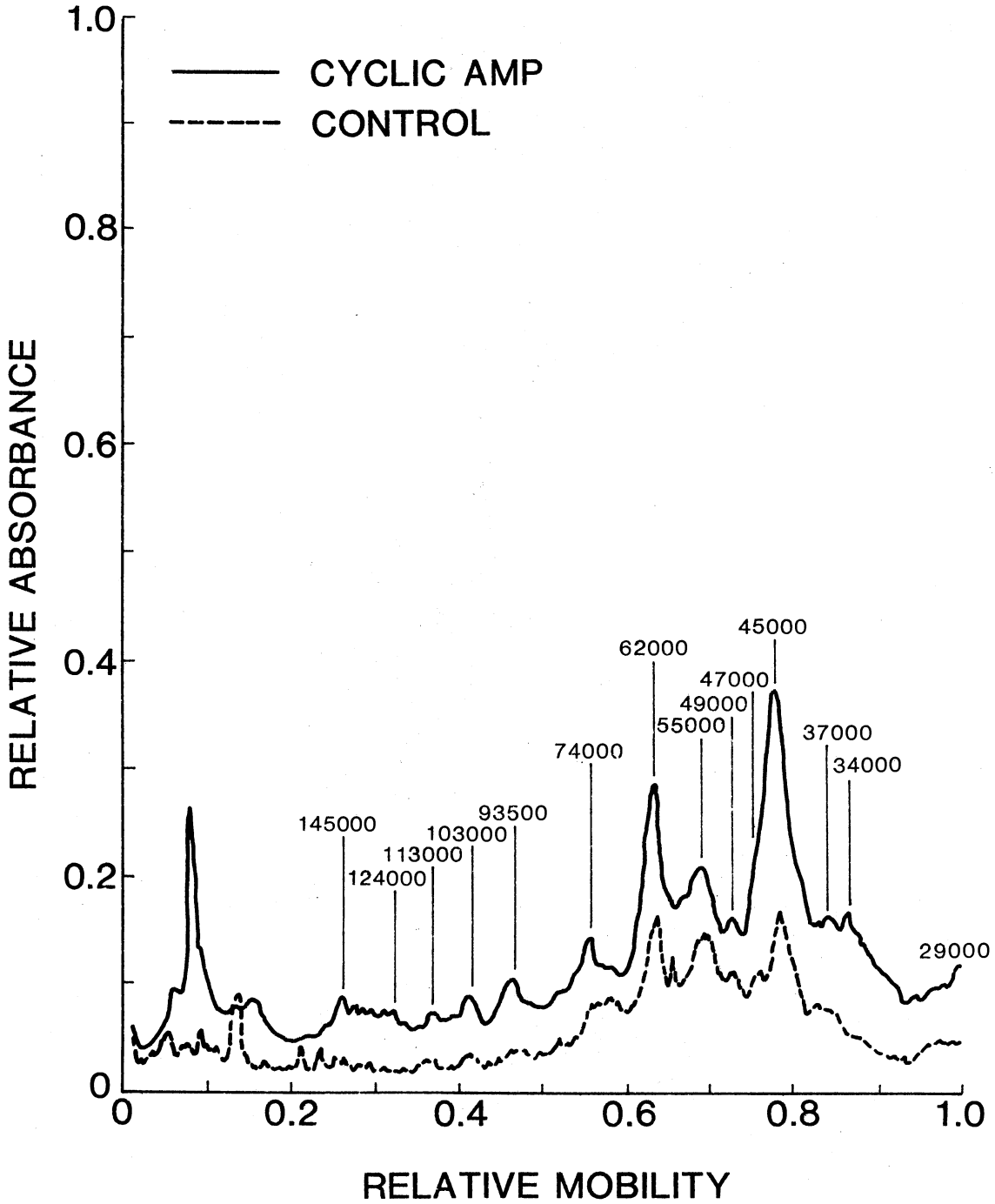

CYCLIC AMP
CONTROL
RELATIVE ABSORBANCE
RELATIVE MOBILITY
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
145000
124000
113000
103000
93500
74000
62000
55000
49000
47000
45000
37000
34000
29000

Figure 3. Dephosphorylation of endogenous proteins during "cold chase" experiments. Phosphate incorporation was measured following incubation with (+) cyclic AMP 10 μM for 5 min and subsequent incubation with (+) unlabelled $10^{-3}$ M ATP for 20 min. Proteins whose state of phosphorylation was affected by unlabelled ATP $10^{-3}$ M are indicated by arrows.

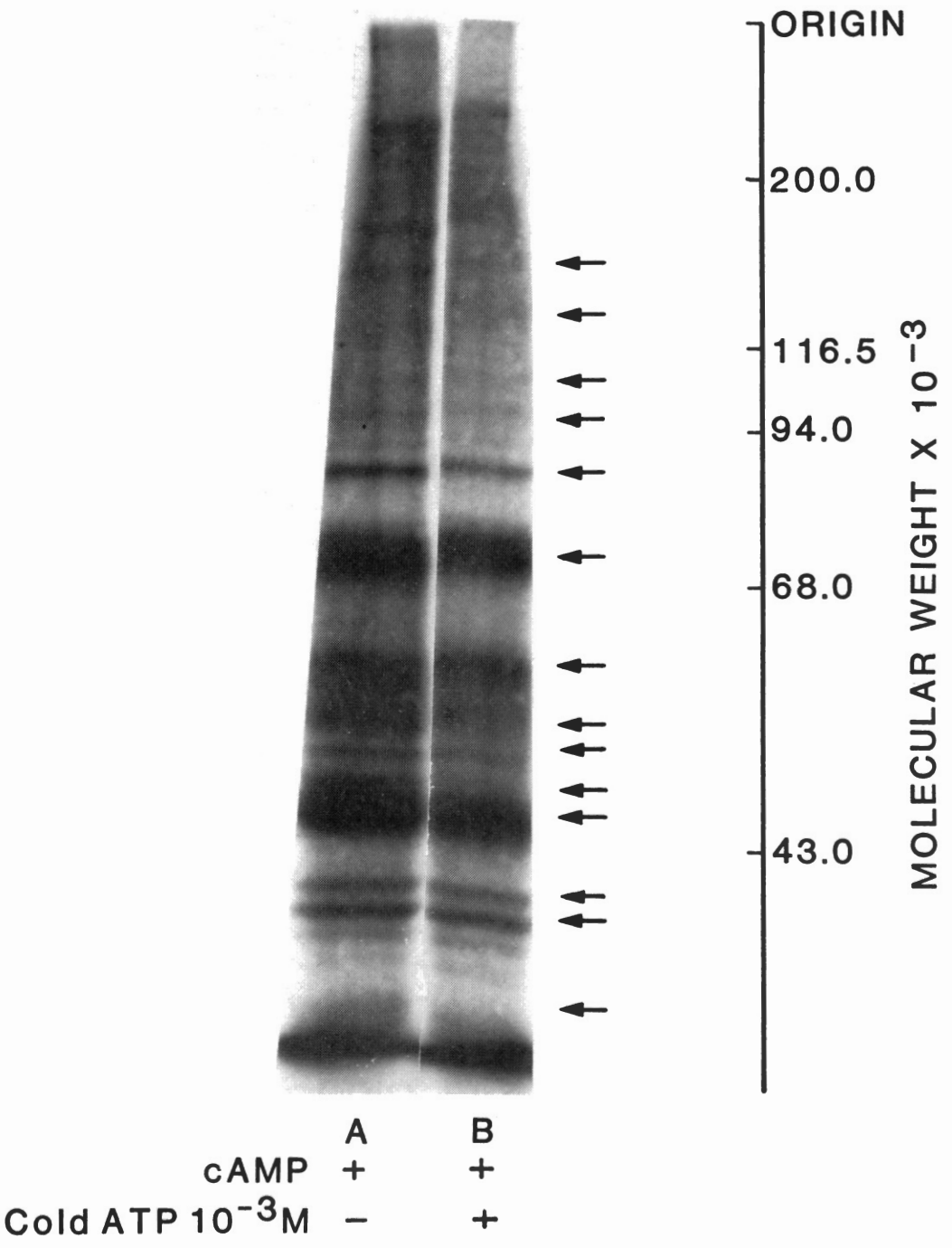

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
cAMP + +
Cold ATP $10^{-3}$M − +

Figure 4. Densitometric scan of the autoradiograph of Figure 3. Incorporation into endogenous proteins of a salivary gland homogenate. _ _ _ _, 10 μM cyclic AMP; _____, $10^{-3}$ M ATP.

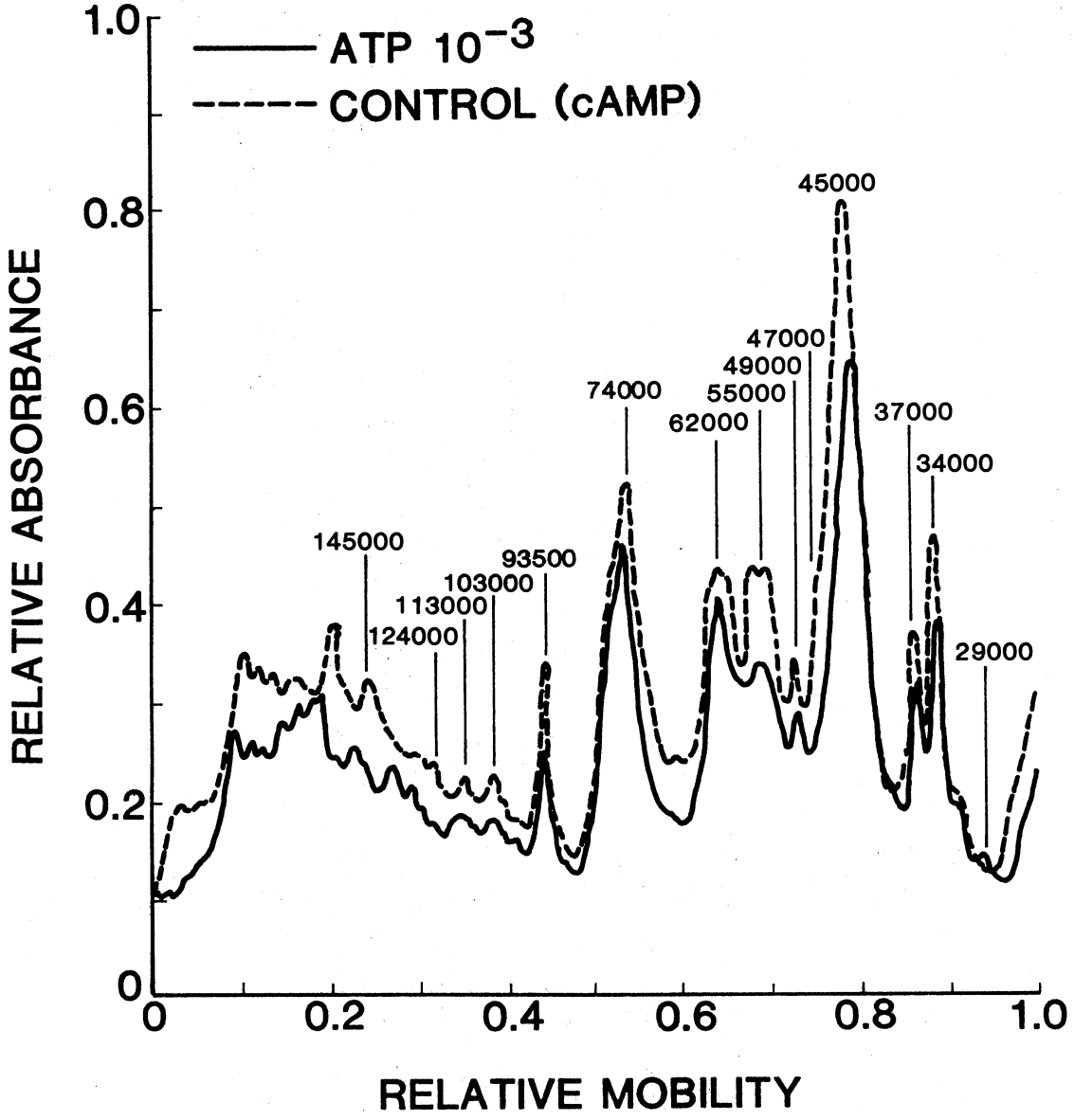

ATP $10^{-3}$
CONTROL (cAMP)
RELATIVE ABSORBANCE
RELATIVE MOBILITY
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
145000
124000
113000
103000
93500
74000
62000
55000
49000
47000
45000
37000
34000
29000

Figure 5. Dephosphorylation of proteins in response to protein kinase inhibitor. Phosphate incorporation was measured following incubation with cyclic AMP (+) and without (-) 4 μg protein kinase inhibitor for 20 min. Proteins whose states of phosphorylation was affected by protein kinase inhibitor are indicated by arrows.

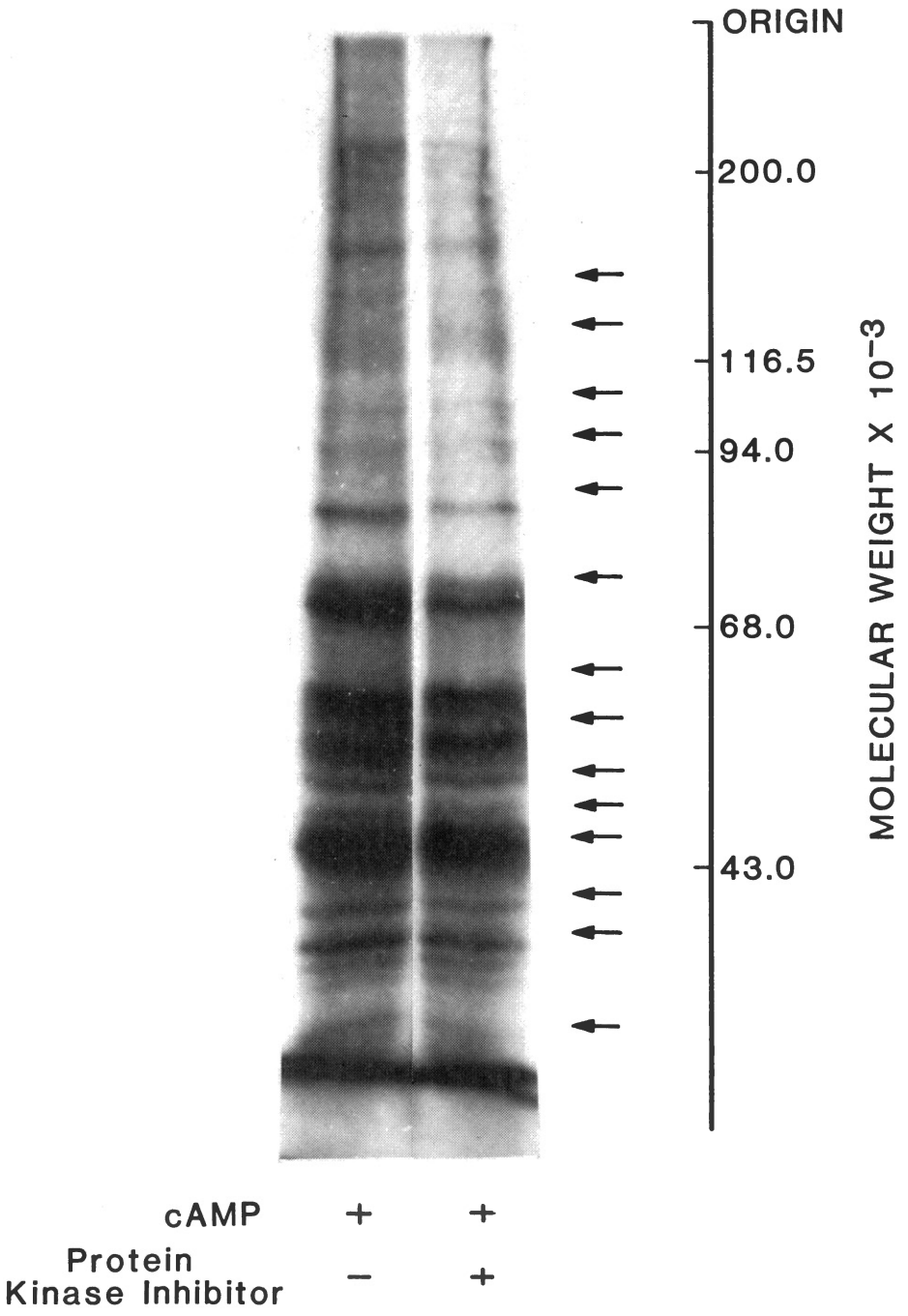

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
cAMP + +
Protein Kinase Inhibitor − +

Figure 6. Densitometric scan of the autoradiograph of Figure 5. ____, protein kinase inhibitor 4 μg per assay; _ _ _ _, cyclic AMP.

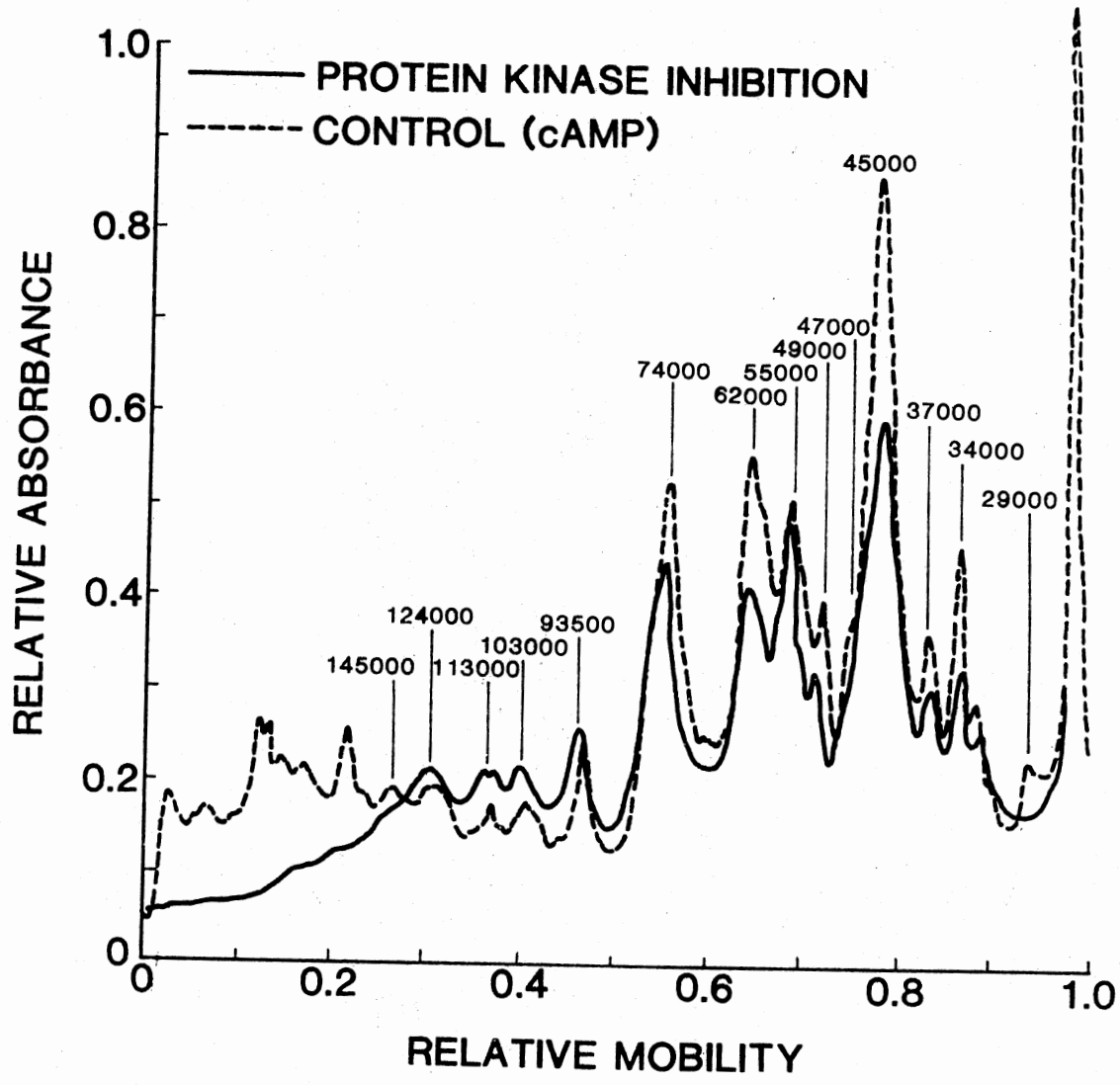
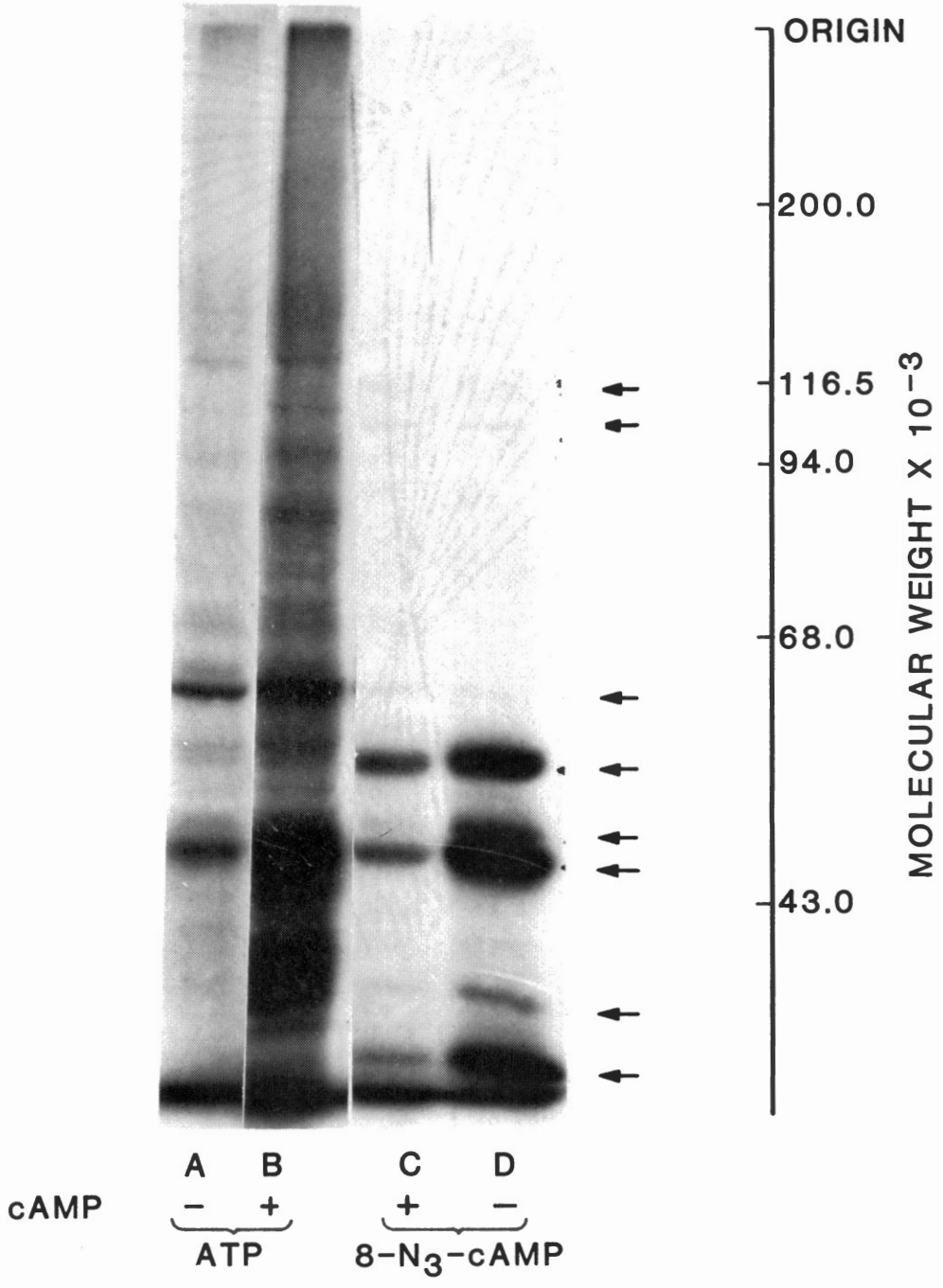

PROTEIN KINASE INHIBITION
CONTROL (cAMP)
RELATIVE ABSORBANCE
RELATIVE MOBILITY
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
145000
124000
113000
103000
93500
74000
62000
55000
49000
47000
45000
37000
34000
29000

Figure 7. Labelling of proteins by [$\gamma$-$^{32}$P]-ATP in the presence or absence of 20 μM cyclic AMP and the photoaffinity analog [$^{32}$P]-8-$N_3$-cAMP in the presence or absence of cAMP. Arrows indicate proteins whose labelling by the analog was depressed by cold cyclic AMP.


ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
A
B
C
D
cAMP
–
+
+
–
ATP
8-$N_3$-cAMP

# PART IV

# SUBCELLULAR LOCATION OF PHOSPHOPROTEINS IN SALIVARY GLANDS FROM *AMBLYOMMA AMERICANUM* (L.)

# SUMMARY

Phosphoproteins in subcellular fractions of tick salivary glands were examined by electrophoresis and autoradiography. Organelles in fractions were identified by electron microscopy and enzyme markers. It is likely that the 100,000 xg supernatant represents the cytosol. The 100,000 xg fraction represents the microsomal fraction. The 900 xg fraction contains smooth and rough membranes and mitochondria. The mitochondria can be separated from the membranes by isopycnic floatation in this fraction. The 11,500 xg pellet apparently was a plasma membrane-rich fraction.

Phosphoproteins with molecular weights of 47,000 and 45,000 daltons appeared to be localized in a plasma membrane-rich 11,500 xg fraction when tissue was pre-incubated with $^{32}P$, stimulated with dopamine, and fractionated. When subcellular fractions were incubated in [$\gamma$-$^{32}P$] ATP in the presence or absence of cyclic AMP, a 45,000 dalton phosphoprotein was present in the cytosol and phosphorylation was inhibited by cyclic AMP. A 45,000 dalton protein appeared in all fractions but phosphorylation appeared to be enhanced by cyclic AMP only in the 11,500 xg fraction. A 55,000 dalton protein was present in the cytosol and 100,000 xg microsomal fraction; its level of phosphorylation was enhanced by cyclic AMP in the microsomal fraction. A 62,000 dalton phosphoprotein was prominant in the cytosol and the 11,500 xg fraction but only in the latter did cyclic AMP enhance phosphate incorporation. Photoaffinity analog studies using 8-$N_3$-[$^{32}P$] cyclic AMP have shown that cyclic AMP binding proteins appear most prominantly in the 900 xg fraction. These proteins have molecular weights of 55,000, 47,000, and 45,000 daltons.

Key words: Cyclic AMP, protein phosphorylation, subcellular fractionation, tick salivary glands.

## INTRODUCTION

The physiology of tick salivary glands has been studied extensively. Sauer et al. [1,2] demonstrated that catecholamines such as dopamine stimulate fluid secretion and increases gland levels of cyclic AMP. A dopamine sensitive adenylate cyclase has also been identified in the glands [3]. Proteins whose states of phosphorylation are affected by dopamine and proteins which bind 8-$N_3$-[$^{32}$P] cAMP have been identified in the glands [4,5]. Adenylate cyclase and the cyclic AMP binding regulatory subunit of protein kinase have been shown to be associated with cell membranes [6,7]. The following experiments were done to identify the subcellular location of specific cyclic AMP stimulated phosphoproteins and cyclic AMP binding proteins (protein kinase subunits) in tick salivary glands.

## MATERIALS AND METHODS

Materials. [$^{32}P_i$] orthophosphate (carrier free) and [$\gamma$-$^{32}$P] ATP was obtained from New England Nuclear, Boston, Massachusetts; 8-$N_3$-[$^{32}$P]cAMP from ICN, Irvine, California. Dopamine and cyclic AMP were obtained from Sigma, St. Louis, Missouri.

Tissue preparation. Male and female lone star ticks Amblyomma americanum (L.) were raised according to the method of Patrick and Hair [8]. Salivary glands from adult female ticks were used in all experiments. Feeding ticks weighing 200 mg or more were removed from the host (sheep) and glands were dissected at 4°C in a medium of

modified, oxygenated TC-199 (Difco) at pH 7.0 containing penicillin and streptomycin sulfate and buffered as described by Needham and Sauer [9]. Approximately 30 pairs of glands were used in each experiment and were homogenized with a small hand tissue homogenizes with a loose fitting pistle in 1 ml medium containing 0.25 M sucrose, 10 mM Tricine buffer (pH 7.2), 10 mM $MgCl_2$, 0.05% para-amino-benzamidine, and 0.1 mM EDTA[1].

Subcellular fractionation. The crude homogenate was centrifuged at 900 xg for 10 min and washed twice. The 900 xg pellet was reconstituted with buffer containing 67% sucrose and placed at the bottom in a 4 ml (1/2" x 1-5/8") centrifuge tube. Various concentrations of sucrose (Figure 1) in Tricine buffer identical to the homogenization buffer except without sucrose were layered on the top of the pellet (0.5 ml aliquots) beginning with the heaviest concentration. The tube was transferred to a swinging bucket rotor and centrifuged at 100,000 xg for 16-18 hours. The fractions were collected in 10 drop increments by pumping 70% sucrose into the bottom of the tube with a ISCO model D gradient fractionator. Absorbance at 254 nm was measured with an ISCO model UA-2 ultraviolet analyzer.

The 900 xg supernatant was centrifuged at 11,500 xg for 10 min and the precipitate washed twice. The 11,500 xg supernatant was centrifuged at 100,000 xg for 60 min and the supernatant collected (soluble fraction) (Figure 1).

Enzyme marker assays. Na+K+ ATPase [10] and adenylate cyclase [11] were used to identify the plasma membranes. Succinate dehydrogenase [12] activity was used to identify mitochondria and glucose-6-phosphatase activity [13] was used to identify microsomes.

---

[1]Ethylenedinitrilotetraacetic acid

Effectiveness of homogenization was determined by assaying for lactate dehydrogenase [14] in soluble and particulate fractions of the crude homogenate.

Electron microscopy. Tissue was collected, homogenized and fractionated as illustrated in Figure 1. Light and heavy subfractions of the 900 xg pellet and total pellet preparations from 11,500 xg and 100,000 xg were routinely processed for electron microscopy.

Fractions of salivary gland tissue were fixed in 2% cacodylate buffered glutaraldehyde for 1 hr (pH 7.4), rinsed in cacodylate buffer, then post-fixed in 2% osmium tetroxide, all at room temperature. The fraction was en bloc stained with 0.5% uranyl acetate at room temperature overnight. Subsequent dehydration of the fraction in a graded series of ethyl alcohol occurred before embedding in polybed (Polysciences, Warrington, Pennslyvania). Appropriate polymerized blocks were chosen for thin sections after viewing initial thick sections (1 μm) stained with Mallory's trichrome. Thin sections (70-90 nm) were obtained with a Sorvall MT-2 ultramicrotome using diamond knives. Sections were placed on 300 mesh copper grids and stained with methanolic uranyl acetate and lead citrate [15]. Sections were examined and photographed with a Philips EM 200 electron microscope.

Electrophoresis and autoradiography: whole gland method. Tissue was dissected as described above. Thirty pairs of salivary glands were incubated for 1 hr at 37° C in buffered, oxygenated in TC-199 (pH 7.0) containing 100 μCi/ml $^{32}P$, total volume of 300 μl. Following incubation, the glands were stimulated for 5 min in 300 l TC-199 containing $10^{-5}$ M dopamine. The glands were then homogenized in 1 ml Tricine buffer as

described and fractionated according to the scheme outlined in Figure 1.

Following subcellular fractionation an aliquot containing 60 μg protein from each fraction and the supernatant was subjected to electrophoresis and subsequent autoradiography following as by McSwain et al. [4].

Broken cell method. Thirty pairs of salivary glands were dissected and homogenized as above. Subcellular fractionation was accomplished following the diagrammatic scheme in Figure 1. Aliquots containing approximately 60 μg of protein from the supernatant, 900, 11,500, and 100,000 xg pellets were incubated in disposable glass test tubes containing 1 ml reaction medium prepared on ice consisting of a 50 mM Hepes[2] buffer (pH 7.0), 10 mM $MgCl_2$, and ± 10 μM cyclic AMP after the method of Rudolph and Kruger [16]. The phosphorylation reaction was initiated by adding 20 μl of 4.8 μM [$\gamma$-$^{32}$P) ATP (specific activity 10-20 Ci/m mole). The tubes were incubated in a shaking water bath for 5 min. The reaction was terminated by adding 100 μl of a stop solution containing 0.625 M Tris HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. The tubes were transferred to a boiling water bath for 2 min and subjected to electrophoresis and autoradiography as described by McSwain et al. [4].

Cyclic AMP binding proteins. To identify the subcellular location of cyclic AMP binding proteins, an aliquot containing approximately 60 μg of protein from each subcellular fraction and the supernatant was incubated in a 100 μl reaction mixture containing 50 mM MES[3] buffer (pH

---

[2]4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

[3]2(N-morpholino)-ethanesulfonic acid

6.2), 10 mM $MgCl_2$, 1 mM isobutyl-methylxanthine, 1 mM ATP, 0.5 mM 2-mercaptoethanol, and 0.2 M 8-$N_3$-[$^{32}$P] cAMP in the absence and presence of 20 μM cyclic AMP following the method of Lohman et al. [7]. The reaction mixture was incubated for 60 min in the dark at 4° C and then radiated for 10 min at 254 mm with a chromate-Vue® ultraviolet light source. Fifty μl of the SDS stop solution described above was then added. The samples were subjected to electrophoresis and autoradiography as previously described [4].

## RESULTS

Electron microscopy. Significant compositional changes were observed between 900, 11,500 and 100,000 xg pellets. The light subfraction of the 900 xg pellet (Figure 2) contained numerous smooth and fewer rough membrane-bround vesicles. Differentiation between the plasma membranes and smooth endoplasmic reticulum was difficult; therefore, membrane-bound vesicles lacking ribosomes were collectively labeled smooth membrane-bound vesicles. Portions of the cytoplasm were also present and contained numerous small rough membrane-bound vesicles (Figure 2).

The heavy subfraction of the 900 xg pellet (Figure 3) had a more heterogenous composition than the light subfraction. It appeared that the number of smooth membrane-bound vesicles had decreased while the rough membrane-bound vesicles appeared to increase slightly. Some intact mitochondria were also present in this subfraction. Numerous small, electron-dense bodies either attached to endoplasmic reticulum or free in the cytoplasm produced large irregular shaped structures (Figure 3). Portions of the cytoplasm also contained rough membrane-bound vesicles.

Fewer isolated organelles as well as fragmented organelles were observed in the total 11,500 xg pellet (Figure 4). Membrane-bound vesicles decreased in size and number. The majority of membrane-bound vesicles present were smooth. Portions of the cytoplasm contained very few, if any, membrane-bound vesicles. Electron-dense bodies were seen in the cytoplasm.

The total 100,000 xg pellet from feeding lone star ticks contained large amounts of small vesicles to which were attached ribosomal-type structures (Figure 5). Other electron-dense material present in this pellet may be chromatin. Smooth membrane-bound vesicles were significantly reduced in size and number. No cytoplasmic fractions were observed in this pellet.

Identification of subcellular fractions by enzyme markers. A cytosolic enzyme, lactate dehydrogenase, was used to determine efficiency of homogenization. Approximately 90% of the enzyme activity appears in the supernatant (Table I) indicating good homogenization.

In initial studies, it appeared that adeylate cyclase and $Na^+K^+$ ATPase activity (plasma membrane markers) was greatest in the 900 xg fraction obtained by differential centrifugation (Table II). Specific activity is elevated in the 11,500 xg pellet due to removal of proteinaceous material. The majority of the adenylate cyclase fraction stimulated by dopamine was in the 900 xg fraction. Similarly the 11,500 xg pellet had a slightly higher specific activity for $Na^+K^+$ ATPase than the 900 xg pellet, but the greatest amount of $Na^+K^+$ ATPase activity is in the 900 xg fraction. This fraction also contained the greatest specific $Na^+K^+$ ATPase activity inhibited by ouabain. The 900 xg pellet was further purified by isopycnic floatation using sucrose gradients

into a light and heavy fraction (Figure 1).

Fractions from the gradient which appeared highest in protein content were assayed for $Na^+K^+$ ATPase and adenylate cyclase (Figure 6). When total enyzme activity was plotted as a function of fraction number, the greater activity for both enzymes appeared in the light fractions. When specific activity of the same enzymes was plotted as a function of fraction number, the maximum appeared in the lightest fraction (Figure 6). Fractions obtained from isopycnic floation of the 900 xg pellet in a different experiment were then assayed for $Na^+K^+$ ATPase and succinate dehydrogenase (Table III, Figure 7). When total enzyme activity for succinate dehydrogenase was plotted against fraction number, the greatest activity appeared in the light fraction as did total protein. In this experiment, the greatest activity of $Na^+K^+$ ATPase was found in the heavy fraction. The greatest specific activity for $Na^+K^+$ ATPase appeared in the heavy fraction while that of succinate dehydrogenase appeared in the light fraction (Figure 7B). The cause for the difference in the two experiments is unclear. Possible explanations are differences in stage of tick feeding from which the glands were obtained or slight variations in hand homogenization techniques. The degree of homogenization could possibly affect the size of vesicles formed and thereby change their location on the gradient. It is also known that the glands undergo remarkable anatomical changes during feeding. However, it is clear that the plasma membranes can be separated from mitochondria by these procedures.

Succinate dehydrogenase (mitochondria marker) assays performed in fractions obtained from differential centrifugation showed little activity in the 11,500 xg pellet (Table II); however, considerable

activity was seen in the 900 xg pellet.

Glucose-6-phosphatase is often used as a marker for endoplasmic reticulum, but its activity is greatly reduced in some cells. In this tissue the activity as well as the subcellular location of this enzyme was variable. The greatest activity was found in the 100,000 xg pellet (Table II). In a different experiment, the greatest activity was found in the supernatant. It is possible that the enzyme may have separated from the microsomal membranes under the conditions of the procedure. The enzyme is known to be unstable.

Electrophoretic distribution of subcellular proteins-whole gland method. When whole salivary glands were incubated in $^{32}P_i$ followed by stimulation with dopamine and subsequent fractionation, little radioactivity was observable in protein bands, possibly because of the time and various separation procedures used after labelling the proteins. The most prominant phosphate incorporation was observed in the 45,000 and 47,000 dalton proteins in the 11,500 xg pellet (Figure 8). These proteins were also shown to incorporate the largest amount of phosphate in previous whole gland and broken cell preparation studies [4,5].

Broken cell method. When salivary gland tissue was homogenized and subcellular fractions obtained by differential centrifugation incubated with [$\gamma$-$^{32}$P] ATP with or without cyclic AMP, significant differences in phosphate incorporation into proteins were observed in the various subcellular fractions (Table IV, Figure 9). Proteins with molecular weights of 62,000, 54,000, 47,000, 45,000, and 29,000 daltons were of particular interest because these incorporated increased amounts of phophate in response to dopamine and cyclic AMP in the previous studies

[4,5]. The 62,000 dalton protein appeared to incorporate phosphate to the greatest extend in the supernatant, as well as being the most labelled protein, and appeared to incorporate phosphate more readily in the absence of cyclic AMP. Phosphate incorporation into this protein was less apparent in the 11,500 xg pellet but was enhance in the presence of cyclic AMP. A phosphorylated 55,000 dalton protein, probably regulatory subunit of Type II cAMP protein kinase [16], was present in the supernatant and 100,000 xg pellet. Cyclic AMP did not appear to affect phosphate incorporation into this protein in the supernatant but did enhance incorporation in the 100,000 xg fraction. The 47,000 and 45,000 dalton proteins correspond in molecular weight to the Type I regulatory subunit of protein kinase [17]. The 47,000 dalton protein appeared to incorporate phosphate only in the supernatant and the incorporation did not appear to be enhanced by cyclic AMP. The 45,000 dalton incorporated phosphate in all fractions but incorporation was enhanced by cyclic AMP only in the 11,500 xg pellet. In other fractions, cyclic AMP appeared to inhibit phosphate incorporation. Incorporation of phosphate in the 29,000 dalton protein appeared in the supernatant and the 11,500 xg pellet. Cyclic AMP enhanced incorporation in the 11,500 xg pellet while it inhibited incorporation in the supernatant. Other proteins with molecular weights corresponding to proteins which incorporated phosphate in response to dopamine and cyclic AMP in previous studies [4,5] were the 102,000, 95,000, 58,000, and 37,000 dalton proteins.

Experiments designed to show the subcellular location of cyclic AMP binding proteins using the photoaffinity analog 8-$N_3$-[$^{32}$P] cAMP (Figure 10) showed that three prominent proteins in the 900 xg pellet, the

11,500 xg pellet, and in the supernatant incorporated the photaffinity analog. These proteins have molecular weights corresponding to 55,000 and 47,000-45,000 daltons. Other proteins incorporated this analog to an extent probably due to non-specific binding or proteolysis of the Type I and II subunits of protein kinase. Binding of the analog in the three proteins was inhibited by additional cyclic AMP.

## DISCUSSION

In preceding papers [4,5], we demonstrated that proteins in tick salivary glands incorporate phosphate in response to dopamine and cyclic AMP. Proteins with molecular weights corresponding to 102,000, 95,000, 62,000, 55,000, 47,000, 45,000 and 37,000 daltons were of particular interest because proteins of similar molecular weights incorporated phosphate during both kinds of experiments.

The 102,000 and 95,000 dalton proteins appeared to be localized in the 100,000 xg fraction and phosphorylation was enhanced by cyclic AMP. The 62,000 dalton protein appears to be localized in the cytosol but phosphorylation was not increased by cyclic AMP in the present experiments as was seen in whole gland or broken cell experiments [4,5]. The 55,000 dalton protein is present in the supernatant and 100,000 xg fraction but phosphorylation is enhanced only in the latter. The phosphorylation of a 47,000 dalton protein was apparent in the supernatant but phosphorylation was not affected by cyclic AMP. Phosphorylation of a 45,000 dalton protein was apparent in all fractions, but was enhanced by cyclic AMP only in the 11,500 xg pellet. Cyclic AMP appeared to inhibit phosphorylation in the other fractions. The 37,000 dalton protein appeared in the supernatant and 900 xg pellet.

Phosphorylation was not affected by cyclic AMP in the supernatant and was apparently inhibited by cyclic AMP in the 900 xg pellet.

Additionally, a 58,000 dalton protein whose phosphorylation was stimulated by dopamine in whole gland studies was found in the supernatant and 900 xg pellet; phosphorylation was enhanced by cyclic AMP only in the supernatant. A 29,000 dalton protein whose level of phosphorylation was enhanced by cyclic AMP in broken cell studies and dopamine in whole salivary glands of unfed ticks was localized in the supernatant and 11,500 xg fraction; phosphorylation was enhanced only in the latter.

Ultrastructural identification of all isolated fractions was difficult due to heterogeneity of the fractions which were obtained from ticks of varying weights. The 900 xg fraction was apparently rich in smooth and rough membranes. The light fraction obtained following isopycnic floatation procedures contained numerous smooth membranes while the heavy fraction had more rough membrane-bound vesicles. Some intact mitochondria were also present in the latter. The light fraction was rich in $Na^+K^+$ ATPase and adenylate cyclase activity. Although location of enzyme activity in the heavy fraction was different, fractions rich in adenylate cyclase and $Na^+K^+$ ATPase were clearly separate from fractions rich in succinate dehydrogenase activity in the 900 xg pellet. From this, it is apparent that even though plasma membranes can be separated from mitochrondria, subcellular organelle experiments should always be accompanied by enzyme marker assays to identify the organelle in the fraction being studied.

Membrane-bound vesicles decreased in size and number in the 11,500 xg pellet. This fraction showed high specific activity for both $Na^+K^+$

ATPase and adenylate cyclase and is probably enriched in plasma membranes. It is of interest to note that several phosphorylated proteins that were more heavily phosphorylated in response to cyclic AMP were present in this fraction.

The 100,000 xg fraction contained large amounts of small vesicles to which were attached ribosomal type structures. Although enzyme markers used in this study were not specific for this fraction, phosphorylation proteins was also stimulated by cyclic AMP.

Photoaffinity analog studies using 8-$N_3$-[$^{32}$P] cAMP show the major cyclic AMP binding proteins to be localized in the 900 xg fraction. These proteins have molecular weights corresponding to 55,000, 47,000, and 45,000 daltons. These are similar to reported weights of Type I and Type II regulatory subunits of protein kinase. Other proteins bound this analog probably due to non specific binding or proteolysis of the subunits.

Tick salivary glands are multicellular and multifunctional organs in which tissue differentiation occurs rapidly following attachment and feeding. The glands are involved in secreting proteins, synthesizing proteins, and secreting fluid. The association of phosphorylated proteins with subcellular location and specific functions appears to be complex. Furthermore, it is clear that other factors which affect phosphorylation of specific proteins in response to dopamine or cyclic AMP in whole gland and broken cell experiments are removed or changed during fractionation procedures. Identification of these factors remains an interesting challenge for future experiments.

# LITERATURE CITED


1. Sauer, J. R., Mincolla, P. M. and Needham, G. R. (1976) Comp. Biochem. Physiol. 53C, 63-66.
2. Needham, G. R. and Sauer, J. R. (1975) J. Insect. Physiol. 21, 1893-1898.
3. Schmidt, S. P., Essenberg, R. C. and Sauer, J. R. (1982) Comp. Biochem. Physiol. 72 C, 9-14.
4. McSwain, J. L., Essenberg, R. C. and Sauer, J. R. (In preparation) Biochem. Biophys. Acta.
5. McSwain, J. L., Essenberg, R. C. and Sauer, J. R. (In preparation) Biochem. Biophys. Acta.
6. Rodbell, J. (1980) Nature 284, 17-22.
7. Lohmann, S. M., Walter, U., Greengard, P. (1980) J. Biol. Chem. 255, 9985-9992.
8. Patrick, C. D. and Hair, J. A. (1975) J. Med. Entomol. 12, 389-390.
9. Needham, G. R. and Sauer, J. R. (1979) J. Parasitol. 65, 531-542.
10. Aldoffsen, R. and Moudrianakis, E. N. (1971) Biochemistry 10, 2247-2253.
11. Schmidt, S. P., Essenberg, R. C. and Sauer, J. R. (1981) J. Cyclic Nucleotide Research 7(6), 375-384.
12. Harmon, H. J. and Crane, F. L. (1976) Biochem. Biophys. Acta. 440, 45-58.
13. Morre, D. J. (1971) Methods of enzymology. Colowick, S. P. and Kaplan, N. D. eds. Vol. XLI, p. 47-52. Acdemic Press, New York.

14. Bernstein, L. H. and Everse, J. (1975) Methods of enzymology. Colowick, S. P. and Kaplan, N. D. eds. Vol. XLI p. 47-52. Academic Press, New York.

15. Venabale, J. H. and Coggeshall, R. (1965) J. Cell Biol. 24, 407-408.

16. Rudolph, S. A. and Krueger, B. K. (1979) Adv. Cyclic Nucleotide Research 10, 107-133.

17. Hofmann, F., Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1975) J. Biol. Chem. 250, 7795-7801.

TABLE I

LACTATE DEHYDROGENASE ACTIVITY IN TICK SALIVARY GLAND TISSUE

| Fraction | Total enzyme activity |
|---|---|
| Crude | 0.165 |
| Supernatant | 0.165 |
| Pellet | 0.02 |

Lactate dehydrogenase activity in tick salivary gland tissue. Enzyme activity is expressed in international units per ml. Numbers in table represent an average at two experiments.

# TABLE II

## CHARACTERIZATION OF SUBCELLULAR FRACTIONS OF TICK SALIVARY GLAND TISSUE

| | | ENZYME MARKERS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $Na^+K^+$ATPase | | | Adenylate Cyclase | | Succinate Dehydrogenase[7] | | Glucose-6 Phosphatase[7] | |
| Fraction | Total Protein (mg) | | Total[1] | Sp.Act.[2] | | Total[3] | Sp.Act.[4] | Total[5] | Sp.Act.[6] | Total[1] | Sp.Act.[2] |
| Crude | 5.66 | Total | 23.88 | 4.22 | Basal | 23.3 | 27.44 | - | - | 5.11 | 0.79 |
| | | Ouabain Inhibited | 8.54 | 1.51 | Dopamine | 45.6 | 53.71 | | | | |
| 900xg | 3.68 | Total | 17.02 | 4.62 | Basal | 29.6 | 26.8 | 0.22 | 0.05 | 2.19 | 0.64 |
| | | Quabain Inhibited | 4.93 | 1.34 | Dopamine | 64.7 | 58.5 | | | | |
| Light Fraction | 0.09 | Total | 1.53 | 17.04 | Basal | 5.9 | 108.1 | - | - | - | - |
| | | Ouabain Inhibited | 0.41 | 4.61 | Dopamine | 11.5 | 210.6 | | | | |
| Heavy Fraction | 0.72 | Total | 5.26 | 7.31 | Basal | 13.7 | 31.7 | - | - | - | - |
| | | Ouabain | 1.34 | 1.87 | Dopamine | 18.2 | 42.1 | | | | |
| 11,500xg | 0.35 | Total | 2.13 | 6.10 | Basal | 7.8 | 74.4 | 0.012 | 0.02 | 0.41 | 1.14 |
| | | Ouabain | 0.49 | 1.42 | Dopamine | 12.9 | 123.0 | | | | |
| 100,000xg | 0.27 | Total | 1.17 | 4.42 | Basal | 3.9 | 29.3 | N.D. | N.D. | 0.17 | 2.00 |
| | | Ouabain Inhibited | 0.45 | 1.73 | Dopamine | 3.4 | 25.6 | | | | |
| Supernatant | 0.69 | Total | 1.10 | 1.60 | Basal | 6.2 | 23.9 | N.D. | N.D. | 0.30 | 0.324 |
| | | Ouabain Inhibited | 0.19 | 0.28 | Dopamine | 2.4 | 9.3 | | | | |

[1] m moles/min in total fraction

[2] m moles/min/mg protein

[3] pmoles cAMP formed

[4] pmoles cAMP/min/mg protein

[5] Δ O.D./min

[6] Δ O.D./min/mg protein

[7] Tissue for this assay was from same separation but different from other assays

-= assay not done

N.D.=None detected

TABLE III

CHARACTERIZATION OF 900xg FRACTION OF TICK SALIVARY GLAND TISSUE OBTAINED FROM ISOPYCNIC FLOATATION

| Fraction | Total Protein | | ENZYME MARKERS $Na^+K^+$ ATPase Tot.Act.[1] | $Na^+K^+$ ATPase Sp.Act.[2] | Succinate Dehydrogenase Total[3] | Succinate Dehydrogenase Sp.Act.[4] |
|---|---|---|---|---|---|---|
| 900xg | | | | | | |
| 10 | 0.11 | Total | .70 | 6.40 | 0.25 | 0.25 |
| | | Ouabain Inhibited | .03 | 0.47 | | |
| 11 | 0.25 | Total | .89 | 3.54 | 0.245 | 0.98 |
| | | Ouabain Inhibited | .09 | 0.39 | | |
| 12 | 0.356 | Total | 1.14 | 3.20 | 0.31 | 0.86 |
| | | Ouabain Inhibited | 0.03 | 1.01 | | |
| 13 | 0.18 | Total | 0.84 | 4.70 | 0.07 | 0.39 |
| | | Ouabain Inhibited | 0.28 | 1.57 | | |
| 14 | 0.07 | Total | 0.67 | 9.07 | 0.05 | 0.68 |
| | | Ouabain Inhibited | 0.13 | 1.85 | | |

[1] m moles/min in total fraction

[2] m moles/min/mg protein

[3] ΔO.D./min

[4] ΔO.D./min/mg protein

TABLE IV

RELATIVE INCORPORATION OF $^{32}P$ INTO ENDOGENOUS PROTEINS OF SUBCELLULAR FRACTIONS OF TICK SALIVARY GLAND TISSUE

| | Supernatant | | 900 xg | | 11,500 xg | | 100,000 xg | |
|---|---|---|---|---|---|---|---|---|
| | Cyclic AMP | | | | | | | |
| Mr | (-) | (+) | (-) | (+) | (-) | (+) | (-) | (+) |
| 260,000 | | | + | ++ | | | | |
| 192,000 | | | | | | | - | + |
| 188,855 | | | | | | | - | + |
| 173,000 | | | - | + | | | | |
| 143,000 | + | + | | | | | + | + |
| 137,000 | | | | | + | + | | |
| 130,000 | + | + | | | | | ++ | + |
| 115,000 | | | - | + | + | ++ | | |
| 108,000 | + | ++ | | | | | | |
| 105,000 | | | - | + | | | | |
| 101,000 | | | | | | | - | + |
| 95,000 | | | | | | | - | + |
| 92,000 | + | ++ | | | | | | |
| 71,000 | | | | | + | ++ | | |
| 62,000 | ++ | + | | | - | + | | |
| 58,000 | + | ++ | + | + | | | | |
| 55,000 | + | + | | | | | - | + |
| 50,000 | + | - | | | | | - | + |
| 47,000 | + | - | | | | | | |
| 45,000 | ++ | + | ++ | + | + | ++ | ++ | + |
| 41,000 | | | | | - | + | - | + |
| 37,000 | + | + | + | - | | | | |
| 34,000 | + | + | | | | | + | - |
| 32,000 | | | - | + | - | + | | |
| 30,000 | | | - | + | - | + | | |
| 29,000 | + | - | | | - | + | | |

Relative incorporation of $^{32}P$ into individual proteins of subcellular fractions of tick salivary gland tissue. Fractions were labelled with [$\gamma$-$^{32}P$] ATP following subcellular fractionation.

- = phosphate incorporation not visible on autoradiogram. + or ++ = relative amount of phosphate visible on autoradiogram.

Figure 1. Subcellular fractionation of tick salivary glands.

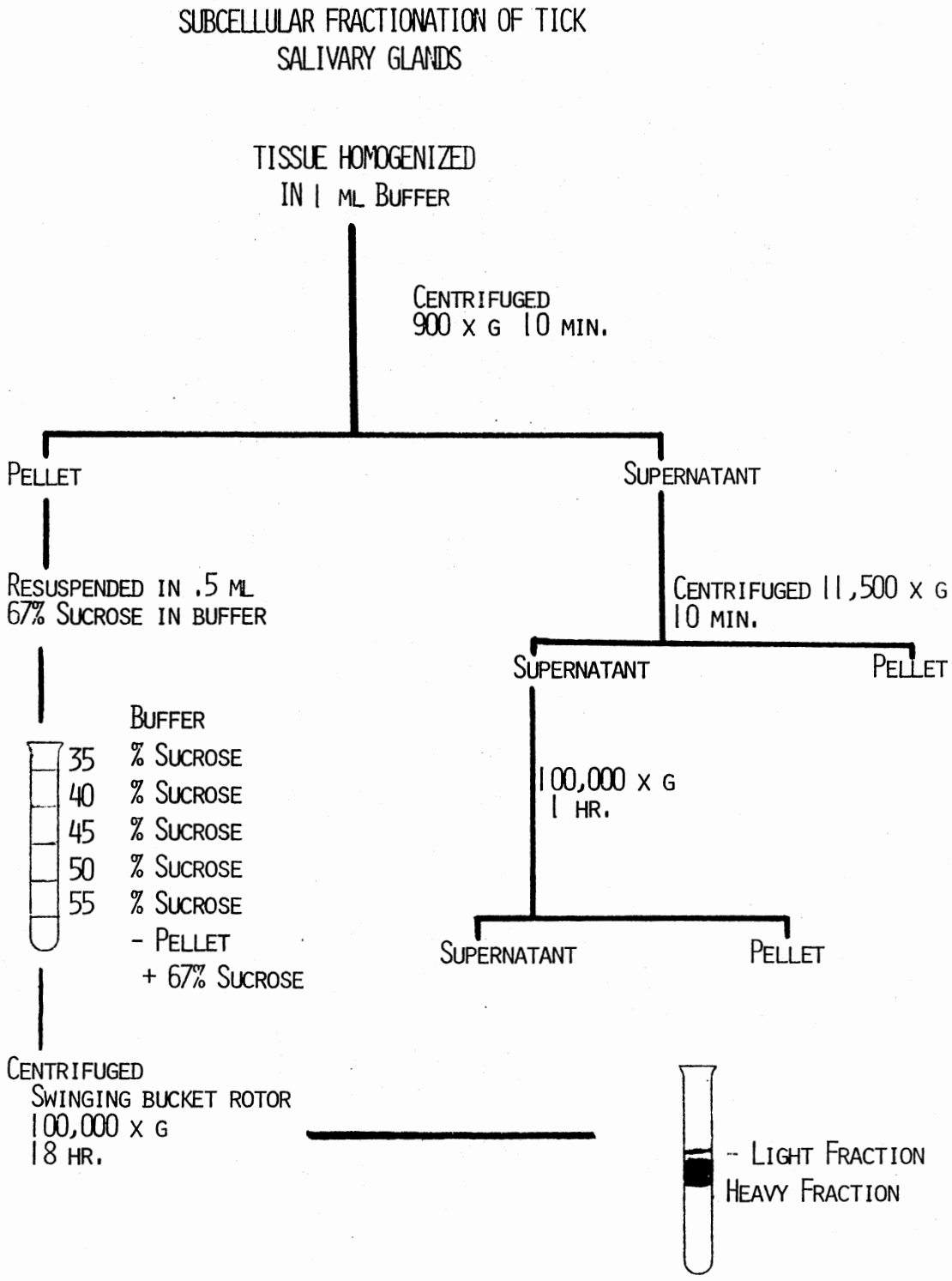

SUBCELLULAR FRACTIONATION OF TICK SALIVARY GLANDS
TISSUE HOMOGENIZED IN 1 ML BUFFER
CENTRIFUGED 900 X G 10 MIN.
PELLET
SUPERNATANT
RESUSPENDED IN .5 ML 67% SUCROSE IN BUFFER
CENTRIFUGED 11,500 X G 10 MIN.
SUPERNATANT
PELLET
100,000 X G 1 HR.
SUPERNATANT
PELLET
BUFFER
35 % SUCROSE
40 % SUCROSE
45 % SUCROSE
50 % SUCROSE
55 % SUCROSE
- PELLET
+ 67% SUCROSE
CENTRIFUGED SWINGING BUCKET ROTOR 100,000 X G 18 HR.
- LIGHT FRACTION
HEAVY FRACTION

Figure 2. Electron micrograph of the 900 xg light fraction from feeding lone star tick salivary gland tissue. Numerous smooth (SM) and fewer rough (RM) membrane-bound vesicles are present in the light subfraction of the 900 xg pellet. Portions of cytoplasm (C) also contained membrane-bound vesicles. X30,735.

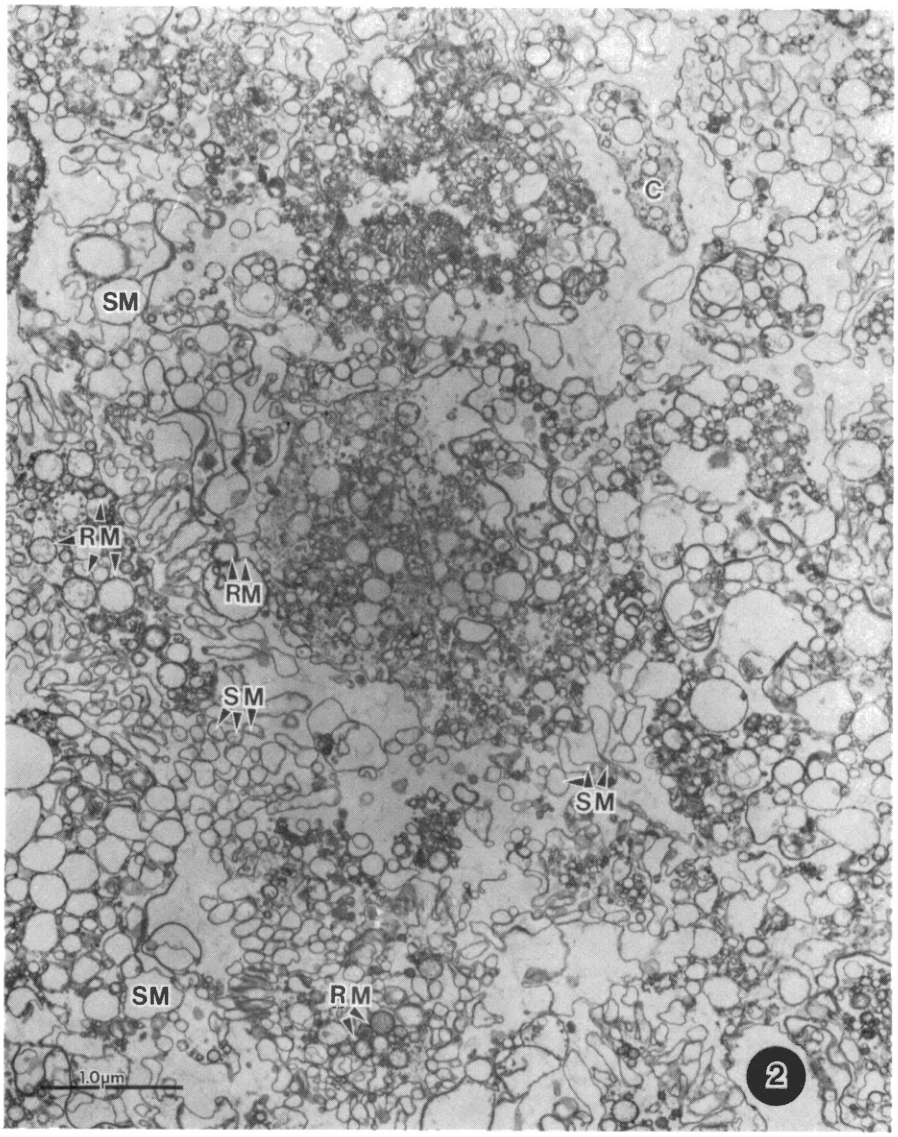

C
SM
RM
RM
SM
SM
SM
RM
1.0μm
2

Figure 3. Electron micrograph of the 900 xg heavy fraction from feeding lone star tick salivary gland tissue. Rough (RM) and smooth (SM) membrane-bound vesicles, partially intact mitochrondria (M) and portions of cytoplasm (C) are present in the heavy subfraction of the 900 xg pellet. The dark electron-dense material in this micrograph may be composed of free ribosomes and ribosomes attached to endoplasmic reticulum as well as cytoplasm. X30,735.

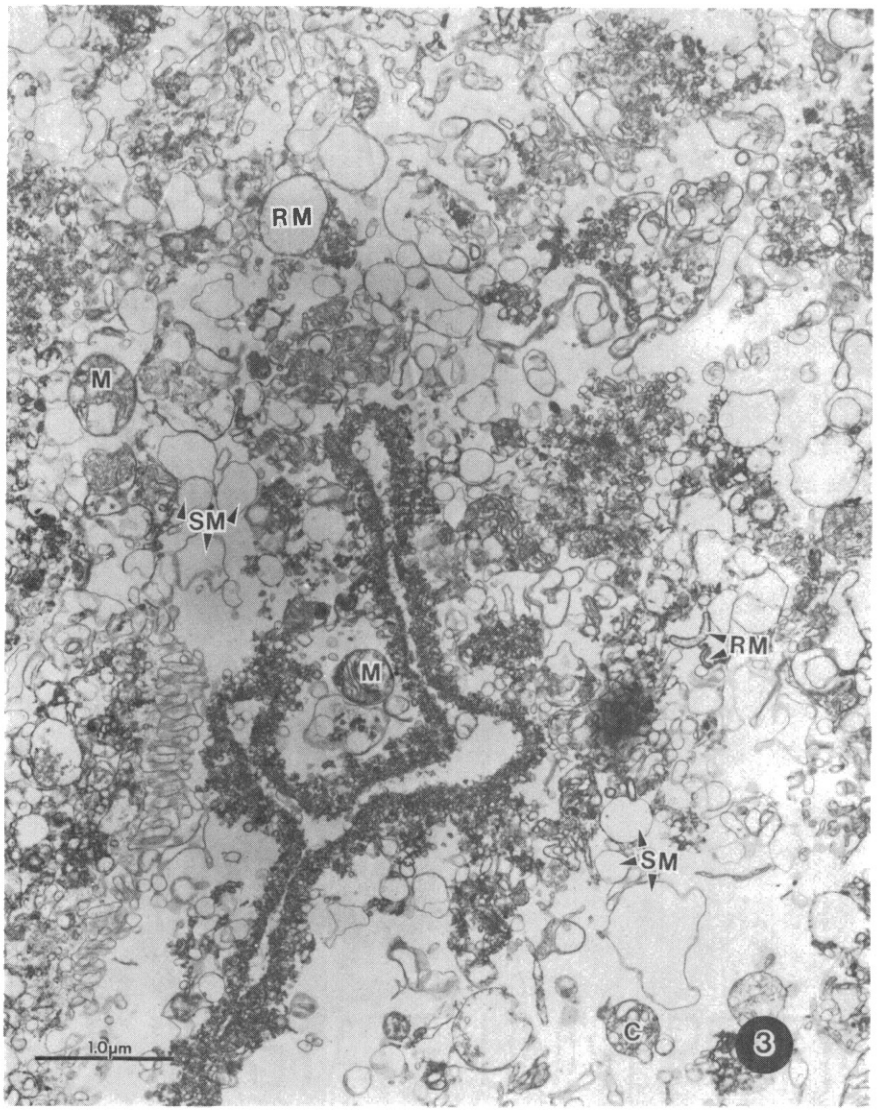

RM
M
SM
M
RM
SM
C
1.0μm
3

Figure 4. Electron micrograph of the total 11,500 xg pellet. Smooth (SM) membrane-bound vesicles and portions of cytoplasm (C) were present in the total 11,500 xg pellet from feeding lone star tick salivary gland tissue. X30,735.

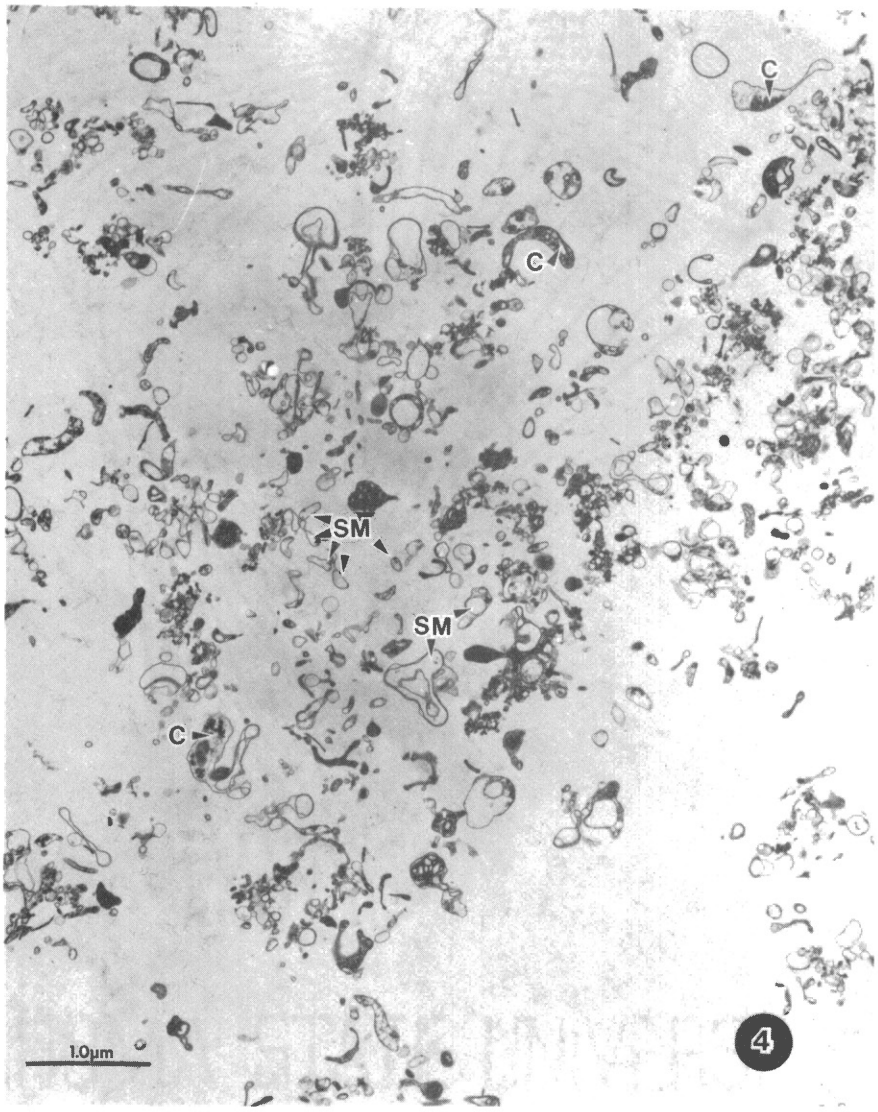

C
C
SM
SM
C
1.0μm
4

Figure 5. Electron micrograph of the 100,000 xg pellet from feeding lone star tick salivary gland tissue. An abundance of free ribosomes (FR) (or possibly attached to endoplasmic reticulum) and/or chromatin (CH), while fewer smooth (SM) membrane-bound vesicles were present in the total 100,000 xg pellet. X30,735.

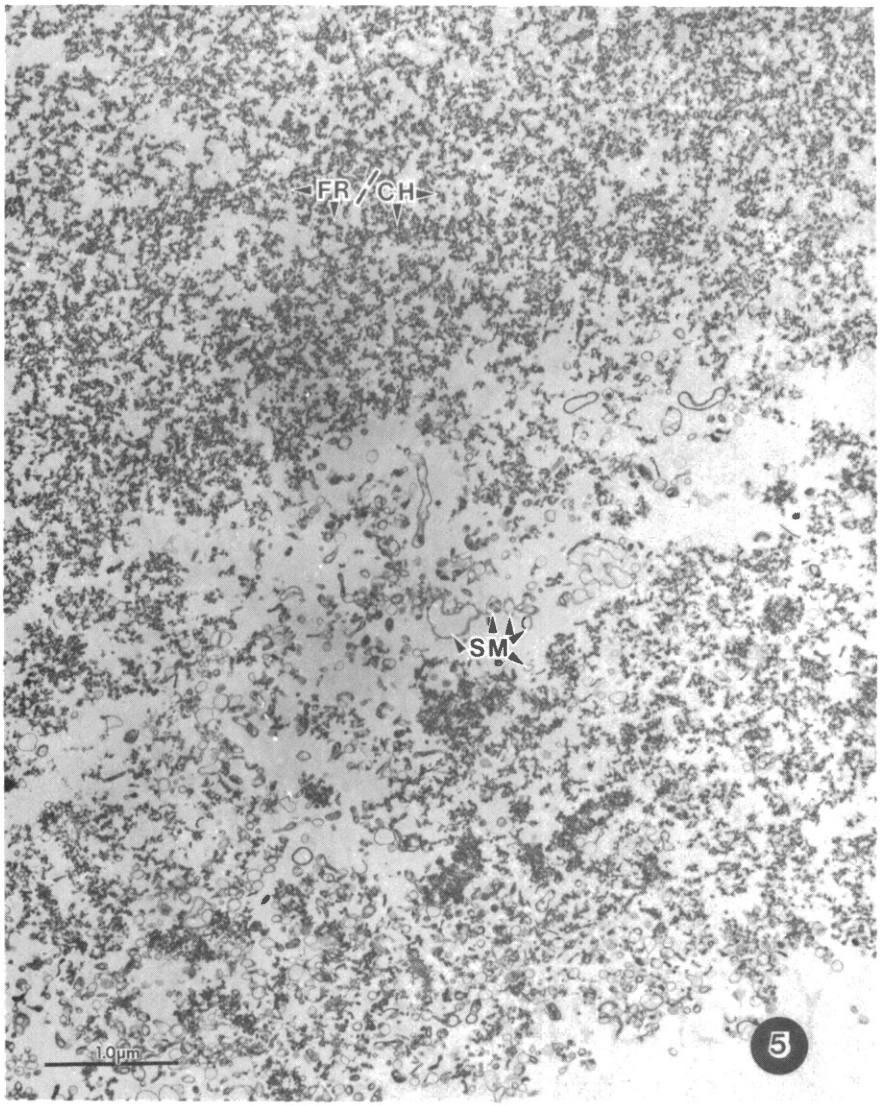
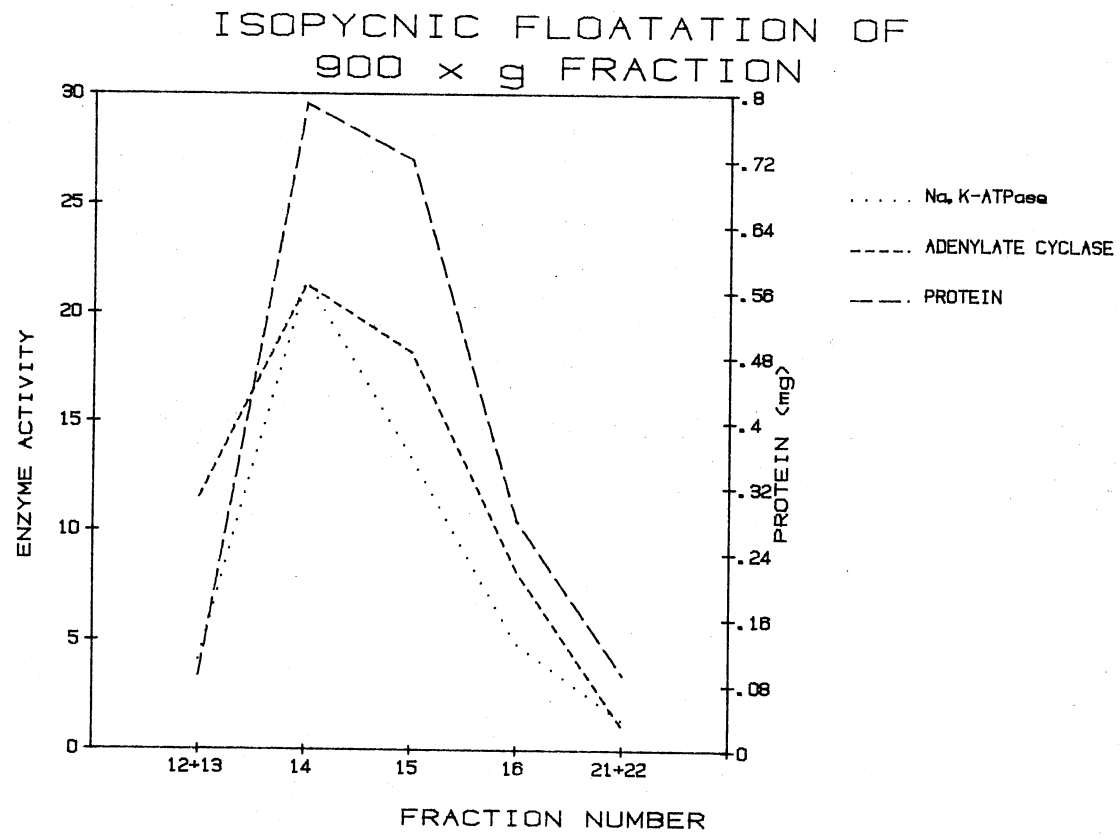

FR
CH
SM
1.0μm
5

Figure 6. Isopycnic floation of 900 xg fraction. Upper. Total $Na^+K^+$ ATPase (mmoles/min in total fraction), adenylate cyclase activity (pmoles formed per fraction), and total protein are plotted against fraction number from isopycnic gradient. $Na^+K^+$ ATPase values are multiplied by 10 to increase peak height. Lower. Specific activity of $Na^+K^+$ ATPase and adenylate cyclase are plotted against fraction number from isopycnic gradient. Specific $Na^+K^+$ ATPase activity is expressed in mmoles/min/mg protein while specific adenylate cyclase activity is expressed in pmoles cAMP/min/mg protein.


ISOPYCNIC FLOATATION OF
900 x g FRACTION
ENZYME ACTIVITY
PROTEIN (mg)
FRACTION NUMBER
12+13
14
15
16
21+22
Na, K-ATPase
ADENYLATE CYCLASE
PROTEIN

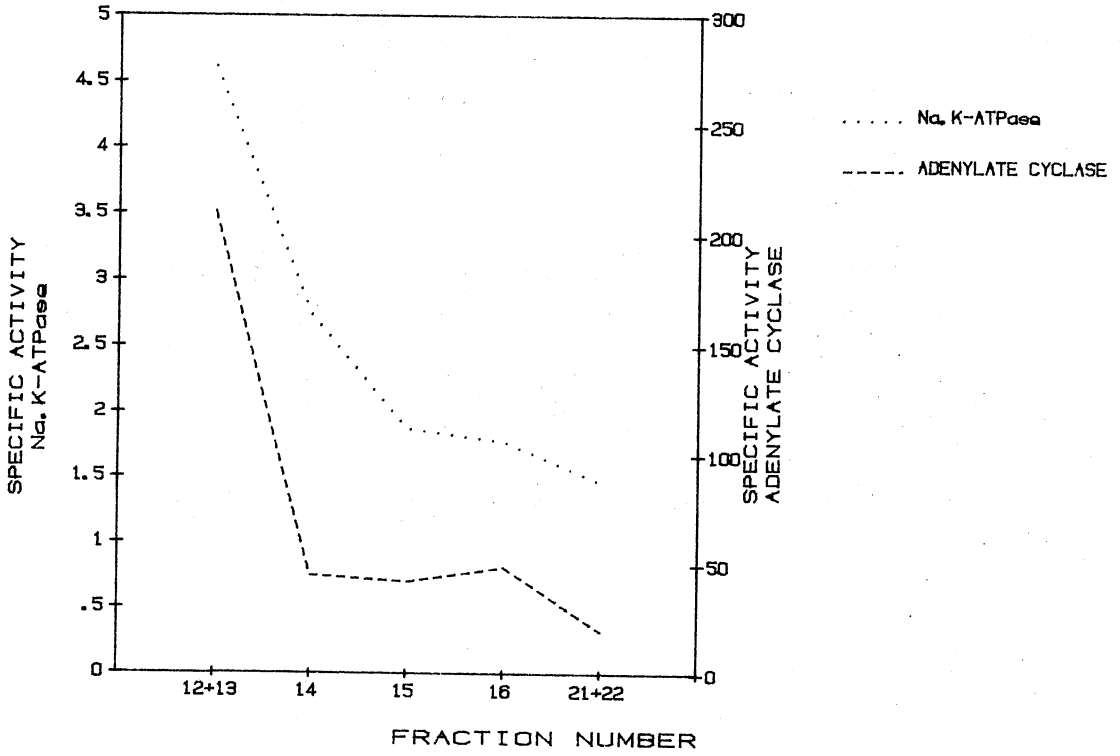
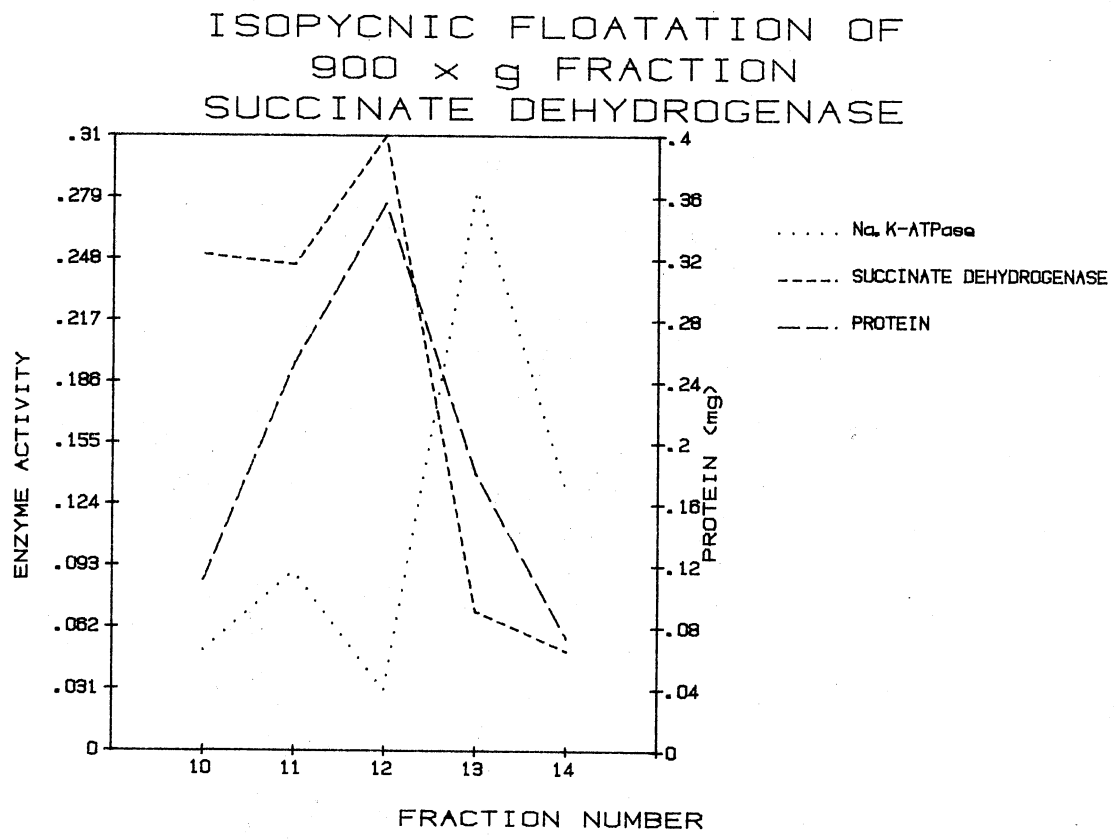

SPECIFIC ACTIVITY
Na, K-ATPase
SPECIFIC ACTIVITY
ADENYLATE CYCLASE
FRACTION NUMBER
12+13
14
15
16
21+22
Na, K-ATPase
ADENYLATE CYCLASE

Figure 7. Isopycnic floation of 900 xg fraction. Succinate dehydrogenase. Upper. Total activities of $Na^+K^+$ ATPase (mmoles/min/in total fraction) and succinate dehydrogenase ($\Delta$O.D./min) and total protein are plotted against fraction number from isopycnic gradient. Lower. Specific activity for $Na^+K^+$ ATPase (mmoles/min/mg protein) and succinate dehydrogenase ($\Delta$O.D./min/mg protein) are plotted against fraction number from isopycnic gradient.


ISOPYCNIC FLOATATION OF
900 x g FRACTION
SUCCINATE DEHYDROGENASE
ENZYME ACTIVITY
PROTEIN (mg)
FRACTION NUMBER
Na, K-ATPase
SUCCINATE DEHYDROGENASE
PROTEIN

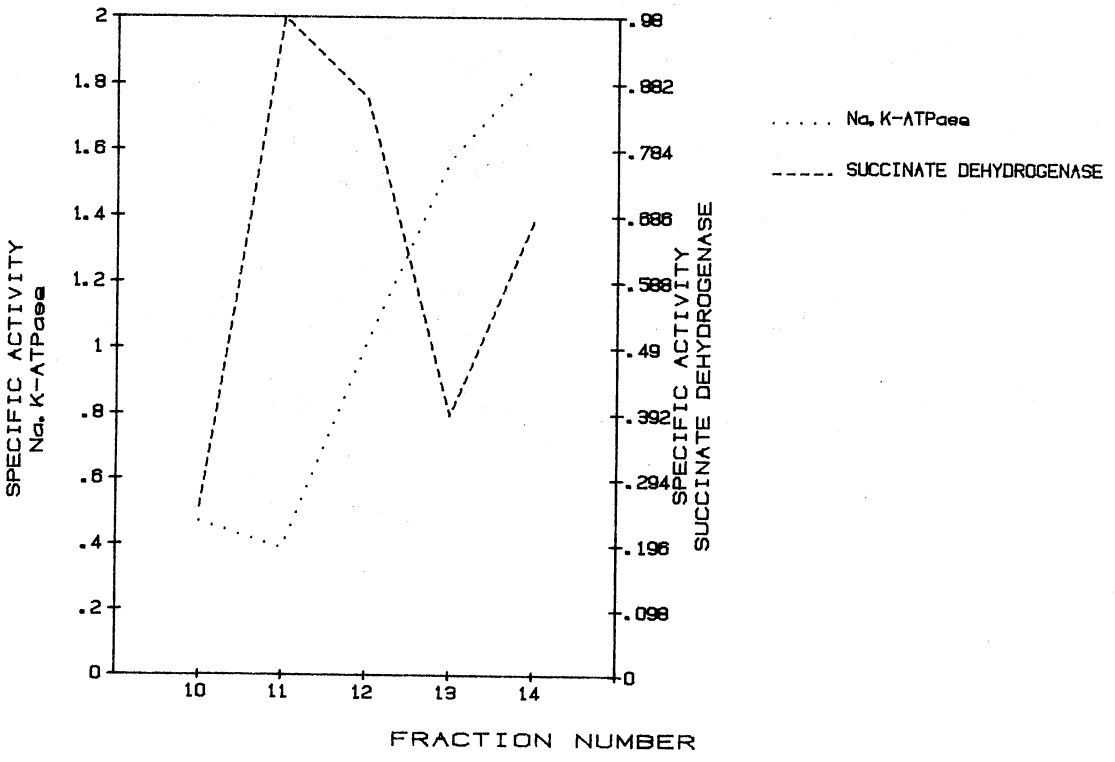

SPECIFIC ACTIVITY
Na, K-ATPase
SPECIFIC ACTIVITY
SUCCINATE DEHYDROGENASE
FRACTION NUMBER
Na, K-ATPase
SUCCINATE DEHYDROGENASE

Figure 8. Autoradiograph of electrophoresed proteins showing incorporation of $^{32}P$ into endogenous proteins of subcellular fraction of tick salivary gland tissue following incubation of whole glands with dopamine $10^{-5}$ M dopamine for 5 min.

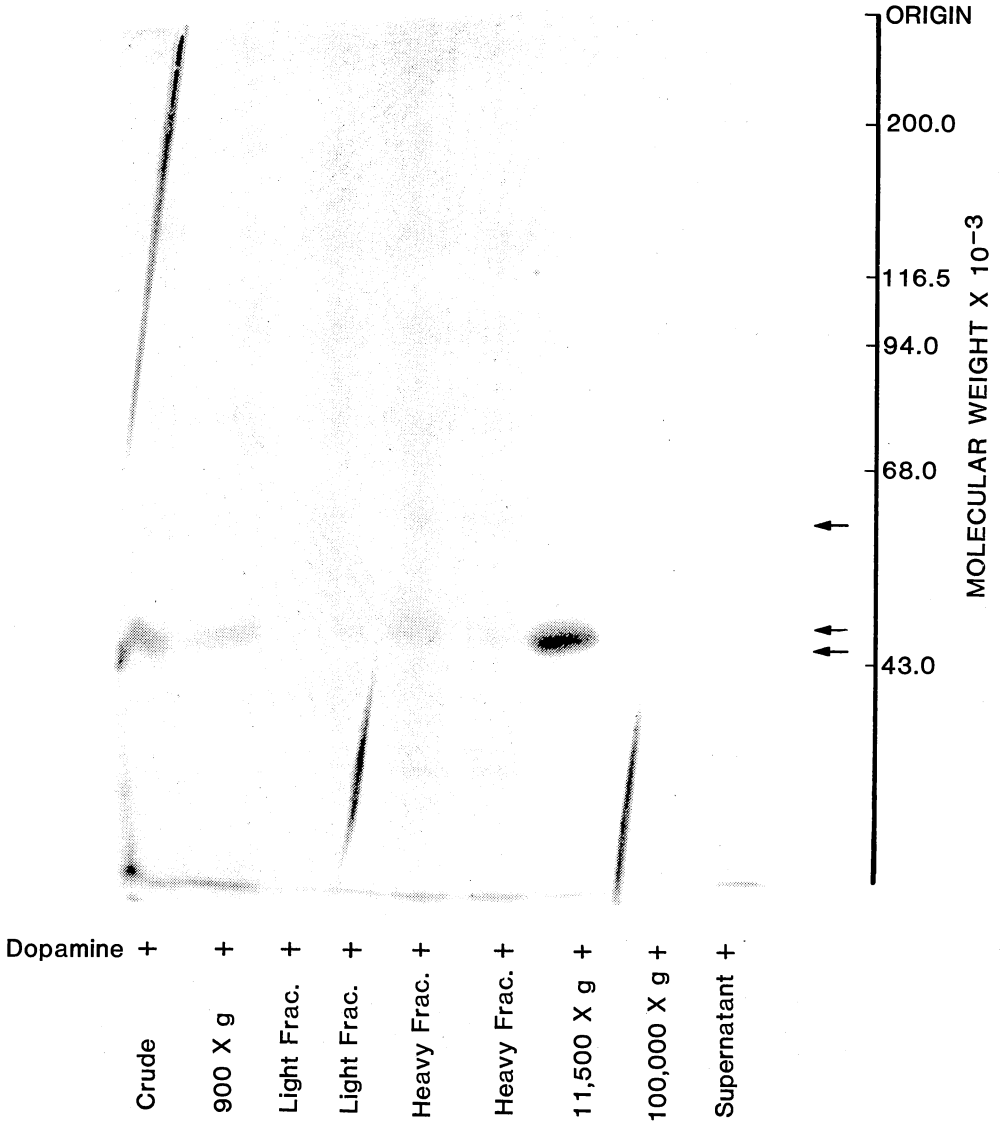

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X 10⁻³
Dopamine + + + + + + + + +
Crude
900 X g
Light Frac.
Light Frac.
Heavy Frac.
Heavy Frac.
11,500 X g
100,000 X g
Supernatant

Figure 9. Autoradiograph of electrophoresed proteins showing incorporation of [$\gamma$-$^{32}$P] ATP into endogenous proteins of subcellular fractions of tick salivary gland tissue. Phosphate incorporation was studied following incubation of subcellular fractions with (+) and without (-) cyclic AMP for 5 min. Proteins whose states of phosphorylation were affected by cyclic AMP are indicated by arrows.

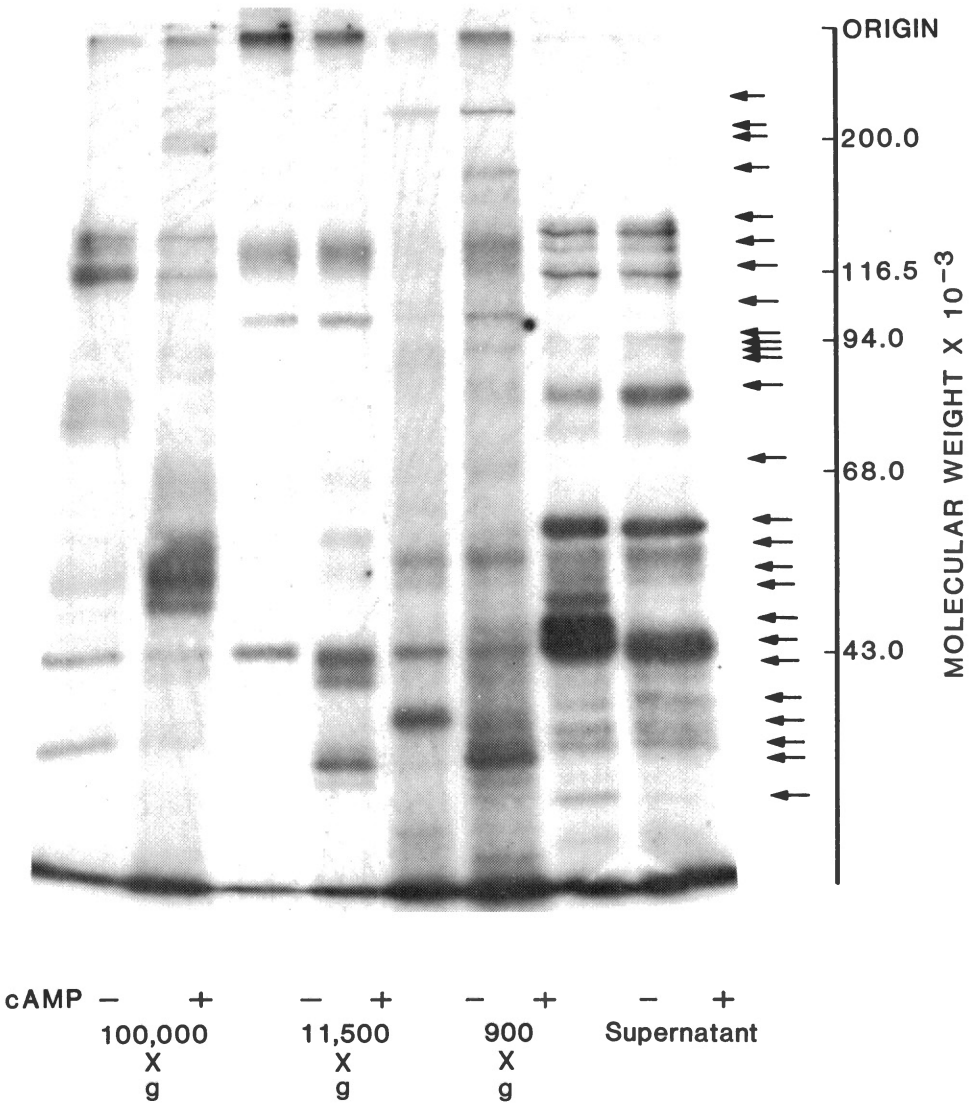
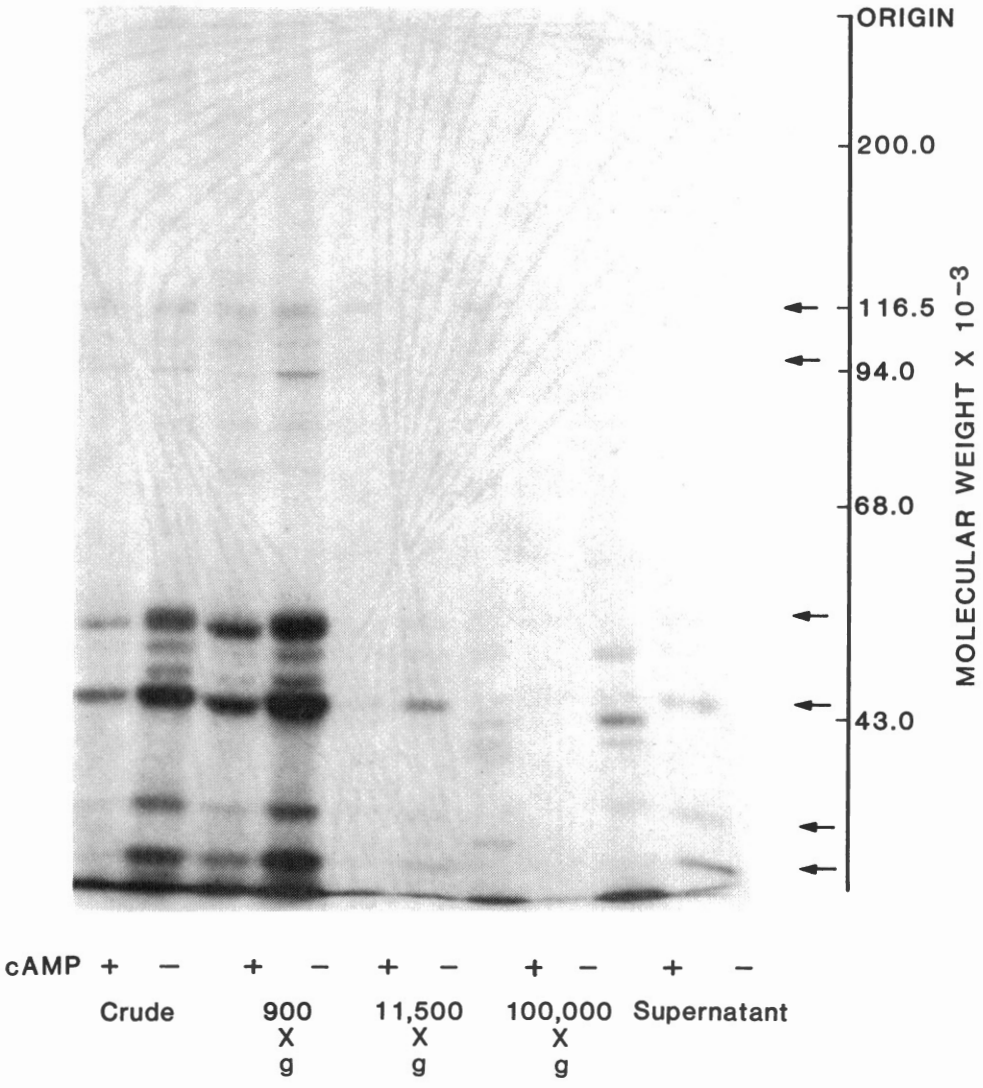

ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X $10^{-3}$
cAMP – + – + – + – +
100,000 X g
11,500 X g
900 X g
Supernatant

Figure 10. Proteins of subcellular fractions of tick salivary gland tissue labelled by a photoaffinity analog of cyclic AMP in the presence (+) and absence (-) of cold cyclic AMP. Arrows indicate proteins whose labelling by the analog was depressed by cold cyclic AMP.


ORIGIN
200.0
116.5
94.0
68.0
43.0
MOLECULAR WEIGHT X 10⁻³
cAMP + − + − + − + − + −
Crude
900 X g
11,500 X g
100,000 X g
Supernatant

VITA

Janis Lee Adams McSwain

Candidate for the Degree of

Doctor of Philosophy

Thesis: ROLE OF CYCLIC AMP MEDIATED PROTEIN PHOSPHORYLATION AND ITS RELATIONSHIP TO FLUID SECRETION IN SALIVARY GLANDS OF AMBLYOMMA AMERICANUM (L.)

Major Field: Entomology

Biographical:

Personal Data: Born in Jackson County, Altus, Oklahoma, September 17, 1944, the daughter of William and Rosa Adams.

Education: Graduated from Altus High School, Altus, Oklahoma, in May 1962; graduated from Wichita General Hospital School of Nursing in May, 1965; received the Bachelor of Science degree in Biology from Central State University in December, 1972; completed requirements for the Master of Science degree in Science Education at Oklahoma State University in July, 1976; completed the requirements for the Doctor of Philosophy degree at Okahoma State University in July, 1983.

Professional Experience: Research Assistant, Department of Entomology, Oklahoma State University, January, 1974 to December, 1982; Science Teacher, Morrison High School, September, 1977 to May, 1978; County School Nurse, Noble County, Oklahoma, September, 1974 to May, 1977; Registered Nurse, Baptist Memorial Hospital, Oklahoma City, Oklahoma, December, 1966 to May, 1968.

Societies and Organizations: Entomological Society of America; National Association for the Advancement of Science; Acarology Society of America; southwestern Association of Parasitologists.