ENDOCRINE CONTROL OF LONE STAR TICK, *Amblyomma americanum* (L.), SALIVARY GLAND DIFFERENTIATION

By

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SALIVARY GLAND DIFFERENTIATION

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PREFACE

Each part of this thesis is a separate and complete manuscript submitted for publication. Part I was published in 1987 in the journal Insect Biochemistry, volume 17(6), 883-890. Part II has been accepted for publication by the Journal of Insect Physiology and will be published early in 1989. Part III will also be submitted to Insect Biochemistry.

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

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I

BIOCHEMICAL DIFFERENTIATION OF LONE STAR TICK, *Amblyomma americanum* (L.), SALIVARY GLAND: EFFECTS OF ATTACHMENT, FEEDING AND MATING

ABSTRACT

Salivary glands of female Amblyomma americanum (L.) are stimulated to differentiate by attachment to a host, subsequent feeding and mating. Incorporation of [^{3}H]uridine into ribosomal and transfer RNAs as well as the synthesis of poly(A+)mRNA and protein parallel the pattern of increasing enzymatic activity and secretory ability of the glands. Unfed ticks contained 3.5±0.47 ng poly(A+)mRNA/gland pr. By the second day of feeding this had increased more than 5-fold. The greatest amount of poly(A+)mRNA found in rapid-feeding phase females (body wt>100 mg) was 370±80 ng/gland pr. Poly(A+)mRNA mass doubles on the final day of feeding, just as the ticks exceeded 100 mg in wt. Ticks attached 1 to 10 days had increasingly greater amounts of salivary monosomes, 60 and 40S ribosomal subunits and polysomes. Polysomal mass/gland pr also attained its maximum above 100 mg tick wt at the slow/rapid-feeding phase boundary; exceeding by 20 times that of unfed ticks. Degenerating glands from replete ticks continued to synthesize protein. In vitro incorporation of [^{3}H]leucine was greatest within 24 hr of attachment. Fluorographs of [^{3}H]leucine labeled protein showed that mating caused a drop in incorporation after the 4th day of feeding. Glands from unmated females attached the same number of days continued to incorporate [^{3}H]leucine at higher levels than those from mated females.

Key Word Index: Protein synthesis, tick salivary glands, ixodid ticks, salivary gland differentiation, RNA synthesis, polyribosomes, poly(A+)mRNA.

INTRODUCTION

Upon initiation of ixodid tick feeding, significant cytological changes are observed in the cells of salivary granular alveolar types II and III (Fawcett et al., 1986) (Fig. 1). Within two days an extensive network of rough endoplasmic reticulum is formed in these cells, some of which appears swollen, suggesting increased protein synthesis by the cell. Simple granular cells of alveolar types II and III form a basolateral labyrinth characteristic of fluid transporting epithelia (Fawcett et al., 1986; Claypool et al., 1987). The cells become enlarged and the alaveolar lumenae dilate, filling with salivary fluid. Although no increase in cell number occurs, the size, mass and protein content of the salivary gland increases about 25-fold during tick feeding (McSwain et al., 1982). The salivary glands gain competence to secrete fluid only after the onset of feeding. This ability is lost early in the postengoregment period, when the glands rapidly degenerate (Kaufman, 1976; Sauer et al., 1979).

Several new polypeptides appear in the glands soon after attachment to a host (McSwain et al., 1982). Other polypeptides, present in unfed ticks, increase in quantity during later stages of tick feeding. Mating stimulates additional feeding and an increase in the amounts but not the number of Coomassie blue stained polypeptides detected (McSwain et al., 1982). Almost all of the polypeptides that disappear do so after mating (i.e. 5-7 days post-attachment). More recently, the expression of salivary gland proteins and several glycoprotein antigens

fouund in the saliva of Hyalomma anatolicum have been found to increase with feeding (Gill et al., 1986). Expression of new salivary antigens by feeding A. americanum has also been demonstrated (Brown, 1986; Brown and Askenase, 1986; Needham et al., 1986).

The elaboration of secretory granules, the numerous changes in morphology, the appearance of new protein bands in polyacrylamide gels after the onset of feeding and increasing concentrations of antigenic proteins all suggest that expression of new genes is induced by feeding and mating stimuli. Little is yet known about the factors controlling macromolecular synthesis and differentiation during early feeding and mating events; nor are the effects upon pathogenic organisms which utilize the tick salivary gland as their route to the vertebrate host known. We report here changes in RNA and protein biosynthesis which occur during differentiation of salivary glands of feeding lone star ticks.

Abbreviations: SDS, Sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; HPRI, human placental ribonuclease inhibitor; MOPS, 3-(N-morpholino) propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; poly(A+)mRNA, polyadenylated messenger RNA; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials

5, 6-[^{3}H]polyuridylic acid was purchased from New England Nuclear (4.5 Ci/mmole UMP). [4, 5-^{3}H]leucine (54 Ci/mmol), [5-^{3}H]uridine (1 Ci/mmol) and Tissue Solubilizer 1 were purchased from Research Products International. HPRI, yeast tRNA, all enzymes, electrophoresis molecular weight standards, poly(A), oligo(dT)-cellulose and other chemicals were purchased from Sigma Chemical Co. Electrophoresis grade reagents were purchased from BioRad. HPLC grade solvents were purchased from Baker Chemical Co.

Animals

Adult female lone star ticks, A. americanum, were reared according to Patrick and Hair (1976). Larvae and nymphs were fed on rabbits and adults on sheep within surgical stockinette sleeves affixed to the skin of ovine hosts. Mating was prevented in some experiments by excluding males from these sleeves. To examine the effects of host proximity on possible salivary biosynthetic acstivity, in the absence of feeding stimuli, groups of females (with and without males) were constrained in screened plastic containers and positioned within sleeves such that feeding was impossible. These non-feeding females were thus subjected to the same temperature and olfactory stimuli as controls in adjacent sleeves (Samish, 1977).

Gland Preparations

Salivary glands were dissected into oxygenated TC-199/MOPS medium

(Needham and Sauer, 1979) and incubated with shaking in vitro at 23 or 37°C with or without isotope for various times. All further manipulations were performed at 4°C. Glands were rinsed three times with 1.5 ml of homogenization buffer (100 mM KCl, 10 mM Mg chloride, 0.1% (v/v) Triton X-100, 50 ug/ml heparin-sulphate, 100 ug/ml yeast transfer RNA, 10 mM Tris-HCl, pH 7.6) and homogenized by six rapid strokes of a Potter-Elvehjem tissue homogenizer. Intactness of nuclei after homogenization was confirmed by phase contrast microscopy. In later preparations 35 U/ml HPRI and 1 mM DTT were substituted for yeast tRNA. The homogenate was centrifuged for 10 min at 500 x g and 10 min at 10,000 x g. Postmitochondrial supernatant protein was determined by the Bradford (1976) method.

RNA Extraction, Fractionation and Measurement

In vitro incorporation of [5-^{3}H]uridine (1 Ci/mmol; 40 uCi/ml) into RNA was measured by incubating 25-50 gland prs each from unfed, 1, 2, 3 and 5-day fed ticks in TC-199/MOPS (Needham and Sauer, 1979) containing the isotope for 90 min at 23°C. The glands were homogenized and postmitochondrial supernatants were prepared, as above. In some experiments aliquots of the postmitochondrial supernatant were TCA precipitated. Total cytoplasmic RNA was isolated from salivary gland tissue by a modification of the method of Weigers and Hilz (1971). Postmitochondrial supernatants were made 0.5% SDS and digested 20 min at room temp with 0.15 ug/ml Proteinase K. The digest was extracted once with phenol-chloroform-m-cresol (9:9:2, by vol) containing 0.1% 8-hydroxyquinoline and twice with chloroform. Extractred RNA was made 200 mM sodium acetate (pH 5.2) and ethanol precipitated. Pelleted RNA

was washed twice with 70% ethanol to remove SDS.

Polyribosomal Preparations

Polysomes were prepared by a modification of the methods of Hosick and Daneholt (1974) and Bantle and Tassava (1974). Groups of 5-10 pairs of glands were homogenized in 1.5 ml of buffer and prepared, as above. In some experiments half of the post-mitochondrial supernatant was treated with 100 ug/ml puromycin-HCl, 10 min at room temperature (Hosick and Daneholt, 1974) or with 5 mM EDTA to dissociate polysomes. Both fractions were layered onto separate 15-40% (w/v) sucrose gradients (buffered with 100 mM KCl, 10 mM Mg chloride, 10 mM Tris-HCl, pH 7.6) and centrifuged 90 min at 208,200 x g_{ave}, 4°C. Absorbance (254 nm) profiles were continuously recorded using an ISCO UA-5 gradient fractionator during fractionation. Profiles were plotted and analyzed according to Duncan and McConkey (1982). Chick leg muscle polysomes were prepared separately (Bantle and Tassava, 1974) to show that intact undegraded polysome profiles could be prepared using this technique.

[^{3}H]Leucine Incorporation

Rates of *in vitro* protein synthesis in salivary glands of unfed and partially fed ticks were measured by incubating groups of glands in TS/15mM MOPS, pH 7.0 (Needham and Sauer, 1979) containing [^{3}H]leucine (54 Ci/mol; 500 uCi/ml) at 37°C. At the end of a 2 hr or shorter incubation the glands were sonicated in 0.5 ml of 10 mM Tris-HCl, pH 7.6. Supernatant from a 10 min centrifugation (12,000 x g) was precipitated with cold 10% TCA onto Whatman GF/C filters. The filtrate was collected and an aliquot counted to determine the amount of TCA soluble material.

Before addition of TCA, aliquots of supernatant were taken for

protein determination and for HPLC determination of leucine precursor pool specific activity, as described by Jones and Gilligan (1983). A Beckman Model 332 HPLC system equiped with an Altex 4.5 x 60 mm guard column packed with ODS 5 um C_{18} reverse phase particles was used. Amino acids were eluted with a stepped, 5-55% methanol gradient. External standards were injected at intervals to calibrate the instrument.

Electrophoresis and Fluorography

To determine sizes of pulse-labeled glandular proteins, two pairs of *in vitro* incubated glands from mated and unmated females allowed to feed for varying amounts of time were rinsed in 0.1 M STE, then lysed and boiled 2 min in 100 ul SDS buffer (Sin, 1982) before freezing. Samples were thawed and subjected to SDS-PAGE (King and Laemmli,1971). A mixture of six molecular weight electrophoresis standards was used: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDA), beta-galactosidase (116 kDa) and myosin (205 kDa). Fluorographs were prepared from the destained, PPO impregnated dried gels by exposing Kodak X-Omat AR film 1-4 days at $-70^{o}C$ (Bonner and Laskey, 1974).

RESULTS

RNA synthesis and measurement

Sucrose gradient absorbance (254 nm) profiles of extracted polysomal RNA showed little degradation (Fig. 2) A(260/280 nm) ratios of extracted RNA ranged from 1.9 to 2.05. To determine if the ability of salivary glands to synthesize RNA changed during the first five days of feeding we measured incorporation of [^{3}H]uridine into gland RNA. Groups of 25 gland prs from unfed and 2 day fed ticks were pulse-labeled *in vitro* for 90 min. After 1.5, 3 and 6 h of incubation virtually all radioactivity was found in the 4 to 5S region of sucrose density gradients; it was probably tRNA (Fig. 2). Newly synthesized 18 and 26S ribosomal RNAs were detectable after 3 and 6 h of pulse-labeling (intermediate in mobility betwen the rRNA standards from *E. coli* and mouse) (Fig. 2). We did not detect [^{3}H]U incorporation into salivary pol(A+)mRNA fractionated by oligo(dT)-cellulose chromatography in any of the stages of feeding examined (data not shown).

To bypass this difficulty poly(A+)mRNA present in glands at the time of dissection was measured by hybridizing the 3'-poly(A) tails of extracted, cytoplasmic poly(A+)mRNA with [^{3}H]poly(U). The size range of this mRNA on sucrose density gradients was 7-30S, consistent with mRNA-like macromolecules (Lewin, 1980). [^{3}H]poly(U) did not hybridize with RNA in the 4S region of the gradients, indicating that there was no detectable mRNA degradation (Fig. 3). By assuming that 3'-poly(A) tails comprised a constant 7% of the poly(A+)mRNA mass during differentiation

(Bantle and Hahn, 1980; Lewin, 1980) we calculated that unfed ticks contained an average of 3.5±0.47 ng poly(A+)mRNA/gland pr (n=4). By the second day of feeding this amount had increased more than 5-fold to 19±0.80 (Fig. 4). The greatest amount of gland poly(A+)mRNA was found in rapidly feeding females (body wt>100 mg), which averaged 370±80 ng/gland pr. Replete, detached ticks showed a decline in poly(A+)mRNA activity to 280±28 ng/gland pr (R1 on Fig. 4). The ratio of poly(A+)mRNA to protein remained constant in feeding ticks (data not shown) suggesting that the synthesis of new protein increased at the same rate as that of mRNA. Total cytoplasmic RNA (pRNA) extracted from glands increased at a smaller rate from 0.60±0.10 ug RNA/gland pr in unfed gland to a high of 5.2±0.68 ug/gland pr in glands from replete females (Fig.4). The percentage of poly(A+)mRNA in extracted cytoplasmic RNA increased 4-fold from 0.54% in glands from unfed ticks to 2.1% after just one day of feeding. This percentage increased to 4.8 % in glands of rapid-feeding phase ticks (body wt>120 mg).

Polyribosomal Aggregation

Possible problems measuring precursor pool sizes and compartmentation often make the interpretation of isotope incorporation experiments difficult. For this reason we measured the mass of active, aggregated polyribosomes, which represented a direct measure of glandular protein synthesis. The mass of polysomes isolated from the salivary gland directly reflects the rate of protein synthesis taking place in the gland. Preliminary experiments using the method of Bantle and Tassava (1976) showed little ribonuclease activity. Unfed ticks had almost undetectable levels of salivary polysome aggregation. Ticks fed

for increasingly longer periods of time had proportionally greater amounts of monosomes as well as polysomes compared to unfed ticks (Figs. 5A and B). The increase in the level of 60 and 40S ribosomal susbunits throughout feeding, as well as an increasing number of monosomes was also evident (Fig. 5A). In this experiment ticks had entered the rapid-feeding phase by the 10th day of feding and were replete by day 11. Polysomal mass/gland pr of 10 day fed, mated ticks exceeded by 20 times that of unfed ticks, 0.21 ± 0.023 A(254 nm)/gland pr vs $9.8 \times 10^{-3} \pm 2.8 \times 10^{-3}$ A(254 nm)/gland pr (Fig. 6). Half of this increase occured after the ninth day of feeding.

The percentage of aggregated ribosomes in the polysomal region of the gradient was highest during the rapid-feeding phase. Ticks fed 10 days atained 70% aggregation; percent aggregation was 50% for all other stages. Polysome mass from one day host-replete females (R1 on Fig. 6) returned to about the level found in 9-day fed ticks. Three day post-replete ticks (R3 on Fig. 6) showed only a slight decline from R1, and had the same polysomal mass as 7-day fed ticks. If mating was prevented, weight gain (feeding) was retarded but polysome aggregation was maintained at a relatively high level. Unmated ticks fed 6 days weighed only slightly less than mated females but had a significantly higher ($P<0.01$, $n=4$) average polysome mass of $7.7 \times 10^{-2} \pm 0.8 \times 10^{-2}$ A(254 nm)/gland pr (6U on Fig. 5). Unmated ticks removed from the host after 11 days of attachment (11U on Fig. 5), coinciding with the time when mated ticks have already attained their full replete weight and dropped from the host (R1 on Fig. 5), contained a larger average polysomal mass of 0.14 ± 0.020 A(254 nm)/gland pr ($P<0.05$, $n=3$) than mated

controls.

Polysomes could not be disaggregated by the chain terminator puromycin, indicating that under the experimental conditions used all polysomes were translationally arrested (Hosick and Daneholt, 1974). Polysomes were, however, disaggregated by 5 mM EDTA. To determine whether the incubation conditions used were causing disaggregation, polysomes were extracted from 20 glands each from several stages of feeding. Paired glands from the same ticks were incubated with [^{3}H]leucine; polysomes were extracted and displayed. The A(254 nm) polysome profiles of control and incubated glands were identical after two hrs of incubation in all stages examined. Incorporated label from fractions of the gradients was precipitated with 10% TCA. The majority of TCA precipitable counts was found at the top of gradients; these were completed polypeptides, pulse-labeled during the incubation, and did not sediment through the gradient (Duncan and McConkey, 1982).

[^{3}H]leucine Incorporation

The extent of [^{3}H]leucine incorporation into TCA insoluble proteins did not parallel changes in salivary gland polysomes. Polyribosome profiles of unfed ticks show almost undetectable aggregation (Figs. 5 and 6), yet glands of this stage incorporated [^{3}H]leucine to an extent equal to glands with six to 10 times more polysomal mass. Labeling experiments showed that [^{3}H]leucine uptake was equal in all stages of feeding up to 4 days (Fig 7), and that intracellular pools equilibrated with exogenous label within 1 h (Fig. 7). HPLC analysis of gland amino acids showed that the level of all amino acids increased markedly with feeding, notably glutamine.

When [^{3}H] leucine incorporation into TCA insoluble protein was corrected for dilution by cold endogenous leucine a sharp increase in synthetic activity within 24 hr of attachment was seen, followed by a return to lower levels of synthesis (Fig. 8). No appreciable amount of TCA precipitable [^{3}H]leucine was found in the spent medium at the end of incubation. Thus, the possibility that [^{3}H]leucine labeled proteins were secreted into the medium and lost from homogenates was unlikely with the conditions used.

Fluorography of [^{3}H]leucine pulse-labeled proteins

Incorporation of [^{3}H]leucine into gland pairs dissected from female ticks fed in the presence of males was most visible in fluorographs on the second, third and fourth days post-attachment (2, 3, 4 on Fig. 9A). The M_r of the most intensely labeled bands were 122,000, 97,600, 47,000, 43,000, 33,000 and 30,000 daltons. The apparent synthetic ability of *in vitro* incubated glands of mated females fed for more than 4 days was much less than those from 1 to 4 day fed ticks (5, 7, 10 on Fig. 9A). At three days post engorgement replete ticks showed no visible incorporation (R3 on Fig. 9A) despite continued high polysomal aggregation (R3 on Fig. 6). In contrast to the mated ticks, glands from unmated ticks on the fourth, fifth, seventh and 10th day post attachment still incorprated [^{3}H]leucine into bands visible on fluorographs (4, 5, 7, 10 on Fig. 9B).

We placed unfed females on ovine hosts in screened containers for one to two days such that they were unable to attach and feed. They did not respond to the raised temperature or olfactory stuimuli by initiating synthesis of feeding-associated salivary proteins (HE on Fig.

9C), than did controls allowed to feed on the same host for the same period (0,1 on Fig. 9C). The presence of unfed males in the same container did not effect [^{3}H]leucine incorporation into glands of host exposed females.

DISCUSSION

After attachment to sheep in the laboratory, the female lone star tick undergoes a slow phase of feeding lasting 8 to 14 days during which its weight increases from 4 to 300 mg, followed by a rapid phase lasting 12-24 h during which the replete weight may reach 500-1000 mg (Sauer et al., 1979). In the absence of mating, the female tick will not increase in weight beyond approximately 35 mg (McSwain et al., 1982). Feeding causes an incarease in rough endoplasmic reticulum (Claypool et al., 1987), the appearance of new proteins in the glands and an increase in total gland protein (McSwain et al.; Schramke et al., 1984). Further, the expression of many new antigens in gland homogenates occurs within the first 4 to 6 days of feeding in A. americanum (Needham et al., 1986; Brown, 19867; Brown and Askenase, 1986) and in Hyalomma anatolicum (Gill et al., 1986). These results suggest that feeding initiates salivary gland protein synthesis.

For protein synthesis to increase there must be ample quantities of messenger (mRNA), ribosomal (rRNA) and transfer (tRNA) synthesized and these components must be aggregated into active polysomes (Boshes, 1970). Maximum protein content of the glands was attained by ticks above 100 mg in weight (Fig. 6), followed by maximum cyclic AMP stimulated secretory capability at 300-400 mg (Schramke et al., 1984; Sauer and Essenberg, 1984). Maximum Na/K-ATPase activity in A. hebraeum salivary glands occurs here also (Kaufman et al., 1976).

Synthesis of poly(A+)mRNA (Figs. 4 and 5) and protein (Figs. 6

and 9) parallel the pattern of increasing enzymatic activity and secretory ability. Synthesis of rRNA, tRNA and 60S and 40S ribosomal subunits indicates de novo synthesis after the stimulation of feeding; it appears unlikely that feeding associated mRNAs are synthesized and stored before attachment to a host. An unused pool of ribosomal subunits is not present in unfed glands (Fig. 5A). Polysome mass and poly(A+)mRNA accumulate most rapidly during the rapid-feeding phase, doubling in mass within the 24 h just prior to repletion (body wt>500 mg) (Figs. 5 and 9). Synthesis of proteins is evident for the maximal rate of secretion seen in the rapid phase of feeding, but less so for gland autolysis which begins soon after repletion. Polysome mass and total protein/gland pr drop precipitously after repletion (Fig. 9). Synthesis continues, albeit at a reduced rat (R1, R3 on Fig. 6). Poly(A+)mRNA mass/gland pr also declines after repletion (R1 on Fig. 4); but may still be present in sufficient quantity to encode proteins necessary for autophagy.

The decrease in in vitro [^{3}H]leucine incorporation into glands taken from ticks after four days of feeding (Fig. 9A) may reflect the absence of a necessary stimulatory factor in the medium. The endogenous pool of amino acids may be diluted significantly by an influx of cold, unlabeled residues from the bloodmeal in late stages of feeding. Unmated females do not take as large a bloodmeal and thus would not saturate endogenous leucine pools to the extent that mated, near replete females do. Polysome mass/gland pr from unmated ticks is actually elevated above the level of mated females on the same day of feeding (6U, 11U on Fig. 6), thus [^{3}H]leucine incorporation is somewhat higher

in the former. This effect can be seen on 10 day fed, unmated vs mated ticks (Fig. 9A and B).

Pathogens present in the tick may become activated by the same regulatory factors used by the tick during differentiation (Van der Ploeg et al., 1985). Samish (1977) found that incubation of infected adult H. excavatum at 37°C or on the host increased the infectivity of Theileria annulata without a bloodmeal stimulus. Kocan et al. (1982) also noted that infectivity of Anaplasma marginale in tick gut homogenates was greatly increased when nymphal D. andersoni were first incubated at 37°C for 2.5 days. To determine if temperature or host proximity induces differentiation-specific polypeptides in A. americanum females we placed unfed, unmated females on ovine hosts for one to two days such that they were unable to attach and feed. Glands from control and host exposed females were pulse-labeled with [^{3}H]leucine in vitro. Host exposed females do not respond to the raised temperature or olfactory stimuli by initiating synthesis of feeding-associated proteins (HE on Fig. 9C), as do controls fed concurrently on the same host (1 on Fig. 9C). The presence or absence of males does not affect this outcome. Perhaps increased temperature upon attachment, in conjunction with bloodmeal components, and mating are primary cues which affect neural and/or endocrine factors that induce differentiation of both the tick and salivary glands and the pathogens they sometimes harbor.

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Figure 1. Diagram illustrating the role of the salivary glands during tick feeding. Secretions include "cement" to help anchor the mouthparts to the host and excess fluid from the concentrated bloodmeal (mostly in the form of excess Na, Cl and water). The glands also secrete anti-hemostatic, antiinflammatory and immunosuppressive materials into the feeding lesion (Ribeiro et al., 1985; 1986). Nevertheless, some salivary products are antigenic and induce resistance to tick feeding by the vertebrate host (Brown and Askenase, 1986; Gill et al., 1986). Most of the disease organisms vectored by ticks are inoculated into the host body by direct introduction of the agent with tick saliva (Sauer, 1977). Adapted from Needham and Sauer (1979).

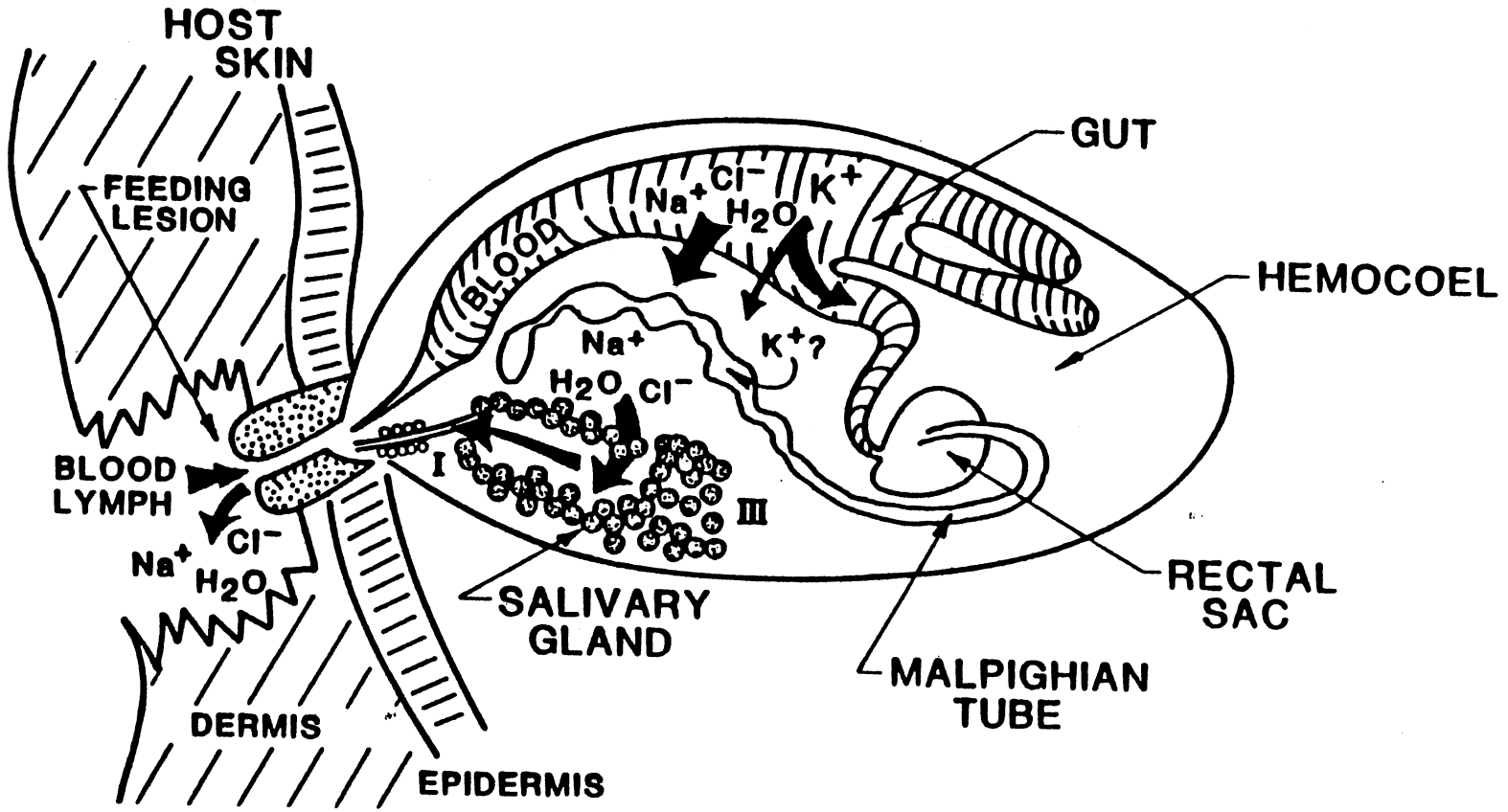

HOST
SKIN
FEEDING
LESION
BLOOD
LYMPH
Cl⁻
Na⁺
H₂O
DERMIS
EPIDERMIS
BLOOD
Na⁺ Cl⁻ K⁺
H₂O
GUT
HEMOCOEL
Na⁺
H₂O Cl⁻
K⁺?
I
III
SALIVARY
GLAND
MALPIGHIAN
TUBE
RECTAL
SAC

Figure 2. Size distrubution of [^{3}H]uridine *in vitro* labeled salivary gland cytoplasmic RNA from ticks fed 2days. Extracted RNA was layered onto a 5-20% (w/v) sucrose gradient and centrifuged 7.5 hr at 208,200 g_{ave}. (■) 1.5, (●) 3 and (▲) 6 h pulse label. (---) Absorbance profile, 254 nm. The large A 254 nm seen in the upper 4-5S region of the gradient was due to unlabeled yeast tRNA added to inhibit ribonuclease.

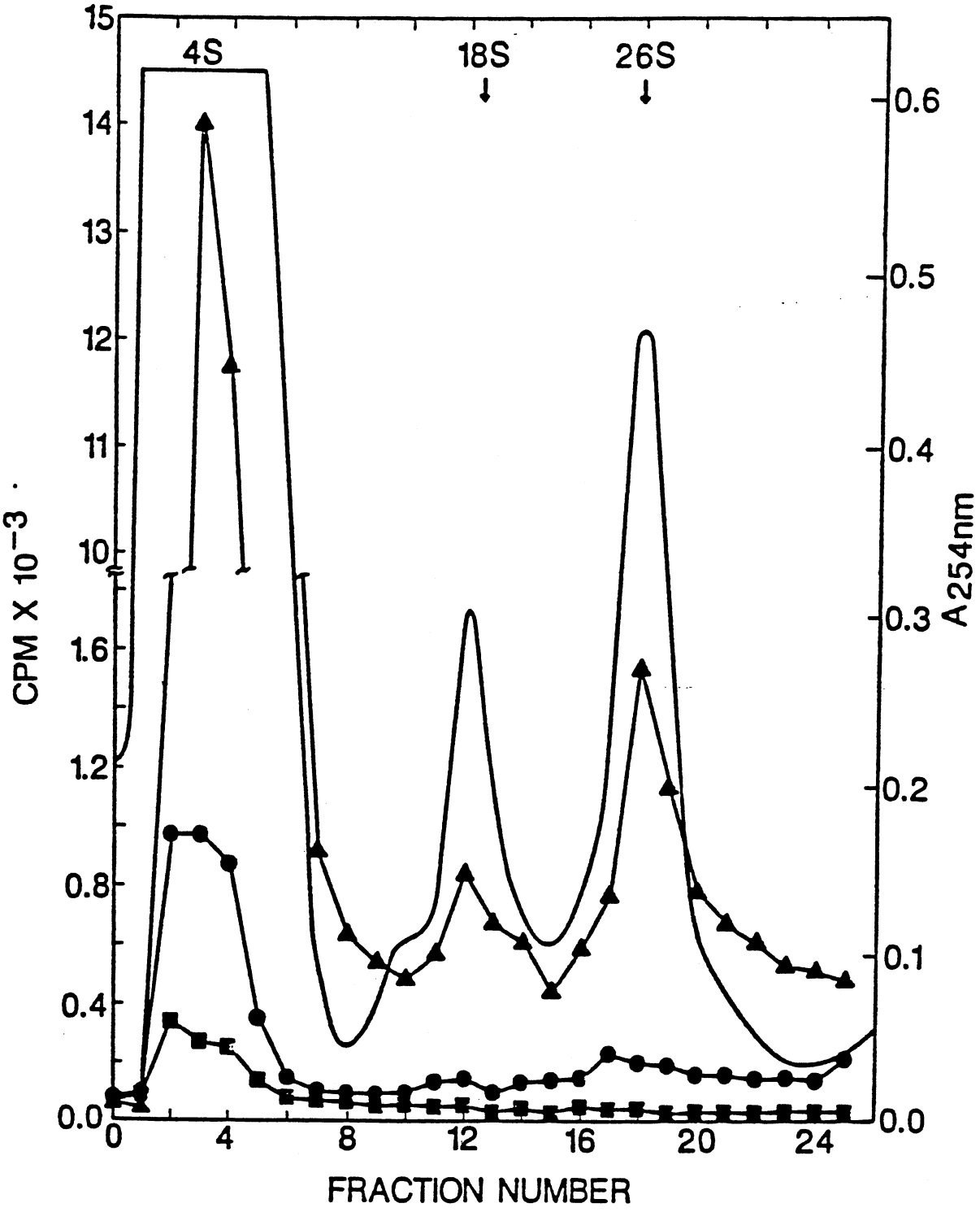

4S
18S
26S
CPM X 10⁻³
A254nm
FRACTION NUMBER
0
4
8
12
16
20
24
0.0
0.4
0.8
1.2
1.6
10
11
12
13
14
15
0.1
0.2
0.3
0.4
0.5
0.6

Figure 3. Size distribution of salivary gland poly(A+)mRNA extracted from 25 gland prs. Fractions from sucrose density gradients like those in Fig. 1 were hybridized with [^{3}H]poly(U) and treated with RNase A (See Materials and Methods). (■), Unfed tick. (◆), 2 day fed tick.

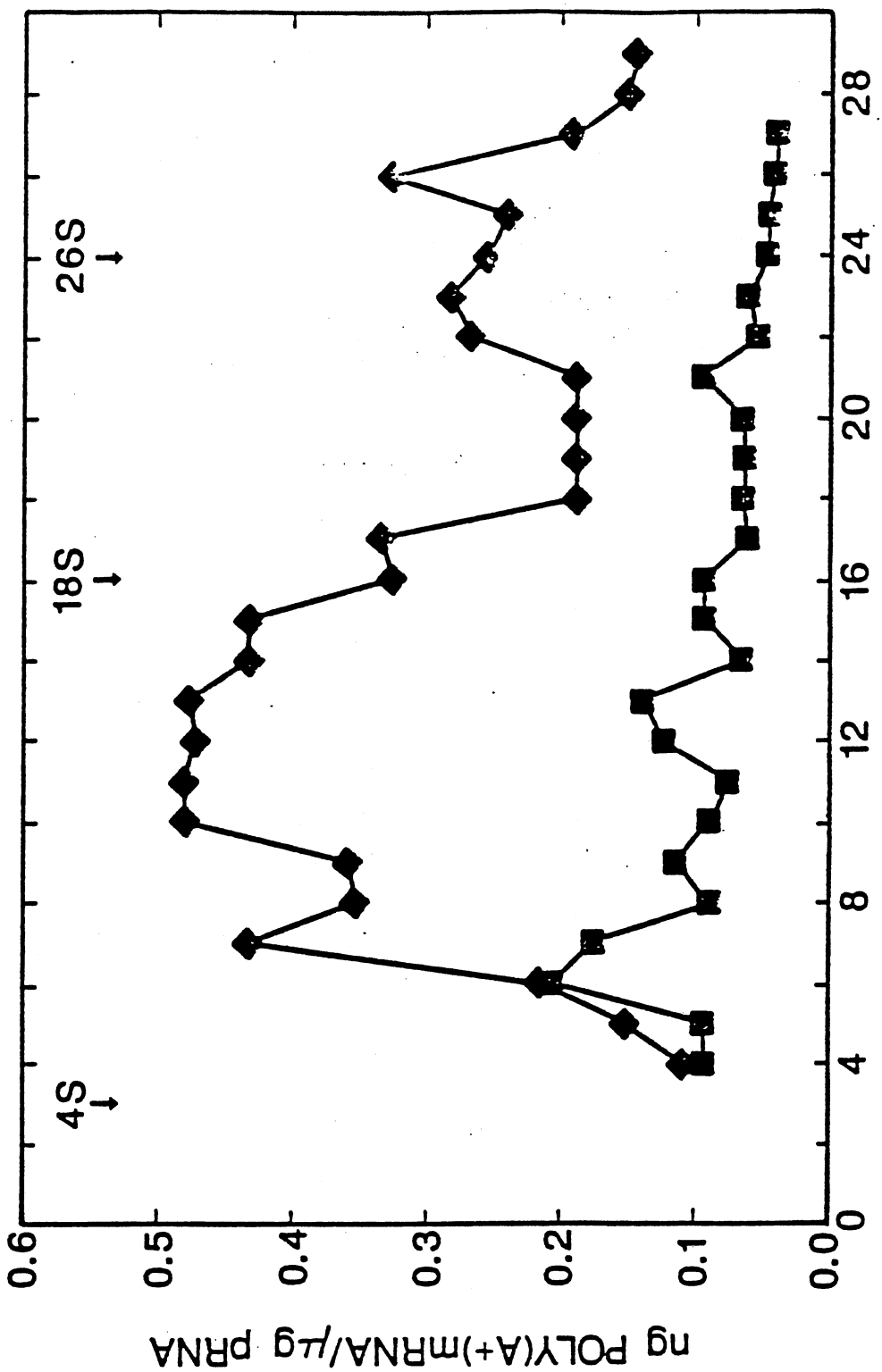

4S
18S
26S
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
4
8
12
16
20
24
28
ng POLY(A+)mRNA/μg pRNA
FRACTION NUMBER

Figure 4. Salivary gland polysomal and poly(A+)mRNA as a function of tick wt. (●), ng poly(A+)mRNA/gland pr as a function of tick wt. Mass estimated by [^{3}H]poly(U) hydbridization to 3'-poly(A) tails of mRNA. Numbers adjacent to points correspond to days on host (all points mean±SEM, n=3-6). R1, one day post-repletion. (▲), ug polysomal RNA extracted/gland pr.

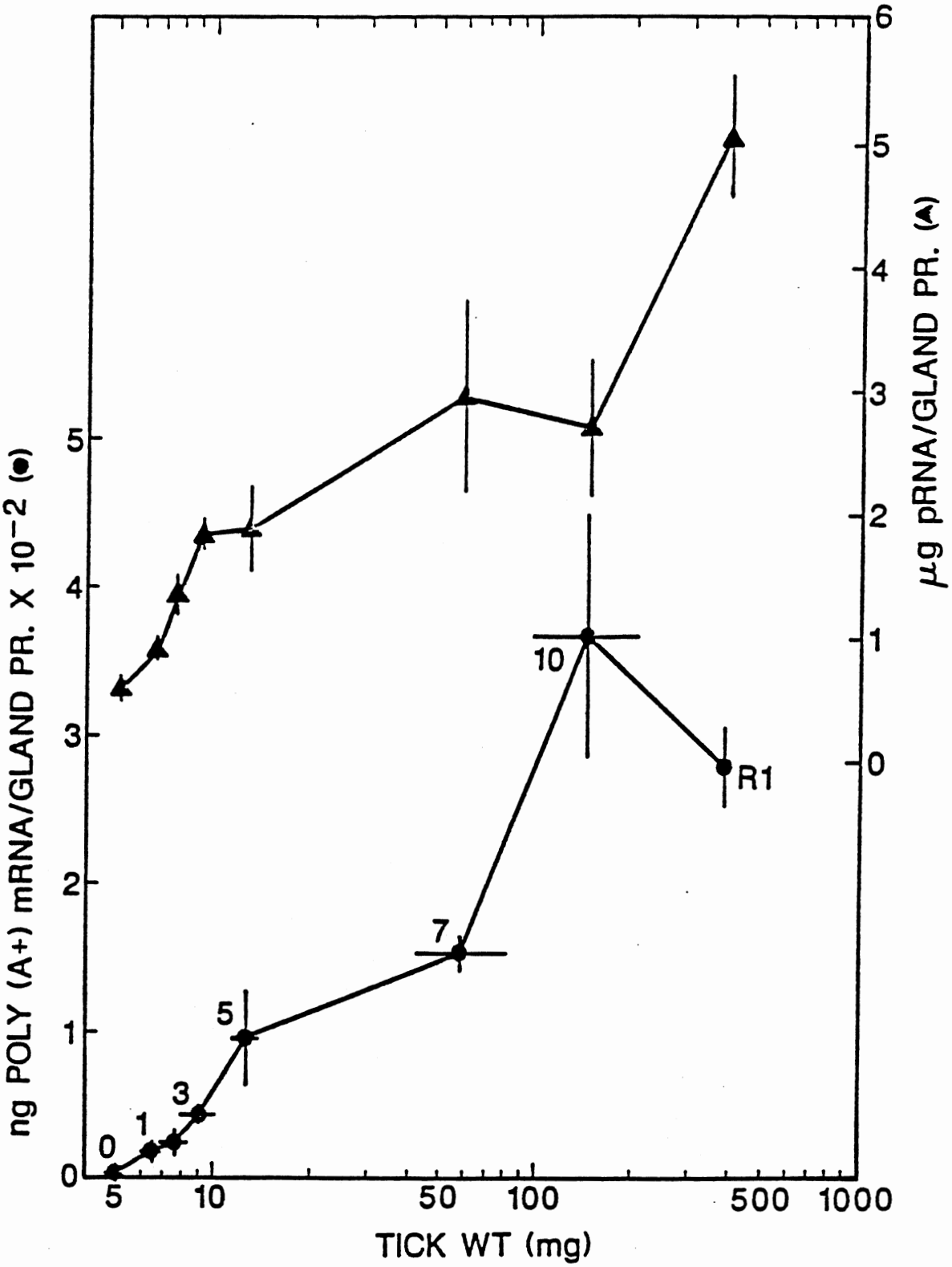

ng POLY (A+) mRNA/GLAND PR. X 10^{-2} (●)
μg pRNA/GLAND PR. (▲)
TICK WT (mg)
0
1
3
5
7
10
R1
5
10
50
100
500
1000
0
1
2
3
4
5
6

Figure 5. Polyribosomal profiles from feeding and nonfeeding salivary glands. Panel A. Salivary monosomal profiles A(254 nm) peaks from 0, one, three, seven, nine and 10 day fed ticks, shown in ascending order. Panel B. Polysomal profiles from same scans.

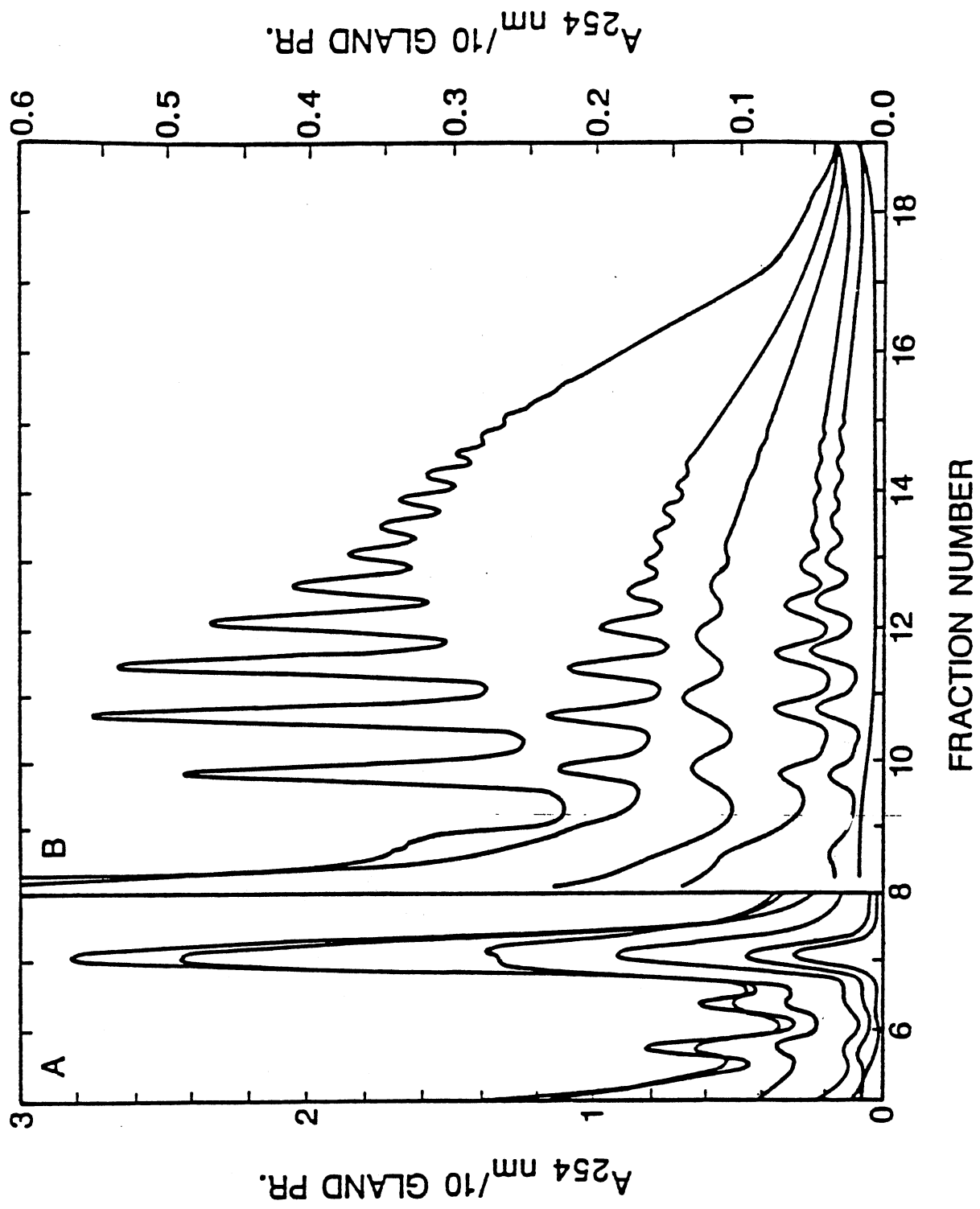

A
B
$A_{254\ nm}$/10 GLAND PR.
$A_{254\ nm}$/10 GLAND PR.
FRACTION NUMBER
0
1
2
3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
6
8
10
12
14
16
18

Figure 6. Increase in salivary gland polysome mass A(254 nm) and protein content as a function of tick wt. Numbers adjacent to points correspond to days fed on host (all points, mean±SEM, n=3-5). (●), polysomal A(254 nm)/gland pr., mated females. (○), polysomal A(254 nm)/gland pr, unmated females; 6U, sixth day of feeding, unmated; 11U, 11 days fed, unmated. R1, one day post repletion, mated. R3, three days post repletion, mated. (▲), postmitochondrial supernatant ug protein/gland pr, mated. (△), postmitochondrial supernatant ug protein/gland pr, unmated.

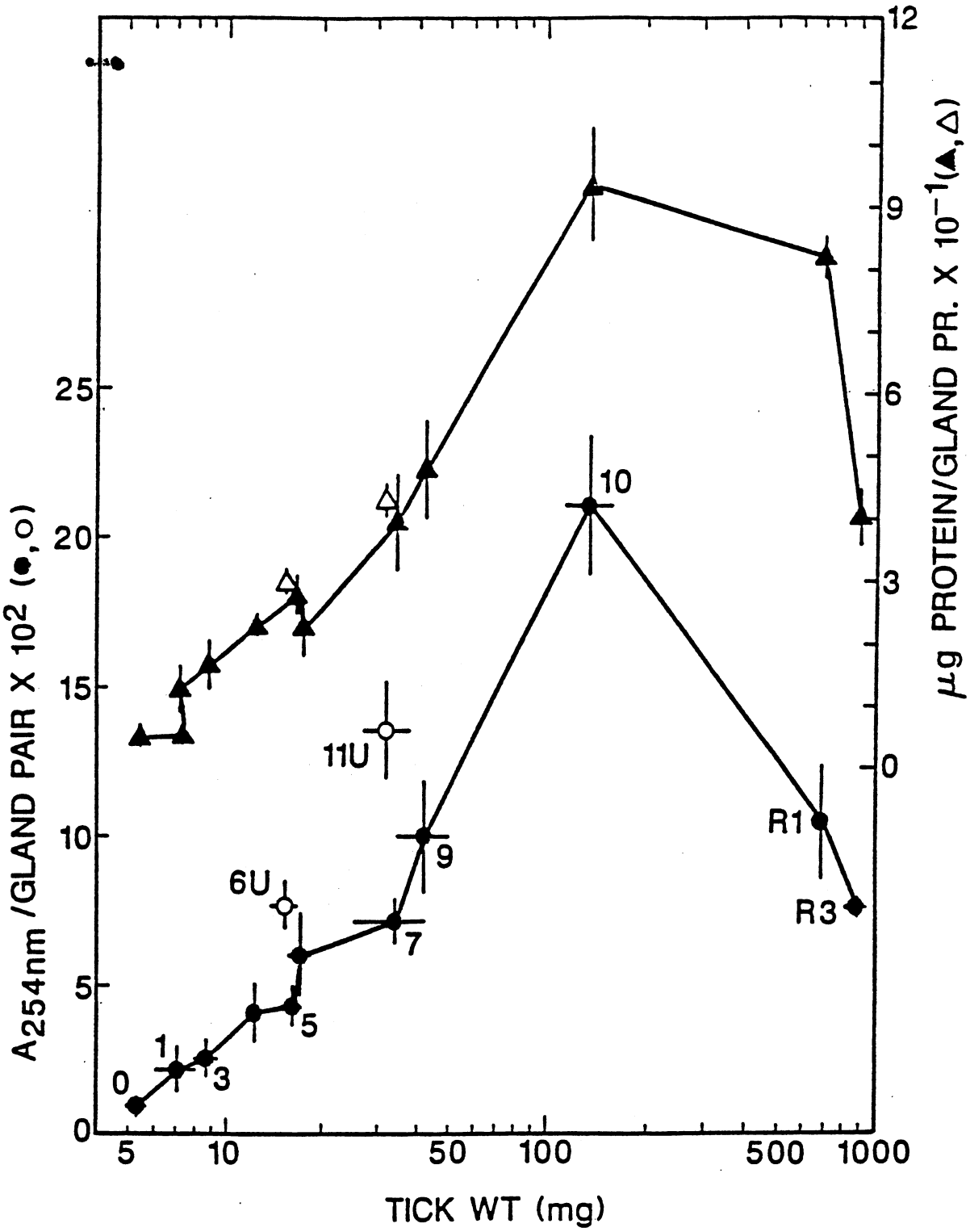

12
9
6
3
0
25
20
15
10
5
0
5
10
50
100
500
1000
TICK WT (mg)
A254nm /GLAND PAIR X 10^2 (●,○)
μg PROTEIN/GLAND PR. X 10^-1(▲,△)
0
1
3
5
6U
7
9
10
11U
R1
R3

Figure 7. Uptake and incorporation of [^{3}H]leucine into salivary glands after different incubation periods *in vitro*. Total cellular dpm (including TCA insoluble and soluble material)/gland pr. Groups of 5 gland prs from unfed (◆), two (■) and 4 day (▲) fed ticks were pulse-labeled 2 hr *in vitro* (See Materials and Methods). (Bars, mean ± SEM, N=4).

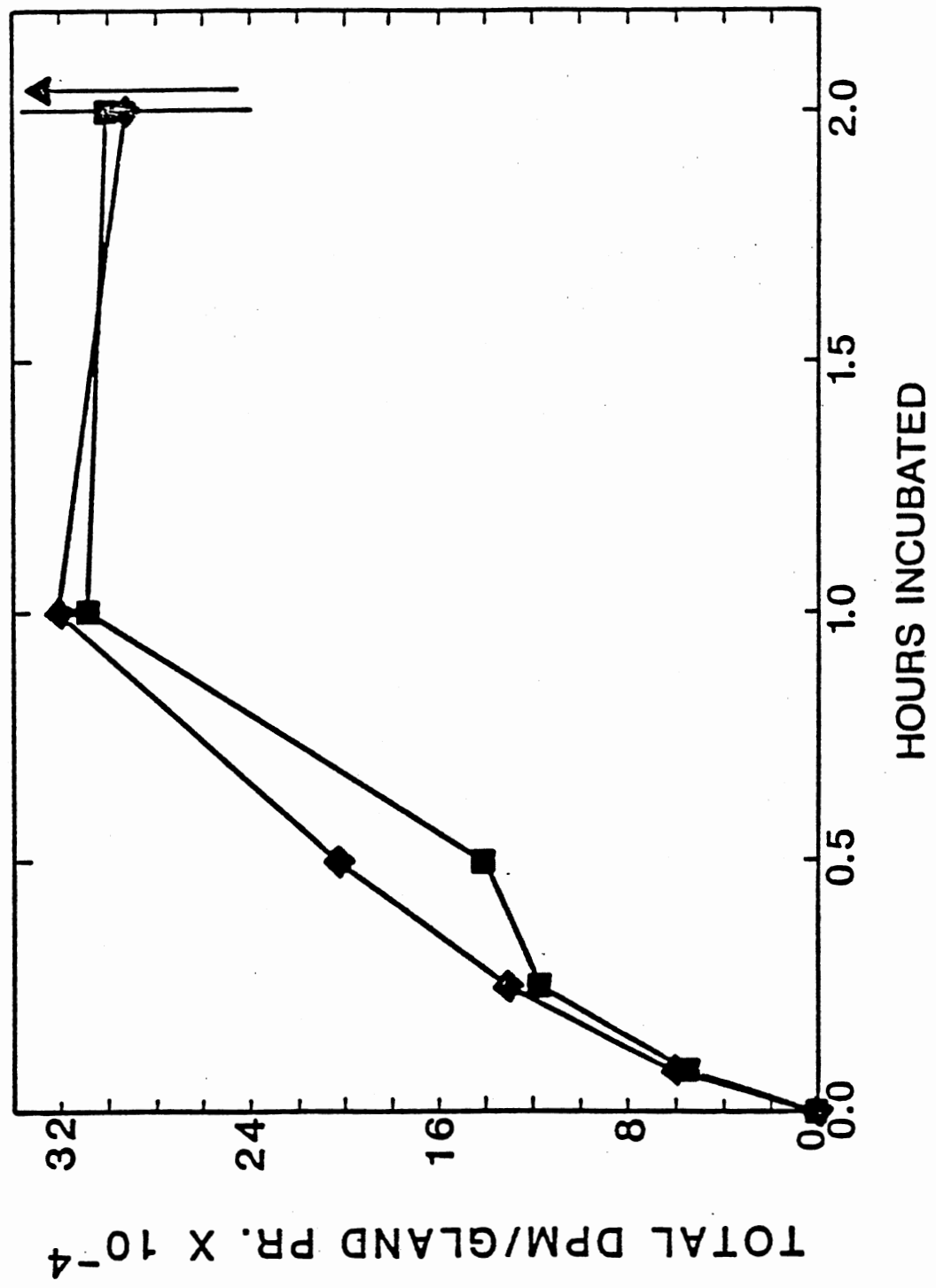

TOTAL DPM/GLAND PR. X 10⁻⁴
32
24
16
8
0
0.0
0.5
1.0
1.5
2.0
HOURS INCUBATED

Figure 8. [^{3}H]leucine incorporation into tick salivary gland protein corrected for variation in leucine pool size (mean±SEM, N=4). Groups of 5 gland prs from unfed, one, two, three and four day fed ticks were puse-labeled 2 hr *in vitro*.

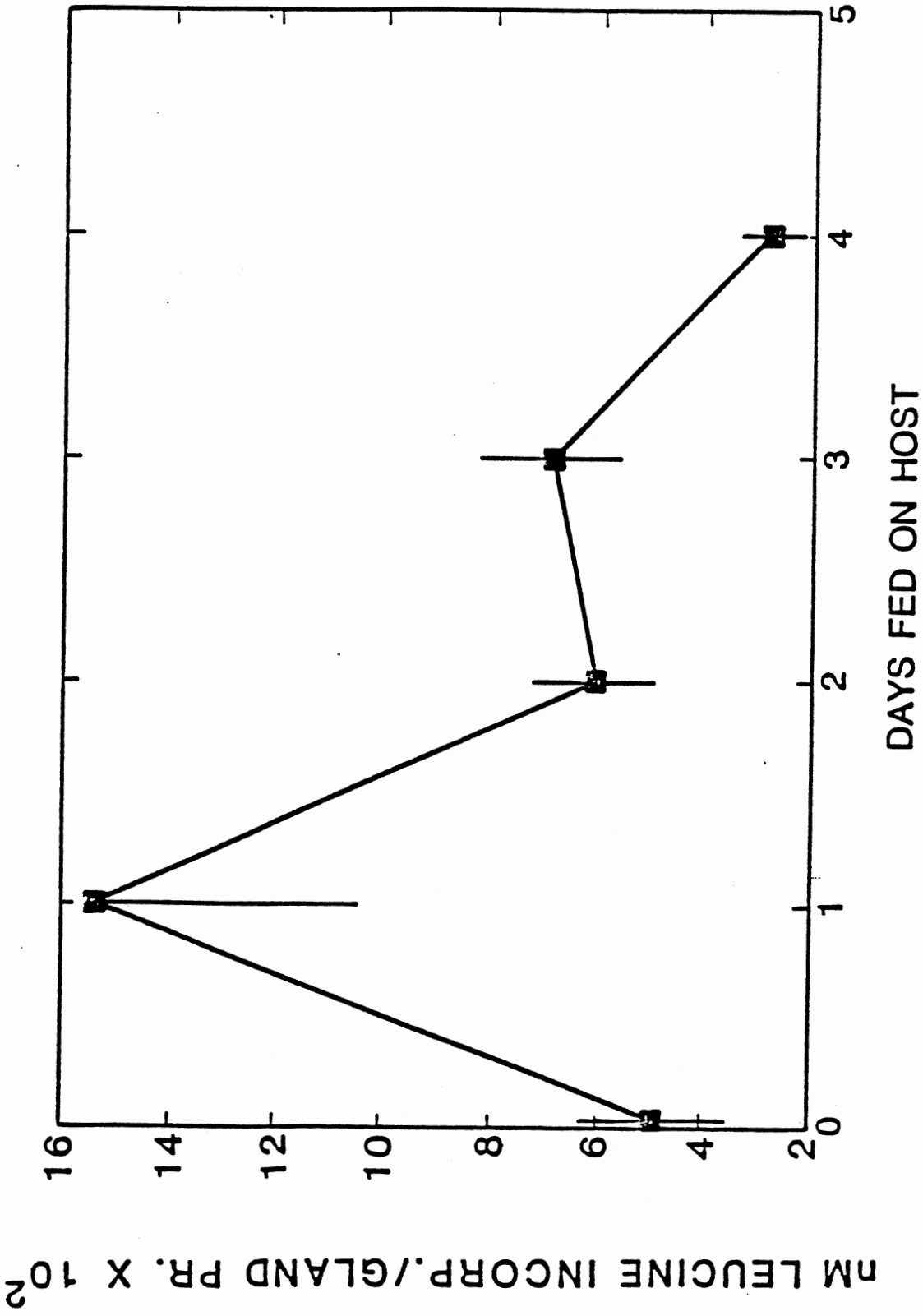
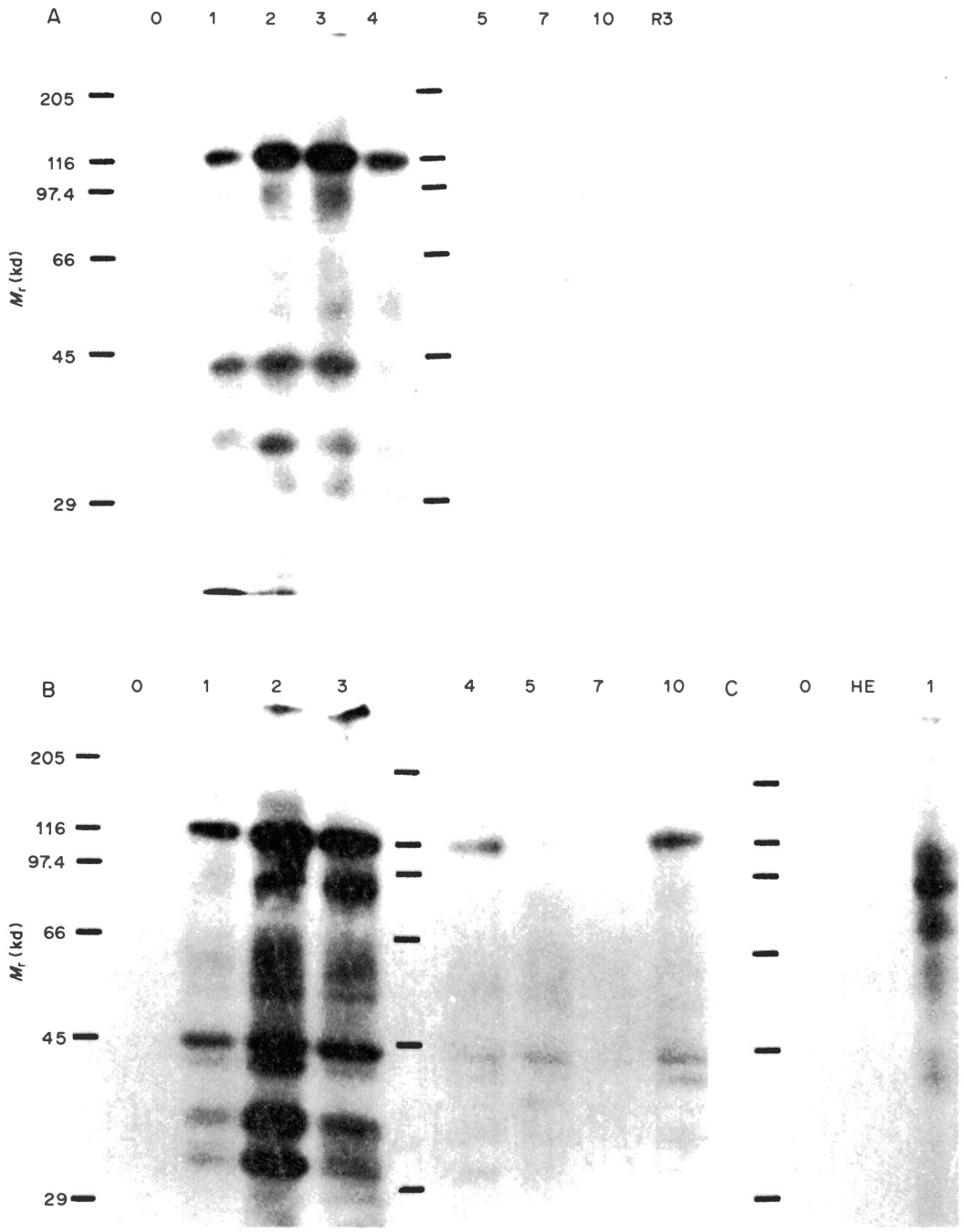

nM LEUCINE INCORP./GLAND PR. X 10^2
16
14
12
10
8
6
4
2
0
1
2
3
4
5
DAYS FED ON HOST

Figure 9. Effect of feeding and mating on [^{3}H]leucine incorporation by salivary glands. Numbers along top of panel indicate number of days fed on the host. Panel A. Fluorograph of labeled proteins present in glands of mated, feeding ticks (four gland pr equivalents per lane). Panel B. Fluorograph of unmated, feeding tick salivary proteins. Panel C. Effect of exposure to host of [^{3}H]leucine incorporation (2 gland pr equivalents per lane). 0, unfed females incubated at room temperature; HE, females exposed to host 24 hr, but prevented from feeding; 1, females allowed to feed on same host for 1 day. In this experiment all ticks were replete by day 12.


A
0
1
2
3
4
5
7
10
R3
205
116
97.4
66
45
29
M_r (kd)
B
0
1
2
3
4
5
7
10
C
0
HE
1
205
116
97.4
66
45
29
M_r (kd)

PART II

EFFECTS OF METHOPRENE AND 20-HYDROXYECDYSONE ON SALIVARY GLAND DEVELOPMENT OF LONE STAR TICK, *Amblyomma americanum* (L.)

ABSTRACT

Topically applied juvenile hormone III analogs 7-S-methoprene and 7-S-hydroprene (50 ug/tick) modestly stimulated attached virgin Amblyomma americanum females to feed and attain average body weights of 27.1 mg (178 % of controls, just 4 % of mated controls); however, tick salivary gland protein levels were stimulated 133 %, and into the range of mated, early-rapid feeding female ticks (>100 mg body weight). Na/K-ATPase activity was also increased to levels seen in salivary glands of mated, early-rapid feeding ticks (212 % of controls) by methoprene. Electron microscopy of salivary glands from treated ticks revealed euchromatic nuclei, partially swollen alveolar lumina, proliferation of basolateral labyrinth and increased rough endoplasmic reticulum, morphological changes normally seen in later feeding stages of mated females, but not in virgins. Treatment of attached, feeding females with 100 ug 20-hydroxyecdysone (20-HOE)/tick also stimulated virgins to gain additional weight (195 %) and total salivary gland protein (144 %), but not Na/K-ATPase activity. Methoprene stimulated protein level was not potentiated by additional treatments with 20-HOE. Tick salivary glands are thus partially, but differentially activated by methoprene and 20-HOE treatment while pre-mating weight arrest (feeding) is released only slightly.

Key Word Index: Tick salivary gland, Methoprene, Hydroprene, 20-hydroxyecdysone, Na/K-ATPase, differentiation.

INTRODUCTION

The salivary glands of ixodid ticks undergo significant ultrastructural and biochemical change after attachment to the vertebrate host and feeding begins (McSwain et al., 1982; Barker et al., 1984; Sauer et al., 1986; Fawcett et al., 1986; Shelby et al., 1987). Control of ixodid tick salivary gland differentiation during feeding seems to be modulated by three separate sets of stimuli:

First, at the onset of feeding, the glands rapidly increase in size and secretory ability. After 3-7 days of feeding, alveolar types II and III continue to increase in size, contain more numerous secretory granules and an extensive basolateral labyrinth than glands of unfed ticks. Rough endoplasmic reticulum proliferates and becomes swollen (Fawcett et al., 1986; Barker et al.,1984). New glandular glycoproteins are expressed by Amblyomma americanum (Brown and Askenase, 1986; Needham et al., 1986) and by Hyalomma anatolicum (Gill et al., 1986). Transplantation experiments suggest a hemolymph borne factor stimulates these changes (Coons and Kaufman, 1988). Protein and mRNA synthesis are triggered but temperature and olfactory stimuli are not sufficient to elicit this response (Shelby et al., 1987).

The second phase begins after mating. Synthetic activities accelerate, the alveoli swell and lumena fill with saliva. Alveoli assume a characteristic hollow, spherical appearance in sectioned glands. Protein and mRNA synthesis rates double in salivary glands of ticks in this stage of feeding (Shelby et al., 1987) while secretory

granules disappear from the complex granular cells. The activities of adenylate cyclase and Na/K-ATPase are highest in salivary glands from rapidly feeding females (Sauer and Essenberg, 1984). Large numbers of gap junctions can be seen joining adjacent plasma membranes (Fawcett et al., 1986).

The final phase occurs as the female attains its replete weight and drops from the host, salivary glands degenerate and autophagic vacuoles fill the cytoplasm. Excess mitochondria, plasma membrane and rough endoplasmic reticulum are eliminated and the glands become unresponsive to dopamine stimulation (Kaufman, 1986). Salivary gland degeneration is initiated in A. hebraeum and A. americanum by 20-hydroxyecdysone infusion, but does not occur in unmated females below a weight threshhold approximately 10 times the unfed weight (Kaufman, 1986; Lindsay and Kaufman, 1988). Dees et al. (1984; 1985) found that whole body ecdysteroids increase during feeding, but most rapidly during the rapid-feeding and replete stages of Dermacentor variabilis and in Hyalomma dromedarii feeding. Protein and messenger RNA synthesis also decline upon repletion (Shelby et al., 1987).

This report presents data on the effects of juvenile hormone and 20-hydroxyecdysone on tick salivary gland differentiation during and after the time of mating. Results suggest that salivary gland differentiation during feeding may be controlled, at least partially, by juvenile hormone-like molecules and by ecdysteroids.

MATERIALS AND METHODS

Materials

All biochemicals, 20-Hydroxyecdysone and enzymes were purchased from SIGMA chemical co. Racemic 7-S-methoprene and 7-S-hydroprene were a kind gift from Gerard Staal (Zoecon-Sandoz, Palo Alto, CA).

Animals

Adult female lone star ticks, Amblyomma americanum (L.), used in experiments were reared and fed at the Oklahoma State University Medical Entomology Laboratory. Larvae and nymphs were fed on rabbits. All experiments in this report used adult ticks fed on sheep within surgical stockinette sleeves affixed to the skin of the ovine hosts. Mating was prevented in most experiments by excluding males from the sleeves.

Hormone applications

Twenty virgin, unfed females were placed in each of two to four sleeves on an ovine host. One day later, unattached females were removed from all sleeves. Six to eight days later one or two sleeves were chosen at random to be controls. All attached ticks within the sleeve were treated with 1 ul of HPLC grade acetone applied directly to the alloscutum. Experimental groups were also assigned at random to sleeves. One ul solutions of methoprene, hydroprene or 20-HOE in acetone were applied to all individuals within the sleeve on the same day controls received acetone. When multiple applications of 20-HOE were applied, control groups also received additional acetone. The locations of particular treatments were varied from anterior to

posterior on the ovine host to prevent a bias towards better feeding areas. On specified days thereafter, usually the 10th or 11th day, four days after hormone treatment, control and experimental groups were removed from the host and individually weighed before dissection.

Gland preparations

All salivary glands from a group were dissected into 0.5 ml of cold homogenization buffer containing 92 mM Tris-(hydroxymethyl)aminomethane-HCl, pH 7.3, 60 mM NaCl, 5 mM Mg chloride, 100 uM Na ethylenediaamine tetraacetic acid (Rutti et al., 1980) and homogenized in a Potter-Elvehjem tissue homogenizer. All other manipulations were performed at 4°C. Aliquots of total and post-mitochondrial supernatants were taken for protein determinations (Bradford, 1976) and enzyme assays.

Enzyme assays

Activity of salivary gland homogenate Na/K-ATPase was assayed using the method of Rutti et al. (1980). Aliquots of homogenate were added to buffer containing 2 mM ATP, with or without 1 mM ouabain and incubated with shaking at 37°C. Experiments were performed in quadruplicate.

Electrophoresis and Immunoblotting

Preparations of salivary were sonicated in 2X SDS sample buffer (Dunbar, 1987) and stored at -20°C. Samples were thawed, boiled and subjected to SDS-PAGE using the Pharmacia PhastSystem. Gradient gels of 10-15% acrylamide were routinely used in analyses, accoring to instructions provided by the manufacturer. In some experiments immunoblots were prepared by diffusion blotting of the finished gels onto ZetaBind (BioRad) blotting membranes at 70°C, as reccommended by

Pharmacia. Membranes were blocked with 5% Carnation nonfat instant milk in Tris buffered saline (TBS; 0.9% NaCl, 10 mM Tris-HCl, pH 7.4) for 2 hr, then incubated with 1/100 dilutions of rabbit antitick hyperimmune antiserum (kindly provided by Debbie Jaworski, Ohio State University Acarology Laboratory, Columbus, OH) for 5 hr before being rinsed, incubated with Protein A-Alkaline phosphatase for 1 hr (Dunbar, 1987). Antisera prepared against bovine brain Na/K-ATPase was purchased from Jackson Immunochemicals.

Microscopy

Control and treated glands from selected ticks were dissected directly into 2 % (v/v) glutaraldehyde/0.27 M Na cacodylate buffer (pH 7.2). Glands were post-fixed with 2 % (w/v) osmium tetroxide, then dehydrated through increasing concentrations of ethanol and propylene oxide before embedding in Epon-Araldite (Venable and Coggeshall, 1965). Thick sections (1 um) from blocks were prepared and stained with methylene blue for light microscopy. Alveolar diameters were measured with an ocular micrometer calibrated with a ruled glass slide. Thin sections (silver reflective) were cut from selected blocks with a Sorvall MT-5000 ultramicrotome using a Dupont diamond knife. Sections were collected on 150 or 200 mesh copper grids, counterstained with 5 % (w/v) uranyl acetate and lead citrate, observed and photographed using a JEOL 100 CX 2 transmission electrom microscope. In one experiment control and treated gland pairs were prepared for scanning electron microscopy by fixing, staining and dehydrating, as above, then critical point dried using CO_2 and sputter coated with gold-palladium. Glands were observed and photographed using a JEOL ASM-35U scanning electron

RESULTS

Effect of Methoprene on Glandular Differentiation

Topical treatments on the sixth or seventh day of feeding were used in most experiments. This corresponds to the time mating normally occurs. Applications of methoprene to unfed females and to females fed less than six days were not effective (data not shown). A topical dose on day 6 or 7 of 50 ug methoprene per tick in acetone stimulated virgins removed after 10 or 11 days of feeding to obtain larger bloodmeals. Four days elapsed between treatment and dissection. There was no difference between virgins treated on day six and dissected on day 10 and those treated on day seven with dissection on day 11. These two groups were pooled. The average body weight of treated virgins was increased to 27.1 mg/tick, 178 % of acetone treated controls, just 4 % of mated controls ($p<0.01$, $n=15$) (Table 1). Treatment with 5 ug methoprene/tick on day 7 of feeding did not significantly increase weight gain over controls after 11 days of feeding. Application of 200 ug/tick methoprene in one experiment also stimulated a small weight gain (21.5 vs 25.1 mg/tick). Fifty ug/tick of another JH analog, hydroprene, elicited weight gain in the same range of stimulation seen with 50 ug methoprene (184 % of controls, $p<0.01$, $n=3$) when applied on day 7 of feeding. Total protein was increased by 50 ug methoprene to 33 % greater in treated vs untreated females ($p<0.01$, $n=11$). Again, 5 ug methoprene treatment did not cause significant increases in glandular protein (Table 1). The activity of Na/K-ATPase was also stimulated to

212 % of paired controls (Table 1) by 50 ug/tick methoprene ($p<0.01$, $n=11$); but not by 5 ug/tick.

To confirm that these changes brought about by methoprene were physiological, we prepared glands for light and electron microscopy. Scanning EM micrographs of stimulated glands showed that methoprene increased alveolar diameter 40±20 % over controls to 139 um ($p<0.01$, $n=3$) (Fig. 1a vs 1b). Transmission EM photomicrographs reveal obvious markers of differentiation: euchromatic nuclei, extensive proliferation of rough endoplasmic reticulum, and basolateral labyrinth. More mitochondria were visible in close association with the basolateral labyrinth. Alveolar lumina become dilated (Fig. 2a, b vs 2c, d). It appears from thick sections that partial degranulation of granule containing cells occurs in methoprene stimulated glands (data not shown).

Effect of 20-HOE applications on gland differentiation

Concern that 20-HOE is rapidly metabolized in conditions such as those used in this study prompted us to apply the hormone several times to each tick. Multiple treatments of attached, feeding ticks with 100 ug 20-HOE/tick on day seven, eight and nine stimulated virgins to gain additional weight (Table 1). Virgins subjected to such a treatment weighed 28.1 mg/tick by day 10 of feeding, compared to a control average of 14.7 (a 195 % gain; $p<0.05$, $n=5$). Total glandular protein was also stimulated by 144 % to 123.2 mg/tick ($p<0.01$, $n=5$). A single application of 100 ug 20-HOE on day eight of feeding did not stimulate weight gain in one pilot experiment (19.5 vs 16.2 mg/tick). Interestingly, 100 ug/tick 20-HOE did not stimulate Na/K-ATPase

activity, as did 50 ug methoprene. In fact, a slight decline of Na/K-ATPase activity in 20-HOE treated ticks is evident.

Methoprene and 20-HOE applications

Methoprene and 20-HOE treatments did not potentiate each other when applied in sequence (Table 1). Virgins treated with 50 ug methoprene on day 6, then with 100 ug 20-HOE on day 8 in each of two experiments gained more weight than acetone treated controls (11.2/17.1 mg and 19.5/33.1 mg/tick). Application of 100 ug 20-HOE on each of three days following 50 ug methoprene treatment on day 6 showed an average gain over controls of 203 % (29.7 vs 14.9 mg/tick), an increase not significantly greater than that effected by either 50 ug methoprene or 100 ug 20-HOE treatment alone (Table 1). Glandular protein was stimulated to 146 % of the control by the combination of methoprene and 20-HOE treatments ($P<0.01$, $n=6$). This was the same range of stimulation seen with either methoprene or 20-HOE alone (Table 1). Interestingly, Na/K-ATPase activity was increased only when methoprene was applied (218 %), not when 20-HOE was given.

Precocene-2 Applications

100 ug/tick P-2 had little affect on tick weight or TSG function when applied on days 0, 2, 6 or 8 of feeding virgins (Table II). 500 ug P-2 on day 6 of feeding, mated females stimulated all three measures of salivary gland differentiation: weight, protein and enzyme activity (304 ± 138, 141 ± 12, and 371 ± 27%, respectively). Subsequent treatment with 50 ug/tick methoprene did not cause any additional increase (Table II).

Electrophoresis and Immunoblotting

Antisera against bovine Na/K-ATPase did not react with tick

salivary gland homogenates at any of the dilutions tested from 1/1000 up to 1/25. However mouse liver homogenates run concurrently as positive controls did show a 120 Kd polypeptide at 1/500 dilution of the antisera (data not shown). Rabbit hyperimmune serum provided by Kathy Jaworski reacted with 12 proteins from salivary gland extracts. Expression of these antigens was not affected by treatment of feeding females with methoprene, hydroprene, 20-HOE or precocene-2 (data not shown).

DISCUSSION

Mechanical stimulation of the gonopore of partially fed virgin female A. americanum (i.e. insertion of sand, agar, bits of rubber and probe) caused large increases in the size of fat body cells, which was attributed to vitellogenin synthesis (Oliver et al., 1984; Oliver, 1986). Factors in male accessory glands and salivary glands have been implicated in stimulating oocyte growth (Connat et al., 1986; Oliver, 1986). Connat et al. (1986) and Sahli et al. (1985) hypothesized that mechanical and hormonal stimuli associated with tick mating stimulate ovary development and vitellogenesis via juvenile hormone-like compounds. If juvenoids are released by mating stimuli, then some of the responses seen in normally mated females should occur when feeding virgins are treated with juvenile hormone, as shown in Fig. 3. We have tested this hypothesis in A. americanum by application of the juvenile hormone analogs to the cuticle of attached, feeding female virgins.

Female A. americanum do not feed to repletion without the stimulus of mating. Normally, unmated females will not exceed 35 mg (McSwain et al., 1982), although weight gain is quite rapid after mating. The day on which mating occurs (days 3-8) is the chief source of variance in the average rate of weight gain. Exclusion of males from the feeding virgins thus eliminates much of the variance that obscures an effect of topically applied compounds or hormones, allowing more sensitive screening of candidate compounds.

Consistant with the effect on tick reproduction we have been able

to induce tick salivary glands to undergo substantial differentiation *in vivo* by treating attached, feeding virgins topically with the juvenile hormone analogs 7-S-methoprene and 7-S-hydroprene. Treatment with 5 ug/tick methoprene did not significantly increase body weight, glandular protein or Na/K-ATPase activity while treatment with 50 ug/tick did stimulate feeding virgins to obtain larger bloodmeals (Table 1). Gland protein and the enzyme Na/K-ATPase were also increased to the range of early/rapid feeding ticks (>100 mg body weight) (Table 1). Methoprene treatment increased the amount of all coommassie blue stained salivary proteins seen on SDS polyacrylamide gels over untreated females (data not shown), an effect noted with unmated and mated female *A. americanum* (McSwain *et al.*, 1982; Shelby *et al.*, 1987) and in *Rhipicephalus evertsi* (Viljoen *et al.*, 1986).

Application of high doses of hormone to the tick cuticle may lead to higher than physiological concentrations in the hemolymph. In order to confirm that the changes brought about by methoprene were the same as late feeding stage ticks we examined treated and control glands using light and electron microscopy. Scanning EM micrographs of stimulated glands showed that methoprene markedly increased alveolar size over controls (Fig. 1a *vs* 1b). Transmission EM micrographs revealed obvious markers of differentiation: extensive proliferation of rough endoplasmic reticulum, extensive basolateral labyrinth and associated mitochondria (Fig. 2a, b *vs* 2c, d). Thick sections showed degranulation of cells in methoprene stimulated glands, accompanied by an average of 141 % increase in alveolar diameter. The lumena become dilated, suggesting the onset of secretory capability (Fig. 2b *vs* 2d). The lumenal dilation

shown in Fig. 2d was only average. In some experiments, a very large dilation was observed.

Mass spectra of extracts from the argasid tick Ornithodorus moubata differed substantially from spectra of authentic insect juvenile hormones. The authors referred to these molecules as JH-like substances (Connat et al., 1986). Kulcsar and Sonenshine have recently shown that hemolymph levels of juvenile hormone binding protein of Dermacentor variabilis (labeled with the photoaffinity analog of JH, [^{3}H]EFDA) were not present in feeding, unmated females, but appeared after mating (in Prestwich, 1987). This indicates that juvenoids may be present at higher concentrations after the mating stimulus. However, the concentration of juvenoids throughout the feeding cycle must be measured to corroborate our results with the salivary gland.

The antiallatal compound Precocene-2 (P-2) has been shown to have inhibitory effects on reproduction of several ixodid and argasid ticks (Booth et al., 1986; Connat et al., 1986; Dees et al., 1985; Pound and Oliver, 1979). In these studies inhibition was reversable by JH III and the JH analog methoprene. Thus we expected that application of P-2 to mated females would block weight gain and TSG activity. Methoprene should restore TSG function in P-2 inhibited ticks. 100 ug/tick P-2 had no affect on tick weight when applied on days 0, 2, 4, 6 or 8 to feeding virgins (Table 2). Subsequent treatments with 50 ug methoprene stimulated weight gain and protein synthesis; an effect similar to that seen in Table I.

Two argasid ticks, Argas persicus and Ornithodorus coriaceus and one ixodid Rhipicephalus sanguineus were sterilized and their

ecdysis inhibited by treatment with precocene (Leahy and Booth, 1980). Precocious metamorphosis was not observed. When JH was applied to precocene-treated nymphs, the percentage remaining in that instar increased and survival was higher. Juvenile hormone alone had no effect. Fumigation of eggs with P-2 disrupted development late in embryogenesis in D. variabilis (Hayes and Oliver, 1981). Subsequent developmental effects included partial failure of stadia to attach, feed, ecdyse and partial-to-complete sterility of adult females. A subsequent application of JH III to P-2 sterilized females enabled them to feed and successfully complete the gonotrophic cycle. Although the endocrine glands in ticks have not been located, Binnington (1986) beleives that the lateral segmental organs located near the synganglion may serve this purpose.

Ecdysteroids have been reported in several species of ixodid and argasid ticks (Dees et al., 1984; Diehl et al., 1986). Dees et al. (1985) reported changes in the titer of whole body ecdysteroids during the feeding cycle of female Hyalomma dromedarii. The titer in feeding virgin females rose gradually but increased markedly after mating, reaching its highest level in replete females. These results suggest that the large increase in ecdysteroids after mating may stimulate or contribute to the increase in salivary gland protein synthesis and secretory ability seen in mated females. Application of 20-HOE to feeding virgins should mimic this natural increase in ecdysteroid titer, leading to gland differentiation.

Topical treatment of feeding virgins with 100 ug 20-HOE/tick stimulates weight gain and protein synthesis into the range seen in

mated females, but does not stimulate Na/K-ATPase activity (Table 1). Indeed, glands from the 20-HOE treated ticks exibited 76 % of control Na/K-ATPase activity. Degeneration does not occur in feeding ticks until after repletion, or forcible removal from the host. Lindsay and Kaufman (1988) report that A. americanum salivary glands incubated in vitro with 20-HOE secrete 64 % less fluid than controls. It is possible that the reduced secretory competence caused by 20-HOE can be attributed to decreased Na/K-ATPase activity.

Provided that juvenoids are released by mating, and ecdysteroid titers also rise we predicted that a combination of methoprene and 20-HOE treatments should potentiate the actions seen with methoprene alone. However, a combination of 50 ug methoprene with 100 ug 20-HOE treatment does not cause additional weight gain or protein synthesis, even when 20-HOE was applied several times (Table 1). Thus, these two hormones do not seem to potentiate each other. In vitro incubation of glands from prefed slow feeding A. americanum with 10 nM 20-HOE for 2 hrs at 37°C increased incorporation of [^{35}S]methionine an average of 30% over paired controls (Shelby et al., unpublished). Ilenchuk and Davey (1983) found a JH I stimulated Na/K-ATPase activity in Rhodnius prolixus follicle cells which did not require synthesis of new enzyme. Our studies do not distinguish activation of Na/K-ATPase from synthesis. This is the first report of tick salivary gland activation by methoprene and 20-HOE treatment. These same treatments do not fully release females from the pre-mating weight arrest.

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Table 1. Effects of Juvenile Hormone Analogs and 20-Hydroxyecdysone on Virgin, Feeding Female Lone Star Tick Weight, Salivary Protein and Na/K-ATPase Activity.

Treatment	Hormone (ug/tick)	Day of Treatment	Day Removed from Host	Mean Wt (mg)		Total Salivary Protein (ug/gland pair)		Na/K-ATPase Activity (nm P_i/min/gland pair)	
				Control	Experimental	Control	Experimental	Control	Experimental
Methoprene	5	7	11	16.2	18.2±0.6 (3)	49.2	51.2±2.5 (3)	431	494±79 (3)
% of Control					112±3		104±5		122±18
Hydroprene	50	7	11	14.3	26.8±1.5 (3)**	69.6	74.6±4.2 (3)	329	497±64 (3)
% of Control					184±9		114±8		152±19
Methoprene	50	6-7[a]	10-11	13.8±0.8	27.1±2.9 (15)**	83.6±6.5	110.0±9.6 (11)**	210±22	412±83 (11)**
% of Control					178±10		133±8		212±33
Methoprene	200	6	9	21.5	25.1	——	——	—	—
20-HOE	100	8	10	19.5	16.2	——	72.1	217	317
20-HOE	100	7,8,9[b]	10	14.7±1.2	28.1±12.4 (5)*	83.3±16.6	123.2±28.5 (5)**	210±54	175±68 (5)
% of Control					195±86		144±14		76±19
Meth + 20-HOE	50 + 100	6 + 8	10	11.2,19.5	17.1, 33.1	——	63.5, 148.6	167,217	179, 896
Meth + 20-HOE	50 + 100	6+7,8,9	10	14.9±1	29.7±3.3 (6)**	83.2±13.6	116.1±14.8 (6)**	169±47	321±94 (6)**
% of Control					203±26		146±18		218±59

* Significant at P<0.05, paired, one-tailed t-test. Values are x±SEM (n)= No. replicates.

** Significant at P<0.01.

[a] Four days elapsed between methoprene application and dissection. Groups 6-10 and 7-11 were pooled.

[b] 20-HOE applied three times to all ticks on days 7, 8 and 9 of feeding.

All hormones applied in 1 ul of acetone to the alloscutum of attached, feeding females. Hormones were applied on the day of feeding indicated in the table. Several days later the feeding females were removed from the host on the day indicated, weighed individually and dissected.

Table 2. Effect of Precocene-2 on Mated and Unmated Feeding Female Lone Star Tick Salivary Glands

Treatment	Hormone (ug/tick)	Day of Treatment	Day Removed from Host	Mean Wt (mg)		Total Salivary Protein (ug/gland pair)		Na/K-ATPase Activity (pm P_i/min/gland pair)	
				Control	Experimental	Control	Experimental	Control	Experimental
Feeding Virgins									
P-2	100	0	10	25.1	22.2,19.4	56.0	58.0, 72.4	856	689, 586
P-2	100	2	10	25.1	17.5,27.8	56.0	65.4, 63.9	856	532, 840
P-2	100	4	10	16.0±4.6	18.3±1.2 (3)	82.3±13.2	105.8±21.5 (3)	404±231	360±61 (3)
% Control					135±18		126±7		199±130
P-2 + Meth	100 + 50	4 + 7	11	12.8,10.1	35.0, 19.3	97.1,93.8	125.8, 112.4	99,257	205, 191
P-2	100	6	11	13.4,15.6	12.7, 15.0	112.9,109.6	99.4, 102.9	123,190	69, 141
P-2 + Meth	100 + 50	6 + 7	11	13.4,15.6	21.8, 13.0	112.9,109.6	122.3, 82.0	123,190	359, 63
P-2	100	8	10	25.1	25.2, 21.9	56.0	58.3, 68.5	856	706, 429
Mated Feeding Females									
Mated Control				159.0		129.0		1321	
" "				96.4		133.1		1085	

*, $P<0.05$, **$P<0.01$, paired, one-tailed t-test; x±SEM; (n) = No. replicates.
Precocene-2 (P-2) was purchased from SIGMA was applied to the alloscutum of attached, feeding females in 1 ul of 20% DMSO.

Figure 1. 1a, SEM micrograph of salivary gland from control, acetone treated, feeding tick. 260 X. 1b, Increase in alveolar size as a result of 50 ug methoprene treatment. 260 X.

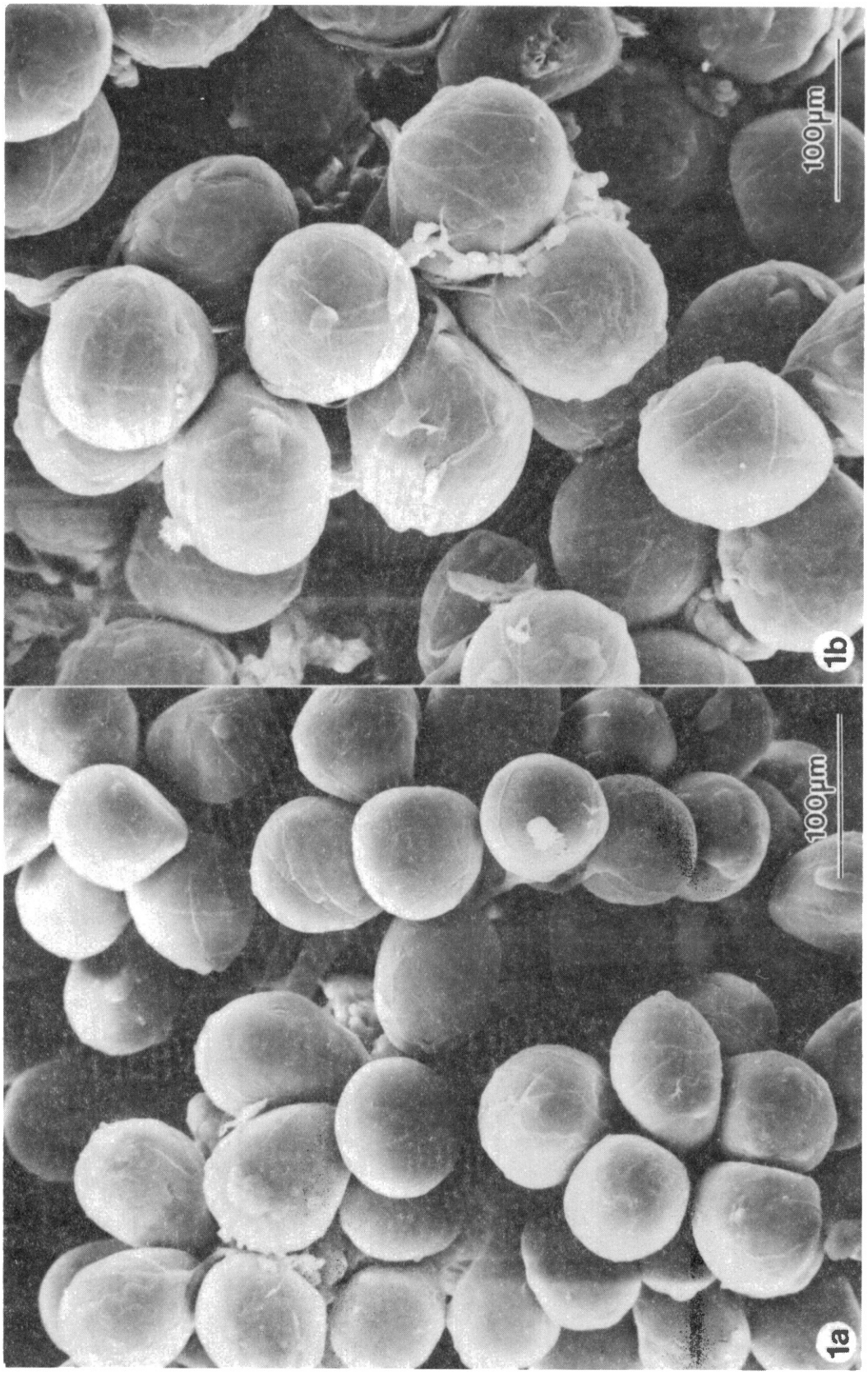

100µm
1a
100µm
1b

Figure 2. 2a, TEM Micrograph of Salivary Alveolus from Control, Acetone Treated, Feeding Tick, 1400 X. 2b, Lumena of control glands are closed (arrows). 5800 X. 2c, ultrastructural changes induced by 4 day methoprene treatment. Salivary alveolus from 50 ug methoprene treated, feeding tick, 1400 X. In vivo methoprene stimulates formation of extensive basolateral labyrinth (BL) and mitochondria. 2d, Lumena (Lu) of methoprene treated, feeding glands exhibit opening and microvillar enlargement (arrows). 5800 X.

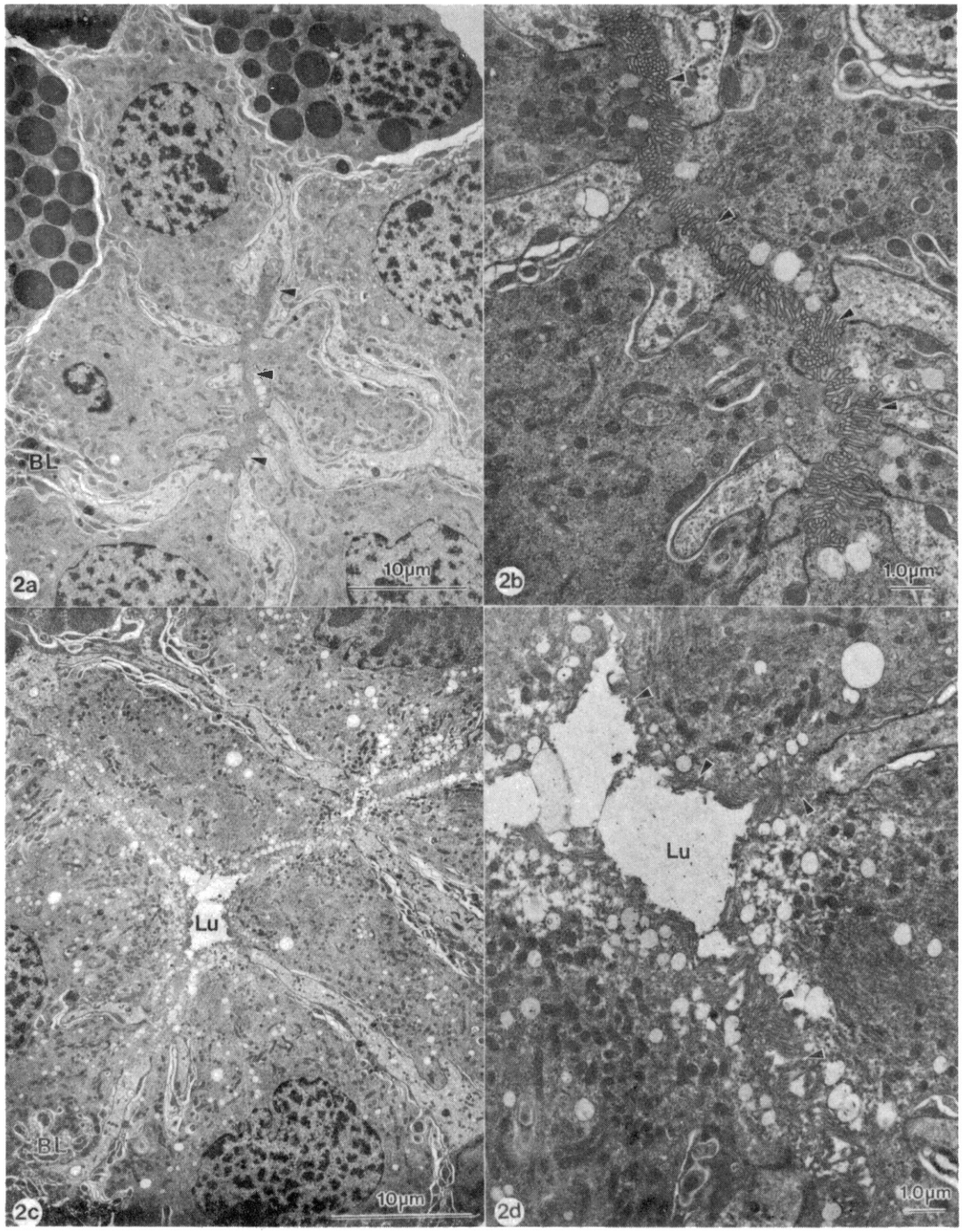
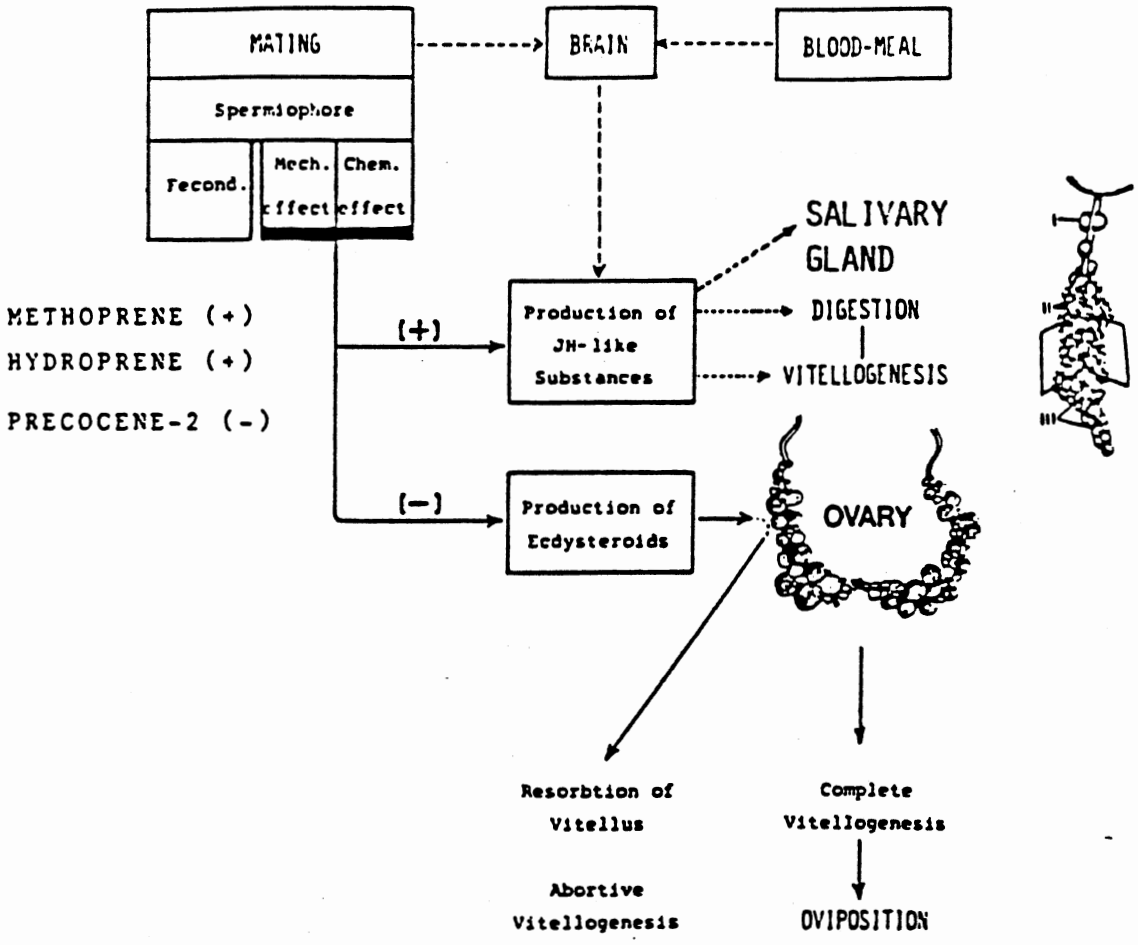

BL
10μm
2a
1.0μm
2b
Lu
BL
10μm
2c
Lu
1.0μm
2d

Figure 3. Scheme of working hypothesis tested by topical application of hormones to feeding tick cuticle. Connat et al. (1986) and Sahli et al. (1985) have suggested that mechanical and hormonal stimuli of mating cause release of juvenile hormones which initiate vitellogenesis. The great increase of salivary gland polysomal mass and mRNA following mating (Shelby et al., 1987) may also be stimulated by release of juvenoids. Adapted from Connat et al. (1986).


MATING
BRAIN
BLOOD-MEAL
Spermiophore
Fecond.
Mech. effect
Chem. effect
SALIVARY GLAND
METHOPRENE (+)
HYDROPRENE (+)
PRECOCENE-2 (-)
(+)
Production of JH-like Substances
DIGESTION
VITELLOGENESIS
(-)
Production of Ecdysteroids
OVARY
Resorbtion of Vitellus
Abortive Vitellogenesis
Complete Vitellogenesis
OVIPOSITION

PART III

REGULATION OF ATPase ACTIVITY IN SALIVARY GLANDS OF LONE STAR TICK, *Amblyomma americanum* (L.)

ABSTRACT

The activity of the salivary gland sodium pump, Na/K-ATPase, increases markedly during feeding of ixodid ticks, concommitant with a steady increase in fluid secretory ability of the glands. Salivary fluid secretion is stimulated by dopamine *in vitro* and *in vivo*. Na/K-ATPase is stimulated in salivary glands of partially fed female lone star tick, *Amblyomma americanum*, salivary glands incubated *in vitro* with dopamine or cyclic AMP/theophylline. Na/K-ATPase activity of gland homogenates is stimulated by cAMP in a dose dependent manner. Micromolar concentrations of calcium inhibit activity. The juvenile hormone analog 7-S-methoprene and the protein kinase C activator OAG inhibit activity of Na/K-ATPase. Added catalytic subunit of cAMP-dependent protein kinase stimulates Na/K-ATPase activity. A bicarbonate stimulated ATPase is present in glands which is not inhibited by vanadate, furosemide or azide.

Key Word Index: Tick salivary gland, Na/K-ATPase, cyclic AMP, Dopamine, Methoprene, Phorbol ester, Protein Kinase.

INTRODUCTION

The salivary glands of feeding ixodid ticks excrete copious amounts of isoosmotic saline from the bloodmeal back into the host (Kaufman, 1986). Understanding the mechanism of fluid transport by the salivary glands has been the goal of several laboratories (Meredith and Kaufman, 1973; Fawcett et al., 1981; Kaufman and Sauer, 1982; Sauer and Essenberg, 1984; Kaufman,1986; Willadsen et al., 1987; Sauer et al., In Press). In brief, salivary fluid secretion appears to be stimulated by direct dopaminergic innervation of alveoli, resulting in increased cytosolic levels of cyclic AMP and the subsequent phosphorylation of unidentified proteins distributed throughout the alveolar cells (McSwain et al., 1985; 1987). Ultrastructurally, ixodid salivary glands during the latter stages of feeding resemble avian salt glands (Fawcett et al., 1986). During feeding an extensive basolateral labyrinth with numerous closely associated mitochondria is formed. The basolateral cells communicate via numerous gap junctions with adjacent cells (Fawcett et al., 1986). Dense septate junctions and desmosomes join cells abutting on the lumen (Meredith and Kaufman, 1973; Fawcett et al., 1981; 1986). The ion "pumps" and channels which mediate fluid transport in tick salivary glands have not been investigated and, based on the extensive work done with insect and vertebrate transporting tissues, it is reasonable to expect that many of these enzymes are closely regulated via second messenger systems (Hootman, 1986; Marty, 1987; Peterson, 1988).

The sodium pump or Na/K-ATPase is directly linked to the process of secretion. Incubation of salivary glands in vitro with the specific inhibitors of the enzyme, ouabain and harmaline, quickly inhibit dopamine stimulated salivation (Kaufman and Phillips, 1973; Needham and Sauer, 1975). During feeding the salivary Na/K-ATPase activity increases markedly (Kaufman et al., 1976; Rutti et al., 1980). The activities of adenylate cyclase and Na/K-ATPase are highest in salivary glands from rapidly feeding females, as determined by body mass (Sauer and Essenberg, 1984). Shelby et al. (1989) have shown that the activity of Na/K-ATPase enzyme is higher in feeding females treated with the juvenile hormone analog methoprene, but declines when females are treated with 20-HOE. Ilunchuk and Davey (1982; 1983; 1987a,b) have demonstrated that Rhodnius prolixus vitellogenic follicle cell preparations contain a juvenile hormone I dependent Na/K-ATPase activity. This was the starting point for our present study. The increased Na/K-ATPase enzyme activity seen in methoprene treated female ticks can conceivably be the result of synthesis, upregulation or direct stimulation via a second messenger transduction system.

Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulphate; DTT, dithiothreitol; cAMP, cyclic adenosine 3'-5' monophosphate.

MATERIALS AND METHODS

Materials

All biochemicals were purchased from SIGMA (St. Louis, MO). Forty eight well microtiter plates were purchased from Falcon (Grand Island, NY). Racemic 7-S-methoprene was a kind gift from Gerard Staal (Zoecon-Sandoz, Palo Alto, CA).

Animals

Adult female lone star ticks, Amblyomma americanum (L.), were reared as described by Patrick and Hair (1976). Larvae and nymphs were fed on rabbits and adults on sheep within surgical stockinette sleeves affixed to the skin of ovine hosts.

Pre-Stimulation of Whole Salivary Glands

Both glands were dissected from individual partially fed female ticks and incubated separately in 100 ul of oxygenated TS/MOPS at 37o C with and without the agonist or inhibitors as indicated in figure legends. The control gland received an equivalent amount of buffer or carrier, where indicated. Methoprene was prepared by sonicating a known weight of the oil in methanol. 1-Oleoyl-2-acetyl-sn-glycerol (OAG) was dispersed in 100% DMSO and stored frozen in aliquots. The calcium ionophore A23187 was dissolved in ethanol and kept frozen until use. At the end of a 5 min incubation, salivary glands were homogenized in Na/K-ATPase buffer (92 mM Tris-HCl, pH 7.3, 60 mM NaCl, 5 mM Mg chloride, 100 uM EDTA) according to Rutti et al. (1980). Aliquots of homogenate were taken for protein determinations (Bradford, 1976).

Pre-Stimulation of Salivary Gland Homogenates

In some experiments 10 ul aliquots of homogenates from one to four glands were added to Na/K-ATPase buffer containing 0.1 mM theophylline and various concentrations of cAMP or calcium. In one set of experiments gland homogenates were preincubated with 20 ug/ml protein kinase inhibitor (PKI) before addition of 10 uM cAMP according to McSwain et al. (1985). Gland homogenates were also incubated with 5 ug/ml catalytic subunit of cAMP-dependent protein kinase (C) according to Mane et al. (1985). At the end of a 5-10 min incubation Na/K-ATPase activity was determined.

Activity of TSG homogenate Na/K-ATPase was assayed using a modification of the method of Rutti et al. (1980). Incubations were started by the addition of 10 ul of homogenate to 250 ul Na/K-ATPase buffer supplemented with 2 mM ATP with or without 1 mM ouabain in a 48 well tissue culture plate and incubated with shaking at 37°C. Reactions were stopped by addition of cold TCA to a final concentration of 10 %. Next, an equal volume of color reagent (2.5 % (w/v) ferrous sulphate, 0.5 % ammonium molybdate in 0.5 N sulfuric acid) was added. The amount of phosphomolybdate complex formed was measured at 700 nm with a Beckman DU spectrophotometer. Experiments were performed in triplicate.

Anion stimulated ATPase was assayed by the method of Gassner and Komnick (1982). Glands were homogenized in a buffer containing 20 mM Tris-Histidine, pH 8.3, 0.2 mM ouabain and 0.5 mM Mg acetate. Anions added to stimulate ATPase activities were 5-100 mM Na bicarbonate or choline-HCl. 10 ul aliquots of homogenate were added to 250 ul of dissection buffer supplemented with 1 mM ATP in a 48 well tissue culture

plate with shaking at $37^{o}C$, as above. The inhibitors furosemide, vanadate, Na azide and NaSCN were added to the medium, as indicated.

Statistics

Data are presented as means $\pm$ standard error of mean (SEM), with n indicating the number of replicates. Statistical analyses were performed using Student's t-test or a paired t-test, where appropriate. A p-value of ≤ 0.05 was considered to represent a statistical difference.

RESULTS

Effects of Dopamine and cAMP/theophylline on Salivary Na/K-ATPase

Storage of salivary glands in the freezer or refrigerator drastically reduced Na/K-ATPase activity. Highest activities of the enzyme were recorded in glands dissected within 1-2 hour of detachment from the ovine host. Thus, due to the availability of partially fed ticks, it proved infeasable to accumulate glands over a period of time for routine assays. An assay requiring a single pair of fresh glands was developed based on Rutti et al. (1980) and miniaturized to fit the 48 well microtiter plate format. The contralateral gland from each tick served as a control. A. americanum salivary gland Na/K-ATPase activity is maximal in mated females above 90 mg body weight (Kaufman et al., 1977). Ticks used in this study were within a range of 60-200 mg body weight.

Incubation of salivary glands from feeding females with 1 uM dopamine in TS/MOPS medium stimulated Na/K-ATPase 130±37 % over controls ($P \leq 0.01$, $n=8$)(Fig. 1A). Incubation in medium supplemented with 1 mM cAMP and 0.1 mM theophylline for 5 minutes stimulated Na/K-ATPase activity 99±30 % over controls ($P \leq 0.01$, $n=6$). Treatment of glands in vitro with 0.1 uM juvenile hormone analog 7-S-methoprene inhibited Na/K-ATPase activity 18 %. The phorbol ester analog OAG also inhibited activity in intact glands by 22 % ($n=5$) (Fig. 1A). Gland homogenates incubated with 1 uM dopamine also exhibited a Na/K-ATPase stimulation of 132=39 % ($P \leq 0.05$, $n=6$) (Fig. 1B). 2.5 mM calcium inhibited Na/K-ATPase

activity of gland homogenates by 72±30 % (Fig. 1B).

Na/K-ATPase activity responds to cAMP in a dose dependent fashion (Fig. 2). Concentrations of 0.001-0.1 uM cAMP appeared to slightly inhibit Na/K-ATPase activity while concentrations above 0.1 uM were stimulatory. 1 mM GABA does not potentiate the effect of 10 uM DA on intact salivary gland Na/K-ATPase (data not shown). This treatment has been shown to potentiate fluid transport in tick salivary glands (Lindsay and Kaufman, 1986).

Effects of PKI and C on Na/K-ATPase activity

Pre-incubation of gland homogenates for five minutes with 20 ug/ml PKI before addition to incubation medium containing 10 uM cAMP did not prevent activation of Na/K-ATPase (Fig. 3). Under the conditions used 10 uM cAMP stimulated Na/K-ATPase 83 % to 3.35 uM Pi/30 min/ug protein. PKI preincubated homogenates averaged 3.34 uM Pi/30 min/ug. Addition of 5 ug/ml C to homogenates increased Na/K-ATPase activity 85 % over the control to 3.37 uM Pi/30 min/ug (Fig. 3).

Anion Stimulated ATPase

An ATPase is present in the salivary gland homogenate which is stimulated by 25 mM or greater K bicarbonate (Fig. 4). However addition of choline-HCl up to 100 mM inhibits the activity. The enzyme is insensitive to levels of vanadate greater than 100 uM (data not shown). Concentrations of azide up to 100 mM did not inhibit this enzyme, nor was it inhibited by up to 10 mM furosemide. Activity was not influenced by dopamine, cAMP/theophylline, OAG or methoprene treatment of intact salivary glands in vitro (data not shown). However, addition of 10 mM NaF to the incubation medium does inhibit activity over control glands

by 75%. Treatment of gland homogenates with 0.1-100 uM dopamine, 0.1-100 uM cAMP/theophylline or 0.1 uM methoprene did not significantly effect ATPase activity (data not shown). Addition of 2.5 mM calcium stimulated a 30.5$\pm$2.2 % change of activity in the absence of bicarbonate.

DISCUSSION

Fluid transporting epithelia depend to a large extent on the enzyme Na/K-ATPase to generate a Na electrochemical potential gradient across the basolateral surface which is used to accumulate Cl, against its electrochemical equilibrium, into the lumen (Hootman, 1986). In vertebrates, Na/K-ATPase is a heterodimer, consisting of a 85-112 kDa ouabain binding, ATPase alpha subunit and a smaller 40-60 kDa regulatory beta subunit. Several isozymes are known for both subunits (Rossier et al., 1987). Na/K-ATPase activity is known to respond to both alpha and beta adrenergic stimulation (Smart and Deth, 1988) and is inhibited by micromolar amounts of calcium via calnaktin and calmodulin (Yingst, 1987). Marver et al. (1986) inferred that the high affinity ouabain binding isozyme of shark rectal gland was the cAMP-stimulated Na/K-ATPase. Protein kinase C (PKC) activators and alpha adrenergic receptor types which activate PKC such as vasopressin and norepinephrine inhibit Na/K-ATPase while beta adrenergic receptor agonists stimulate the enzyme (Simpson and Hawthorne, 1988).

The role of Na/K-ATPase in insect cell volume regulation has been explored by Ilunchuk and Davey (1982; 1983; 1987a, 1987b). Rhodnius prolixus vitellogenic follicle cells shrink when treated with juvenile hormone, opening the paracellular spaces for easier penetration and uptake of vitellogenin. The fact that ouabain prevented this shrinkage led them to examine the regulation of Na/K-ATPase by juvenile hormone. Microsomal preparations contain a juvenile hormone I stimulated

Na/K-ATPase activity. Treatment of vitellogenic follicle cell preparations with 4 uM JH I caused a 6 fold increase in Na/K-ATPase activity. Membrane preparations from previtellogenic follicles and brain do not respond to JH I (Ilunchuk and Davey, 1987a; 1987b).

Shelby et al. (1987) reported substantial increases in the rate of salivary protein and messenger RNA during feeding. Treatment of feeding virgins with the juvenile hormone analog methoprene increased the amount of salivary Na/K-ATPase activity. Activity declines when females are treated with 20-hydroxyecdysone (Shelby et al., 1989). Na/K-ATPase activity from Amblyomma americanum salivary glands is very labile. Activity of the enzyme rapidly declines after the tick is removed from the host. Within 1-2 hours more than 90 % of the ouabain sensitive activity disappears. The increased Na/K-ATPase enzyme activity seen in methoprene treated female ticks can concieveably be the result of synthesis, upregulation or direct stimulation via a second messenger transduction system.

The stimulatory effect of 10 uM dopamine on salivary fluid secretion of Amblyomma hebraeum is maximally potentiated by 1 mM GABA (Lindsay and Kaufman, 1986). Using salivary glands of partially fed female A. americanum in an identical assay we were unable to demonstrate a potentiating effect of GABA on Na/K-ATPase activity. This may indicate the tick GABA receptor acts directly on the Cl channel during secretion, not via the phophotidylinositol pathway (Eldefrawi and Eldefrawi, 1987). Rutti et al. (1980) were unable to stimulate salivary gland homogenate Na/K-ATPase of A. hebraeum with dopamine. We have been able to stimulate Na/K-ATPase in intact glands and in homogenates with 1

uM dopamine (Fig. 1). High concentrations of cAMP, which bypass the dopamine receptor and adenylate cyclase stimulate Na/K-ATPase activity in whole glands (Fig. 1). When gland homogenates are treated with 1 uM dopamine the variability is high (Fig. 1). This is also true of cAMP stimulation of homogenate Na/K-ATPase, especially at the lower concentrations of cAMP used (Fig. 2).

Nakagaki and Sasaki (1988) demonstrated that methoprene can depolarize cells of the posterior silk gland of Bommbyx mori. The methoprene stimulation of Na/K-ATPase reported by Ilunchuk and Davey (1987a, 1987b) may be explained by its similarity to lipid components of phosphatidylinositol, namely diacylglycerol which is a potent activator of PKC. More recently Yamamoto et al. (1987) have presented evidence that juvenile hormone, and its analog methoprene may activate PKC. Results of Ilunchuk and Davey may be reconciled with those of Yamamoto et al. (1988) by postulating that JH and its analogs methoprene and hydroprene activate PKC, which in turn stimulates Na/K-ATPase activity.

Activation of Na/K-ATPase by a cAMP dependent pathway would involve phosphorylation of the enzyme or a regulatory protein by the catalytic subunit of protein kinase A. The efficacy of cAMP and phorbol ester stimulated phosphorylation of proteins has been demonstrated by McSwain et al. (1985; 1987; In Press). Addition of cAMP or catalytic subunit to gland homogenates should stimulate Na/K-ATPase activity, while preincubation with PKI shold block the effect of cAMP activation. Incubation of intact salivary glands with 1-10 uM dopamine stimulates ouabain sensitive ATPase activity (Fig. 1). Incubation with high extracellular concentrations of cAMP also stimulates the enzyme. The

diacylglycerol analog OAG inhibits slightly the activity of Na/K-ATPase, as does methoprene (Fig. 1). Broken cell preparations incubated according to McSwain et al. (1985) with dopamine and cAMP show higher activities than control (Fig. 1). The response to cAMP is dose dependent (Fig. 2). A 5 minute preincubation of gland homogenates with 5 ug/ml catalytic subunit stimulates Na/K-ATPase to the same extent as 10 uM cAMP. Paradoxically preincubation with PKI does not block the stimulatory effect of 10 uM cAMP (Fig. 3). Turi and Samogyi (1988) found that calcium inhibition of rat myometrial microsomal Na/K-ATPase was dependent on cAMP-dependent catalytic subunit. Removal of kinase activity from microsomal membranes by SDS treatment relieved calcium inhibition. Similarly, PKI inhibition of Na/K-ATPase was dependent on calcium. The salivary gland homogenates used in the present study contained no added calcium. Higher levels of calcium may be required to sensitize inhibition of Na/K-ATPase.

Chamberlin and Phillips (1988) demonstrated that peptidergic stimulation of chloride transport by locust rectum was mediated by cAMP. The short circuit current across the recta is raised by treatment with cAMP, theophylline and forskolin. Ixodid tick gut tissue responds similarly to added adrenergic agents such as noradrenaline and adrenaline (Guenther et al., 1977; Barker et al., 1977). Adrenaline, noradrenaline, cAMP, cGMP and theophylline raise the transepithelial potential, while propanolol and pilocarpine lower the potential difference (P.D.) across the gut epithelium. Whether or not the tick salivary gland responds to dopamine stimulation with an increased P.D. or short short circuit current is not known.

Leichleitner and Phillips (1988) reported a bicarbonate stimulated anion ATPase in microsomes prepared from the chloride transporting tissue, locust rectal epithelium which also responded to chloride and sulphite. This enzyme was inhibited by thiocyanate, azide and oligomycin, but not by levamisole or vanadate; indicating a possible mitochondrial origin of the activity. Gassner and Komnick (1982) found that the loop diuretic furosemide inhibited both bicarbonate and chloride stimulated ATPase activity of dragonfly rectum. Tick salivary glands posses a bicarbonate stimulated ATPase activity which is insensitive to azide, vanadate and furosemide and which is not stimulated by choline chloride (Fig. 4). Since these experiments were conducted with total gland homogenates the subcellular location of the bicarbonate stimulated ATPase is, at present, unknown. A number of pharmacological agents, agonists and antagonists, have been tested for their effect on fluid secretion by the salivary glands (Kaufman, 1986; Sauer et al., In Press). None of the known inhibitors of chloride or bicarbonate transport, such as furosemide or bumetanide, have yet been tested. The importance of bicarbonate in Na and Cl secretion by salivary glands has yet to be determined.

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Figure 1. Activity of agonists on salivary gland Na/K-ATPase in intact and broken cell preparations. Panel A. Salivary glands incubated *in vitro* in TS/MOPS medium with 100 uM OAG, 1 uM dopamine, 1 mM cAMP/0.1 mM theophylline and 1 uM methoprene. Contralateral glands from the same tick served as a control in each replicate. (means $\pm$ SEM, n=6-8, asterisks denote significance at $P \leq 0.05$). Panel B. Salivary gland homogenates incubated with 1 uM dopamine or 2.5 uM calcium.

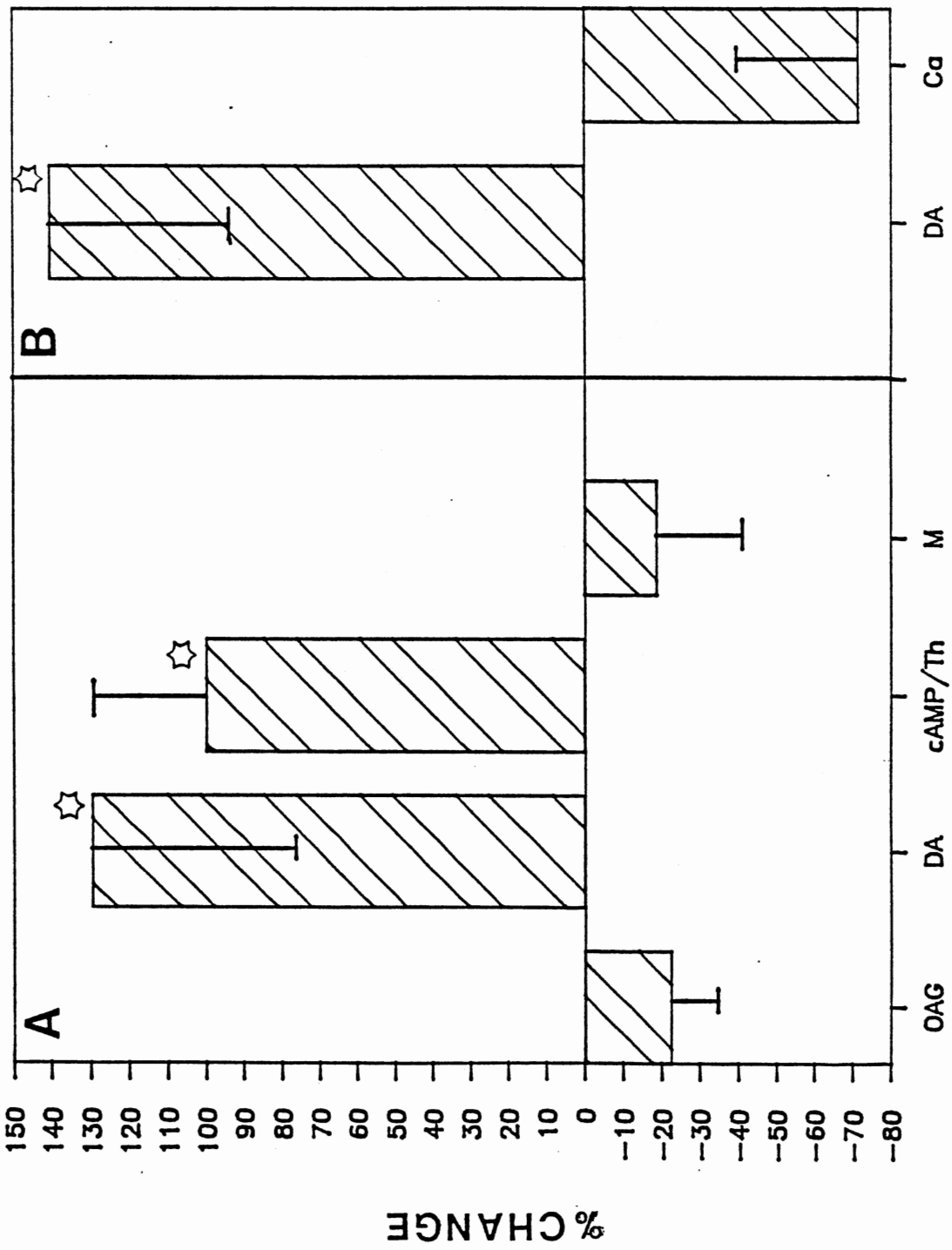

% CHANGE
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
A
B
OAG
DA
cAMP/Th
M
DA
Ca

Figure 2. Effect of increasing cAMP concentration on homogenate Na/K-ATPase activity. (Means±SEM, n=6-10).

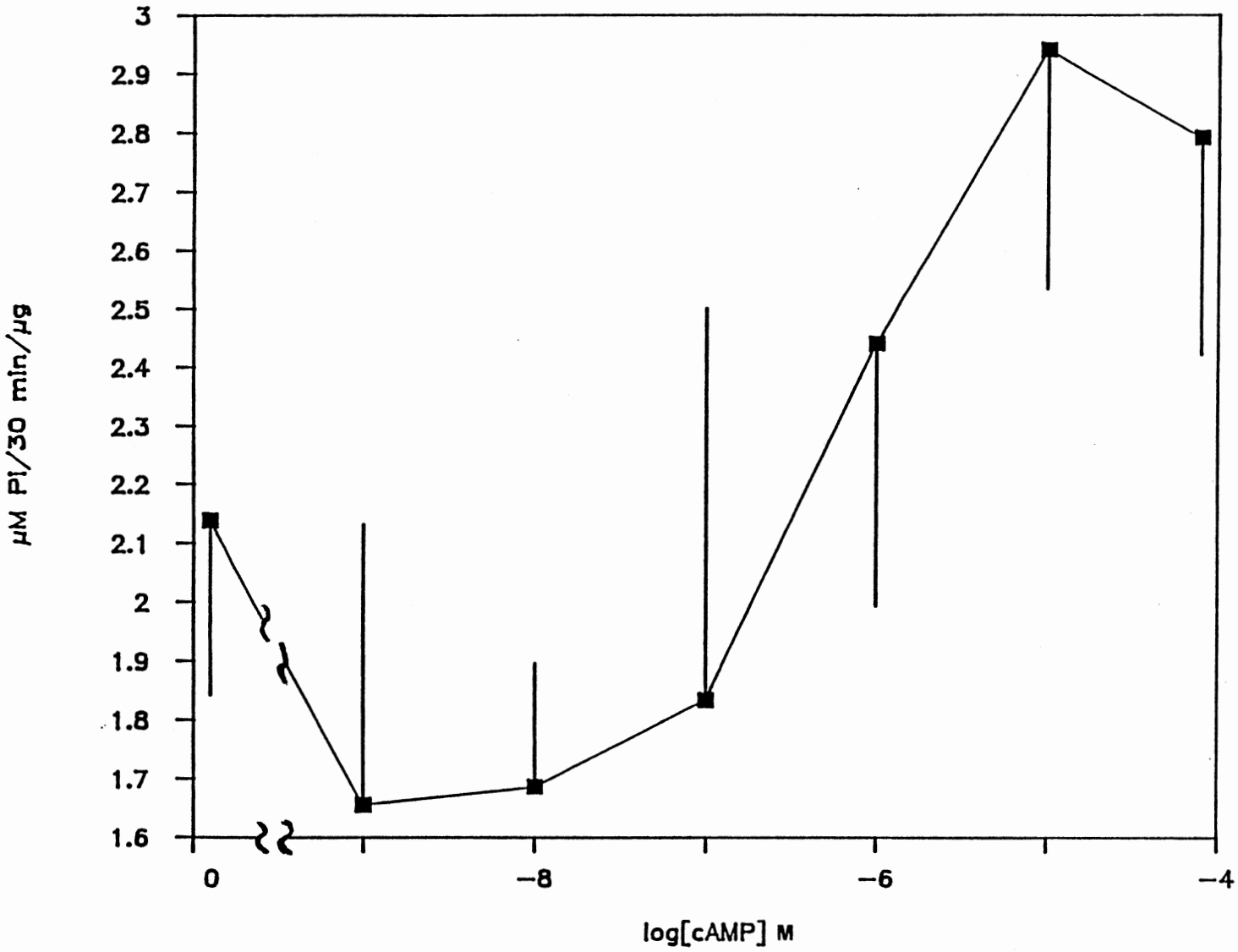

3
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
1.9
1.8
1.7
1.6
µM Pi/30 min/µg
0
−8
−6
−4
log[cAMP] M

Figure 3. Effect of 10 uM cAMP, 20 ug/ml protein kinase inhibitor (PKI) and 5 ug/ml cAMP-dependant protein kinase catalytic subunit (C) on Na/K-ATPase activity of salivary gland homogenates. (n=3).

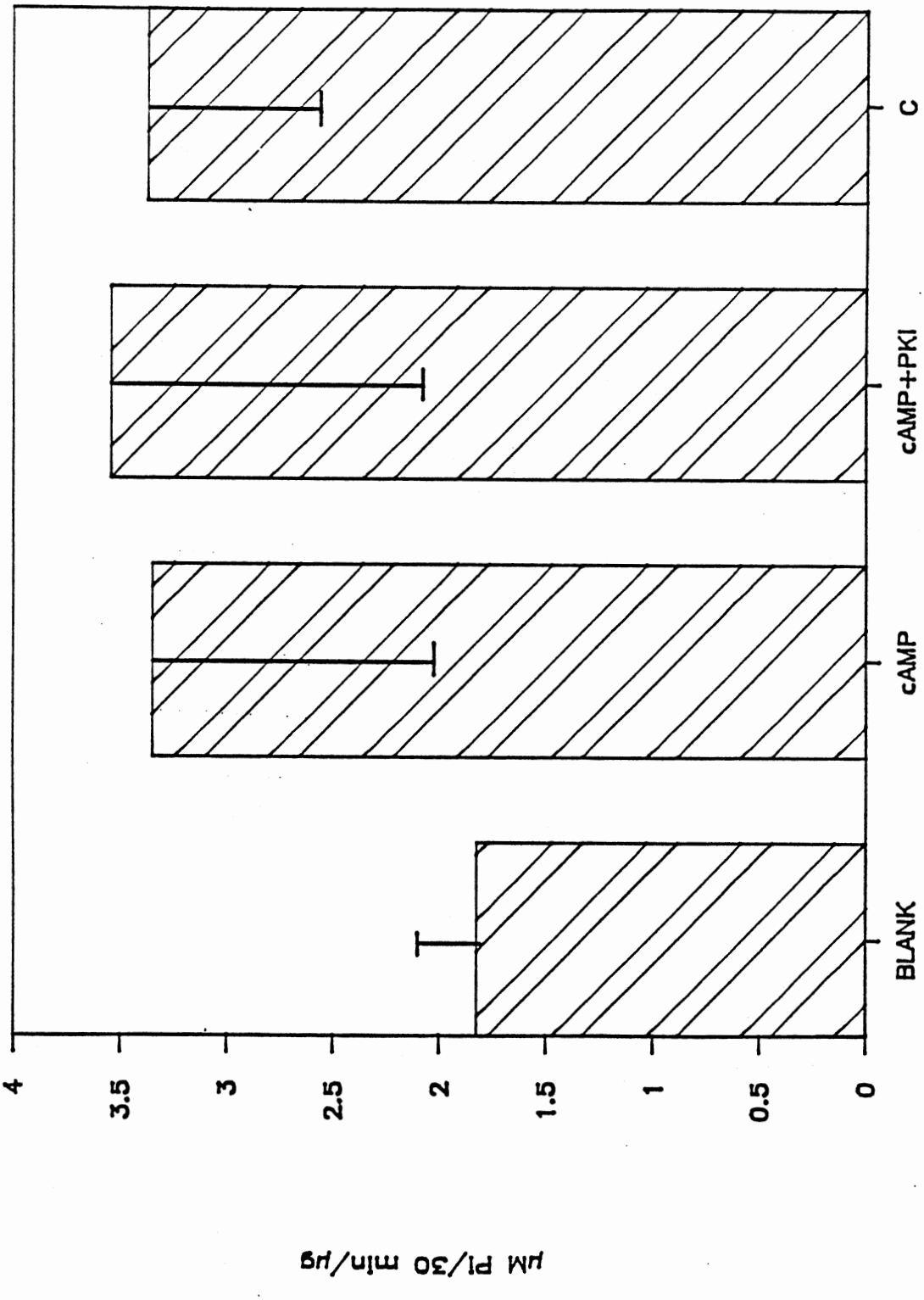
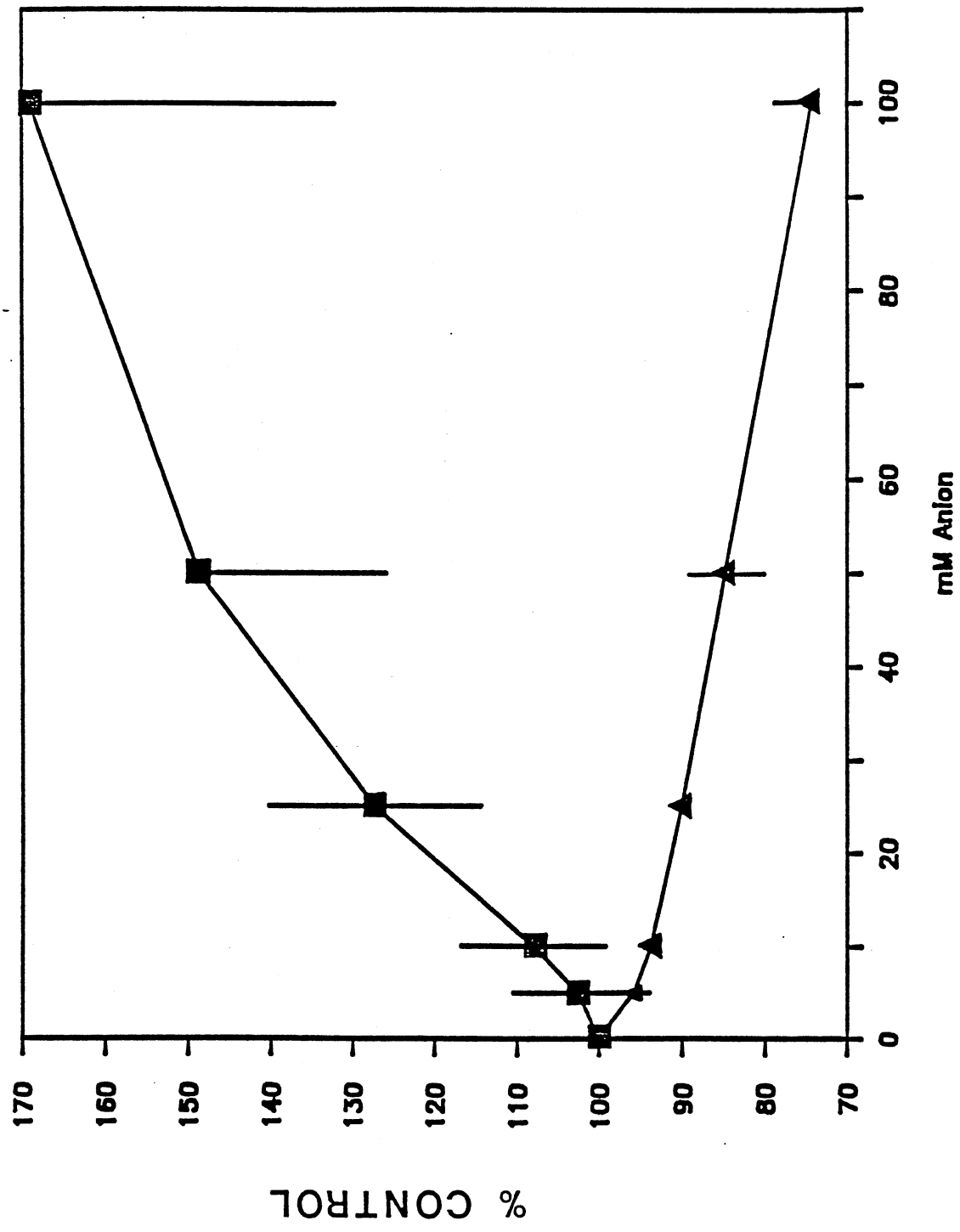

μM PI/30 min/μg
4
3.5
3
2.5
2
1.5
1
0.5
0
BLANK
cAMP
cAMP+PKI
C

Figure 4. Effect of bicarbonate and chloride on activity of anion stimulated ATPase of salivary gland homogenates. (■) Potassium bicarbonate. (▲) Choline chloride. ($\underline{n}$=3).


% CONTROL
170
160
150
140
130
120
110
100
90
80
70
0
20
40
60
80
100
mM Anion

VITA

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