CHAPTER - VIII

INHIBITION OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE NERVE CORD, MIDGUT AND FAT BODY OF THE FIFTH INSTAR CASTOR SEMILOOPER FOLLOWING THE FENITROTHION TREATMENT.
Transport of alkali metal cations into and across the nerve membrane and epithelia has been studied in several groups of organisms (Glynn, 1957; Skou, 1964; Baker and Norris, 1971; Dunham and Hoffman, 1971; Vaughan and Cook, 1972; Gardner and Frantz, 1974; Needham and Sauer, 1975). This transport is catalyzed by sodium-potassium dependent adenosine triphosphate phosphohydrolase (Na\(^+\)-K\(^+\) ATPase; EC 3.6.14) (Skou, 1964). These studies as well as cation transport in vertebrate systems led Keynes (1969) to propose that all epithelial cells possess Na\(^+\)-K\(^+\) ATPases. One of the universal features of Na\(^+\)-K\(^+\) ATPase is its sensitivity to ouabain (Glynn, 1957). Ouabain inhibition of alkali metal transport thus occurs via an effect on the Na\(^+\)-K\(^+\) ATPase. In insects ouabain sensitive Na\(^+\)-K\(^+\) ATPase has been implicated in Na\(^+\) transport (Baker and Norris, 1971; Rivera, 1975; Takeda and Hasegawa, 1975).

In Lepidopterous insects, the major monovalent cation present in the diet and in hemolymph is potassium and sodium is present in negligible amount (Jungreis et al., 1973; Florkin and Jeuniaux, 1974; Harvey et al., 1975; Jungreis, 1977). In these insects, movement of potassium occurs in the absence of sodium counter-transport (Harvey and Nedergaard, 1964; Jungreis and Harvey, 1975). The activity of Na\(^+\) K \(^-\) dependant ATPase could not be determined in midgut from larval and adult stages of *Cecropia* silkmoth, tobacco hornworm and Monarch butterfly. This result was confirmed by observations of selective binding of \(^3\)H-ouabain to homogenates of neuronal tissues but not of midgut epithelium.

The biochemical process by which various kinds of insecticides affect membrane-bound ATPases activities has been examined in nervous system
and other tissues of insects (Narahashi, 1976; Wilkinson, 1976; Matsumura and Ha$$suddin, 1979; Brooks, 1980; Osborne, 1980; Treherne, 1980; Cutkomp et al. 1980; Chefurka, 1983; Deaton, 1984). Generally, it has been accepted that insecticides act directly on the nervous system and disrupt normal ion permeabilities in the nerve membrane that are implicit in the generation and conduction of nerve impulses (Shanes, 1951; Wang et al. 1972; Osborne, 1980; Rubin et al. 1980; Treherne and Pichon, 1972).

Of the various classes of insecticide chemicals, the organochlorines and particularly DDT is the most widely studied insecticide with reference to its inhibitory action on the membrane bound ATPases. In recent years the selectivity and diversity of OP compounds have attracted the attention of biochemists to study the mode of action of OP compounds on cellular metabolism. Surprisingly the investigation on OP compounds has centered around the AChE inhibition. No attempts have been made to gain further information on the possible action of OP insecticides on the ATPases the key enzymes in cellular metabolism. The OP compounds have been widely used against several lepidopteran pests. However, the biochemical phenomena associated with its toxic effect(s) have not been studied in a single insect. In the present investigation, we have examined the effect of topical application of fenitrothion on the activities of ATPases system of different tissues of 5th instar castor semilooper Achaea janata.
MATERIALS AND METHODS

Insects:
The castor semilooper larvae used in these experiments were from an inbred laboratory colony. The details of the rearing techniques and maintenance of the culture has already been described elsewhere (Chapter-I).

Chemicals:
Adenosine triphosphate disodium salt and ouabain were obtained from Sigma (St.Louis, USA). All other salts and solvents were of analytical grade from British Drug House (India).

Insecticide Treatment:
Ten microliter of acetone containing desired concentration of insecticide was topically applied. The detailed procedure has already been described elsewhere (Chapter-II). 10 and 30 µg of insecticide concentrations were used as sublethal and lethal dose respectively. Twenty five larvae treated with lethal/sublethal dose of insecticide showing prostration stage of poisoning sequence were used for the determination of enzyme activity. Larvae treated with equal volume of acetone alone served as controls.

Tissue Source:
Larvae were chilled on crushed ice for 20 min to make them immobile, surgically opened and intact gut was removed. Gut contents were discarded along with the peritrophic membrane following dissection. Adhering hemolymph was removed.
by gently blotting the tissue with filter paper. The nerve cord along with the brain was removed by separating it from the adhering tissues and stored at 0°C until used (Fig.1)

Pooled tissues (5-6 larval midgut, 8-10 larval nerve cord along with brain) were homogenized with ice-cold buffer in potter Elvehjem glass homogenizer. The homogenizing buffer contained 10 mM magnesium chloride, 200 mM sodium chloride and 50 mM Tris HCl buffer. Homogenates were centrifuged at 8000 g for 5 minutes at 2°C to remove cell debris. The supernatant was used as source of enzyme. All the procedures were carried out at 2 to 4°C. The enzyme source was used almost immediately.

Assay of ATPases activity:
The enzyme activities presented in this study were of maximum obtainable under the conditions mentioned. The enzyme velocity, temperature and pH optima have already been reported for midgut and fat body of castor semilooper larvae (Holihosur, 1985).

Enzyme activity was measured indirectly using the method of Bonting (1970). The incubation medium prepared for different cation stimulated ATPases in a final volume of 1 ml contained the following ingredients.
The contents were mixed and reaction was initiated by the addition of ATP. Incubation was carried at 30°C with constant shaking for 60 min. The reaction was stopped by adding 750 µl of 20 percent trichloroacetic acid. Inorganic phosphate (P\textsubscript{i}) released following ATP hydrolysis was measured spectrophotometrically at 660 nm.

**Estimation of Inorganic Phosphate**

The procedure followed was according to the method of Bartlett (1959) described by Dittmer and Wells (1969). The method was based on the determination of inorganic phosphate in perchloric acid digest of the tissue lipid. The inorganic phosphate was allowed to react with ammonium molybdate (5%) to form phosphomolybdic acid. The latter was reduced by treating with 200 µl of reducing
Fig. 1. NERVOUS SYSTEM OF Achaea janata
reagent (30 gm sodium bisulfite, 6 gm sodium sulfite and 0.3 gm 1, 2, 4-
amnonaphthol sulfonic acid in 250 ml distilled water). Potassium dihydrogen
phosphate was used as internal standard.

**Protein Content Determination:**
The protein content of the tissue was determined according to the method of
Lowry et al. (1951) (for details see Chapter VI).

**RESULTS**

Distribution of Na\(^+\)-K\(^+\), Mg\(^{2+}\) and K\(^+\) stimulated ATPases in various
tissues of the castor semilooper was presented in the Table-1. It has to be
emphasized, however, that these activities were based mainly upon selective
ion stimulation of adenosine triphosphate (ATP) hydrolytic enzymes. Any of the
groups could consist of more than one ATPase. Nevertheless for the purpose
of understanding of insecticide effect, one could distinguish different kinds of
ATPases if they show no sensitivity to insecticide.

Among the three tissues, the nerve cord compared to midgut and fat
body exhibited significantly higher concentration of Na\(^+\)-K\(^+\) and Mg\(^{2+}\) stimulated
ATPases on the basis of the total activity obtained. The nerve cord demon-
strated higher K\(^+\) stimulated ATPase compared to Na\(^+\)-K\(^+\) and Mg\(^{2+}\) stimulated
ones. However, between the Na\(^+\)-K\(^+\) and Mg\(^{2+}\) stimulated ATPases, the former
one was found considerably higher in nerve cord and midgut tissues. The
results of the present study also revealed that all the three tissues demonstrated
higher K\(^+\) stimulated ATPase than those of Na\(^+\)-K\(^+\) and Mg\(^{2+}\) stimulated ATPases
(Table-1). The K\(^+\) stimulated ATPase in the fat body and nerve cord was found
significantly higher than that of the midgut.
Table 1. Distribution of Na\(^+\)-K\(^+\), Mg\(^{2+}\) and K\(^+\) stimulated ATPases activity in the various tissues of the 5th instar castor semilooper A. janata.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Na(^+)-K(^+) ATPase</th>
<th>Mg(^{2+})ATPase</th>
<th>K(^+)ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve cord</td>
<td>2.81 ± 0.30*</td>
<td>1.63 ± 0.14</td>
<td>3.16 ± 0.18</td>
</tr>
<tr>
<td>Fat body</td>
<td>0.516 ± 0.06</td>
<td>0.80 ± 0.04</td>
<td>4.12 ± 0.13</td>
</tr>
<tr>
<td>Midgut</td>
<td>0.497 ± 0.07</td>
<td>0.30 ± 0.02</td>
<td>1.44 ± 0.23</td>
</tr>
</tbody>
</table>

*The results are Mean ± SE of five experiments.

\(n = 5\)

ANOVA

<table>
<thead>
<tr>
<th></th>
<th>P &lt; 0.05</th>
<th>P &lt; 0.05</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve cord vs. Fat body</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Fat body vs. Midgut</td>
<td>&lt; NS</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Nerve cord vs. Midgut</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
Table 2. Total Sodium and Potassium concentration in hemolymph of the *Achaea janata* 5th instar larva.

<table>
<thead>
<tr>
<th></th>
<th>Na⁺ (ppm)</th>
<th>K⁺ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.27 ± 0.23</td>
<td>56.38 ± 3.58</td>
</tr>
</tbody>
</table>

*The results Mean ± SE of live experiments.

n = 3.
Fig. 2

THE pH PROFILE OF ATPase ACTIVITY FROM V INSTAR Achaea janata NERVE CORD

- o o Na⁺ K⁺ ATPase
- • • K⁺ Stimulated ATPase
- △ △ Mg⁺⁺ ATPase

Each point indicates the mean of five determinations.
The activities of ATPases as a function of pH is shown in Fig. 2. The pH optima of the Na\(^+\)-K\(^+\) ATPases as well as of K\(^+\) stimulated ATPase of the nerve cord showed almost the same pH requirement (7.6), whereas the pH optimum of Mg\(^{2+}\) activated ATPase was found to be 8.2. The latter enzyme seemed to retain a high level of enzyme activity at higher pH. On the other hand the activities of both Na\(^+\)-K\(^+\) ATPase and K\(^+\) stimulated ATPase were reduced at higher pH (Fig. 2).

It is known that the ATPase hitherto mentioned are actively involved in the transport of monovalent cations across the epithelial/nerve membrane. The activity of these enzymes would then dependant upon the bathing medium, the hemolymph. The Na\(^+\)\(\text{K}^+\) concentration of the hemolymph were summarized in Table-2. Like any other phytophagous lepidopteran species, the hemolymph of the castor semilooper was found to be very high in potassium (56.38 ppm) and low in sodium (2.27 ppm).

The effect of the organophosphorus compound fenitrothion on the activities of various ATPases were summarized in Tables 3 to 6. The activity of Na\(^+\)-K\(^+\) ATPase was significantly inhibited by sublethal dose (10\(\mu\)g/larva) in the nerve cord only after 48 h from the time of the application of the insecticide (Table-3). Further, the larvae treated with lethal dose (30\(\mu\)g) of fenitrothion demonstrated significant inhibition of Na\(^+\)-K\(^+\) ATPase in the nerve cord and midgut tissues only (Table-4).

The most interesting observation of the present study was that the fenitrothion treatment did not affect the K\(^+\) stimulated ATPase in all the
Table 3. Effect of sublethal dose (10 μg) of fenitrothion on the nerve cord Na\(^+\)-K\(^+\) ATPase of 5th instar castor semilooper *A. janata* at 24 h\(\text{post treatment}\) and 48 h\(\text{post treatment}\).

<table>
<thead>
<tr>
<th></th>
<th>μm Pi released/mg protein/h</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.47 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>24 h(\text{post treatment})</td>
<td>2.95 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>48 h(\text{post treatment})</td>
<td>1.30 ± 0.26</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

The results are Mean ± SE of five experiments.

n = 5.
Table 4. Na\(^{+}\)-K\(^{+}\) ATPase activity in the various tissue homogenates of 5th instar castor semilooper *A. lanata* after lethal dose (30 \(\mu\)g) treatment of insecticide.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>(\mu)m Pi released/mg protein/hr</th>
<th>Control</th>
<th>Treated</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve cord</td>
<td>2.81 ± 0.30</td>
<td>0.848 ± 0.04</td>
<td>P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Fat body</td>
<td>0.516 ± 0.06</td>
<td>0.374 ± 0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Midgut</td>
<td>0.497 ± 0.07</td>
<td>0.165 ± 0.01</td>
<td>P &lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>

The results are Mean ± SE of five experiments.

\(n = 5\).
Table-5. Effect of lethal dose (30 μg) of fenitrothion on the K⁺ stimulated ATPase activity of the various tissues of 5th instar *A. janata*.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control (μm P\textsubscript{i} released/mg protein/h)</th>
<th>Treated (μm P\textsubscript{i} released/mg protein/h)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve cord</td>
<td>3.16 ± 0.18</td>
<td>3.13 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Fat body</td>
<td>1.44 ± 0.23</td>
<td>1.12 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>Midgut</td>
<td>4.12 ± 0.13</td>
<td>4.38 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

The results are Mean ± SE of five experiments.
n= 3.
Table 6. Effect of lethal dose of fenitrothion on the Mg$^{2+}$ stimulated ATPase activity in various tissue homogenates of the 5th instar *A. janata* larvae.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control $\mu$m P$_i$ released/mg protein/h</th>
<th>Treated $\mu$m P$_i$ released/mg protein/h</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve cord</td>
<td>1.63 ± 0.14</td>
<td>0.67 ± 0.14</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Fat body</td>
<td>0.80 ± 0.04</td>
<td>0.37 ± 0.03</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Midgut</td>
<td>0.30 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

The results indicate Mean ± SE of five experiments.

n = 3.
three tissues tested for (Table-5). The activity of Mg$^{2+}$ stimulated ATPase revealed significant depletion in the activity of all the three tissues following the lethal dose of fenitrothion treatment (Table-6).

**DISCUSSION**

It has been recognized that extracellular concentrations of monovalent cations in the central nervous system (CNS) of certain insects must be regulated at levels different from those that appear in the hemolymph (Treherne and Schofield, 1979). Part of this regulatory capacity results from the physical barrier imposed by the perineurium and inner glial cells which restrict access of hemolymph components to the CNS. This probably acts to buffer the neurons from abrupt changes in the hemolymph ion concentrations. However, it is clear that membrane transport mechanisms must also be operative to maintain these ion gradients over extended periods. It has been shown that intact cockroach ventral nerve cord will conduct only in the presence of sodium and sodium will be slowed down if ethacryninc acid, a sodium transport inhibitor and dinitrophenol, a metabolic poison are added to the bathing medium (Schofield and Treherne, 1975). Pichon et al. (1972) found that the concentration of sodium in the hemolymph of *Manduca sexta* (2.5 mM) will not support nervous activity and that sodium must be concentrated in the intracellular spaces surrounding the axon.

The enzyme most commonly associated with sodium transport is the Na$^+$.K$^+$ ATPase which I have demonstrated in the ventral nerve cord, midgut
and fat body of the 5th instar castor semilooper (Table-1). The Na\(^+\)-K\(^+\) ATPase of castor semilooper was similar to Na\(^+\)-K\(^+\) ATPase reported for other lepidopteran species of insects in several basic parameters like pH and requirement for magnesium ions and cardiac glycoside (Ouabain). Like other phytophagous insects (Florkin and Jeuniaux 1974) the hemolymph of castor semilooper contain very high concentration of potassium (56.38 ppm) compared to low level of sodium (2.27 ppm). The presence of Na\(^+\)-K\(^+\) ATPase in the neural tissue is further evidence that the castor semilooper larvae rely upon the same mechanisms for the maintenance of action potentials as do other insects whose body fluids possess high sodium and low potassium levels (Treherne, 1976). The results of the present study also revealed that the nerve cord compared to fat body and midgut contained significantly higher level of Na\(^+\)-K\(^+\) and Mg\(^{2+}\) stimulated ATPases. The present observations also showed that the nerve cord of castor semilooper larvae contained (on the basis of the total activity obtained) high level of K\(^+\) stimulated ATPase than that of Na\(^+\)-K\(^+\) ATPases which may reflect an adaptation to the high potassium-low sodium composition of the hemolymph (Tables 1 & 5). The hemolymph levels of sodium and potassium of three lepidopterans studied, appear to be regulated primarily by the midgut (Irvine, 1969; Jungreis et al. 1973; Jungreis and Vaughan, 1977; Harvey et al. 1973 and Jungreis 1977). The in vitro studies of active transport of potassium across the larval midgut of H. cecropia, M. sexta and A. pernyi takes place in the absence of sodium in the bathing medium (Harvey and Nedergaard, 1964; Harvey and Zerahn, 1972; Wood, 1972 and Blankemeyer, 1977). These studies suggest that K\(^+\) is precisely regulated in the phytophagous lepidopteran insects. The midgut of 5th instar castor semilooper also demonstrated considerably higher K\(^+\) stimulated ATPase than Na\(^+\)-K\(^+\) consistent with the important role of the former enzyme in the potassium transport across the midgut.
The participation of Na⁺-K⁺ ATPase in alkali metal transport across epithelia was first demonstrated by Skou (1957). Later Glynn (1957) observed that cardiac glycosides like ouabain would inhibit this enzyme complex. The degree of inhibition is dependant upon the concentration of potassium levels in excess of 20 mM to block the ouabain inhibition (Vaughan and Cook, 1972; Gardner and Frantz, 1974; Jungreis and Vaughan, 1976). Midgut epithelium of three Lepidopteran species has been demonstrated to lack Na⁺-K⁺ ATPases which are normally associated with the binding of ouabain (Vaughan and Cook, 1972). In the present study the midgut as well as fatbody tissues of 5th instar castor semilooper revealed the presence of ouabain sensitive Na⁺-K⁺ ATPase in the presence of potassium ions in the incubation medium. Jungreis and Vaughan (1977) are of the opinion that the presence of potassium at that concentration would block ouabain inhibition, hence sensitivity to ouabain inhibition cannot be determined at such times. Further studies are required to characterize the ouabain sensitive Na⁺-K⁺ ATPase activities in the midgut tissue of the castor semilooper A. jananata.

Effect of Fenitrothion on Na⁺-K⁺, Mg²⁺ and K⁺ stimulated ATPases:

Most, if not all insecticides attack the CNS of insects (Narahashi, 1976). Recently, it has become evident that insecticides such as DDT and pyrethroids directly disturb the transmission of action potentials. Both pyrethroids and DDT induce multiple spiking and pyrethroids also block axonal transmission. In either case synaptic transmission will be disrupted. The OP insecticides on the other hand seemed to interfere with neurochemistry of synapses (Osborne, 1980). The best understood action of OP is that of cholinesterase inhibition. Inhibition increases the level of acetylcholine in the synaptic cleft, thus potentiating synaptic conduction which leads to multiple spiking in the postsynaptic element.
Several organochlorine insecticides have been shown to inhibit Na\(^+\)-K\(^+\) ATPase (Koch et al. 1969; Holan, 1969; Matsumura and Patil, 1969; Bradkowski and Matsumura, 1972; Davis and Wedemeyer, 1971; Desaiah et al. 1974; Cutkomp et al. 1976 and 1980; Younis et al. 1978; Brooks, 1980; Chefurka, 1982 and Mourelle et al. 1985). Fenitrothion an OP compound also showed a strong inhibition of the ATPase of various tissues tested in the present study. These results are contrary to the reports of Koch (cited by Koch et al. 1969) who has reported that malathion an OP compound has no inhibitory effect on Na\(^+\)-K\(^+\) ATPase of the nerve cord. Both nerve cord and midgut of 5th instar castor semilooper larvae showed high level of Na\(^+\)-K\(^+\) ATPase inhibition following the fenitrothion treatment. The glial cells of insect CNS are implicated in regulation of the ionic composition of the fluid bathing the nerve cells (Treherne and Pichon, 1972) and with sequestration of neurotransmitters (Houk and Beck, 1977). It appears that the damage to glial cells by fenitrothion could upset their roles in ionic regulation and metabolism of neurotransmitters leading to increased nervous activity as well as nerve membrane conductance (Narahashi and Hille, 1968).

Similarly, the Mg\(^{2+}\) stimulated ATPase also demonstrated significant reduction following the fenitrothion treatment (Table-6). Mitochondrial Mg\(^{2+}\) ATPase has been shown to participate in the ATP production through oxidative phosphorylation (Racker, 1970; Sando and Packer, 1973). In the present study Mg\(^{2+}\) activated ATPase was determined in the total homogenate and it was assumed that much of the observed enzyme activity may represent mitochondrial one. It may be recalled here that the various neuromuscular symptoms like hyperactivity and tremors showed by fenitrothion treated larvae could certainly
be the result of an imbalance of Mg$^{2+}$ ATPase involved in energy regulation in a muscle tissue. It may be of considerable interest to note that the fat body, the major energy storage organ of the larva, showed significant inhibition of Mg$^{2+}$ stimulated ATPase following the insecticide treatment. It appears that fenitrothion upset energy regulating mechanism in the fat body. The most interesting finding of the present study was that the fenitrothion had no inhibitory effect on K$^+$ stimulated ATPase in all the three tissues tested for (Table-5). This may be attributed to the high concentration of potassium in the hemolymph. However, further experiments are needed to support this assumption.

The roles of these insect ion specific ATPases in the transport functions of the midgut could not be assessed. The inhibition of enzyme activities were similar to the level of inhibition found in the nerve cord. However, we have no direct physiological evidence which implicates the various ATPases in the transport of ions by the midgut.

The lack of fundamental knowledge on the functional roles of the ATPases in the insect nerve cord and midgut pose a major problem. At this stage, the only tangible conclusion one could draw from this work was that Na$^+$.K$^+$ ATPase as well as Mg$^{2+}$ ATPase were inhibited by the topical application of the fenitrothion. It is believed that the fenitrothion interacts with membrane proteins thereby destabilizing membrane bound enzymes. Indeed such disruptive effect on plasma liver membrane of rats treated with DDT and toxaphene has been suggested (Mourelle, et al. 1985). Further research is required to elucidate the effect of OP compounds on the membrane proteins of insects.