PHYSIOLOGICAL ROLE OF V-ATPASE AND DETECTION OF IT'S ACTIVITY IN THE SALIVARY GLANDS OF THE LONE STAR TICK *AMBLYOMMA AMERICANUM*(L.)

By

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LIST OF ABBREVIATIONS

APS	Ammonium Persuphate
DMSO	Dimethyl Sulphoxide
Buffer A	Homoginization buffer for the SDS PAGE
S-B	Stacking gel buffer
R-B	Running gel buffer
RPS	Rabbit Pre immune Serum
PBS	Phosphate Saline Buffer
Ab	Antibody
D-B	Dissecting Buffer
Buffer B-1	Homoginizing buffer 1
Buffer B-2	Homoginizing buffer 2
DMSO	Dimethyl Sulfoxide
Pi	Inorganic phosphate
TCA	Trichloro acetic acid
EtOH	Ethanol
MES	2-(*N*- morpholino)ethanesulfonic acid
PEP	Phosphoenolpyruvate
RSO	Right Side Out
ISO	In Side Out
DCCD	N,N'-dicyclohexylcarbodiimide
OD	Optical Density
PMSF	Phenylmethylsulphonyl flouride
TEMED	N,N,N',N'-Tetramethylethylenediamine

CHAPTER I

Introduction

Ticks are found in all terrestrial regions of the earth and are important vectors of human and animal diseases . They are blood feeding arthropods and second only to mosquitoes as vectors of life threatening human and animal diseases. Ticks transmit a greater variety of infectious agents than any other arthropod group. Among many pathogenic organisms transmitted by ticks are fungi, viruses, rickettsia, bacteria and protozoa. Rocky mountain spotted fever, a severe life threatening rickettsial disease, is widespread throughout the eastern United States. Lyme disease, caused by a spirochete, is now the most important vector borne disease in the United States and Europe. Other examples of tick transmitted diseases include Human babesiosis and human ehrlichiosis. Other tick borne diseases affect livestock and these include heart water, East coast fever, louping ill and cattle fever. Worldwide losses due to tick feeding and tick borne diseases on livestock are estimated to amount to several billion dollars each year (1).

Ticks are classified into two major families, the Ixodidae, or hard ticks, and the Argasidae, or soft ticks. A third minor family, Nuttalliellidae, consists of only one species. The ixodid life cycle includes larval, nymphal, and adult stages,each with a single instar. In contrast, argasid ticks have two or more nymphal instars. Most argasid ticks feed rapidly, ingesting 5-10 times their initial body weight in minutes to a few hours. Ixodid ticks spend more time attached to the host. Adult female ixodids undergo a slow feeding phase for up to 48 hours. They detach only after ingesting enough nutrients for egg production. The

ixodid female may ingest more than 100 times her initial body weight. *Amblyomma americanum* (L) increase from an unfed weight of 4 mg to a weight of about 250 mg in 8-14 days (slow feeding stage) and then enter a state of rapid feeding attaining a weight of 600-900 mg in 12-48 hr. (2). To be successful as ectoparasites, ticks have developed some remarkable features. These include their ability to live for a relatively long period (up to several years) off the host ; large bloodmeal size ; slow bloodmeal digestion ; and prolific egg production (1).

The salivary glands of the tick have many functions and are vital to the biological success of ticks and play a critical role in the transmission of pathogens. Among important functions of the salivary glands is the production of ameliorating substances that are necessary to overcome the problems associated with prolonged feeding and attachment to the host. These substances exhibit antiinflammatory, analgesic, anti-coagulant, anti-hemostatic, vasodilatory, and/or immunosuppressive properties (3, 4). The SGs are the route of transmission for most pathogens and the development site for many. Another important function is to concentrate the blood meal by returning excess water and ions to the host. Conversely, during periods when they are not attached to the host, ticks can avoid desiccation by trapping water vapor using SG secretions. They also play a primary role in osmoregulation. As a result, to perform their required functions, the SGs undergo marked and rapid structural reorganization after the tick attaches to the host.

The salivary glands of the female ticks consist of three multicellular alveoli types, type I, II, and III. (4).Type II and III undergo major changes in

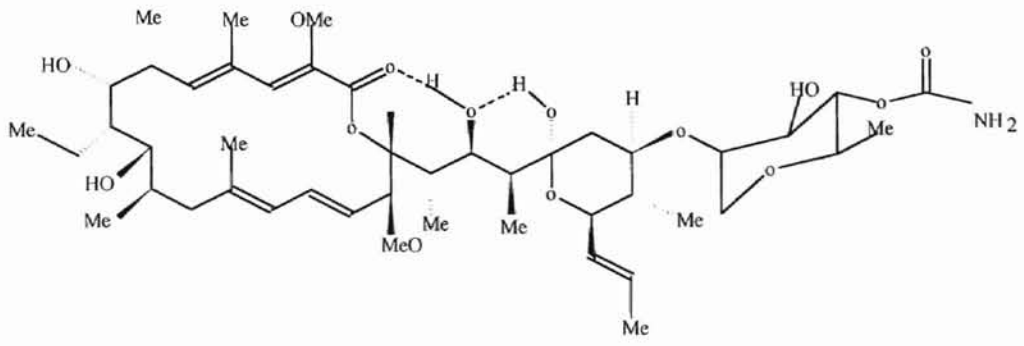
morphology after few a days of feeding. In the final stages of feeding, most of the granular material seen in the early stages has disappeared. Cells in the type III alveoli, transform to resemble fluid transporting epithelia, consisting of a complex mass of plasma membranes and closely associated mitochondria (reviewed by 4). The actual mechanism of salivary secretion is not well understood. However, it is known that the salivary glands are controlled by nerves and the neurotransmitter at the neuroeffector junction is dopamine. Type I acini are located in the anterior part, attached to the main duct and contain no granules. The morphology of the type I alveolus in unfed female ticks, *Amblyomma americanum* (L.) consists of several peripheral pyramidal cells, a central cell, a constrictor cell, and numerous peritubular cells (5). These alveoli may also secrete hygroscopic material (6, 7) that is involved in water absorption from unsaturated air (8, 9, 6, 10). The salivary glands of the ticks more closely resemble the acinar, multicellular salivary glands of the cockroach *Periplaneta americana* (11). The distal duct cells of the cockroach is known to contain V-ATPase (12), and are similar to the type I acini of the tick salivary glands.

Currently there are three classes of ATP hydrolyzing proton pumps, or H^+-ATPases, namely, P-ATPase, F-ATPase, and V-ATPase. H^+ translocating P-ATPases as well as Na^+/K^+-ATPases of plasma membranes and the Ca^{2+}-ATPase of sarcoplasmic reticulum, form phosphoaspartyl intermediate and are inhibited by the phosphate analog orthovanadate. F-ATPases are the proton translocating ATP synthases of mitochondria, chloroplast and bacterial plasma membrane and are inhibited by azides. V-ATPases were originally found in vacuoles and other intracellular vesicular organelles (hence the V for vacuolar),

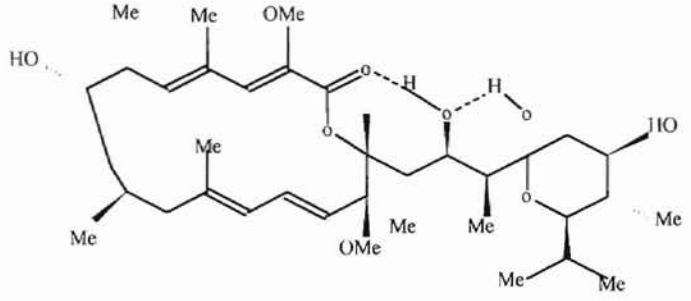
but have subsequently also been found in the plasma membranes in the midgut and sensory epithelia (13, 14). This enzyme is specifically inhibited by bafilomycin A1 (15), and concanamycins (16).

Bafilomycins and concanamycins, which can be distinguished in structure and function, are closely related compounds that belong to the group of "unusual macrolides" (17), which are characterized by a 10 to 48 membered macrocyclic lactone ring and which are produced by bacteria. Together with related, highly unsaturated macrolides produced by *Streptomyces* species, bafilomycins and concanamycins encompass the group of plecomacrolides (18). Their most pronounced structural element is the six membered hemiacetal ring, which is connected to the macrolactone ring by a C3 spacer (19). Structures of bafilomycin A1 and folimycin are shown in figure 1.

Overall the V-ATPase has a large number of functions. Compartments receiving protons from V-ATPase can be rendered acidic, neutral or even alkaline, depending upon the selectivity of other carriers (Porters) and channels present upon the strength of gegenions and upon cellular and compartmental geometry. In mammalian cells these enzymes help make Golgi vesicles mildly acidic, clathrin coated vesicles more acidic and lysosomes highly acidic (20, 21, 22). V-ATPases are involved in endocytosis and exocytosis (23, 24). In yeasts and in other fungi this enzyme acidifies vacuoles used as storage sites for



a.



b.

Figure 1. Structures of folimycin (a.) and bafilomycin A1 (b.).

amino acids, Ca^{2+}, carbohydrates, phosphate and hydrolases (25, 26). They energize the salt regulation and osmotic balance in tonoplast, which is the membrane surrounding the central vacuole in plant cells (27, 28). V-ATPases also energize accumulation of neurotransmitter amines by synaptic vesicles and chromaffin granules (29). In osteoclast membranes they acidify the extracellular fluid and provide an environment for the optimum performance of osteoclasts leading to bone reabsorption in both normal and abnormal conditions (30). V-ATPases play an essential role in the acid base balance by energizing renal plasma membranes leading to acidification of urine and reabsorption of bicarbonates (31, 32, 33).

In all of the above cases V-ATPase pumps protons into acidic compartments. However, a V-ATPase in the apical plasma membrane of phagocytic cells alkalizes the external medium; similarly a V-ATPase in the apical plasma membrane of certain insect epithelial cells can produce a lumen pH exceeding 11. How this enzyme can produce many of these diverse roles depends on the other transporters present in the membrane.

Fluid and ion transport in the midgut and the Malphigian tubules of many insects have also been shown to depend on the action of V-ATPase (13, 14, 34, 35, 36). The action of V-ATPase pumps protons away from the cytoplasm thus creating a proton gradient which can lead to an electrical or chemical gradient depending on the other ion channels present in the membrane. In fluid transporting membranes protons are exchanged with cations in the apical membrane and this creates a net movement of anions into the cell through the basolateral membrane and eventually moving out into the lumen through anion

channels due to the charge separation (Fig 2.). This movement of ions creates the necessary osmotic difference for the movement of fluid through the membranes.

These enzymes are multimeric complexes with cytoplasmic V1 and membrane bound V0 analogous to the F1 and F0 domains of F-ATPases. Cytoplasmic ATP is hydrolyzed in the V1 domain, forcing protons away from the cytoplasm through the V0 domain across the membrane. The large, hydrophilic V1 domains protrude as particles on the cytoplasmic face of vacuolar and plasma membranes. Like F1 particles V1 particles are approximately 9-10 nm in diameter and are always present on cytoplasmic surfaces of cation transporting membranes.

The catalytic sector (V1) contains six different polylpeptides denoted as subunits A to F. The stoichiometry of subunits A, B, C, D and E in the catalytic sector of V-ATPase from bovine clathrin coated vesicles was determined to be 3:3:1:1:1, respectively (37). Subunit A of V-ATPase is the NEM sensitive catalytic ATP hydrolyzing subunit that is equivalent to the ß subunit of F-ATPase.(38, 39, 40, 41). The amino acid sequence of this subunit contains a glycine rich motif that is common for ATP binding proteins (42).

Subunit B of V-ATPase may contain a regulatory ATP binding site (43). This subunit binds ATP analogs only under restricted conditions (44). Sequence analysis has revealed extensive homology to the α subunit of F-ATPase (45). Subunit B, however, contains no glycine rich sequence, normally found in nucleotide binding site. These and other observations suggest that subunit B may function in regulating the activity of V-ATPase (43).

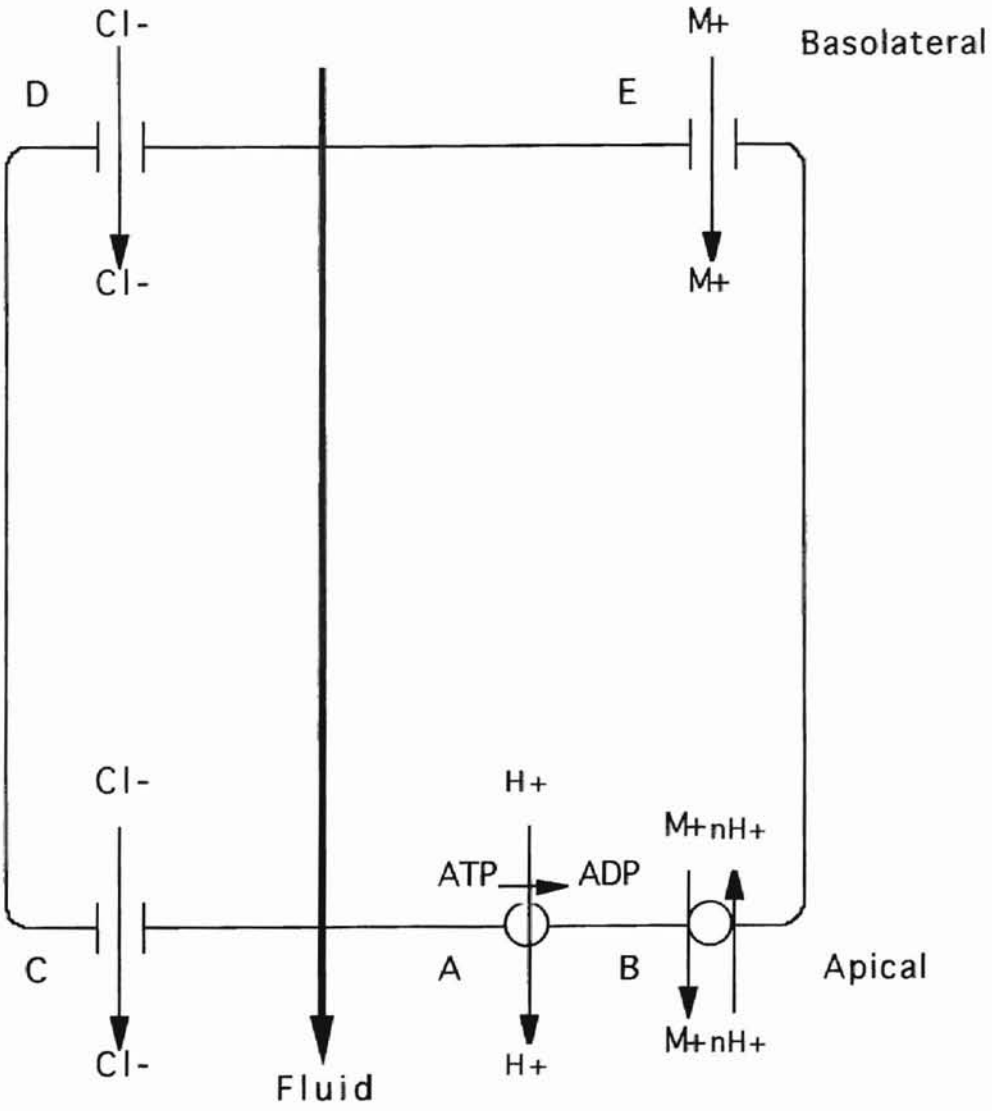


Figure 2. Model of fluid transport and involvement of V-ATPase.

The remaining subunits in the catalytic sector of V-ATPase have no homology to F-ATPase subunits. No functions have been assigned to subunits C, D, E and F.

The function of the membrane sector is to conduct protons across the membrane. This sector of V-ATPase is also composed of multiple subunits, and the 16 kDa proteolipid (subunit c) is the principal subunit of this sector. Subunit c is a highly hydrophobic protein containing about 160 amino acids. This subunit binds to N,N'-dicyclohexylcarbodiimide (DCCD) which blocks the proton conductance across the membrane and inactivates the enzyme.

The M 115 is thought to bind the catalytic sector of the enzyme and M 16 subunit has high homology to the b subunit of F-ATPase. The functions of most of the subunits in the membrane sector are unknown and need to be established in the future. Based on the work done on mammalian and yeast V-ATPase the current view of the enzyme is shown in figure 3.

An unusual property of F-ATPase is it's sensitivity to cold (46), and it is shown that the V-ATPase is sensitive to cold too and inactivated at 0 ºC (47). When reconstituted V-ATPase from chromaffin granules was incubated on ice, the enzyme lost it's capability to pump protons. This was dependent on the presence of Mg^{2+}, Cl^- and ATP during incubation. Approximately 1 mM ATP, 1 mM Mg^{2+}, and 200 mM Cl^- were required for maximum inactivation and incubation for about 10 min was required to achieve 50% inactivation (47).

Recently the complete cDNA sequence of the C subunit of this enzyme has been cloned in the tick salivary glands of the female tick, *Amblyomma americanum* (L) (11), suggesting the presence of a V ATPase in the female tick

salivary glands. The purpose of this project was to see if the functional enzyme is present in the salivary glands of the tick and if V-ATPase plays a role in the fluid secretion in the female tick *Amblyomma americunum* (L) salivary glands.

Subunit	M.W. kDa
A	68
B	57
C	44
D	28
E	26
F	14
a	20
c	16
M 16	14
M 115	96
M 48	51
M 39	36

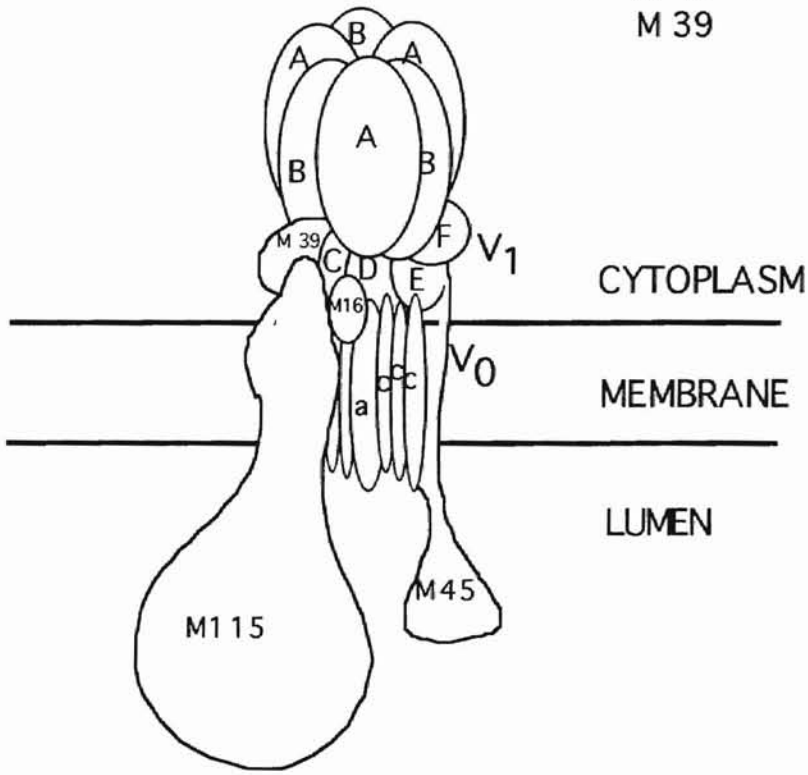


Figure 3. Overall subunit structure of a representative V-ATPase. M. stands for membrane bound subunits.

CHAPTER II

Materials and methods

Fluid secretion assays

Amblyomma americanum ticks were fed and maintained as described by (48), in the tick rearing facility at Oklahoma State University. Salivary glands were dissected and secretion was assayed by two methods, the salivary duct ligation method (49, 50), and open duct technique method (51, 52).

Salivary duct ligation method

Salivary glands from partially fed females *Amblyomma americanum* were dissected and placed in TC-199 MOPS containing 0.01 g/l streptomycin sulphate and 0.03 g/l penicillin G (52) with and without ATPase inhibitors dissolved in media containing 3% propylene glycol:ethanol: DMSO (1:1:1,v:v:v). Inhibitors included bafilomycin A1 and folimycin which are specific V-ATPase inhibitors obtained from Sigma (St Louis, MO) and Calbiochem (Lajolla, Calif.). Stock solutions of 100μM bafilomycin and folimycin were made and the final concentration of these inhibitors in the assay was 100 μM. Ouabain (specific inhibitor of Na^+/K^+-ATPase), and N-ethyl maleimide (NEM) (an inhibitor of P-ATPases and V-ATPases) were obtained from Sigma (St Louis, MO). NEM was prepared as a 10 mM stock solution and the final concentration of NEM in the assay was 10 μM. Ouabain was prepared as a 100 mM stock solution and from this stock solution a 10 mM solution was prepared and the final concentration of

ouabain in the assay was 100 μM. The main salivary ducts were ligated and severed anterior to the ligation. One gland was treated as the control and the other as the experimental. After ligation the control gland was preincubated for 15 min in TC-199/MOPS with the appropriate amount of the solvent DMSO. The experimental gland was treated the same way as the control except it was incubated with the appropriate inhibitor. After the preincubation the glands were stimulated with 1 μM dopamine and weighed at 5 minute time intervals. The percent weight increase was calculated based on the initial weight of the gland. Weight increases are proportional to the salivary fluid secretion (49, 50, 52).

Open duct method

Salivary secretion from the glands was monitored by using the open duct method (51, 52). Ticks were dissected in the TC-199 MOPS medium and the glands were isolated carefully with some mouthparts attached to the duct. Glands were then placed in a small indentation of wax filled with a drop of TC-199 MOPS in a petri dish. The buffer droplet was covered with mineral oil. The main salivary duct was drawn into the surrounding mineral oil and anchored via still attached mouthparts onto a metal wire. Glands were pre incubated for 15 min in either the control or the experimental media and then stimulated with 1μM dopamine. Secreted saliva was collected using a capillary tube at 5 minutes intervals and transferred to another region of the mineral oil. At each interval the glands were rinsed several times before the next stimulation. Finally the volume of the secreted saliva was calculated by using the formula $4/3\pi r^3$. Secretion is expressed in nl/min at room temperature.

Western blotting

SDS-PAGE

Tissue preparation: The ticks were dissected and the glands were isolated in the dissecting buffer (D-B) containing 20 mM EGTA and 0.1 M MOPS at pH 7.6. Glands were then homogenized using dual 20 homogenizer in the homogenization buffer (buffer A) containing 10 mM Tris-HCL, 1 mM EDTA and 10 mM NaCl. The protease inhibitor Phenylmethylsulphonyl flouride (PMSF) was added in 1 mM final concentration to the homogenization buffer just before the glands were homogenized. PMSF was prepared as a 100 mM stock solution in 100% EtOH to prevent it from hydrolysis, and stored at 4^0C.

After homogenization the homogenate was centrifuged at 2000 rpm in a bench top centrifuge for 5 minutes to remove the crude particles. The supernatant was then centrifuged at 11750 rpm in the same centrifuge for 10 min to separate the plasma membrane fraction (53). The pellet containing the plasma membrane fraction was resuspended in buffer A. Protein concentration of both the plasma membrane fraction and the supernatant was then determined using the Bradford method (1976). If the SDS gel electrophoresis was not done on the same day the tissue was stored at -70^0C.

As a positive control the midgut of *Manduca sexta* was dissected and homogenized in buffer A, and the protein concentration was determined using the Bradford method. The Manduca tissues was also stored at -70^0C.

Gel preparation

Stock solutions: A 30% stock solution of acrylamide/bis form Bi-Rad was

used for all the experiments. Upper stacking gel buffer (S-B)contained 0.5 M Tris-HCl, pH 6.8, and the resolving gel buffer (R-B) contained 1.5 M Tris-HCl, pH 8.8. These buffers were stored at 4 ^{0}C. A 10% SDS solution was prepared by dissolving 10 g SDS in 90 ml of H_2O and bringing the volume up to 100 ml, and a 10% solution of ammonium persulphate (APS) was prepared by dissolving 100 mg of APS in 1 ml of deionized water. Sample buffer 8 ml was prepared by mixing 3.8 ml of deionized water, 1.0 ml of S-B, 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4 ml of 2-mercaptoethanol, and 1% (w/v) bromophenol blue. Sample buffer and the SDS solution were stored at room temperature. A 10X running buffer was prepared by mixing 30 g of Tris base, 144 g of glycine, and 10 g of SDS in 1 L of deionized water. This buffer was stored at 4 ^{0}C and was brought to room temperature before each run. A 1X solution of running buffer was prepared for each electrophoretic run by diluting 50 ml of 5X buffer with 450 ml of deionized water.

Each electrophoretic run was done on a 4% stacking gel and a 12% separating gel. The 4% gel was prepared by adding 3.05 ml of deionized water, 1.25 ml of S-B, 50 μl of 10% SDS 0.665 ml of 30% acrylamide/bis, 25 μl of 10% ammonium persulphate, and 5 μl of TEMED. The 12% separating gel was prepared by adding 3.35 ml of deionized water, 2.5 ml of R-B, 100 μl of 10% SDS solution, 4 ml of 30% acrylamide/bis, 50 μl of 10% APS, and 5 μl of TEMED.

SDS-PAGE was done using Bio-Rad mini-PROTEAN II electrophoresis cell, and on 1 mm thick 12% separating gel and a 4% stacking gel. All the samples were diluted 2X with the sample buffer and boiled for 3 minutes at 100

^{0}C. The samples were centrifuged briefly for about 30 seconds and 100μg of protein was loaded onto each well. A constant voltage setting of 200 volts for 20 min was used for all the runs.

After electrophoresis the gel was cut into two halves, one half was stained with coomassie blue and the other half was used to transfer the protein onto a nitrocellulose membrane. The gel was usually stained overnight and destained for about 2 hours using a constant shaker.

Transfer and Blotting

The transfer buffer was prepared containing 25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3, and stored at 4 ^{0}C. Following electrophoresis, the gel was rinsed in the transfer buffer for about 1 minute. The transfer was done using a Bio-Rad mini trans-blot electrophoretic transfer cell, as described in the Bio-Rad instruction manual. The transfer was completed within in 1 hour at a constant voltage of 100 V and a variable current that started at about 250 mA and increased up to about 350 mA after 1 hour.

After transferring, the membrane was placed in a petri dish and blocked for 45 minutes in blotto containing 5% non fat dry milk in PBS (Phosphate Saline Buffer) with gentle agitation at room temperature. Then the blotto was removed and the membrane was incubated with the primary Ab (Antibody) diluted 1:200, overnight with gentle agitation at 4 ^{0}C. The primary Ab was raised in rabbit against the B subunit of V-ATPase of *Culex quinquefasciatus*, that was kindly provided by Dr. Sarjeet S. Gill, University of California, Riverside. The membrane was then washed 2 times with blotto, and 3 times with blotto and

0.05% Tween-80 for 5 minutes each. After washes, the membrane was incubated for 60 minutes at room temperature with diluted (1:5000) secondary Ab (Goat anti rabbit) conjugated to horseredish peroxidase. The membrane was again washed twice with blotto and 3 times with blotto and Tween for 5 minutes each.

The bands were visualized using 4-chloro-1-naphthol as the substrate. The substrate was dissolved in 10 ml of methanol and was added to a container containing 60 ml of PBS and 90 μl of 3% H_2O_2. The membrane was then incubated with the substrate up to 30 minutes until the color is developed.

Assay for V-ATPase activity in tick salivary glands

Colorimetric assays

Ticks were reared and maintained as described above. Partially fed female ticks (50-150 mg) were dissected and the glands were isolated in the dissecting buffer (D-B). Glands were then homogenized in Buffer B-1 (homogenizing buffer 1) containing 10 mM Tricine (pH 7.2), 0.25 M sucrose, 10 mM $MgCl_2$ and 0.05% Benzamidine. The homogenate was centrifuged at 2000 rpm in a bench top centrifuge for 5 minutes and the supernatant was used for the assay. Protein concentration was determined using the Bradford method (1976). A 2x assay buffer included 2 mM $MgCl_2$, 40 mM KCl, 100 mM Tris MOPS, 0.2 mM EDTA, 2 mM Mercaptoethanol, 0.6% BSA pH 8.0 (54).

Inhibitors: 50 mM sodium azide in H_2O stored at 4 ^{0}C, 5 mM sodium orthovanadate in H_2O stored at room temperature, I mM NEM in EtOH (ethanol)

stored at 4 ^{0}C, 5μg/μl oligomycin in EtOH stored at -20 ^{0}C,1 mM ouabain in H_2O stored at -20 ^{0}C, and 100 μM Bafilomycin A1 dissolved in DMSO (dimethyl sulfoxide) stored at -20 ^{0}C. A stock solution of 50 mM Tris ATP was prepared. and used as substrate.

The assay contained 75μl of 2x assay buffer, and 150 μg of protein in the crude homogenate. After the addition of inhibitors and the homogenate (enzyme), the final volume was brought up to 150μl by the addition of distilled and deionized water. Before the reactions were started, homogenate and the inhibitors were pre-incubated for 10 minutes. Each assay was then started by adding ATP to a final concentration 2.0 mM and the assays were carried out at room temperature. Most inhibitors were assayed individually, but in order to detect the specific V-ATPase activity, bafilomycin was assayed in the presence of oligomycin and ouabain, or azide and vanadate. However, in most experiments V-ATPase activity was detected by assaying ATPase activity in the presence of bafilomycin A1 with and without azide and vanadate. Initial velocity conditions were also tested for the enzyme (p. 42). The Final concentrations of inhibitors were 2.8 nM oligomycin, 10 μM NEM, 5 mM sodium azide, 0.1 mM orthovanadate, 10 μM bafilomycin A1 and 0.1 mM ouabain. The reactions were stopped by the addition of 30 μl of 50% TCA (trichloro acetic acid). Each assay contained a zero time control where, the reaction was stopped immediately by adding TCA. All other reactions were carried on for 30 minutes. Controls for DMSO and EtOH, without inhibitors were also conducted. After 30 minutes, reactions were stopped by TCA, and then centrifuged at 6000 rpm in a bench top centrifuge for 2 minutes. ATPase activity was measured by monitoring the

release of inorganic phosphate (Pi) using the Method of Fiske and Subbarow (55). Acid molybdate and the Fiske reagent were purchased from Sigma St. Louis, MO. A stock solution of 0.2 mM NaH_2PO_4 was prepared and used to generate a standard curve with a concentration range of 8-56 nmol. Color development was started by the addition of Fiske reagent and absorbance at 660 nm was read after the addition of the reagent.

Other tick salivary gland tissue was prepared as described for *Manduca sexta* (see below). Tick salivary glands were dissected and isolated in the dissecting buffer (D-B). Glands were homogenized in B-2 buffer and were centrifuged at 2000 rpm for 5 minutes. The supernatant was then diluted in 2 ml of B-2 buffer and centrifuged for 60 minutes at 100,000xg. The pellet was then resuspended in the assay buffer or the resuspension buffer (resus buffer) containing 5 mM Tris MOPS, 3.2 mM mercaptoethanol, 0.32 mM EGTA, 0.25% ethanol, 0.1% BSA at pH 7.5. The protein concentration was determined and was corrected for the 0.1% BSA present in the buffer. 150 μg enzyme was used for the assays. V-ATPase activity was then measured as for M. sexta, and the same controls were included.

Spectrophotometric method

ATPase activity was measured using a coupled reaction as described in (56). Continuous hydrolysis of ATP by ATPases was monitored by coupling the production of ADP to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase reactions. NADH breakdown was monitored using a Thermo max micro plate reader from Molecular Devices. The reactions are coupled as

shown in the Fig. 4.

The following reagents were prepared. The ATPase stock assay medium contained 7.05 mM ATP sodium salt, 10.6 mM $MgSO_4$ and 59 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH, pH 6.0. The solution was stored at -20 °C. Stock solutions of 45 mM NADH in 10 mM Tris-MES, pH 7.5, and 125 mM phosphoenolpyruvate (PEP) were prepared. Lactate dehydrogenase in glycerol with a activity of 4.56 units/μl, and 3.8 mg of Pyruvate kinase in 3.4 ml of glycerol with an activity of 400 units/mg was purchased from Sigma St. Louis, MO.

The reaction mixture contained, 6 mM ATP, 9 mM $MgSO_4$, 50 mM MES-KOH, pH 6.0, 0.45 mM NADH, 1.25 mM PEP, 1.2 units of lactate dehydrogenase, and 25 units of pyruvate kinase in a volume of 200 μl. V-ATPase activity was measured with and without 10 μM folimycin in the presence of 100 μM sodium orthovanadate and 500 μM sodium azide. The requirement of the pyruvate kinase for a monovalent cation is satisfied by the KOH used to adjust the pH. The oxidation of NADH was recorded at 30 °C by monitoring the absorbance at 340 nm using a thermomax micro plate reader from Molecular Devices. A blank was included in the assay which was subtracted from all the readings. The blank contained everything of the above reactants except NADH. Another consisted of all the reactants except the homogenate. Other controls included the solvent DMSO without inhibitors.

All the components in the assay, including the inhibitors were first added to the wells and thoroughly mixed, and incubated for 10 minutes at room

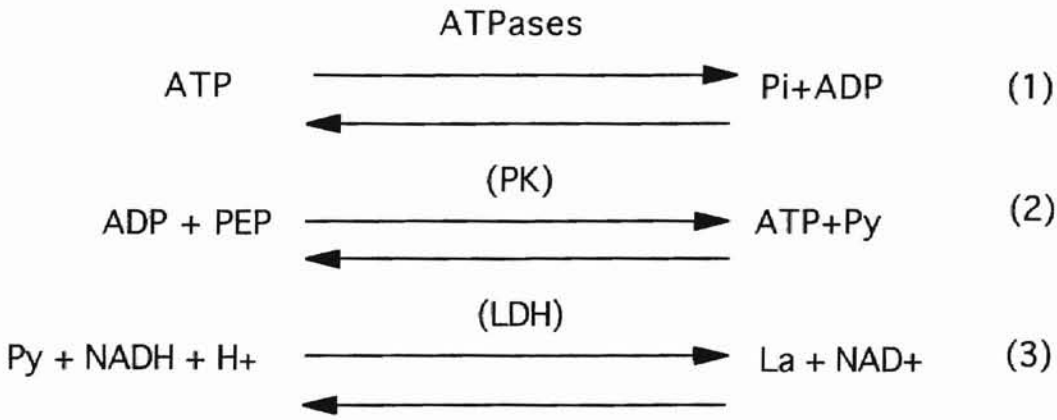


Figure 4. The reactions in the coupled ATPase assay
Pi, inorganic phosphate; PEP, phophoenolpyruvate; PK, pyruvate kinase; Py, pyruvate; LDH, lactate dehydrogenase; La, lactate. Reaction (1) requires Mg^{2+}; reaction (2) requires Mg^{2+} and K^{+}. During the assay all the reactions proceed to the right. The rate of reaction (3) is determined as dA_{340}/dt. The rate measured in reaction (3) is the reaction rate of reaction (1) (57).

temperature. The total volume of the assay was brought to 200 μl by the addition of deionized water. After 10 minutes, the assay was started by the addition of NADH and its oxidation was monitored at 340 nm at 30 °C. The reaction was monitored for 15 minutes and readings were recorded automatically every 15 seconds. The reactants were stirred before each absorbance reading.

Solvent and Batch Affects on V-ATPase activity

Stock solutions of 100 μM and 1 μM bafilomycin were prepared using EtOH and DMSO as solvents. To obtain 10 and 0.1, and 1 and 0.01 μM bafilomycin in the assay reactions, 15 and 1.5 μl were taken respectively from the stock solutions. The assays were performed and the inorganic phosphate was determined as described for the colorimetric assays. Controls for the solvents DMSO and EtOH, and a control without bafilomycin were included in the assays. Since the most solvent added was 15 μl (10% of the total assay volume) the solvent control included 15 μl of either DMSO or EtOH.

In other experiments, in order to avoid the variation of having different amounts of solvent, even amounts of the solvent was added to all the reactions. Serial dilutions of 10, 1 and 0.1 μM bafilomycin was prepared from the 100 μM bafilomycin stock. For each reaction, bafilomycin A1 was diluted 10-fold by adding 15 μl (10% of the total assay volume) of the appropriate solutions to the total reaction volume of 150 μl to achieve 10, 1, 0.1, and 0.01 μM final concentrations of bafilomycin. To see the effect of adding only 1% of the solvent, 1.5 μl was taken from the 100 and 1 μM stock

Table 1. Standard curve generation and Inorganic phosphate determination of the assay samples.

	Blank	Standards (μl)							Sample
	(μl)	1	2	3	4	5	6	7	(μl)
Acid Molybdate	200	200	200	200	200	200	200	200	200
Fiske Reagent	80	80	80	80	80	80	80	80	80
Pi-Solution		40	80	120	160	200	240	280	
Sample									20
H_2O	720	680	640	600	560	520	480	440	700

solutions to test the effect of 1 μM and 0.01 μM bafilomycin. Azide and vanadate were added to all the reactions except the control. Ethanol and zero time controls were included in the assays. The concentrations of azide and vanadate and the assay conditions were kept the same as described above.

The effect of 10 μM bafilomycin A1, folimycin and 1% DMSO was tested. New bafilomycin A1 and folimycin were purchased from the Wako chemicals, Richmond, VA. Stock solutions of 1 mM bafilomycin and folimycin were prepared from 100 μg of bafilomycin A1 and folimycin. One hundred μM and 10 μM solutions were prepared from the 1 mM stock and stored at -20 ^{0}C. The effect of 10, 1 and 0.1 μM bafilomycin and folimycin on ATPase activity were tested. Volume of 1.5 μl from the appropriate stock solutions were added to the reaction mixture to obtain these concentrations. Azide and vanadate were added to all the reactions except the control. Similar controls were included in these assays as well.

V-ATPase activity in the midgut of *Manduca sexta*

Fourth instar larvae of *M.sexta* were purchased as second or third instar larvae from the Carolina Biological Supply Co., and reared at 26 °C on the artificial diet supplied with the larvae. The tissue was prepared as described in (54).The posterior portion of the larval midgut was removed and cut into small squares of tissue. The tissues were suspended in homogenizing buffer 2 (B-2) containing 0.26 M sucrose, 5 mM EDTA and 5 mM Tris (pH 8.1) and homogenized for 3 minutes in an ice cold Duall 20 homogenizer. The homogenate was diluted with 4 ml of B-2 buffer and centrifuged at 100,000xg

for 60 min at 4 °C.The supernatant was discarded and the pellet was resuspended in 500 µl of assay buffer. The protein concentration with correction for the 0.6% BSA in the assay buffer of the resuspended pellet was determined by the Bradford method. Azide and vanadate insensitive, bafilomycin sensitive ATPase activity was measured as described above. Zero time and no inhibitor controls were performed.

Characterization of Azide and vanadate insensitive V-ATPase activity

Effect of enzyme concentration: Tissue preparation and experimental conditions were as described earlier, except the amount of salivary gland enzyme was variable (25, 50, 100 and 150 µg). Zero time controls to correct for the basal amount of Pi were included in all the experiments. Azide and vanadate were included in all the reactions. The homogenates were preincubated with the inhibitors for 10 minutes before the reactions were started. The effect of bafilomycin(V-ATPase activity) was tested in the presence of azide and vanadate. Inorganic phosphate was determined as described above.

Effect of reaction time: The assays were performed as before with 150 µg of protein, 2 mM ATP, azide and vanadate in the assay mixture. The reactions were stopped at 0, 10, 20 and 30 minutes by adding 50% TCA and the Pi was determined as described above.

Effect of substrate concentration: Tissue preparation and the experiments

were performed as described for the colorimetric assay except the reaction was carried on for only 15 minutes. Substrate (ATP) concentrations of 0.5, 1, 1.5, 2.0 and 2.5 mM were tested in the presence of azide and vanadate. As a control a reaction that did not have any ATP was also included in the assays.

Effect of pH on insensitive ATPase: Using KOH and HCl, the pH of the assay buffer was adjusted to obtain different pHs of 6.09, 6.57, 7.08, 7.53, 8.06, 8.51 and 9.18. Azide and vanadate insensitive ATPase activity was then measured at these pHs as described above.

Effect of cold on V-ATPase activity: After homogenization, the homogenate was divided into two, one was kept on ice while the other was kept at room temperature. Both were tested for V-ATPase activity with and without bafilomycin in the presence of azide and vanadate as described above.

Effect of deoxycholate on V-ATPase activity.

Salivary gland tissue was prepared as previously described except the B-2 homogenizing buffer contained 0.05% benzamidine. the amount of protein used in the assays were 150 μg. A 10% solution of sodium salt of deoxycholate was prepared by dissolving 5 g of the detergent in 45 ml of deionized water and adjusting the volume to 50 ml. Different amounts of the detergent per mg protein were tested in the presence of azide and vanadate with and without folimycin. In one assay, 0,1, 2, 4, and 6 mg/mg protein were tested and in another assay 0, 1, 3, 6 and 9 mg/ mg protein were tested. The homogenate was incubated for 10 minutes with appropriate amounts of the detergent before adding buffer and

inhibitors. After adding inhibitors the mixture was incubated for 5 minutes before the reaction was started by the addition of ATP. A control consisted of everything in the reaction medium except the substrate. Reactions were monitored for 15 minutes.

V-ATPase in different regions of the Salivary glands

Salivary glands were dissected and isolated In the dissecting buffer. The anterior portion of the glands containing the Type I acini were carefully separated from the remainder of the gland. Both the anterior portion and the rest of the gland were homogenized in separate Duall 20 homogenizers in the homogenizing buffer B-2 containing 0.05% benzamidine. Both the anterior and the posterior regions of the salivary glands were prepared as previously described , and assayed for V-ATPase activity with and without folimycin.

CHAPTER III

RESULTS AND DISCUSSION

ATPase activity in tick salivary glands as assessed by specific inhibitors

Colorimetric method

Bafilomycin A1 and folimycin are specific inhibitors of V-ATPase at nM concentrations (15 and 16), while sodium azide and orthovanadate are inhibitors of F- and P-ATPases respectively. Oligomycin is a specific inhibitor of mitochondrial F-ATPase, and ouabain a specific inhibitor of Na^+/K^+-ATPase. V-ATPase is also inhibited by NEM but NEM is not a specific inhibitor of V-ATPase.

To identify and obtain a general idea of ATPases present in the tick salivary gland many different inhibitors were tested (fig. 5). The Pi measured in the 0-time control was subtracted from the rest of the reaction readings to account for the basal level of Pi in the homogenate. 100% activity of ATPase was measured in the reaction in which there were no inhibitors added and the % activity in the other reactions were calculated and plotted based on the 100% activity.

There is about 12-15% inhibition due to oligomycin and bafilomycin, and ouabain shows almost no inhibition. Azide, NEM and EtOH control shows about 10% inhibition while vanadate showed only about 5% inhibition. Bafilomycin, ouabain, and oligomycin together shows about 30% inhibition of total ATPase

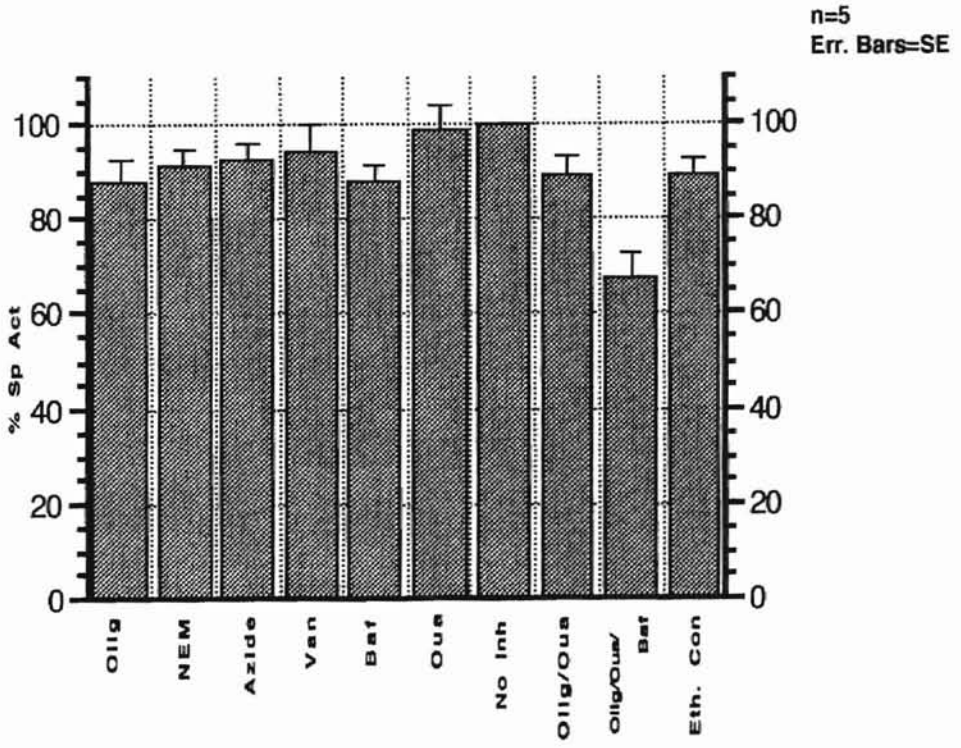


Figure 5. Effect of different inhibitors on ATPase activity & bafilomycin sensitivity to ouabain and oligomycin insensitive ATPase activity. Concentrations of inhibitors were 2.8 nM oligomycin, 10 μM NEM, 5 mM sodium azide, 0.1 mM orthovanadate, 10 μM bafilomycin A1 and 0.1 mM ouabain.

activity.

The little inhibition by ouabain and vanadate is possibly due to the lack of Na^+ in the assay conditions for Na^+/K^+-ATPase to function. The 30% inhibition due to all 3 inhibitors is in agreement with the 12-15% inhibition observed with oligomycin and bafilomycin.

To further establish the inhibition of V-ATPase due to bafilomycin, the classic azide and vanadate insensitive ATPase activity was tested in the presence of bafilomycin (fig. 6).

There is about 15% inhibition due to azide, about 10% inhibition due to vanadate, and about 20% inhibition due to bafilomycin. Azide and vanadate together inhibited ATPase activity about 20%. Azide, vanadate, and bafilomycin inhibited activity about 40%. There seems to be about 20% inhibition due to bafilomycin, and another 20% inhibition due to azide and vanadate. This inhibition due to bafilomycin may be an indication of the V-ATPase activity. however, the bafilomycin concentration used was 10 μM, and V-ATPase in other tissues is sensitive to nM bafilomycin A1. Therefore, I tested various concentrations of bafilomycin and the effect of DMSO on ATPase activity(fig. 7).

There is about 12% and 8% inhibition due to 10 and 0.1 μM bafilomycin respectively. Almost no inhibition was seen with 1 and 0.01μM concentrations of bafilomycin A1. The 10 and the 0.1 μM bafilomycin A1 were tested with 10% of the solvent whereas, the 1 and 0.01 μM bafilomycin A1 concentrations were assayed with only 1% of the solvent. The DMSO control was tested with 10% total DMSO in the assay reaction since the maximum amount used was 10%. The apparent inhibition due to 10 μM bafilomycin A1 was due mostly to the

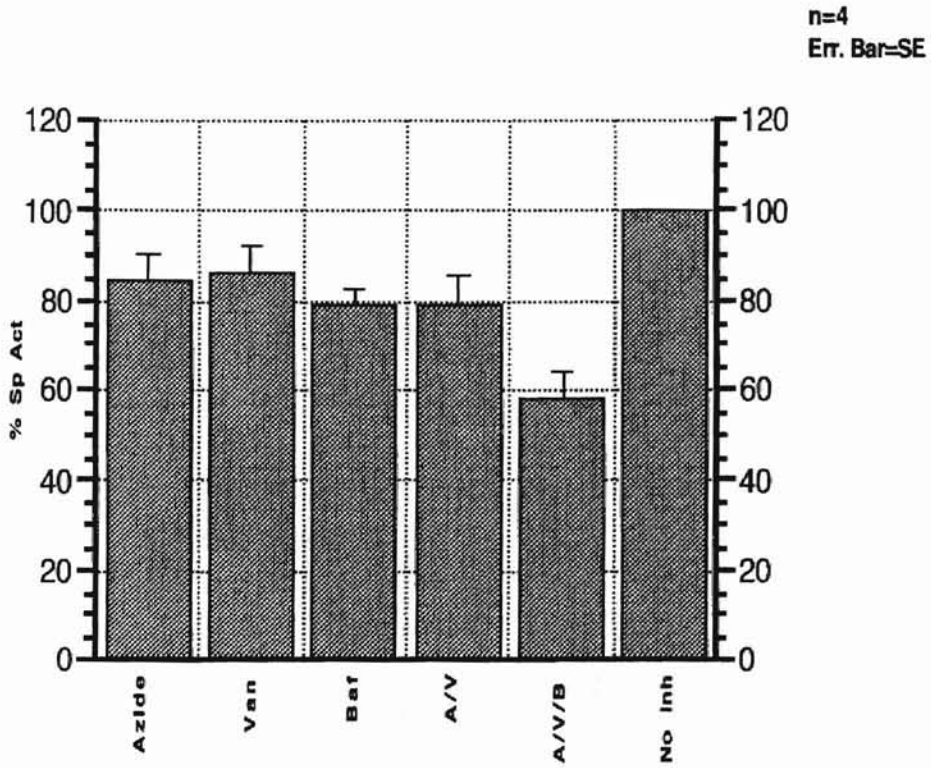


Figure 6. Effect of bafilomycin A1 on azide and vanadate insensitive ATPase activity.
The experiment contains 10% solvent DMSO.Concentrations of inhibitors were 0 5 mM sodium azide, 0.1 mM orthovanadate and 10 μM bafilomycin A1.

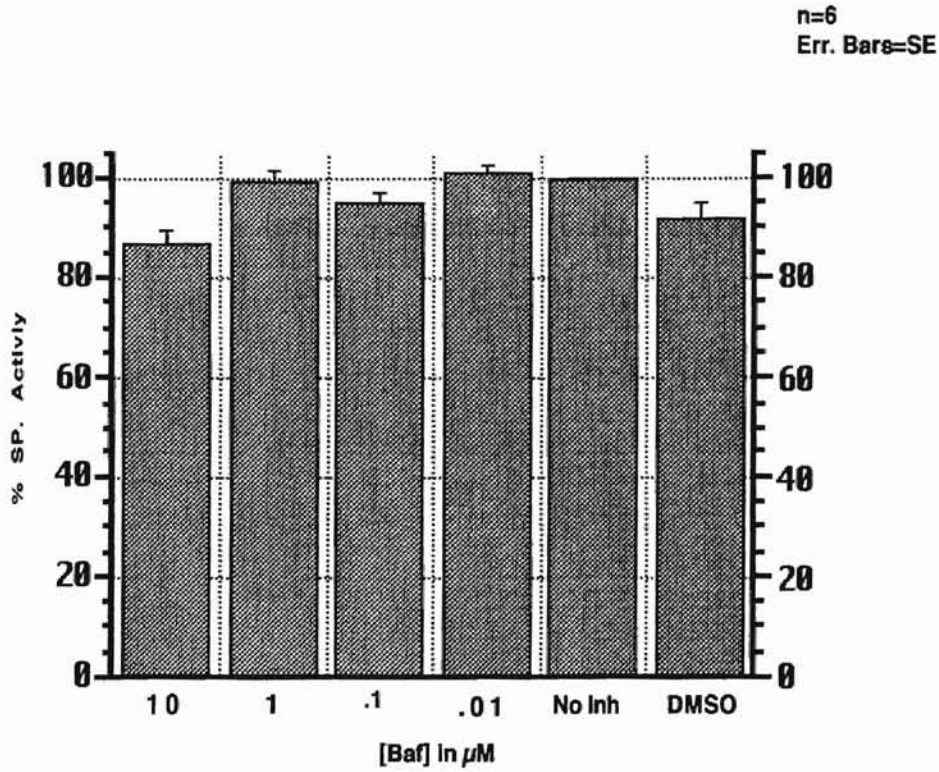


Figure 7. Dose response to bafilomycin A1.
10% of the solvent added to the 10, 0.1 μM bafilomycin and the control DMSO. Only 1% of the solvent added to the 1 and 0.01 μM bafilomycin A1.

solvent DMSO.

Since the solvent DMSO is interferes with the assay, EtOH was tested as the solvent for bafilomycin (Fig. 8). Again 10 and 0.1 μM bafilomycin appeared inhibitory because 10% EtOH was added to the reaction tubes, and the others showed no inhibition. The solvent control inhibited activity about 10%.

To eliminate the variation due to adding different amounts of the solvent in serial dilutions, 10% of the solvent was added to all the reactions (Fig. 9). There was about 20% inhibition due to the EtOH control and about the same amount of inhibition with bafilomycin A1. It is clear that the EtOH at this high level inhibits ATPase activity. Instead of adding 10% of the total volume, only 1% of the solvent EtOH was tested in the presence of azide and vanadate (Fig. 10).

Only 1, 0.1 and 0.01 μM concentrations were tested. The stock concentration of bafilomycin could not be increased since only 10 μg was available from both Sigma St. Louis, MO and Calbiochem. Hardly any inhibition was observed at any of the concentrations tested. Since bafilomycin is expected to inhibit V-ATPase in the nM range, I expected some inhibitory effect even at low concentrations. It is possible that tick V-ATPase is not sensitive to these concentrations of bafilomycin A1.

100 μg of both bafilomycin A1 and folimycin were purchased from Wako Bio Products, Richmond, VA, and 1 mM stock solutions of the drugs were prepared in DMSO. The effect of 10, 1 an 0.1 μM bafilomycin A1 and folimycin were tested in the presence of azide and vanadate, and only 1% of the solvent was added to all the reactions (Fig. 11). Bafilomycin A1 at 10 μM seemed to

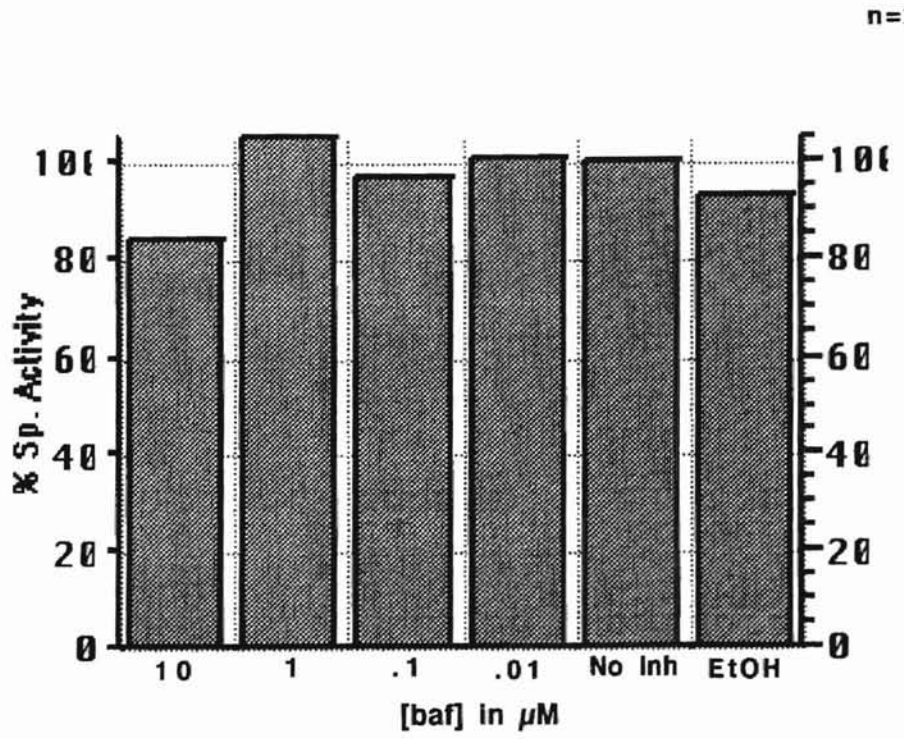


Figure 8 Dose response to bafilomycin A1 using EtOH as the solvent. 10, 0.1 μM bafilomycin A1 and the control received 10% of the solvent while 1 and 0.01 μM bafilomycin A1 received only 1% of the solvent.

n=2

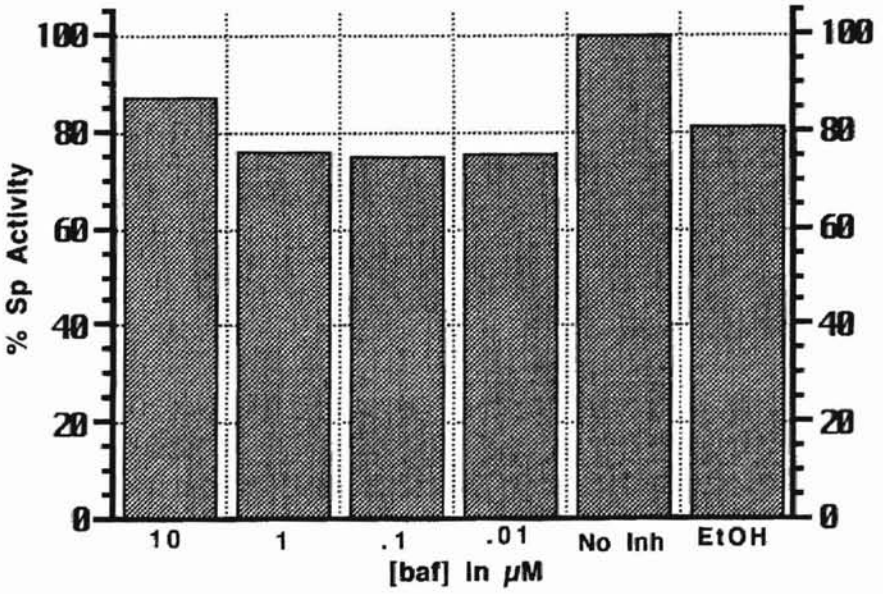


Figure 9. Dose response using EtOH as the solvent
10% of the solvent was added to all the reactions in the presence of 0.5 mM azide and 0.1 mM vanadate.

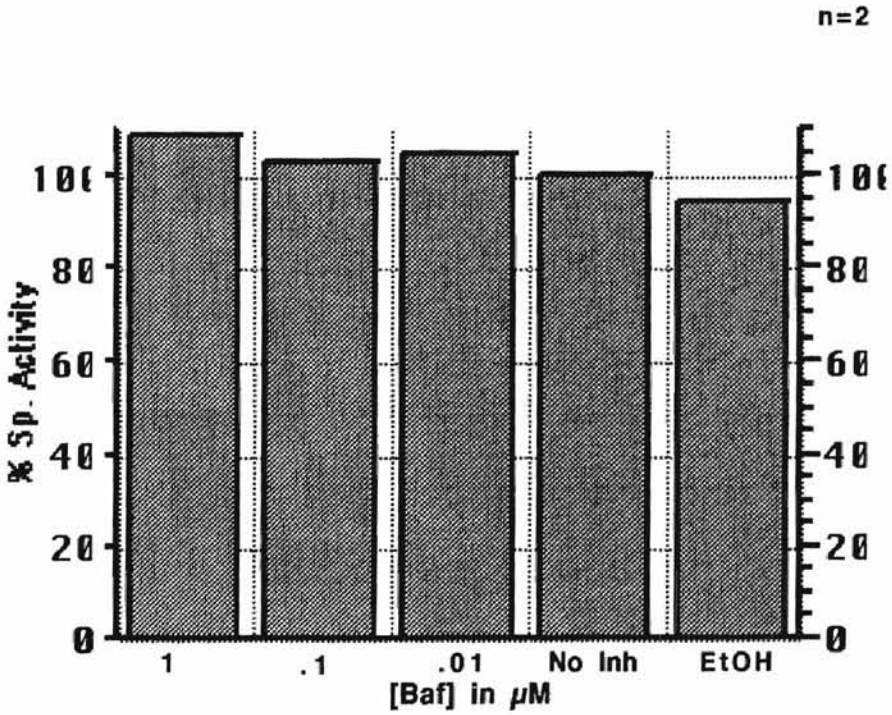


Figure 10. Dose response using EtOH as the solvent
1% of the solvent was added to all the reactions in the presence of 0.5 mM azide and 0.1 mM vanadate.

inhibit about 5% ATPase activity, whereas, folimycin at that concentration inhibits about 20% of the ATPase activity. At 1 and 0.1 μM concentrations of inhibitors there is no inhibition with bafilomycin A1 and only about 5% inhibition with folimycin. DMSO at 1% does not seem to have any inhibitory effect on ATPase activity. The inhibition due to 10 μM folimycin may be due to the inhibition of V-ATPase. It has been documented that folimycin is a more potent inhibitor of V-ATPase than bafilomycin A1 (Drose, et al., 1993).

Is V-ATPase inactivated when kept on ice?

V-, and F-ATPases share many subunits that are very similar to each other (39 and 45). An unusual property of F-ATPase is their sensitivity to cold (46). It was also known that V-ATPase is inactivated at 0 °C (47). Incubation of the reconstituted V-ATPase from chromaffin granules are inactivated on ice in the presence of Mg^{2+}, Cl^- and ATP (47). Tick salivary gland, homogenate was incubated on ice prior to the assay. The homogenization buffer contained 10 mM $MgCl_2$. Since it has been difficult to inhibit azide and vanadate insensitive V-ATPase activity with bafilomycin A1 and folimycin it is possible that the enzyme is inactivated when kept on ice. This was tested by keeping one sample on ice while the other remained at room temperature (fig. 12).

There appears to be no significant difference on ATPase activity between the homogenates incubated at room temperature and on ice. The experiment showed almost no inhibition due to bafilomycin.

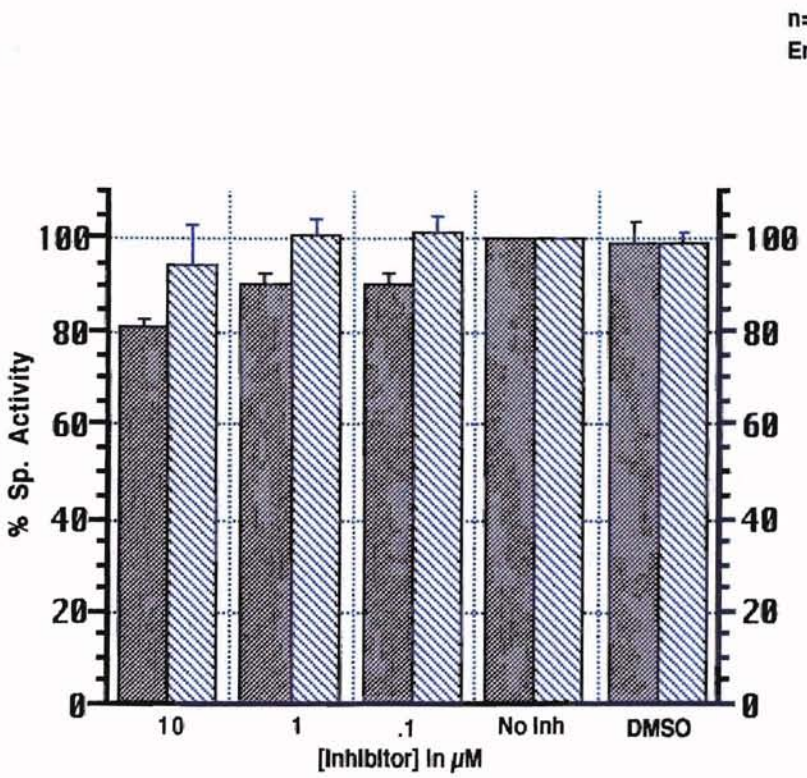


Figure 11. Dose response with bafilomycin A1 and folimycin using DMSO as the solvent.
Total of 1% of the solvent was added to all the reactions in the presence of azide and vanadate. Blue bars represent bafilomycin and the grey bars represent folimycin.

V-ATPase activity in the midgut of *Manduca sexta*

Since there was difficulty showing substantial bafilomycin A1 or folimycin sensitive ATPase activity, the posterior half of the midgut of *M. sexta* was used as a positive control to test the V-ATPase assay system. Goblet cells of the *M. sexta* are known to have high V-ATPase activity and function in creating a high pH gradient making the lumen of the midgut very alkaline reaching pH of about 12 (13). The posterior half of the midgut was tested for V-ATPase activity using bafilomycin as an inhibitor in the presence of azide and vanadate (fig. 13).

There is about 50% inhibition due to bafilomycin compared to azide and vanadate. However, the solvent DMSO also seems to have an inhibitory effect of about 25%. Therefore the true inhibition observed due to bafilomycin is about 25%. There is a clear inhibition of V-ATPase activity by bafilomycin A1 detected in the presence of azide and vanadate. The inhibition due to bafilomycin can be increased by optimizing the conditions for *M. sexta*.

Although the tissue preparation for *M. sexta* was different from the tick salivary gland preparation the assay system was identical. Since the positive control was successful, the assay conditions and the system seem to be working. Since, the positive control works and yet it is hard to show substantial V-ATPase activity in the tick salivary glands, further experiments were required.

Even though it was difficult to obtain substantial inhibition with specific V-ATPase inhibitors, there is still considerable ATPase activity in the presence of azide and vanadate. Since azide and vanadate are supposed to inhibit all the P-and the F-ATPases, the ATPase activity seen in the presence of azide and vanadate is unknown.

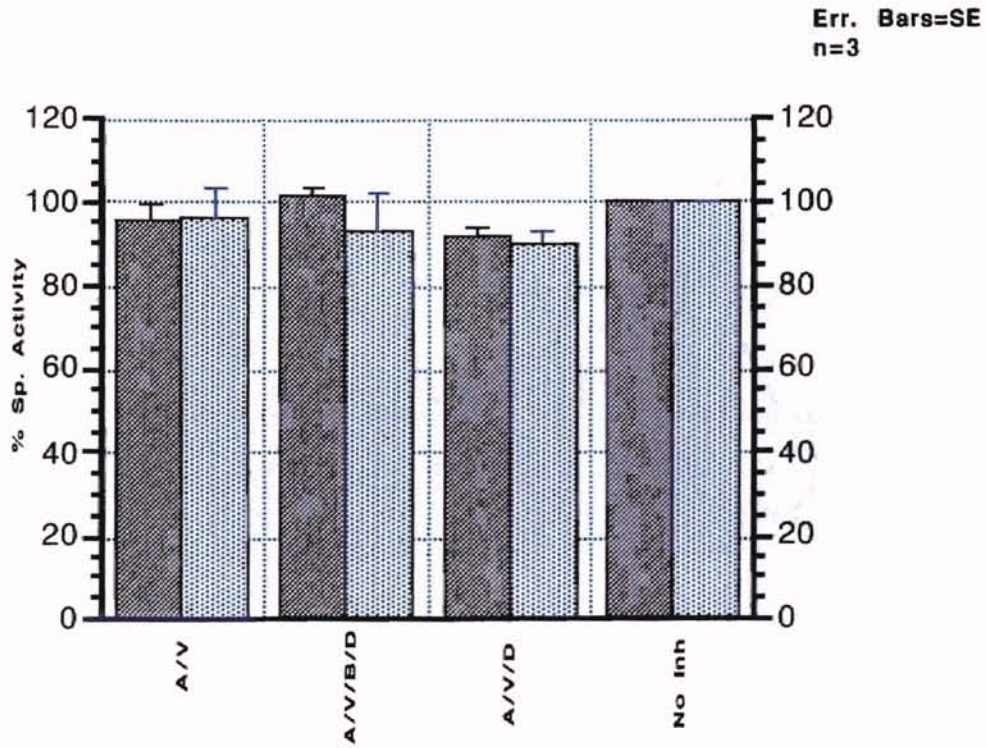


Figure 12. The effect on V-ATPase activity when salivary gland homogenate was incubated on ice and at room temperature.
Blue represents the homogenate incubated at room temperature and grey represents the homogenate incubated on ice. A, V, B and D stands for azide, vanadate, bafilomycin A1 and DMSO respectively.

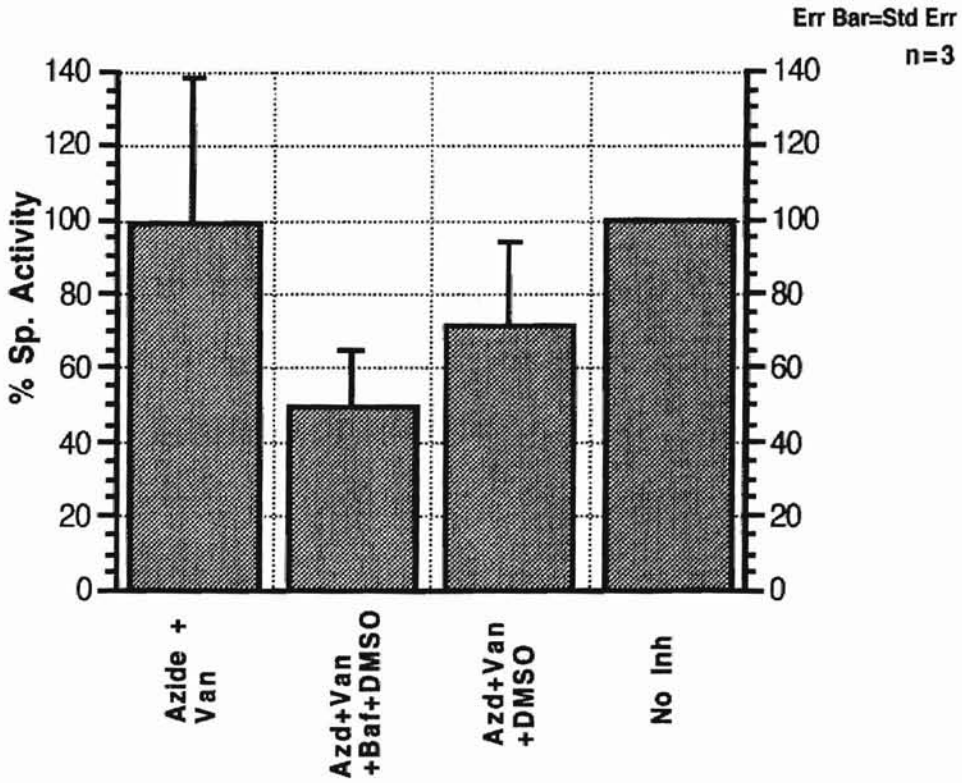


Figure 13. Effect of 10 μM bafilomycin A1 on *M. sexta* midgut in the presence of 0.5 mM azide and 0.1 mM vanadate..

Characterization of Azide and vanadate insensitive ATPase activity

Affect of enzyme concentration The enzyme concentration was varied by adding different amounts of the homogenate while all other constituents in the incubation medium were constant. The reactions were carried out in the presence of azide and vanadate (Fig. 14).

A linear relationship can be seen between the amount of inorganic phosphate released and the amount of protein added. The amount of protein used in subsequent experiments was 150 μg which is in the linear range. Clearly there is substantial azide and vanadate insensitive activity. Since azide and vanadate are supposed to inhibit most of the P- and F-ATPases, this release of Pi with increasing protein may be V-ATPase activity. This possibility was tested by doing the same experiment with and without bafilomycin A1 in the presence of azide and vanadate (fig. 15). There was no inhibition due to 10 μM bafilomycin added to the incubation medium.

Affect of time To further understand the azide and vanadate insensitive ATPase activity, the amount of Pi released was measured at 0, 10, 20 and 30 minutes incubation (fig. 16). A linear relationship was observed in the amount of Pi found and the time of incubation of enzyme with substrate.

Affect of substrate (ATP) concentration Whether the time dependent increase in Pi measured is due to ATP hydrolysis was tested by varying the amount of ATP. Since the positive control *M. sexta* midgut worked well, tick salivary gland tissue was prepared as for *M. sexta* (14). Since most of the ATPases are known to

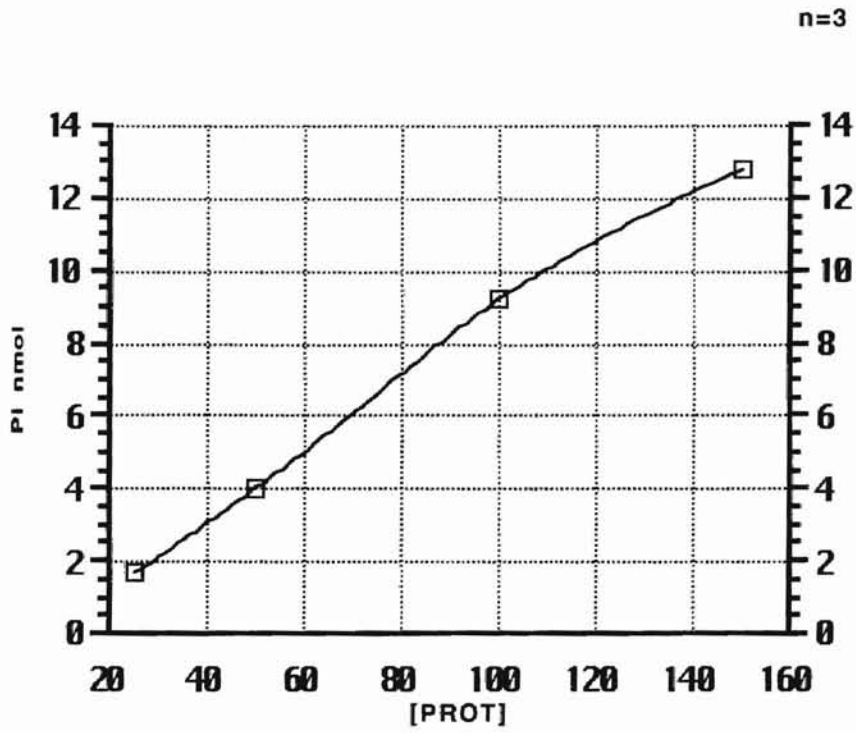


Figure 14. Effect of enzyme concentration on 0.5 mM Azide and 0.1 mM vanadate insensitive ATPase activity.
The reaction was carried on for 30 min and contained 2 mM ATP.

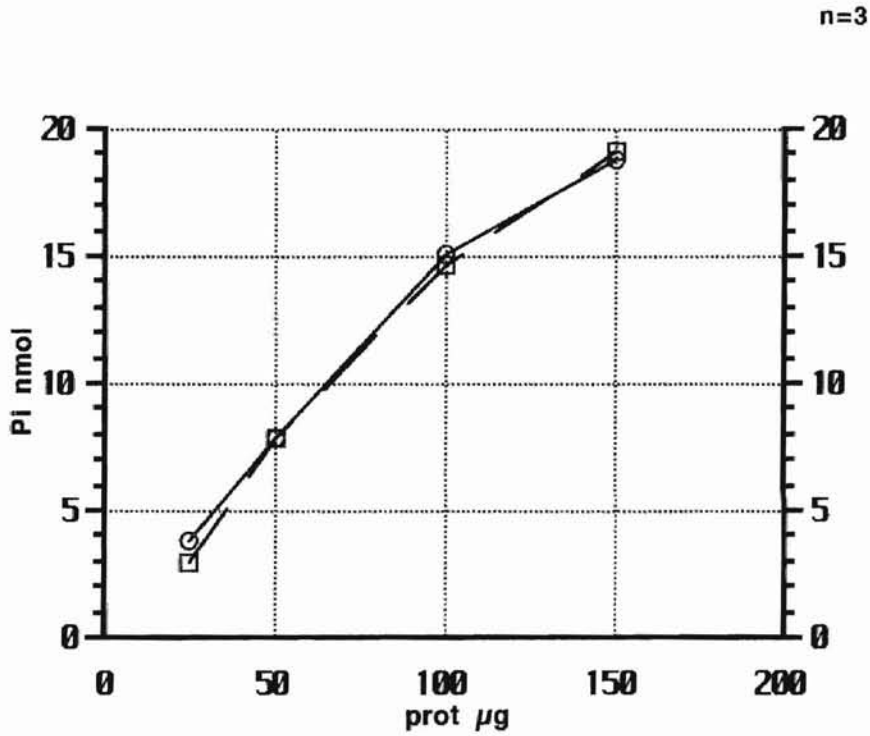


Figure 15. Effect of enzyme concentration and bafilomycin sensitivity to 0.5 mM Azide and 0.1 mM vanadate insensitive ATPase activity.
Reaction was carried on for 30 min. and contained 2 mM substrate ATP. Blue line represents with bafilomycin A1 and red line represents without bafilomycin A1.

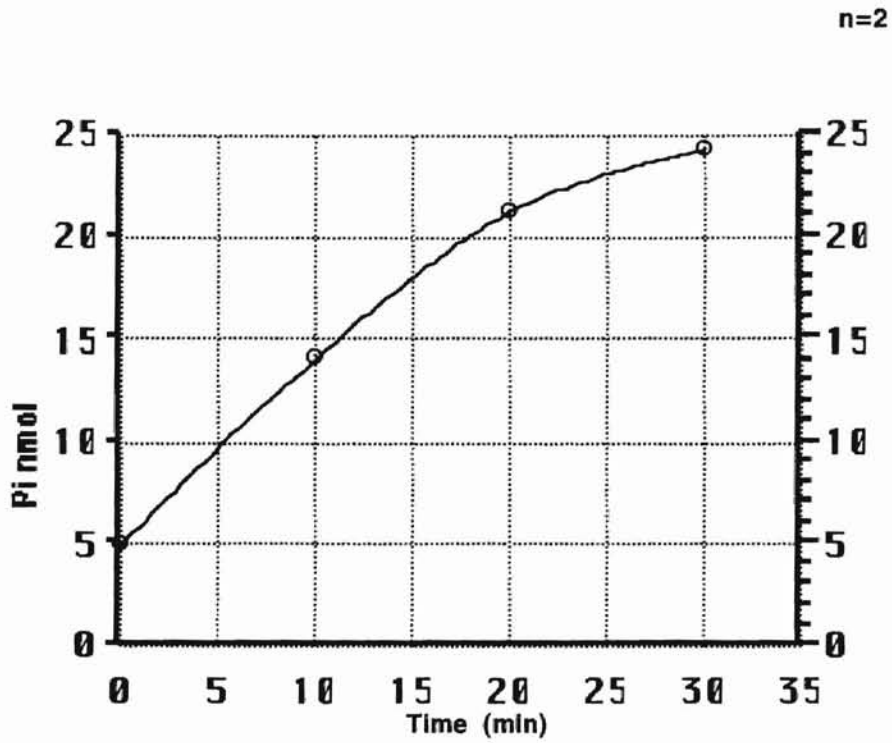


Figure 16. Reaction product vs time of 0.5 mM azide and 0.1 mM vanadate insensitive ATPase activity.
Enzyme and substrate concentrations were constant at 150 μg and 2 mM respectively.

be membrane bound, the microsomal fraction of the salivary glands containing the membranes were used as enzyme source. ATP concentrations of 0, 0.5, 1, 1.5, 2 and 2.5 mM were tested. The specific activity (Pi nmol/ min/ mg protein) was plotted against the substrate concentration (fig. 17).

The specific activity increased linearly up to 1 mM ATP and plateaued. In all previous experiments, the ATP concentration used was 2 mM, so the concentration of substrate was not a limiting factor, and bafilomycin A1 is not a competitive inhibitor (19). This experiment shows that the increasing Pi measured is actually due to break down of ATP by ATPase, as increasing concentrations of the substrate increased the amount of Pi released. It appears that another azide and vanadate insensitive ATPase activity, not effectively inhibited by specific V-ATPase inhibitors, is active in the salivary glands under these conditions.

pH optimum for the azide and vanadate insensitive ATPase activity.

The pH optimum was measured for the azide and vanadate insensitive ATPase activity by using a range of pHs from 6.09-9.18 (Fig. 18). The optimum pH is between pH 8.0 and 8.5. Subsequent assays were performed at a pH of 8.1.

Bafilomycin A1 sensitivity of tick salivary glands as compared to *M. sexta*.

Since bafilomycin A1 inhibited activity in *M. sexta*, the salivary glands were tested in the same way. The salivary gland homogenate was centrifuged

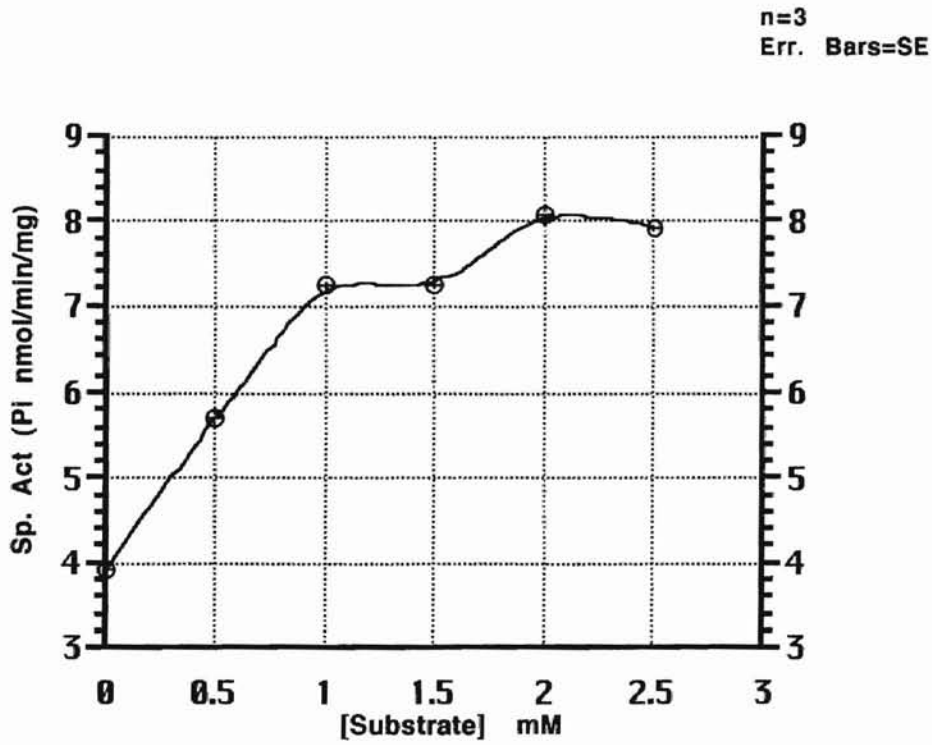


Figure 17. Azide and vanadate insensitive ATPase activity vs substrate concentration
Reactions were with 150 μg enzyme, 0.5 mM azide and 0.1 mM vanadate for a 30 min reaction period.

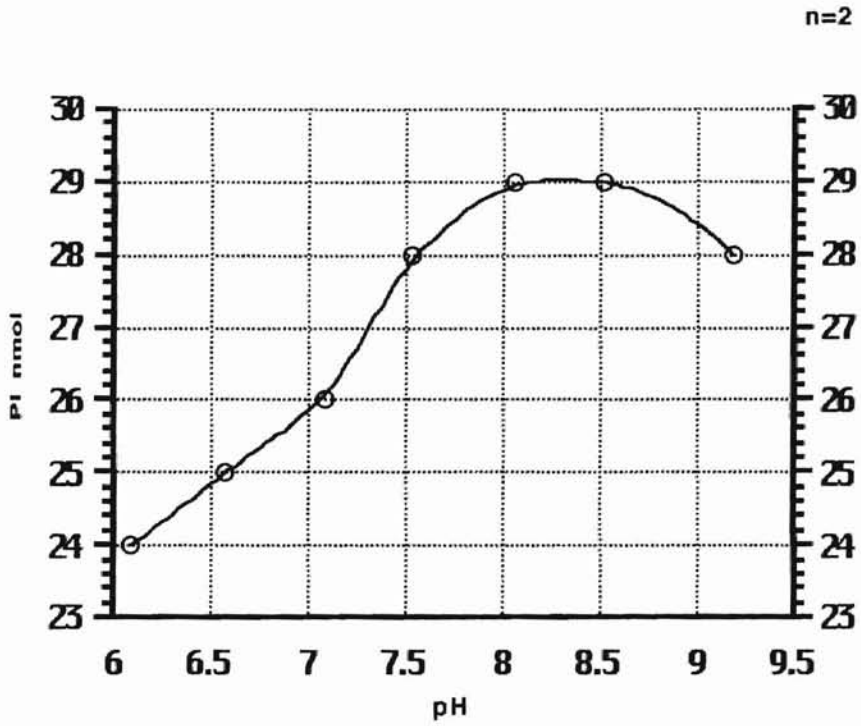


Figure 18. The effect of pH on azide and vanadate insensitive ATPase activity.
Reactions were with 150 μg enzyme, 2 mM ATP, 0.5 mM azide and 0.1 mM vanadate for a 30 min reaction period.

at 100,000xg and the pellet resuspended in the assay buffer (Fig. 19). There was no apparent inhibition due to 10 μM bafilomycin A1.

According to the protocol for testing V-ATPase activity in the insect midgut, the 100,000xg pellet should be resuspended in the Resus buffer without 2 mM $MgCl_2$ and 40 mM KCl. V-ATPase can be inactivated on ice in the presence of Mg^{2+}, Cl^- and ATP (41). Using this protocol salivary gland azide and vanadate insensitive activity was not inhibited by 10 μM bafilomycin A1 (Fig. 20).

Membrane solubilization agent and effect of V-ATPase inhibitors

When tissue pellets containing the microsomal fraction are resuspended in buffer, vesicles will be formed due to hydrophobic interactions of the membranes. These vesicles could be either right side out (RSO) or inside out (ISO). The solvent DMSO and the inhibitors of V-ATPase are lipid soluble and would be able to access the enzyme regardless of the orientation of the vesicles. ATP on the other hand, will not be able to access the ATPases depending on the orientation of the enzyme and its vesicles and hence V-ATPase activity might not be possible.

In order to test this possibility, deoxycholate was used as a detergent to disrupt the membrane so that the substrate and the inhibitors would have ready access to the enzyme. If too much detergent is added, the enzyme will dissociate from the membrane and will lose it's activity. Deoxycholate concentrations of 0, 1, 2, 4 and 6 mg/mg protein, and 0, 1, 3, 6 and 9 mg/mg

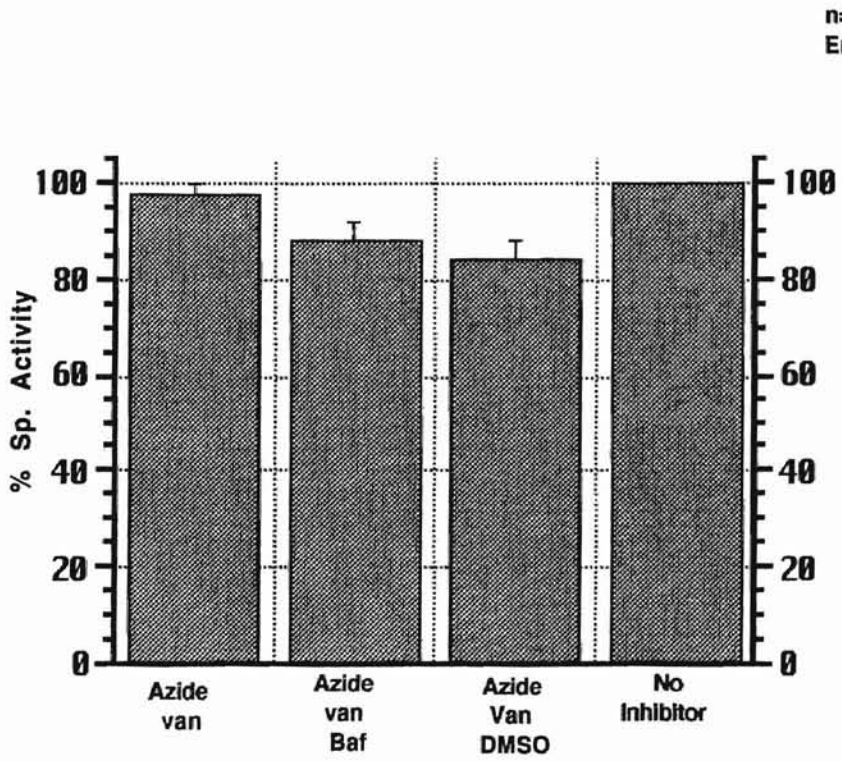


Figure 19. Bafilomycin sensitivity of salivary glands prepared by method of (12).
The 100,000xg pellet was resuspended in the assay buffer. Reactions were with 150 μg enzyme, 2 mM ATP, 0.5 mM azide and 0.1 mM vanadate for a 30 min reaction period.

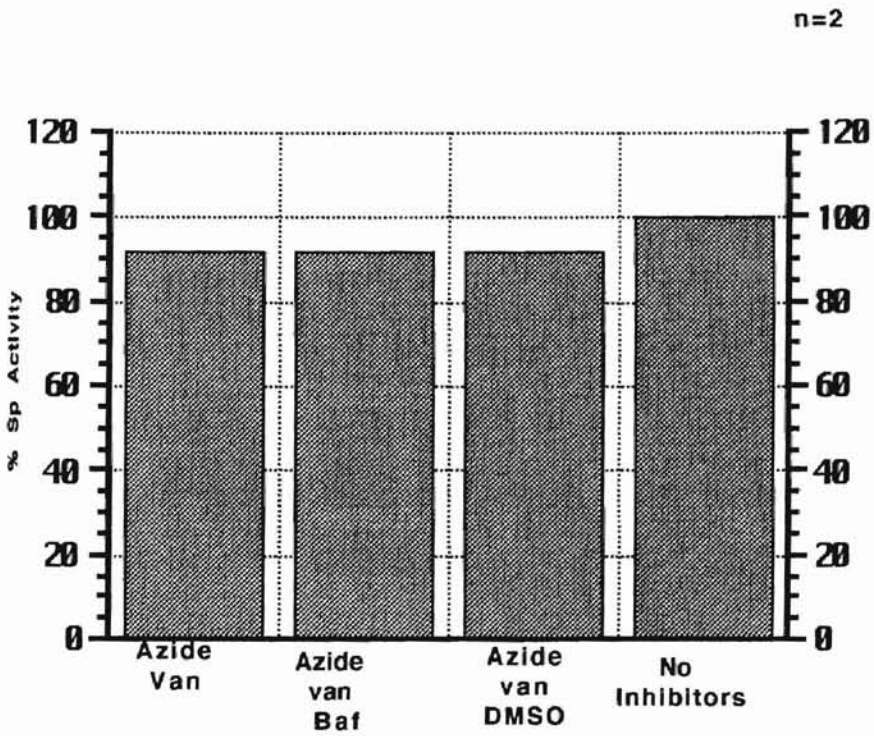


Figure 20. Bafilomycin sensitivity of salivary glands prepared by method of (12).
The 100,000xg pellet was resuspended in the resus buffer. Reactions were with 150 μg enzyme, 2 mM ATP, 0.5 mM azide and 0.1 mM vanadate for a 30 min reaction period.

protein were tested in two different experiments (Fig. 21 & 22). The experiments were carried out for 15 minutes in the presence of azide and vanadate, and the effect of folimycin was tested by having one set of reactions with azide and vanadate and the other set with azide, vanadate and folimycin.

The specific activity was inhibited 50% when the detergent was increased to 9 mg/mg protein. In the first experiment there was a slight inhibition seen due to folimycin at a detergent concentration of 1 mg/mg protein. However, at 6 mg/mg protein folimycin appeared to be stimulatory. In the second experiment (Fig. 22) there is a slight inhibition seen due to folimycin.

Is V-ATPase localized in type I acini?

Since it has been difficult to demonstrate much inhibition using the known V-ATPase inhibitors in whole salivary gland homogenate or fractions, I sought to determine if V-ATPase was localized in one type of acini. The salivary glands of the ticks more closely resemble the acinar, multicellular salivary glands of the cockroach *Periplaneta americana* (11). The distal duct cells of the cockroach are known to contain V-ATPase (12), and are morphologically similar to the Type I acini of the tick salivary glands. To test this possibility, the Type I acini were isolated from the tick salivary glands, by carefully isolating the most anterior part of the glands with the main duct. The anterior portion containing the Type I acini and the posterior portion were both tested for folimycin sensitive ATPase activity in the presence of azide and vanadate (Fig. 23). Again, there was no inhibition due to folimycin seen either in the anterior or the posterior regions of the glands.

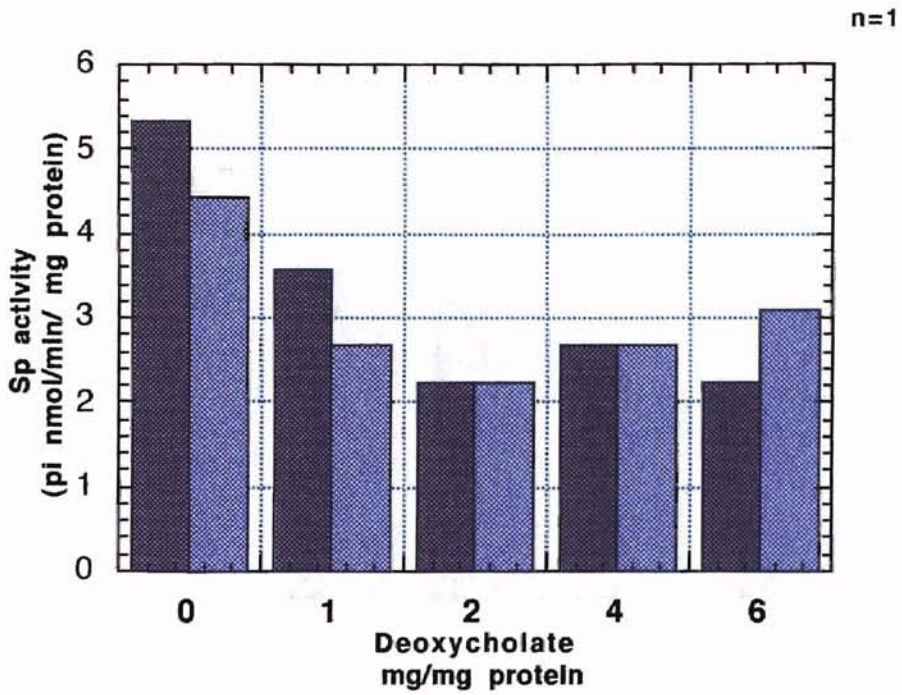


Figure 21. The effect of the detergent deoxycholate on 10 μM folimycin sensitivity to azide and vanadate insensitive ATPase activity. Reactions were with 150 μg enzyme, 2 mM ATP, 0.5 mM azide and 0.1 mM vanadate for a 15 min reaction period. 1, 2, 4 and 6 mg of detergent/mg protein were tested. The blue bars represent the effect of folimycin in the presence of the detergent, while the black bars represent the effect without folimycin in the presence of the detergent.

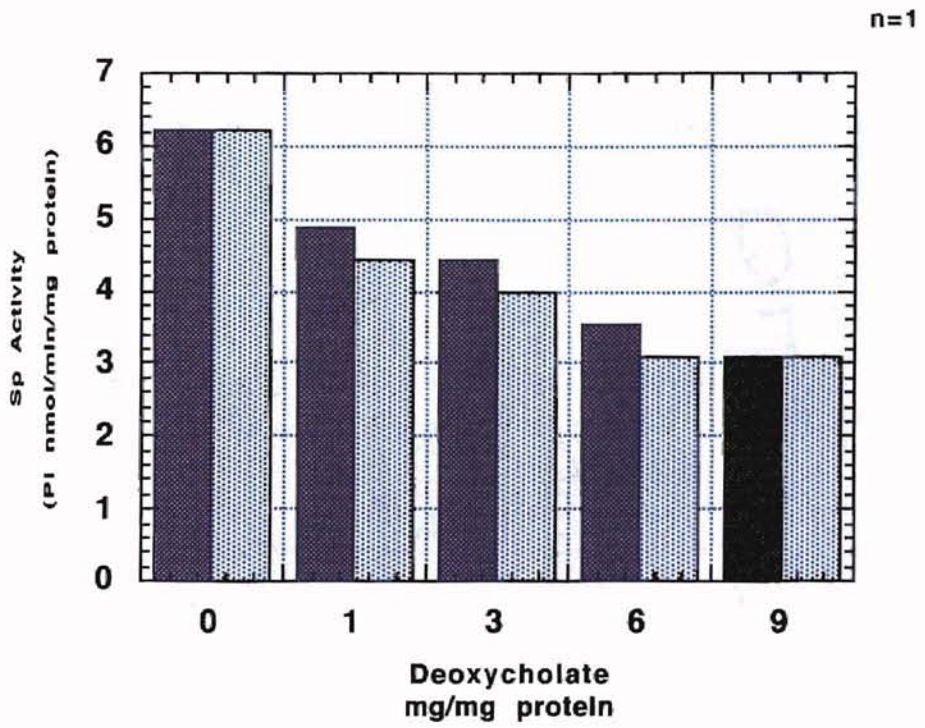


Figure 22. The effect of the detergent deoxycholate on 10 μM folimycin sensitivity to azide and vanadate insensitive ATPase activity. Reactions were with 150 μg enzyme, 2 mM ATP, 0.5 mM azide and 0.1 mM vanadate for a 15 min reaction period 1, 3, 6 and 9 mg of detergent/mg protein were tested. The blue bars represent the effect of folimycin in the presence of the detergent, while the black bars represent the effect without folimycin in the presence of the detergent.

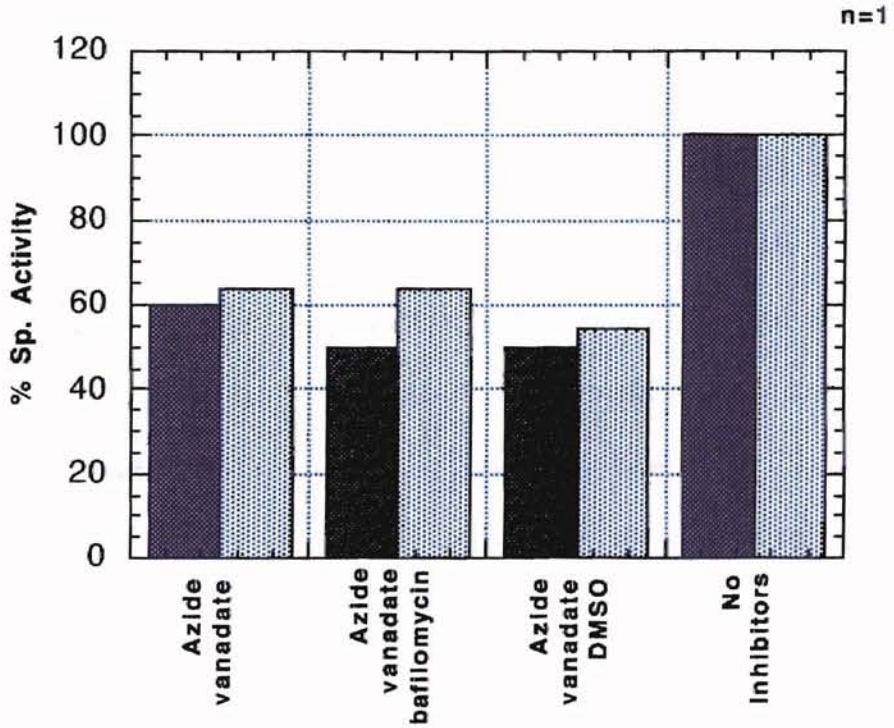


Figure 23. Difference between the anterior portion and the posterior portion of the salivary glands on 10 μM folimycin sensitivity to azide and vanadate insensitive ATPase activity.
Reactions were with 100 μg enzyme, 2 mM ATP, 0.5 mM azide and 0.1 mM vanadate for a 15 min reaction period Blue bars represent the anterior portion while the black bars represent the posterior.

Spectrophotometric method

Difficulty to show inhibition due to bafilomycin in the colorimetric assays may be due to the background Pi present in the homogenate. To eliminate this, a coupled enzyme assay, which will monitor only the hydrolysis of ATP by ATPases was used. Here the hydrolysis of ATP is measured by coupling the pyruvate kinase and lactate dehydrogenase reactions. The pyruvate kinase reaction utilizes phosphoenolpyruvate and ADP from ATP hydrolysis to form pyruvate. Lactate dehydrogenase then utilizes pyruvate and NADH to form lactate. The oxidation of NADH can be monitored spectrophotometrically at 340 nm. Oxidation of NADH depends only on the availability of ADP.

Since the concentrations of LDH and pyruvate kinase and the substrates are at high concentrations, the rate limiting reaction is the production of ADP by ATPases, and the rate measured by monitoring the oxidation of NADH is directly proportional to the rate of hydrolysis of ATP.

Reaction rates were calculated by the plate reader as rate of change of absorbance. The plate reader calculates the rates from each set of maximum 10 sequential data points within the period of time given. The maximum of these rates is reported as V_{max}. A control where no homogenate was added to the incubation medium was included in the assay to monitor nonspecific oxidation of NADH.

Inhibition due to 10 μM folimycin was measured in the presence of 0.5 mM azide and 0.1 mM vanadate to detect the V-ATPase activity (Fig 24). The reaction rates reported by the plate reader were summarized, and the 100%

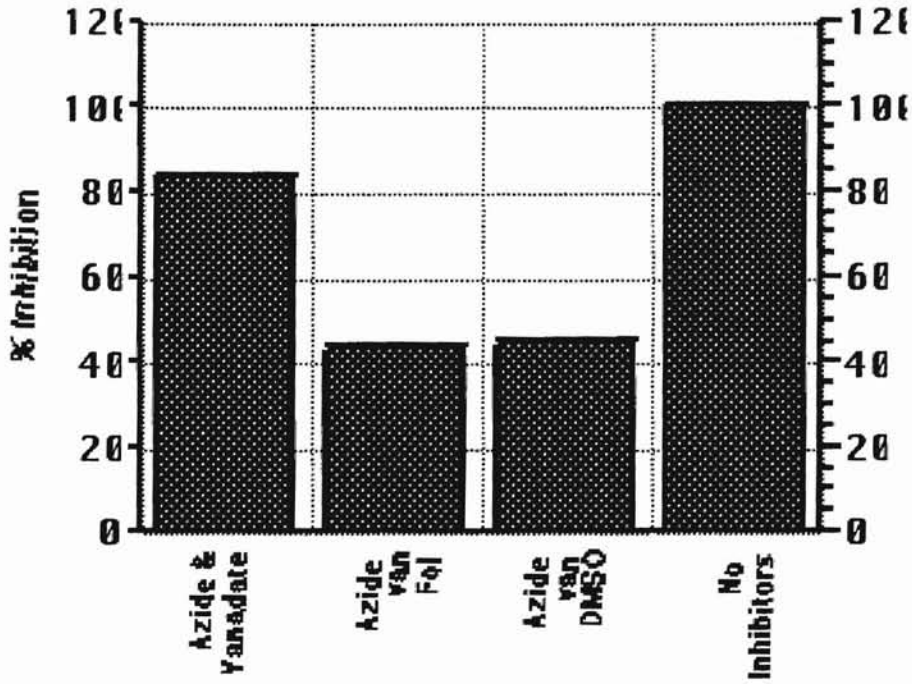


Figure 24. 10 μM folimycin sensitivity in the presence of 0.5 mM azide and 0.1 mM vanadate, tested spectrophotometrically using the coupled assay.

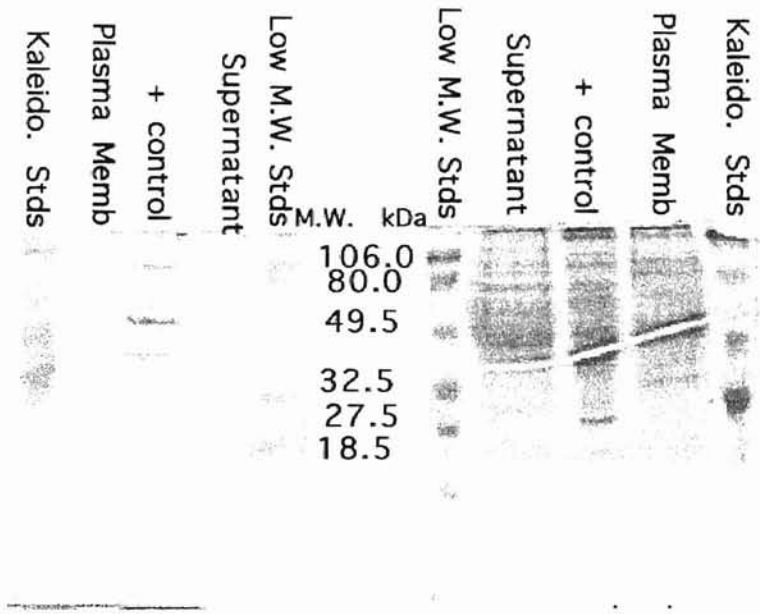
activity was calculated as the activity measured with no inhibitors present. Percent activity was then plotted against different inhibitors.

About 15% of ATPase activity was inhibited by azide and vanadate, and about 50% by folimycin and DMSO. Although DMSO causes 50 % inhibition, if folimycin inhibits V-ATPase significantly, there should be an additive effect seen when both folimycin and DMSO are present together. The solvent concentration in the reactions was 1%, and that is the lowest possible concentration of the solvent possible in order to test 10 μM folimycin. Again there is no apparent inhibition was observed due to folimycin, since almost the same inhibition was seen with DMSO.

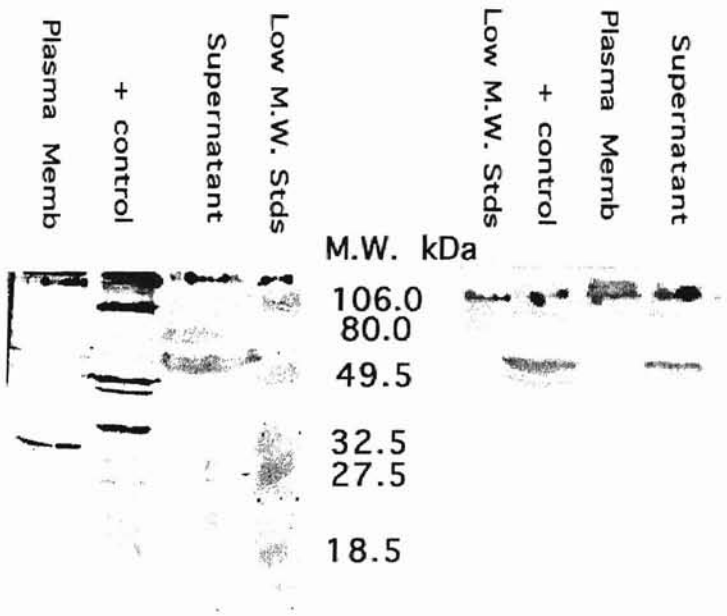
Identification of the B-subunit of V-ATPase in tick salivary glands by Western blot analysis

An antibody against the B-subunit of the mosquito *Culex quinquefasciatus* V-ATPase was used to identify the B-subunit of the tick *Amblyomma americanum* V-ATPase. The tick salivary gland plasma membrane fraction and the supernatant as well as *Manduca sexta* midgut were tested as a positive control.

The positive control showed 3 strong bands while both the plasma membrane fraction and the supernatant showed 2 to 3 very faint bands (fig. 25 a).



a immuno blot coommassie stained gel



b.

Anti B Ab. RPS

Figure 25. Western blot of the anti B subunit Ab. and the gel with coommassie blue stained proteins(a.).
Negative control with rabbit preimmune serum (RPS) along with the anti B subunit Ab. (b.) shows that the bands in the tick samples are non-specific binding due to a factors present in the RPS.

As a control, rabbit pre-immune serum (without the Anti B subunit Ab) was tested and the same bands from the tick tissue samples with the same intensity appear after the color development. However, the positive control only shows very faint band (fig. 25 b.). The bands seen with the positive control when anti B Ab was used show molecular weights of 40, 55, and 92 KDa. The expected MW of the B-subunit is about 57 KDa and the 55 KDa band may be the B-subunit. It is not clear however, what the 92 Kda and the 40 KDa bands are. The 92 KDa band may be a dimer of either the 40 or the 52 KDa bands seen. The plasma membranes of the tick salivary glands show 3 faint bands that correspond to 100, 60, and 64 KDa bands, and the supernatant fraction shows 4 faint bands that correspond to 40, 50, 64, and 95 KDa bands.

There is a considerable amount of conservation in the B-subunit within species. For example, the B-subunit of *Helicoverpa virescens* is most homologous to human kidney B-subunit (58). In addition very high homology is observed with other B-subunits in the data bases. In most cases there is divergence in the N- and the C-terminals. However, the putative ATP binding site of the B-subunit of *H. virescens*, NGSIT (amino acids 329-333), is identical in Neurospora, yeast and chromaffin granules (58).

The Ab against the B-subunit of V-ATPase of *Culex quinquefasciatus* either does not cross react with the tick B-subunit or the subunit is not present in detectable amounts in tick salivary glands. The bands seen in the experiment are due to some IgG binding to tick proteins. The B-subunit of the positive control *M. sexta* on the other hand, seems to cross react with the antibody.

Effect of V-ATPase inhibitors on fluid secretion

The possible role of V-ATPase in fluid secretion was investigated using specific V-ATPase inhibitors 1μM bafilomycin and folimycin, 10 μM NEM and Na^+/K^+-ATPase inhibitor 100 μM ouabain. Salivary glands were stimulated with 1 μM dopamine and the fluid secretion was monitored by two different methods: the main salivary duct ligation method (49, 50) and the salivary output method (52). NEM inhibited fluid secretion about 20% (fig. 26), while bafilomycin A1 and folimycin inhibited secretion about 18% and 30% (figs. 27 and 28) respectively. 1 μM bafilomycin A1 inhibited secretion approximately 25% as measured by the open duct salivary output method (fig. 29), in agreement with the results of the ligated duct method. In comparison, 100 μM ouabain inhibited dopamine stimulated secretion about 70%. Bafilomycin A1 did not potentiate inhibition of secretion by ouabain (fig. 30)

Fluid and ion transport in the midgut and the Malphigian tubules of many insects have been shown to depend on the action of V-ATPase (13, 14, 34, 35, 36). The mechanism of fluid secretion in ticks is unknown and complicated by the complex and changing morphology of the salivary glands during tick feeding (59). Fluid secretion by tick salivary glands is partially inhibited by specific V-ATPase inhibitors. Ouabain, a specific inhibitor of the Na^+/K^+-ATPase, is a more potent inhibitor of secretion than the V-ATPase inhibitors. Ouabain and bafilomycin A1 together are no more effective than ouabain alone in inhibiting secretion. Na^+/K^+-ATPase activity has been demonstrated in the tick salivary glands and has been shown to increase during tick feeding (60, 61). Inhibitors of Na^+/K^+-ATPase have been shown previously to inhibit tick salivary

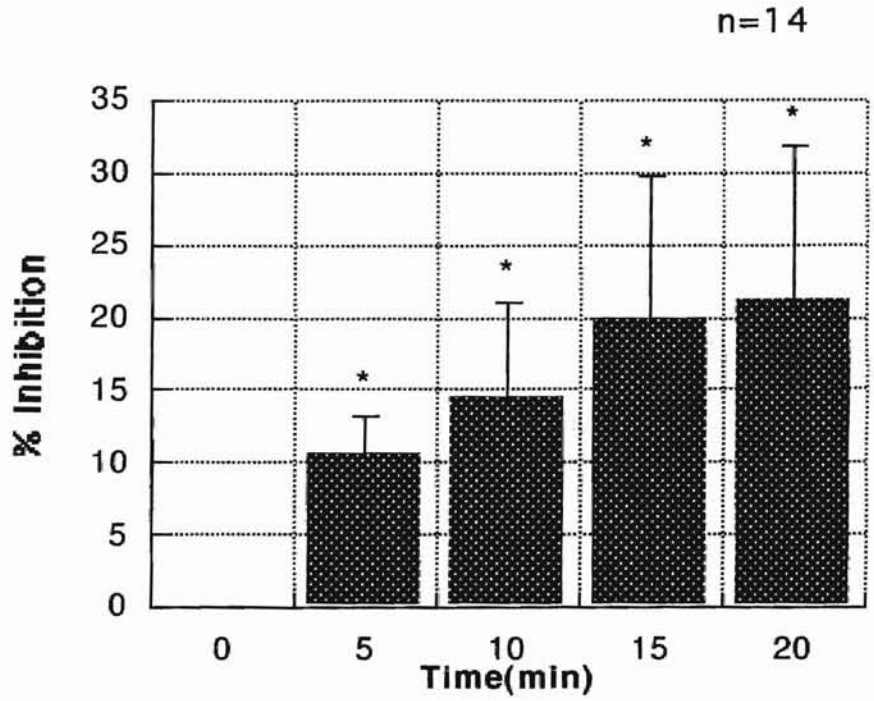


Figure 26. Effect of 10 μM NEM on fluid secretion by isolated salivary glands
Fluid secretion was measured by the ligated duct technique in salivary glands stimulated with 1 μM dopamine.
Bars and points indicate mean ± standard error, and * indicates a significant difference from control ($P<0.05$, paired sample t test)

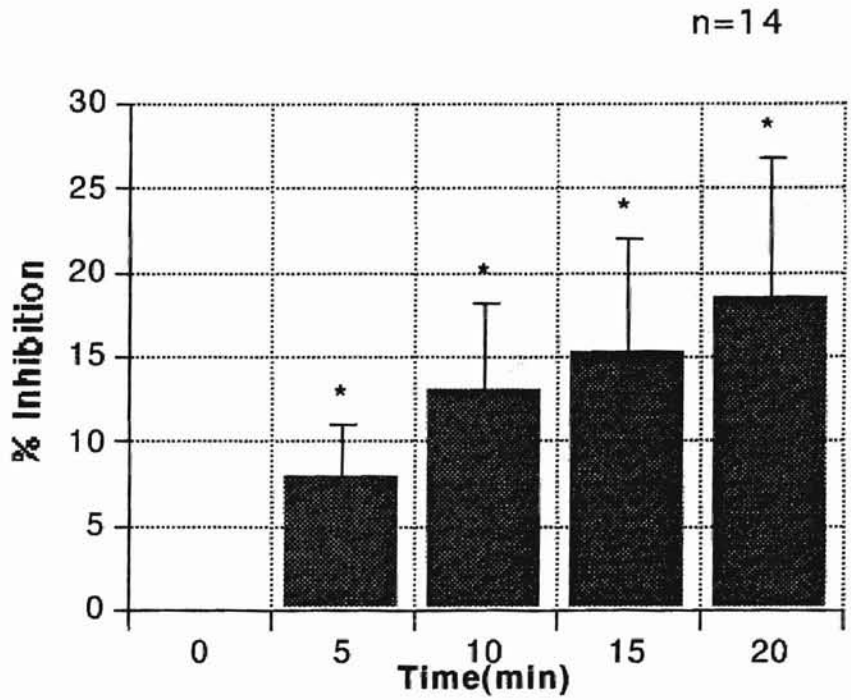


Figure 27. Effect of 1 μM Bafilomycin on fluid secretion by isolated salivary glands. Fluid secretion was measured by the ligated duct technique in salivary glands stimulated with 1 μM dopamine.
Bars and points indicate mean ± standard error, and * indicates a significant difference from control ($P<0.05$, paired sample t test)

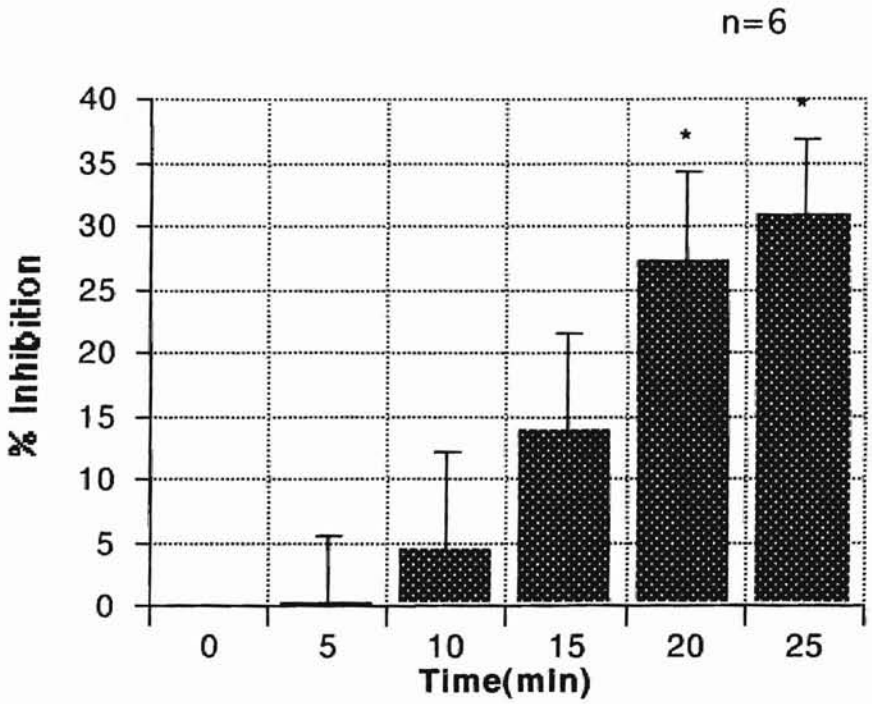


Figure 28. Effect of 1 μM folimycin on fluid secretion by isolated salivary glands Fluid secretion was measured by the ligated duct technique in salivary glands stimulated with 1 μM dopamine.Bars and points indicate mean ± standard error, and * indicates a significant difference from control ($P<0.05$, paired sample t test)

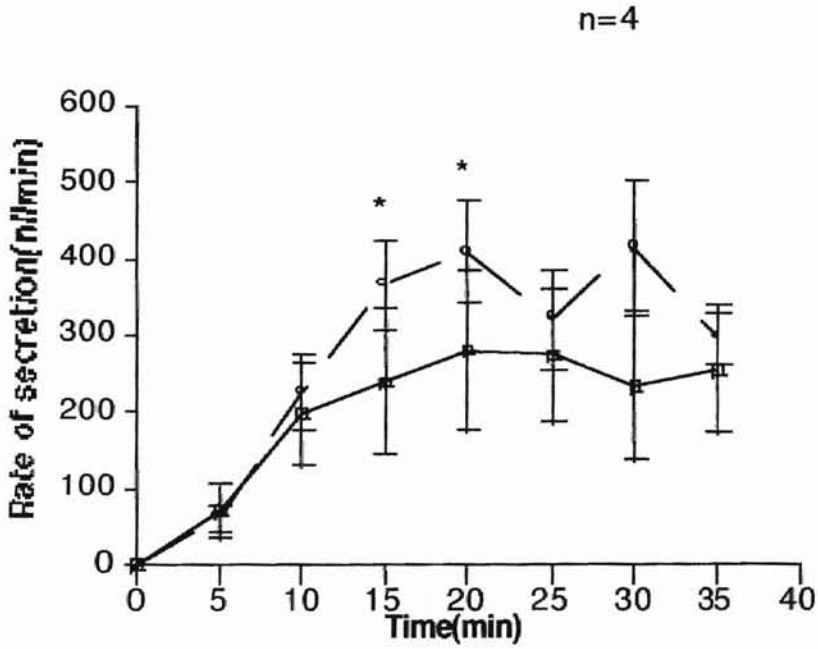


Figure 29. Secretion measured by the open duct technique. Glands were stimulated with 1 μM dopamine alone (black) and dopamine + 1 μM bafilomycin A1 (blue).
Bars and points indicate mean ± standard error, and * indicates a significant difference from control ($P<0.05$, paired sample t test)

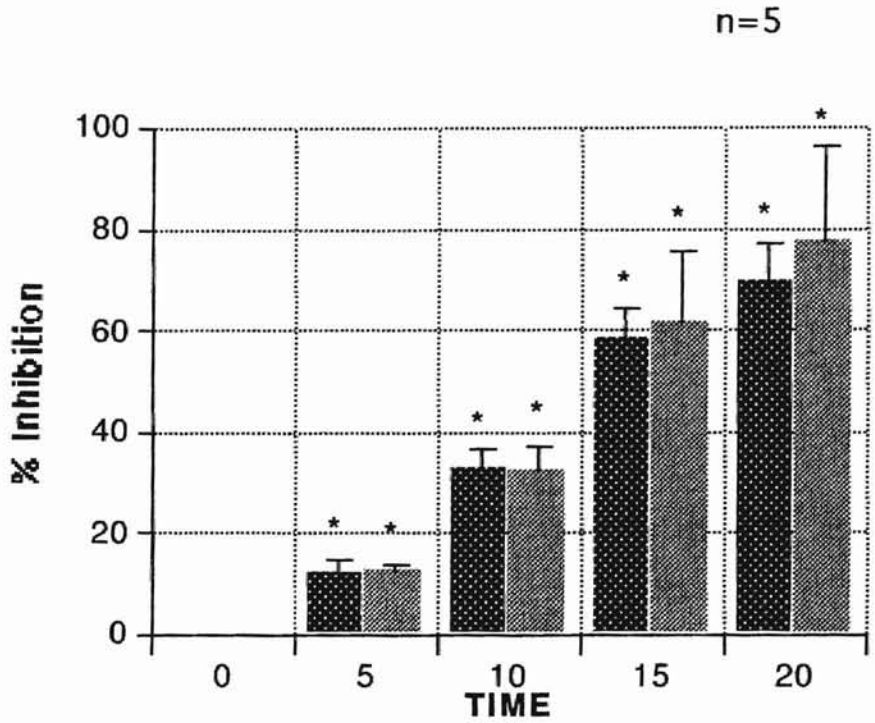


Figure 30. Effect of ouabain on secretion by isolated salivary glands. Fluid secretion was measured by the ligated duct technique in salivary glands stimulated with 1 μM dopamine and treated with 100 μM ouabain (right bars, n=5) or 100 μM ouabain + 1 μM bafilomycin A1 (left bars, n=6).
Bars and points indicate mean ± standard error, and * indicates a significant difference from control ($P<0.05$, paired sample t test)

gland secretion (51, 62). The partial inhibition of fluid secretion by NEM, folimycin and bafilomycin suggests that either the role of V-ATPase in fluid secretion is not very significant in the tick salivary glands, or the partial inhibition of fluid secretion is really not due to an inhibition of V-ATPase but rather the overall toxicity of the drugs on the glands or, even that bafilomycin A1 and folimycin are partially able to inhibit a P-ATPase. Bafilomycin A1 And folimycin are known to inhibit P-ATPases in the μM range (19).

CHAPTER IV

SUMMARY AND CONCLUSIONS

Ticks are important vectors that transmit many pathogens to humans as well as livestock. They cause serious damage to both human health and economy. The multifunctional salivary glands of the ticks are vital to their success as parasites and is the major site of pathogenic transfer. Therefore, it is important to study the molecular processes and physiology of these glands to gain a better understanding in order to reduce or eliminate the problems caused by these parasites.

V-ATPase is found in almost all species studied and has diverse functions. Due to its ability to pump protons, this enzyme is able to create a proton gradient, thus creating either a chemical or electrical gradient depending on the other channels present in the membrane. This chemical or electrical gradient is then used to achieve many functions. In insect Malphigian tubules, for example, the enzyme is known to play an important role in fluid secretion, in bones they provide an acidic medium for the osteoclasts to function and in renal epithelia they help in the reabsorption of Na^+ and bicarbonate. The C subunit of V-ATPase was cloned from the tick *Amblyomma americanum* (L.) salivary glands giving evidence for the presence of this enzyme (11).

V-ATPase activity was assayed using known inhibitors, bafilomycin A1 and folimycin. DMSO and EtOH were used as solvents for the inhibitors and the effect of the inhibitors on the assay was tested. It was found that both the solvents are toxic when assay contained 10 % of the solvent. For most of the

assays 1% of the solvent showed no toxic effect. However, there is an unusual inhibitory effect seen in spectrophotometric assays due to the solvent even at 1%. This may be due to a the effect of the solvent on the coupling enzymes. The assays for V-ATPase shows no inhibition due to either bafilomycin A1 or folimycin in either assay systems. The possible reasons for this were tested. For example, the possibility of enzyme inactivation at cold temperatures was tested, no difference was found in incubating the enzyme on ice as compared to incubating at room temperature. The possibility of substrate inaccessibility was also tested using deoxycholate as a detergent. Detergent had insignificant effect on the sensitivity of ATPases to bafilomycin A1 in the presence of azide and vanadate.

Manduca sexta was used as a positive control and fair inhibition was observed in response to bafilomycin A1, thus validating the assay conditions. The possibility that the tick tissue preparation may not be optimal was also tested by using assay conditions optimal for assaying the *M. sexta* enzyme. Neither 10 μM bafilomycin A1 nor folimycin in the presence of 0.5 mM azide and 0.1 mM vanadate were inhibitory when assaying the salivary gland enzyme under these conditions.

Even though there was no inhibition due to the specific V-ATPase inhibitors, there was ATPase activity in the presence of 0.5 mM azide and 0.1 mM vanadate. This ATPase activity was characterized and the optimum pH was found to be between 8 and 8.5. The ATPase activity was linear up to 30 minutes with 150 μg of enzyme in the presence of 2.5 mM ATP substrate. The activity was also linear up to 1 mM ATP substrate and plateaued at 1 mM ATP, with 150

μg of enzyme for 30 minute reaction time. Enzyme activity was linear up to 20 minutes with 150 μg of protein and 2 mM ATP.

The assays for V-ATPase activity were performed within the linear range of time and the enzyme concentration, and still no inhibition was observed. The anterior portions and the posterior portions of the salivary glands were isolated and tested for the V-ATPase activity to test the possibility of enzyme localized in type I acini of the glands. Still no inhibition was detected in response to specific inhibitors of V-ATPase.

Is the ATPase activity observed in the presence of azide and vanadate due to V-ATPase? To further establish the presence of a V-ATPase, Western blots were performed using an antibody against the B subunit raised against the mosquito Culex *quinquefasciatus.* Despite the fact that the B subunit is highly conserved between species, the antibody was not able to recognize a B subunit in the tick. However, the positive control *M. sexta* did cross react with the antibody giving a positive result and confirming that the conditions of the experiment were valid (fig. 25).

There is a C subunit of V-ATPase cloned from the tick salivary glands but no activity of the enzyme was detected. Western blot analysis also failed to recognize the tick B subunit. However, there is ATPase activity in the presence of azide and vanadate. Does this ATPase activity have a physiological role? Maybe this ATPase can be inhibited with bafilomycin A1 in the intact salivary glands.

Since V-ATPase is known to play a role in fluid secretion in insects, and the major function of the salivary glands of the ticks are fluid secretion, the

possibility of V-ATPase involved in fluid secretion was tested using both the specific inhibitors of V-ATPase and a specific inhibitor of Na^+/K^+-ATPase, ouabain. Two methods were used to assay this and about the same results were obtained from both (fig 25-30). Dopamine stimulated fluid secretion was inhibited 25-30% by bafilomycin and folimycin respectively. However, ouabain inhibited dopamine stimulated secretion about 70%. This obviously shows that the Na^+/K^+-ATPase is more involved in the process of fluid secretion than V-ATPase.

The inability of specific V-ATPase inhibitors to inhibit the azide and vanadate insensitive ATPase activity in the tick salivary glands is quite puzzling. In many other organisms it is only nM concentrations of bafilomycin A1 or folimycin are required to inhibit azide and vanadate insensitive ATPase activity. For example, the concentration of bafilomycin A1 required for 50% inhibition of V-ATPase in the vacuolar membranes of *Neurospora crassa* is 0.4 nmol/mg (15). The V-ATPase in vesicles from goblet cell apical membranes of *M. sexta* is 20±10 nmol/mg (63), and vacuolar membranes from *Saccharomyces cerevisiae* is 0.6 nmol/mg (64). However, H^+-ATPases derived from the bacterium Archaea, are inhibited only at micromolar concentrations of bafilomycin A1, and are believed to be intermediate between F and V-ATPases(65).

The C subunit of V-ATPase has been cloned form the tick *Amblyomma americanum* (L.) salivary glands, and about 20-30% of fluid secretion in vitro was inhibited by bafilomycin, suggesting a V-ATPase is present in the tick salivary glands. However, an antibody against the B subunit of V-ATPase in the mosquito *Culex quinquefasciatus* failed to recognize the B subunit in the tick,

and no significant and consistent inhibition was detected in the presence of azide and vanadate with both bafilomycin A1 or folimycin with two different assay systems.

In addition, V-ATPase may play a significant role in inhibiting fluid secretion in the tick salivary glands and bafilomycin A1 and folimycin inhibited secretion only 25-30% and this inhibition could be caused by the inhibition of Na^+/K^+-ATPase by bafilomycin A1 and Folimycin, since they are inhibitory to P-ATPases at μM concentrations(19). Furthermore, ouabain inhibits fluid secretion about 70% suggesting that Na^+/K^+-ATPase is the important ATPase in the mechanism of fluid secretion.

In conclusion, there are three possibilities for these confusing results. One, is that there is no V-ATPase present in the tick salivary glands. If this is so, why is there a gene for the C subunit of the enzyme, and why is fluid secretion inhibited by bafilomycin A1? The C subunit could be a part of another type of ATPase or a protein system, and the bafilomycin A1 may have inhibited Na^+/K^+-ATPase. Second, is that V-ATPase may be present only in minute quantities, that is not detectable both by Western blots and biochemically assaying it's activity. The third and the most likely possibility is that the tick V-ATPase may be quite different from other V-ATPases and may not be sensitive to known V-ATPase inhibitors. This explains results from both the Western and the biochemical assays. Also to further strengthen this hypothesis, there is definitely ATPase activity detected in the presence of azide and vanadate.

In my opinion future studies for this enzyme in the tick salivary glands should begin by attempting to clone the proteolipid subunit of V-ATPase. The

proteolipid subunit is found in all the V-ATPases found so far, and the amino acid sequence is fairly well conserved. This will strengthen or weaken the presence of V-ATPase in the tick salivary glands of *Amblyomma americanum*(L.).

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