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PYRETHROID INHIBITION OF NEURAL ATPases

By

John Marshall Clark II

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ABSTRACT

PYRETHROID INHIBITION OF NEURAL ATPases

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Various ATPases present in an axon-rich, membrane preparation from the retinal nerves of the squid, Loligo pealei, were studied. In each case, the activity specific to each enzyme can be recognized by the different activity in the presence and absence of specific ion(s). Other criteria used in classifying these ATPases are: appropriate ATP substrate concentrations (e.g., Km), temperature dependence, protein concentration, and the effect of inhibitors such as ouabain, CN^-, EGTA, EDTA, ruthenium red and lanthanum.

As a result, six distinctly recognizable ATPase systems have been found; (1) ouabain insensitive Na^+-K^+ ATPase, (2) ouabain sensitive Na^+-K^+ ATPase, (3) Ca^{2+}-Mg^{2+} ATPase, (4) Ca^{2+} ATPase, (5) Mg^{2+} ATPase and (6) a nerve myosin.

The biochemical process by which various pyrethroid insecticides affect membrane-bound ATPase activities of the squid nervous system was then examined. Of the six recognized ATPases, only Ca^{2+}-stimulated ATPases are seen to be clearly sensitive to pyrethroid inhibition. These enzymes are Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase. Both were shown to be located in subcellular fractions of the retinal nerve and optic lobe of the squid. It was found that pyrethrin and its closely related synthetic analog, allethrin, primarily inhibit Ca^{2+} ATPase, whereas highly modified pyrethroids such as cypermethrin and decamethrin mainly inhibit Ca^{2+}-Mg^{2+} ATPase. Permethrin, which is considered

to possess structural similarities to both naturally-occurring pyrethrins and highly modified pyrethroids, was found to have an intermediate property in terms of its inhibitory potency to both Ca^{2+} and Ca^{2+}-Mg^{2+} ATPase.

An effort was made to relate calcium regulation resulting from inhibition of these Ca^{2+}-stimulated ATPases to the electrophysiological symptoms induced both in the axonic and synaptic regions of the nervous system by these insecticides.

For this purpose, Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase were identified by similar means in the microsomal and synaptosomal fractions of the brain of the American cockroach, Periplaneta americana. Both enzymes were found to be highly sensitive to the action of pyrethroid insecticides. Ca^{2+} ATPase was clearly more sensitive toward the action of allethrin when compared to the more highly modified compounds such as cypermethrin. Conversely, cypermethrin was determined to be more inhibitory to Ca^{2+}-Mg^{2+} ATPase activity than was allethrin. Overall, the synaptic preparation appeared to be the more sensitive site of pyrethrin action. This was particularly true in the case of nonmitochondrial Ca^{2+}-Mg^{2+} ATPase which was the most sensitive enzyme examined.

These in vitro findings have been substantiated by in vivo experiments where similar pyrethroid inhibition patterns of Ca^{2+}-stimulated ATPases were observed in roaches which elicited pyrethroid poisoning symptoms. More importantly, in vivo inhibition occurred in the presence of a similar amount of insecticide which had been determined to cause a substantial amount of inhibition in vitro.

By utilizing specific modifiers of ion flux, it was determined that at least a proportion of the ATPase activity may be related to Ca^{2+} transport. An effort was made to correlate Ca^{2+}-stimulated ATPase activity which was clearly

sensitive to pyrethroid action and Ca^{2+} transport mechanisms which control Ca^{2+} regulation in the nerve and hence its excitability.

The author would like to dedicate this work to Professor Carolyn Burdick, Brooklyn College of the City University of New York, without whose help and continual scientific generosity this project would never have been completed.

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The author wishes to express his deep appreciation to Professor Fumio Matsumura for so many things that it is impossible to adequately summarize them here. In particular, the author would like to thank Mrs. Teruko Matsumura and her wonderful family for always providing such genuine hospitality and good feeling in their home wherever that may be.

John Clark is especially grateful to Ms. Katie Pointer for containing her cosmic spirit, particularly during periods of complete lunar light.

The author also wishes to acknowledge Mrs. Alice Ellis for her excellent assistance in the preparation of this dissertation.

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ATPases IN THE AXON-RICH MEMBRANE PREPARATION FROM THE RETINAL NERVE OF THE SQUID, LOLIGO PEALEI

INTRODUCTION

Squid axons have been extensively studied by physiologists for their electrophysiological properties, and yet, surprisingly very little data are available regarding their biochemical characteristics. The major reason for this lack of information is that the giant axons which are usually used for electrophysiological experiments have only a small ratio of the actual axonic membrane to other neural matters, and are unsuited for biochemical studies (Ritchie, 1973).

It was originally found by Canessa-Fisher et al. (1967) that by using a large Chilean squid species, the retinal nerve (optical nerve) of the squid consists of small axonic fibers with a favorable axon to glial cell ratio (10:1). Such small axons have relatively large membrane surface areas which facilitate biochemical studies. An earlier report (Matsumura, 1977) showed that such retinal nerve preparations from the most commonly studied squid species in North America, Loligo pealei, had high ATP-dependent membrane phosphorylation activities which were greatly influenced by the ionic environment of the assay medium. Because of the requirement of ATP as the substrate, most of the above phosphorylation activities have been considered to be related to ATPases. In this context, ATPase is defined as an enzyme which utilizes ATP as a substrate and results in a phosphorylated form of the enzyme itself. Dephosphorylation is required prior to binding another ATP molecule as substrate. Protein kinase and phosphoprotein phosphatase activities were not determined in the present study

due to previous reports that such activities were not susceptible to inhibition by chlorinated hydrocarbon insecticides (Doherty and Matsumura, 1975). Information on ATPases in the squid axonic membrane is incomplete, the only enzyme which has been extensively studied being Na^+-K^+ ATPase (Brinley and Mullins, 1968; Sjodin, 1974) which has been acknowledged to work as a Na^+ pump.

Recently there has been an upsurge of interest in the regulatory mechanisms for Ca^{2+} (for example DiPolo, 1974; Mullins and Brinley, 1975) and Mg^{2+} (de Weer, 1976) which require ATP as their energy source. Clearly, there must be other ATPases than Na^+-K^+ ATPase in the squid axon. The present study was undertaken to gather more information on such ATPases.

MATERIALS AND METHODS

Animals. The North American species of squid, Loligo pealei, was used in the preparation of the cell-free membrane fraction of retinal nerves which comprises the assay material used throughout this study. All squids were captured and kept alive until ready for use at the Marine Biological Laboratory at Woods Hole, Massachusetts during the summer months of 1977 and 1978. Squid dissection and storage techniques for nerve tissue have been previously reported (Matsumura, 1977). Approximately 40 mg (wet wt) of retinal nerve tissue was obtained from each squid.

Chemicals. A tetraethylammonium salt of gamma (γ)-^{32}P-labeled adenosine triphosphate (ATP), purchased from New England Nuclear, Boston, Massachusetts, was utilized as the substrate for all assays and had a starting specific activity of approx 26.2 mCi/μ mol. The ionic strengths of individual assay buffers were adjusted with the chloride salts of sodium, potassium, magnesium, calcium and lithium (all reagent grade). Potassium cyanide was used for the tests on the effects of CN^-. Lanthanum chloride (La^{3+}) was obtained from Alfa Products, Beverly, Massachusetts, and activated charcoal (Norite

brand) was purchased from the University of Wisconsin Stores, Madison, Wisconsin. The following chemicals were all purchased from Sigma Chemical Company, St. Louis, Missouri: ouabain, ethyleneglycol-bis-N,N'-tetracetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), ruthenium red, the sodium salt of adenosine diphosphate (ADP, Grade III), bovine serum albumin (A4378), and dithiothreitol (DTT). All agents to be tested were prepared in the corresponding buffer of the assay and unless otherwise stated, all inhibition experiments were carried out by preincubation of the agent for 10 min at 25°C prior to the addition of [γ^{32}P]ATP substrate.

Preparations of ATPase Fractions. When membrane preparations were needed, homogenates of retinal nerves were made in 750 mM sucrose plus 1 mM EDTA and 0.1 mM dithiothreitol (DTT) at 40 mg/ml by using a hand-operated glass-glass homogenizer at 0°C. The homogenate was diluted to a final concentration of 250 mM sucrose with distilled-deionized water at 0°C and centrifuged at 8000 g for 10 min at 4°C in a swing bucket rotor (SW 25.1, Spinco). The supernatant was saved at 0°C and the pellet resuspended in an equal volume of 250 mM sucrose and centrifuged as before. The supernatants were combined and centrifuged at 90,000 g for 2.5 hr at 4°C. After discarding the supernatant, the pellet was resuspended into the original volume of 750 mM sucrose containing 0.1 mM DTT and stored frozen. This 90,000 g fraction was used exclusively in all enzymatic assays unless otherwise indicated. Protein determination was by the method of Lowry et al. (1951).

ATPase Assay. The extent of inorganic phosphate (Pi) production from ATP was determined as follows: to 0.9 ml of 30 mM Tris-HCl buffer (at pH 7.1 unless stated otherwise) with various ion combinations, 0.1 ml of the above nerve preparations was added. The ionic compositions of various buffers used are summarized in Table 1. Various inhibitors were added to the enzyme-buffer

Table 1. Standard Buffer Compositions

ATPase studied	Concentrations, mM Na^+	K^+	Mg^{2+}	Ca^{2+}	Inhibitors	ATP
Na^+-K^+, ouabain insensitive	160	40	10	0	ouabain[b]	5.6×10^{-8}M
Na^+-K^+, ouabain sensitive	160	40	10	0	-	10^{-4}M
Total Ca^{2+} and Mg^{2+} ATPase	160	160	10	0.2	ouabain	5.6×10^{-8}M
Ca^{2+}-Mg^{2+} ATPase	0	160	10	0.01[c]	ouabain EGTA	5.6×10^{-8}M
Basal Mg^{2+} ATPase	0	160	10	0	ouabain	5.6×10^{-8}M
Basal Ca^{2+} ATPase	0	160	0	0.01	ouabain EGTA	5.6×10^{-8}M
Ca^{2+} ATPase	160	160	0	2	ouabain EDTA (1 mM)	5.6×10^{-8}M
Li^+-Ca^{2+} ATPase	0 (Li = 160)	160	0	2	ouabain EDTA (1 mM)	5.6×10^{-8}M
Total Mg^{2+} ATPase	160	160	10	0	ouabain KCN (2 mM)	5.6×10^{-8}M
Myosin-type ATPase (a) neurofilament (10 nm)	0	60	1[a]	0	ouabain EGTA	10^{-3}M
(b) Myosin	0	600	1[a]	0	ouabain EDTA (2.5 mM)	10^{-3}M

[a] $MgSO_4$ instead of $MgCl_2$. 1 mM Ca^{2+} was sometimes used in place of Mg^{2+}.

[b] Ouabain and EGTA concentrations were 10^{-4}M and 5×10^{-4}M, respectively.

[c] This level of free Ca^{2+} concentrations were established with a Ca EGTA buffer system, Portzehl et al. (1964).

mixture and equilibrated for 10 min at 25°C. [γ-^{32}P]ATP in 10 µl of distilled water was added and the system thoroughly stirred using a Vortex-mixer. Approximately 3 x 10^5 dpm were used per assay tube. The final concentration of ATP was 5.6 x 10^{-8}M unless otherwise stated in the text. The system was incubated at 30°C for 10 min and the reaction stopped with 0.3 ml of 10% trichloroacetic acid (TCA) at 0°C. Membrane protein was co-precipitated with 1.0 mg of bovine serum albumin (BSA) plus 1.35 mg KH_2PO_4 in 0.1 ml distilled water, and after mixing, the system was allowed to equilibrate for 5 min at 25°C. The system was centrifuged at 3000 g for 3 min, and the aqueous supernatant sans protein was quantitatively transferred to a clean assay tube. Approximately 100 mg of activated charcoal was added to bind unreacted labeled ATP and the tube thoroughly mixed. The sides of the tube were rinsed with 0.2 ml of ethanol and after mixing was again centrifuged to precipitate the charcoal at 3000 g for 3 min. A 0.5 ml aliquot was taken for liquid scintillation counting for analysis. The counting efficiency was approximately 65 to 70%. The experimental variability is reported as the means $\pm$ standard error of at least two separate experiments (two replicates each). Standard error was determined as the standard deviation divided by the square root of N where N equals the number of replicates.

The extent of γ-^{32}P incorporation into nerve protein (Pi-E production) was determined similarly but after the addition of TCA, the precipitate was collected by centrifugation at 3000 g and redissolved by using 0.2 ml of 2N NaOH. After mixing, the system was reprecipitated with 8 ml of 4% TCA and the precipitate recollected by centrifugation as before. This washing procedure was repeated once more and the final precipitate, after being dissolved in NaOH, was transferred in 10 ml of scintillation counting solution for analysis.

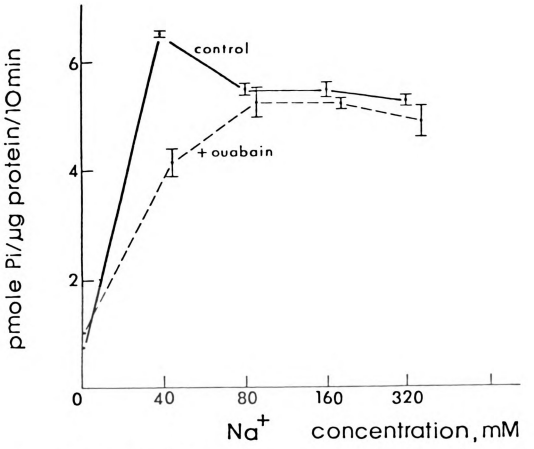

Figure 1. Effect of Na^+ concentration on the level of Na^+-K^+ ATPase activity in the presence (dotted line) and absence (solid line) of ouabain in the squid retinal nerve 90,000 g precipitate as determined by ^{32}Pi production (i.e., γ-^{32}P-ATP hydrolysis). The composition of other ions and incubation conditions remained unchanged (e.g., K^+ = 40 mM, Mg^{2+} = 10 mM, Tris-HCl = 30 mM at pH 7.1 and ATP = 5.6×10^{-4} M. Incubation was for 10 min at 30°C. Ouabain concentration was 10^{-4} M. Experimental variability reported as standard error.

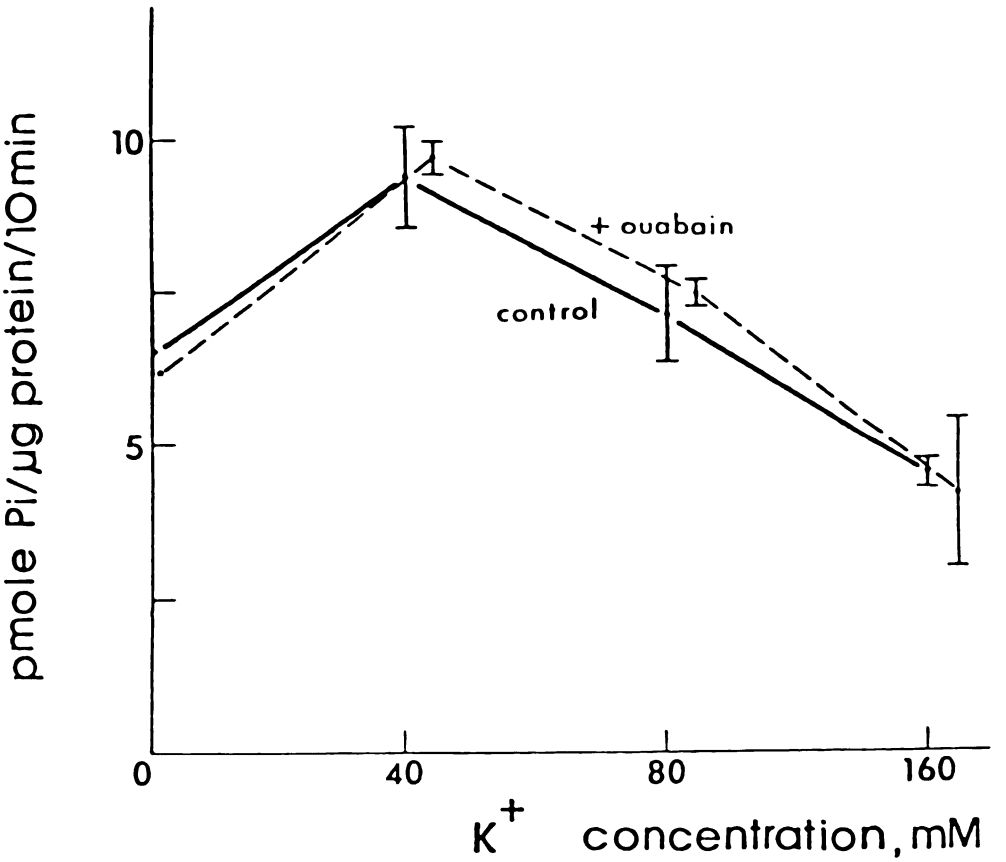

Figure 2. Effect of K^+ concentration on the level of Na^+-K^+ ATPase activity in the presence (dotted line) and absence (solid line) of ouabain (10^{-4}M). Other ionic compositions were: Na^+ = 160 mM, Mg^{2+} = 10 mM. ATP concentration was 5.6 x 10^{-8}M. Ouabain concentration was 10^{-4}M. Experimental variability reported as standard error.

RESULTS

Ouabain Insensitive Na^+-K^+ ATPase. Axonic membranes, devoid of axoplasm, are expected to contain a low level of ATP. For instance, Mullins and Brinley (1969) estimate that the concentration of ATP in the internally dialyzed giant axon is on the order of 10^{-6}M. Therefore, our initial efforts were made to study ATPases which operate at low substrate concentrations.

In the experiment shown in Figure 1, the concentration of Na^+ was varied, while those of K^+ and Mg^{2+} were kept constant (40 mM and 10 mM, respectively). ATP concentration was 5.6×10^{-8}M. It is noted that the ATPase activity increased as Na^+ concentration was raised. A similar experiment (Figure 2), in which Na^+ (160 mM) and Mg^{2+} (10 mM) were kept constant and K^+ was varied, gave essentially the same result, whether in the presence or absence of 10^{-4}M ouabain. To test the possibility that such ion sensitivity comes from the increase in total monovalent, alkali cation (i.e., Na^+ plus K^+), or from changes in concentration of each specific cation, the ratio of Na^+ to K^+ was varied, while keeping the total concentration of Na^+ plus K^+ (200 mM) and other ionic conditions constant (Figure 3). The result clearly indicates that the ratio of Na^+ to K^+ is important in determining the optimum ATPase activities.

A question must be raised whether such ATPase activity is due to the well established Na^+-K^+ ATPase pump or not. The sensitivity of the ATPase toward 10^{-4}M ouabain (Figures 1, 2 and 3) indicates that the activity due to ouabain sensitive Na^+-K^+ is generally low, implying that the contribution of such an ATPase to the total ATPase activities is rather small under these experimental conditions. The percentage of ouabain sensitive Na^+-K^+ ATPase to ouabain insensitive Na^+-K^+ ATPase varied from one preparation to another, but averages approximately 20% of the total activity. This is similar to the case with the lobster nerve preparations when they are tested at low ATP concentration

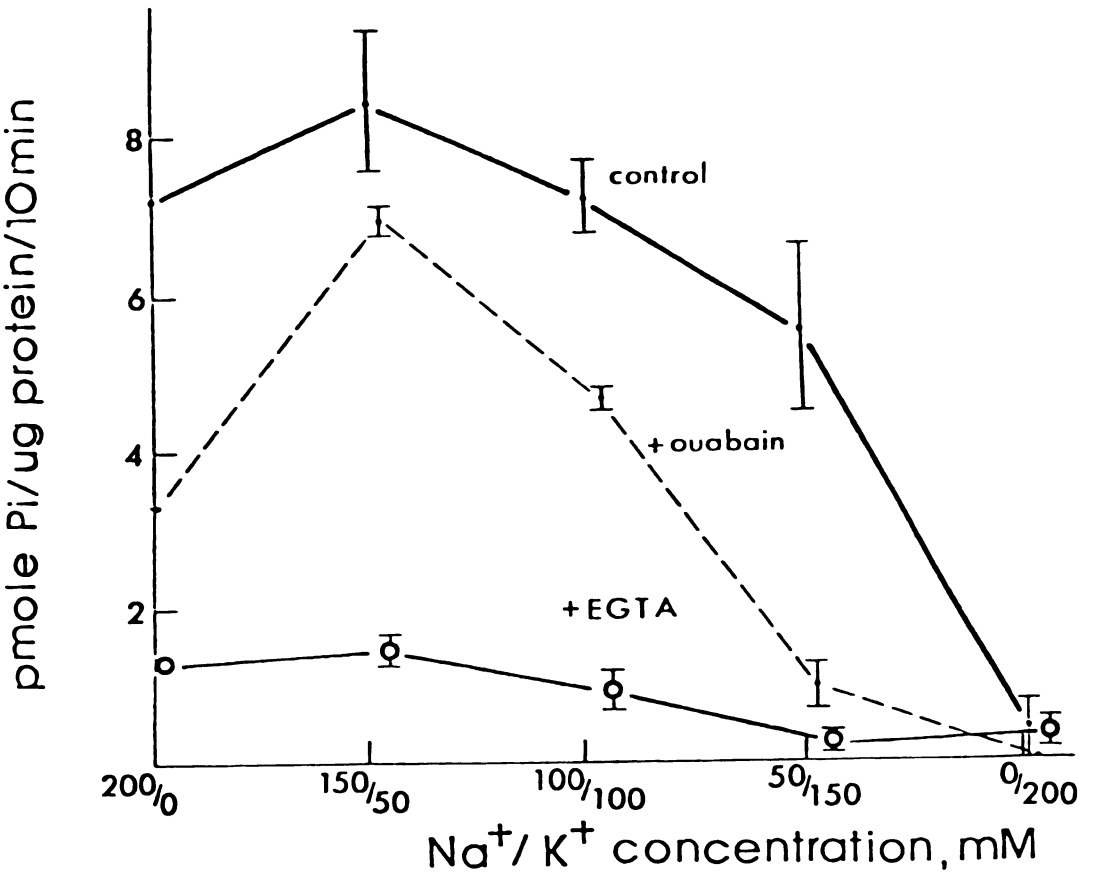

Figure 3. Effect of the change in Na^+:K^+ ratio on the level of Na^+-K^+ ATPase activity in the presence (dotted line) and absence (solid line) of ouabain (10^{-4}M) and with EGTA (1 mM) plus ouabain (circle line). Other incubation conditions were: Mg^{2+} = 10 mM and ATP = 5.6 x 10^{-8}M. Experimental variability reported as standard error.

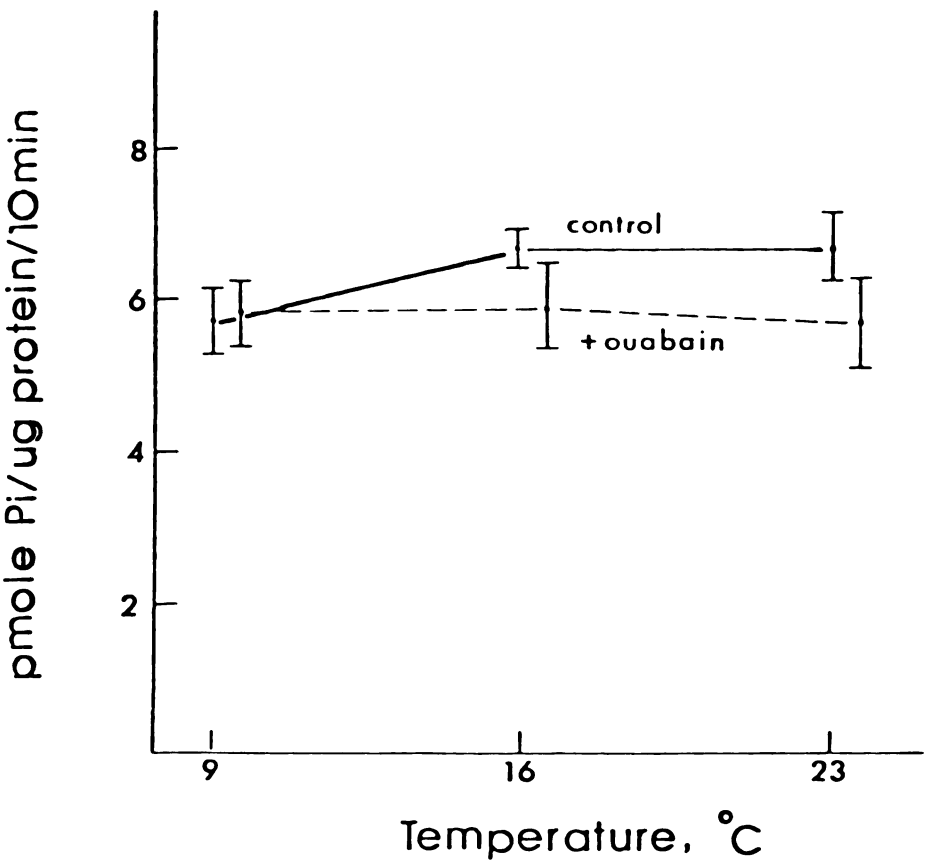

Figure 4. Effect of temperature changes on Na^+-K^+ ATPase activity in the presence (dotted line) and absence (solid line) of ouabain (10^{-4}M). Incubation conditions were: Na^+ = 160 mM, K^+ = 40 mM, Mg^{2+} = 10 mM, and ATP = 5.6 x 10^{-8}M. Experimental variability reported as standard error.

Table 2. Effect[a] of ATP Concentration on the Activity of Na^+-K^+ ATPase in the Presence of Ouabain and CN^-

Treatment	ATP concentration	
	Low (5.6×10^{-8}M)	High (2.0×10^{-5}M)
	pmol Pi/μg protein/10 min ± SE[b]	nmol Pi/μg protein/10 min ± SE[b]
Control	1.64 ± 0.65	1.09 ± 0.28
Ouabain (10^{-4}M)	3.28 ± 0.72	0.78 ± 0.15
CN^- (2 mM)	4.75 ± 0.38	1.80 ± 0.04

[a]Values reported are average of two identical experiments.

[b]SE = standard error.

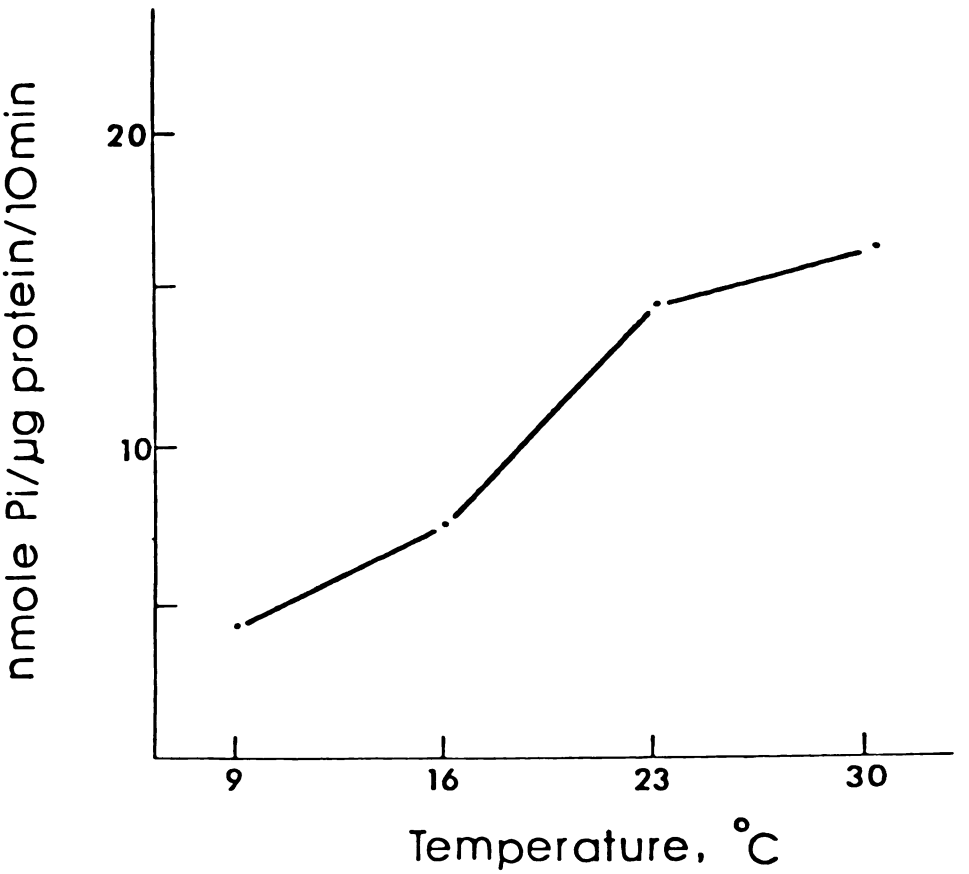

Figure 5. Effect of temperature changes on ouabain sensitive Na^+-K^+ ATPase activity plotted as the difference between the total activity minus the ouabain insensitive activity (i.e.,Δ) at high ATP concentration (ATP = 10^{-4}M). Other incubation conditions were: Na^+ = 160 mM, K^+ = 40 mM, Mg^{2+} = 10 mM, and ouabain = 10^{-4}M.

(Doherty and Matsumura, 1974). Another characteristic of this ouabain insensitive Na^+-K^+ ATPase is that it is not temperature sensitive (Figure 4).

Ouabain Sensitive Na^+-K^+ ATPase. According to Doherty and Matsumura (1974), ouabain sensitive Na^+-K^+ ATPase is more active at high ATP concentrations. To test this possibility, the inhibitory patterns of ouabain and CN^- were examined at a high and a low ATP concentration. It can be seen in Table 2 that the level of ouabain and CN^- inhibition increases at the high ATP concentration as expected. Also in accordance with the above observation, the temperature dependency of these ATPases also increases with increased ATPase concentration (compare Figure 4 and Figure 5). In view of the abundance of information concerning ouabain sensitive Na^+-K^+ ATPases, our efforts were directed to study other types of ATPases.

Total Ca^{2+} and Mg^{2+} ATPase Activities. Examination of Figure 3 reveals that the overall ATPase activity is greatly inhibited by addition of EGTA, a specific calcium chelator. To examine the effect of calcium on squid retinal axons, the Ca^{2+} concentration was varied, while Na^+, K^+ and Mg^{2+} concentrations were kept constant at 160, 160 and 10 mM, respectively. The results illustrated in Figure 6 show that there is an ATPase(s) whose activity is stimulated by Ca^{2+} at concentrations less than 0.1 mM. The enzyme shows temperature sensitivity (Figure 7) in accordance with Duncan's (1976) observations as well as the susceptibility toward EGTA (Figure 8). However, the level of EGTA inhibition varied from one enzyme preparation to another, probably reflecting the variation in bound Ca^{2+} in the membrane.

Duncan (1976) has suggested that the active Ca^{2+} extrusion processes (i.e., Ca^{2+}-pump) are operated by an ATPase requiring both Ca^{2+} and Mg^{2+} for its maximum activity. It is generally designated as Ca^{2+}-Mg^{2+} ATPase and has

been found in brain microsomal preparations which include axonic membranes (Robinson, 1976).

$\underline{Ca^{2+}\text{-}Mg^{2+}\ ATPase}$. The above characteristics of the Total Ca^{2+} and Mg^{2+} ATPase activity indicate the probable presence of Ca^{2+}-Mg^{2+} ATPase. To prove its presence, a Ca^{2+} buffer containing K^+, Mg^{2+}, EGTA and Ca^{2+} (Table 1) was adopted. In this buffer, increase in free Ca^{2+} (Portzehl et al., 1964) results in a drastic increase (46%) over basal Mg^{2+} ATPase activity (Figure 9). Ca^{2+}-Mg^{2+} ATPase activity can then be measured by subtracting the sum of the basal Mg^{2+} (Ca^{2+}-Mg^{2+} buffer minus Ca^{2+}) and the basal Ca^{2+} (Ca^{2+}-Mg^{2+} buffer minus Mg^{2+}) activities from the total activity seen with the Ca^{2+}-Mg^{2+} buffer system (Table 1). That is, only that ATPase activity apparent from the coupled stimulation of Ca^{2+} and Mg^{2+} ions is defined as Ca^{2+}-Mg^{2+} ATPase. The result (see insert, Figure 9) shows that the optimum Ca^{2+} concentration for this enzyme is relatively low (i.e., on the order of 10^{-5}M). To be sure, there are other ATPases which require the presence of both Ca^{2+} and Mg^{2+} (Weber and Murray, 1973; Mahendran et al., 1974). However, the most notable characteristic of Ca^{2+}-pump ATPase (=Ca^{2+}-Mg^{2+} ATPase) is its high temperature sensitivity indicative of metabolic type of ATPases (Duncan, 1976). The effect of temperature on the Ca^{2+}-Mg^{2+} ATPase in squid membrane preparation is also profound with a Q_{10} of 3.78 between 37^{o} and 27^{o}C (unpublished data).

$\underline{Ca^{2+}\ ATPase}$. Another ATPase which could play some role in Ca^{2+} regulatory processes is Ca^{2+}-ATPase (Trams et al., 1976; Lauter et al., 1977; Matsumura and Ghiasuddin, 1979). The enzyme is generally considered to be located at the outer surface of the membrane, and requires a relatively high level of Ca^{+} (greater than 0.1 mM) to be fully activated. In the experiment summarized in Figure 10, we have varied the Ca^{2+} concentration in the absence

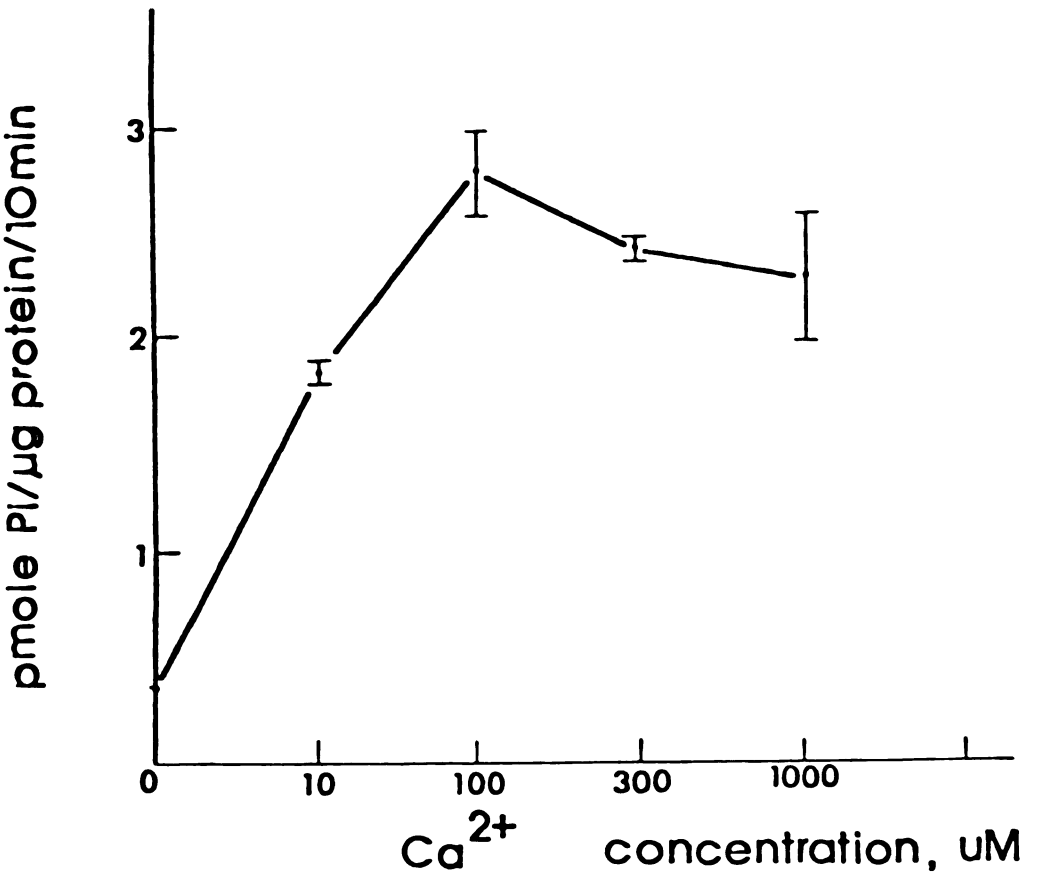

Figure 6. Effect of Ca^{2+} concentration on the level of Total Ca^{2+} and Mg^{2+} ATPase activity. Other ionic compositions were Na^{+} = 160 mM, K^{+} = 160 mM, and Mg^{2+} = 10 mM. ATP concentration was 5.6 x 10^{-8}M. Ouabain concentration was 10^{-4}M. Experimental variability reported as standard error.

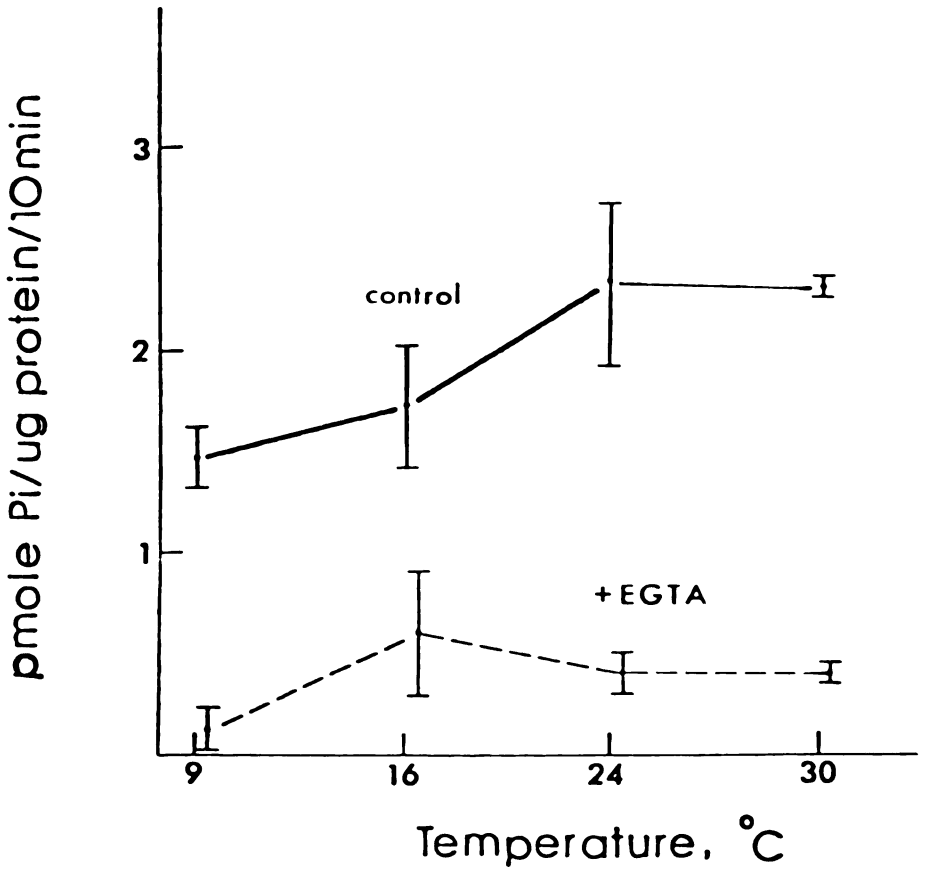

Figure 7. Effect of temperature changes on Total Ca^{2+} and Mg^{2+} ATPase activity in presence (dotted line) and absence (solid line) of EGTA (1.25 mM. Ionic compositions were: Na^{+} = 160 mM, K^{+} = 160 mM, Mg^{2+} = 10 mM, and Ca^{2+} = 0.2 mM. ATP concentration was 5.6 x 10^{-8} M. Experimental variability reported as standard error.

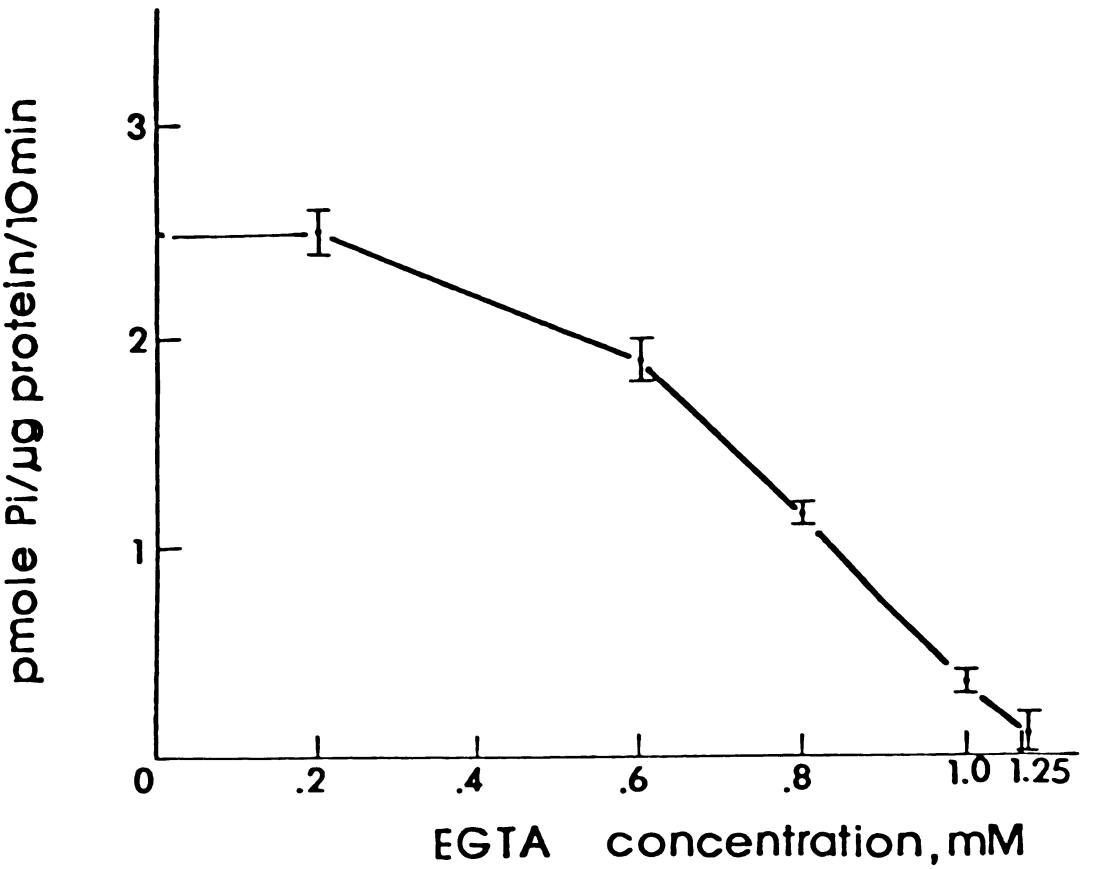

Figure 8. Effects of EGTA concentration changes on Total Ca^{2+} and Mg^{2+} ATPase activity. Ionic compositions were: Na^{+} = 160 mM, K^{+} = 160 mM, Mg^{2+} = 10 mM, and Ca^{2+} = 0.2 mM. ATP concentration was 5.6×10^{-8} M. Experimental variability reported as standard error.

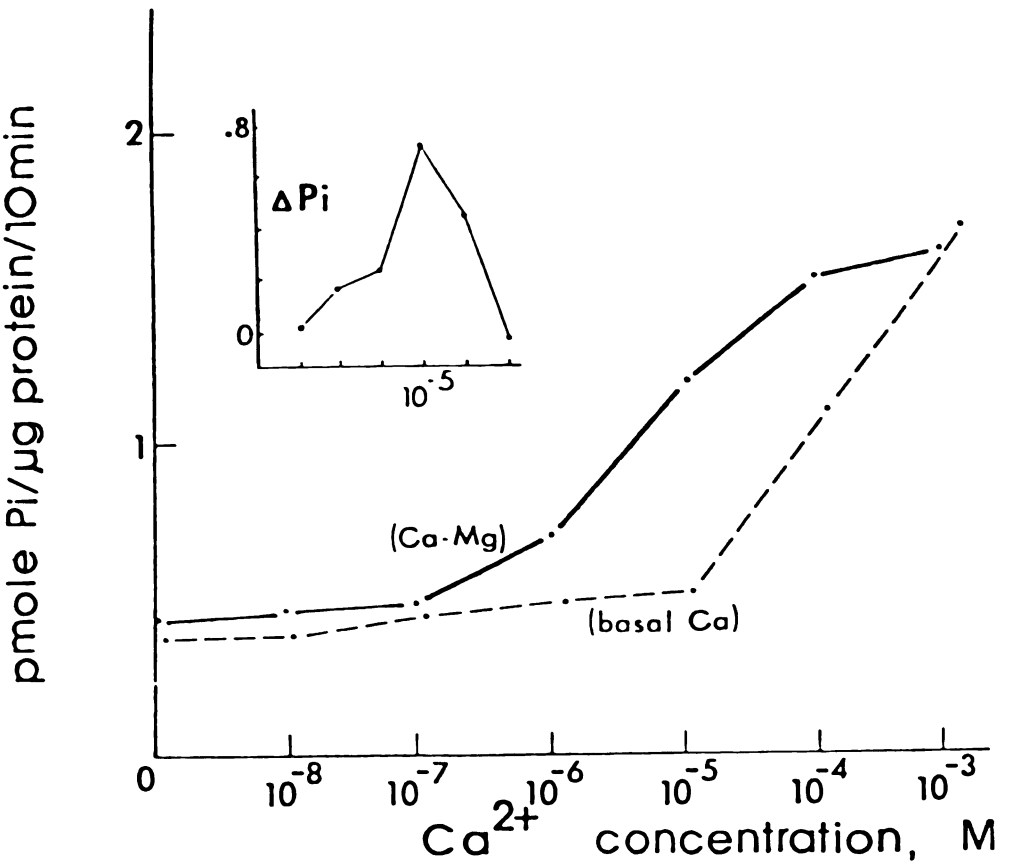

Figure 9. Effect of Ca^{2+} concentration on the level of Ca^{2+}-Mg^{2+} ATPase activity. Other ionic compositions were: K^{+} = 160 mM, Mg^{2+} = 10 mM. ATP concentration was 5.6×10^{-8} M.

of Mg^{2+} and found that the stimulation of ATPase activity occurs only at high Ca^{2+} concentrations in contrast to the case with Ca^{2+}-Mg^{2+} ATPase as described above. Clearly then, these two Ca^{2+}-stimulated systems are different ATPases. The Ca^{2+} ATPase activities are also affected by changes in Na^{+} but not K^{+} concentrations as shown in Figures 11 and 12. The temperature sensitivity of this ATPase in terms of Q_{10} between 16-26°C was found to be 2.23.

Other Ca^{2+}-stimulated ATPases. According to Baker et al. (1969), there is a Ca^{2+}-stimulated ATPase system which can operate in the presence of Li^{+} in place of normally used Na^{+} in the squid axon (Li^{+}-Ca^{2+} ATPase, Table 1). On the other hand, the ouabain sensitive Na^{+}-K^{+} ATPase and Ca^{2+}-Mg^{2+} ATPase do not operate with Li^{+} as a substitute for Na^{+} (Sjodin, 1974; Robinson, 1976). The change in Ca^{2+} concentration in the presence of Li^{+} (160 mM) and K^{+} (160 mM) brings a small, but a significant increase in the ATPase activity (Figure 13). The Ca^{2+} concentration to stimulate the ATPase activity in this case was of the same magnitude as that for the Ca^{2+}-ATPase (see Figure 10) and also falls within the range of Ca^{2+} concentrations reported for the stimulation of enzyme systems studied by Lauter et al. (1977), Matsumura and Ghiasuddin (1979) and DiPolo (1974). Increased Li^{+} concentration produced a mild stimulatory effect on Ca^{2+} ATPase activity (i.e., Ca^{2+}-K^{+} stimulated) only at low Li^{+} concentrations (see Baker et al., 1969) but was inhibitory on ouabain insensitive Na^{+}-K^{+} ATPase (i.e., Mg^{2+}-K^{+} stimulated) activity (Figure 14).

To further aid the classification attempts on various ATPases, certain known inhibitors were examined for their action in each ATPase assay medium. The results are shown in Table 3.

Total Mg^{2+} ATPases. Since Mg^{2+} is the most commonly required ion for ATPases activity, specific identification of individual ATPases of this class of

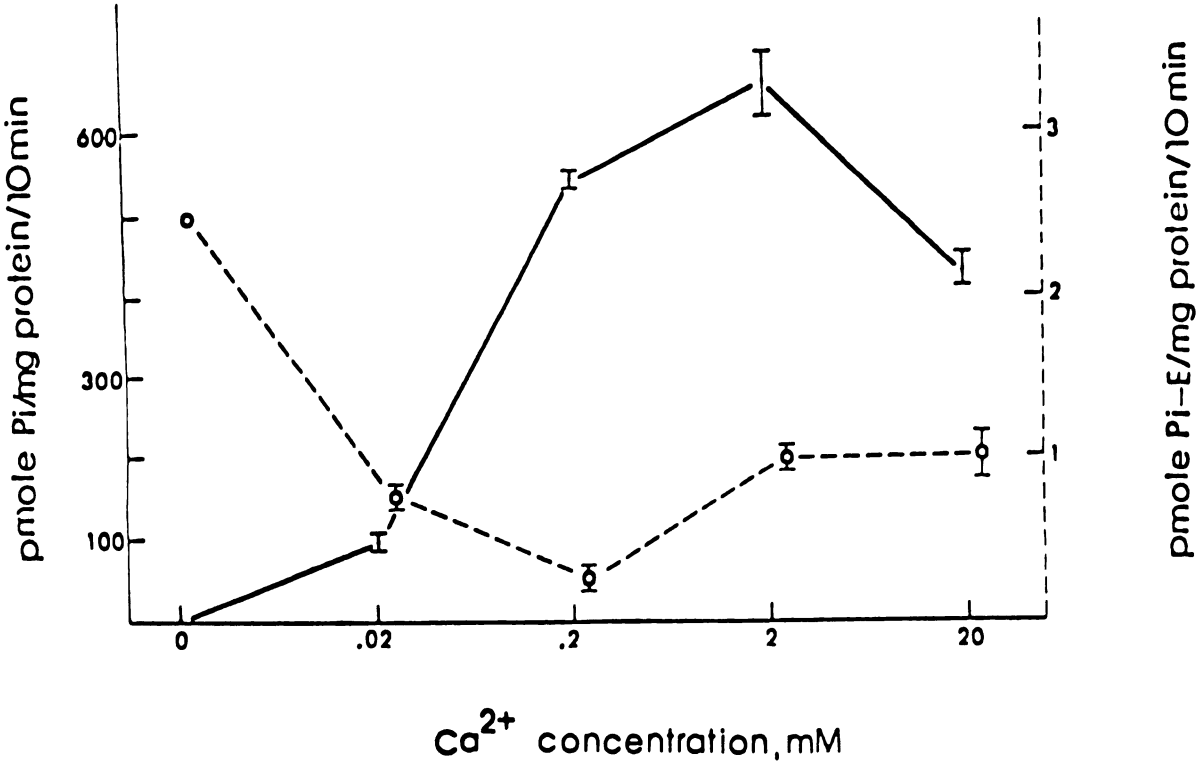

Figure 10. Effect of Ca^{2+} concentration on Ca^{2+} ATPase Pi production (solid line) and phosphorylation (= Pi-E, dotted line) activity. Other ionic compositions were: Na^{+} = 160 mM, K^{+} = 160 mM. ATP concentration was 5.6×10^{-8}M. Experimental variability reported as standard error.

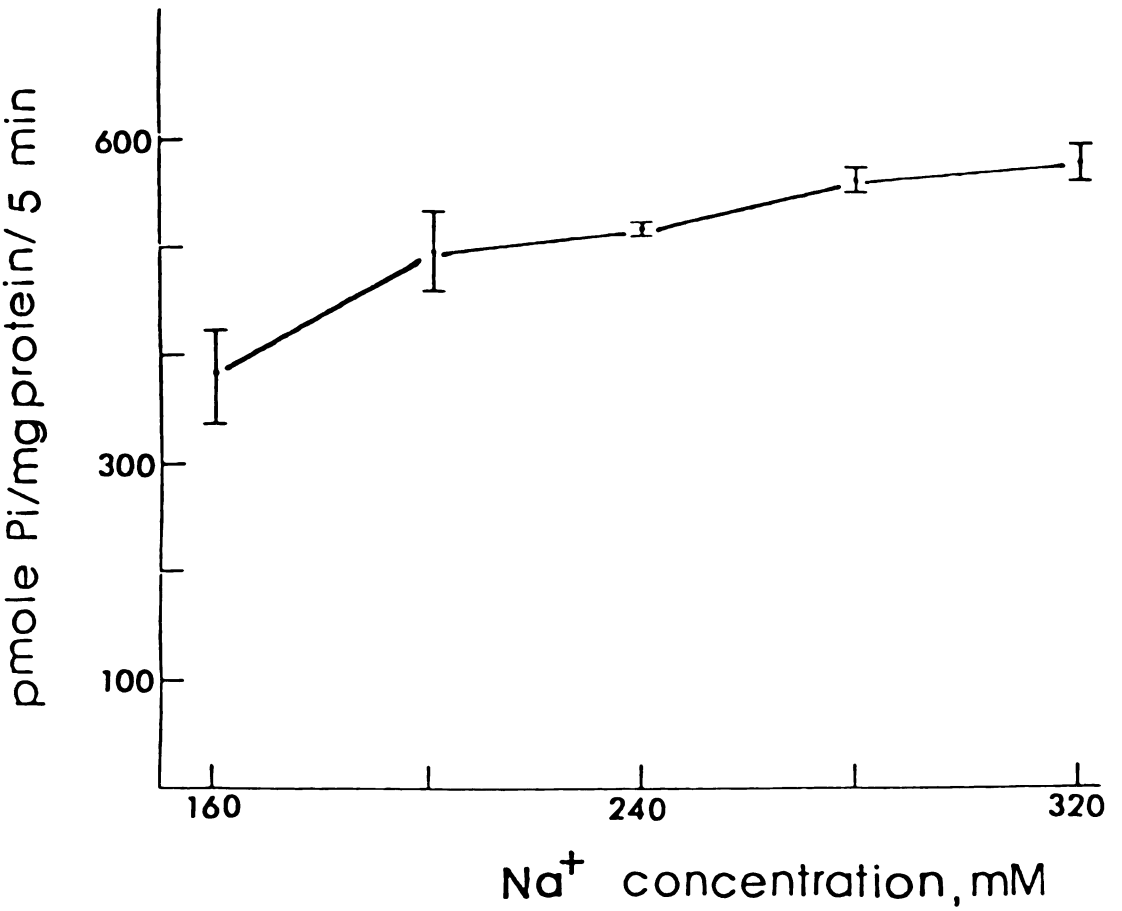

Figure 11. Effect of Na^+ concentration on Ca^{2+} ATPase activity. Other ionic compositions were: K^+ = 160 mM, Ca^{2+} = 0.2 mM. ATP concentration was 5.6×10^{-8} M. Experimental variability reported as standard error.

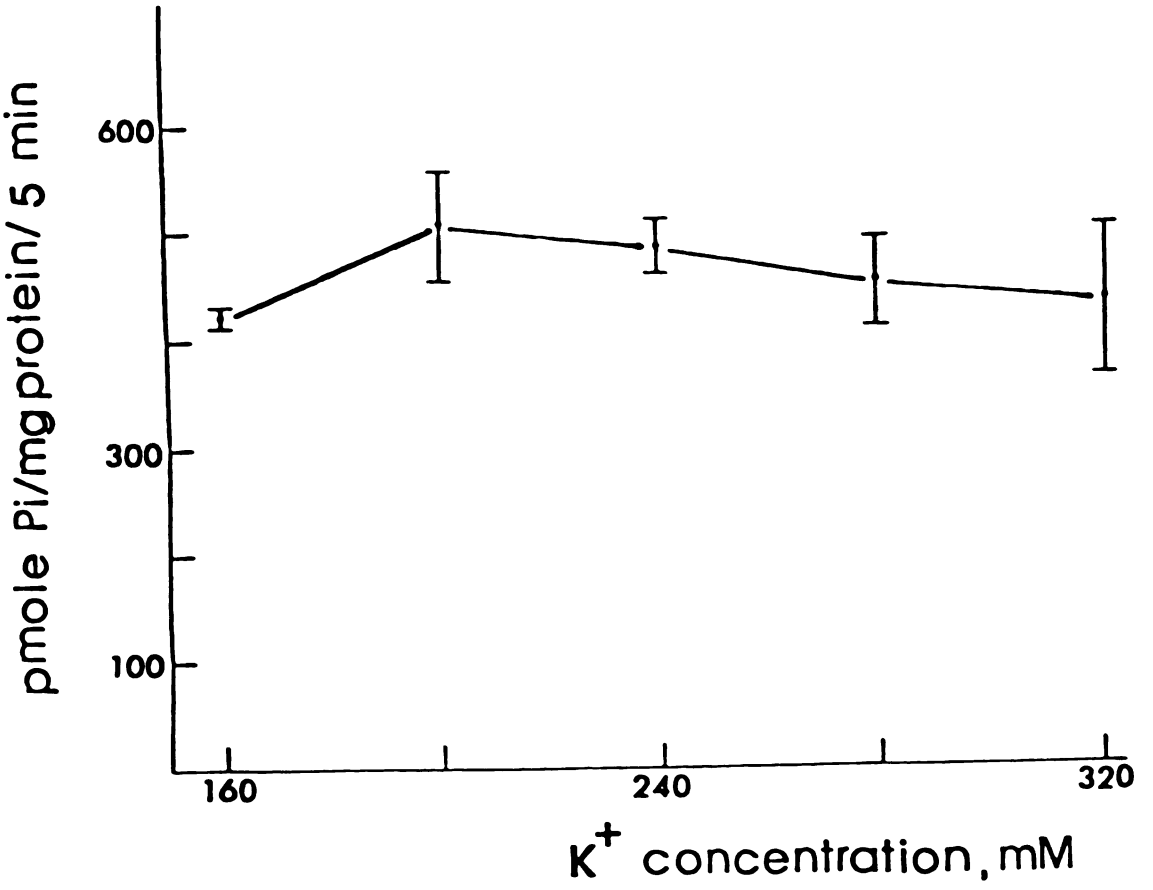

Figure 12. Effect of K^+ concentration on Ca^{2+} ATPase activity. Other ionic compositions were: Na^+ = 160 mM, Ca^{2+} = 0.2 mM, and ATP concentration was 5.6×10^{-8} M. Experimental variability reported as standard error.

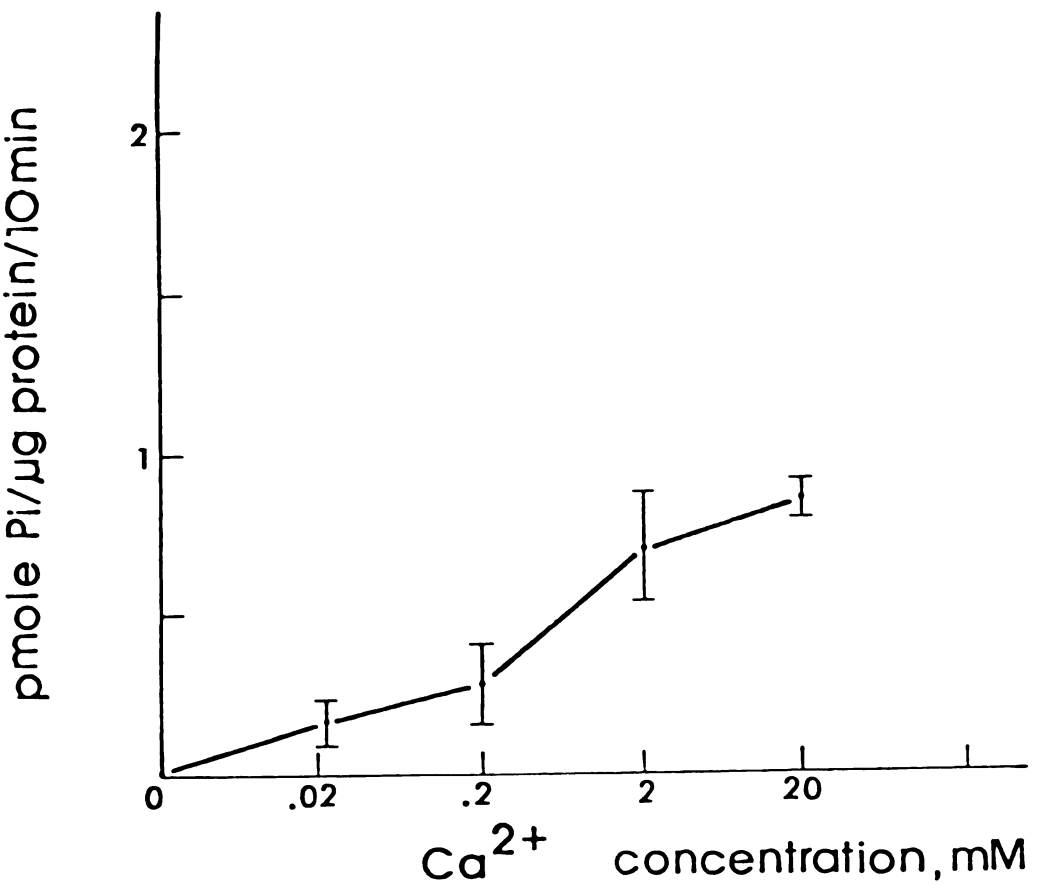

Figure 13. Effect of Ca^{2+} concentration on Li^{+} and K^{+} stimulated ATPase activity (i.e., Ca^{2+} ATPase like). Other ionic compositions were Li^{+} = 160 mM and K^{+} = 160 mM. ATP concentration was 5.6×10^{-8} M. Experimental variability reported as standard error.

enzymes by using a Mg^{2+} containing medium would be most difficult. Here, the condition of assay was arbitrarily set to study nonmitochondrial ATPases by the addition of KCN at low ATP concentration (see Table 1). Under these conditions, the total ATPases activity shows a maximum response at 10 mM Mg^{2+} (Figure 15). On the other hand, the system is less responsive to the change in K^+ concentration showing somewhat inhibitory tendencies at higher concentrations (Figure 2, ouabain curve). The response to Na^+ showed a bell-shaped curve with the maximum being in the neighborhood of 160 mM (Figure 16).

Lanthanum has been known to act as a specific inhibitor of Mg^{2+} and Ca^{2+} ATPases. Indeed, the total Mg^{2+} ATPase of this preparation was found to be highly sensitive to lanthanum (Figure 17).

Myosin-type ATPases. As for myosin-type ATPases, two relevant works on squid nerves are available; one by See and Metuzals (1976) on squid brain myosin and the other by Shecket and Lasek (1978) on the squid neurofilaments in the axoplasm of giant axons. To investigate the presence of these enzymes, the effect of Mg^{2+} concentration was examined both at high (10^{-3}M) and low (5.6×10^{-8}M) ATP concentrations. The result shown in Figure 18 indicates that the overall relationship between Mg^{2+} concentration is similar to the one obtained by Shecket and Lasek (1978). On the other hand, the effect of Ca^{2+} concentration (Figure 19) was not similar to theirs. In our nerve preparation, there was an optimum at 1 mM Ca^{2+}. The effect of K^+ was similarly studied (Figure 20) and showed an optimum concentration of K^+ of 60 mM.

According to See and Metuzals (1976), squid brain myosin has a characteristic to lose the stimulatory property of Mg^{2+} at 600 mM K^+. Instead, at this concentration of K^+, the removal of Mg^{2+} by EDTA stimulates the ATP hydrolyzing activity of myosin. Shecket and Lasek (1978) have shown that there

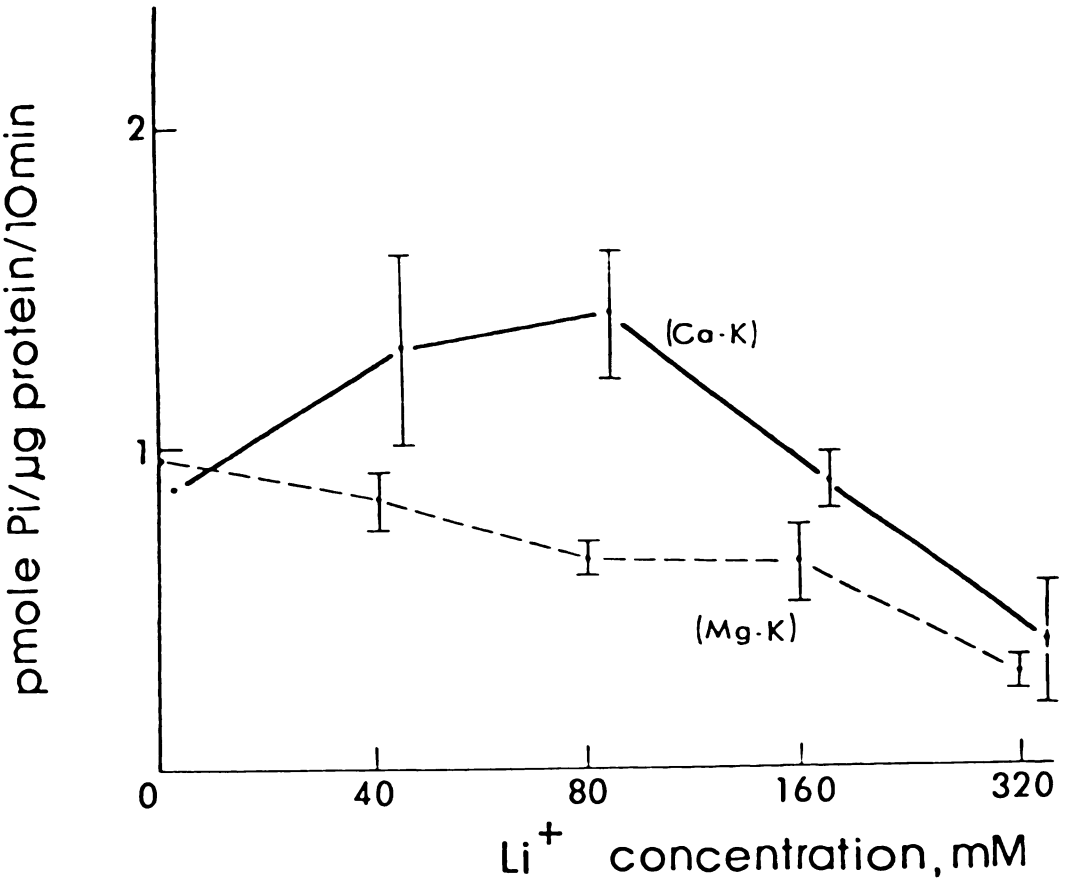

Figure 14. Effect of Li^+ concentration on Mg^{2+} plus K^+ stimulated (dotted line), and Ca^{2+} plus K^+ stimulated (solid line) ATPase activities. Concentration of ions were: Mg^{2+} = 10 mM, K^+ = 40 mM, and Ca^{2+} = 2 mM, K^+ = 160 mM, respectively. ATP concentration was 5.6×10^{-8} M in each case. Experimental variability reported as standard error.

Table 3. Effects of Various Inhibitors on ATPase Activities

Treatment[a]	Na^+-K^+ ATPase: High ATP (10^{-4}M)	Na^+-K^+ ATPase: Low ATP (5.6×10^{-8}M)	Mg^{2+} ATPase	Ca^{2+} ATPase
	(% of control)[b]			
Control	100	100	100	100
Ruthenium red				
(60 μM)	96	89	100	75
(180 μM)	-	63	61	47
Lanthanum (10 mM)	79	21	62	10
EGTA (1 mM)	0	-	-	0
ADP (0.1 mM)	81	38	26	29

[a] All (+) 10^{-4}M ouabain.

[b] All percentages reported as means ($\bar{x}$) of two replicates.

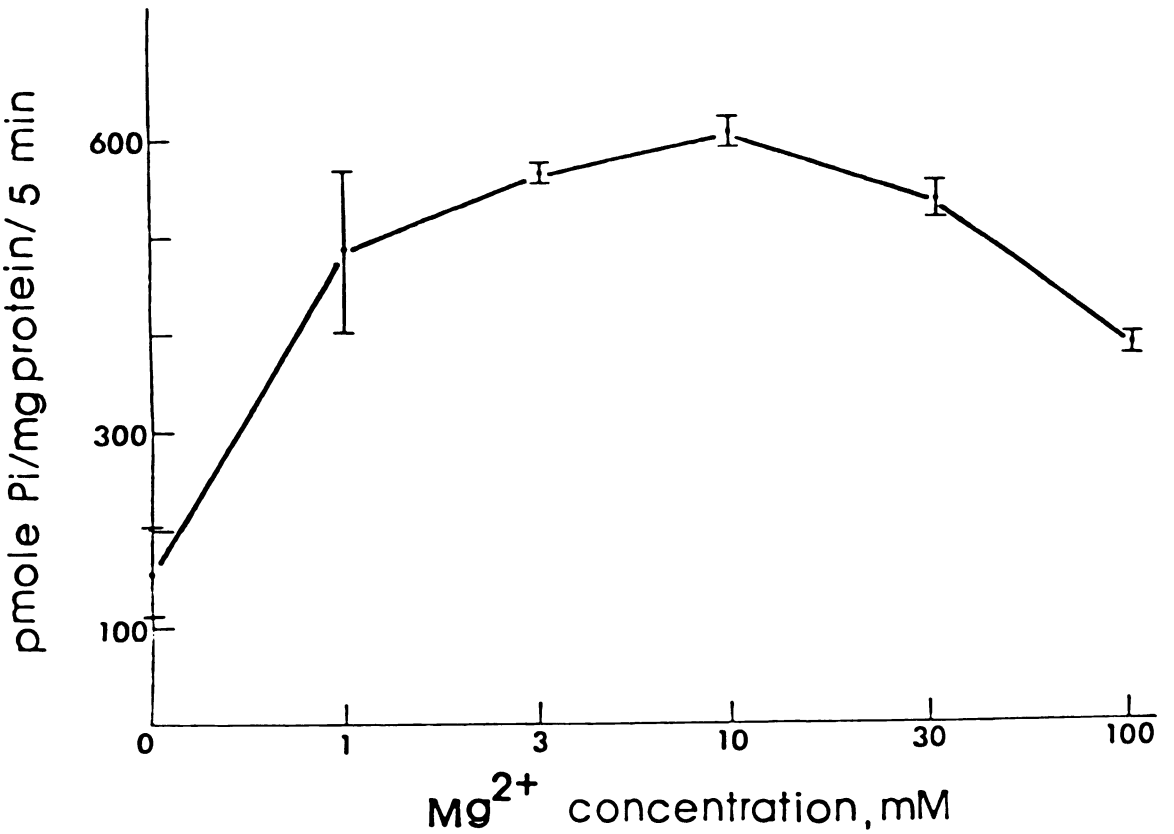

Figure 15. Effect of Mg^{2+} concentration on total Mg^{2+} ATPase activity. Other ionic compositions were: Na^+ = 160 mM, K^+ = 160 mM. ATP concentration was 5.6×10^{-8} M. KCN concentration was 2 mM. Experimental variability reported as standard error.

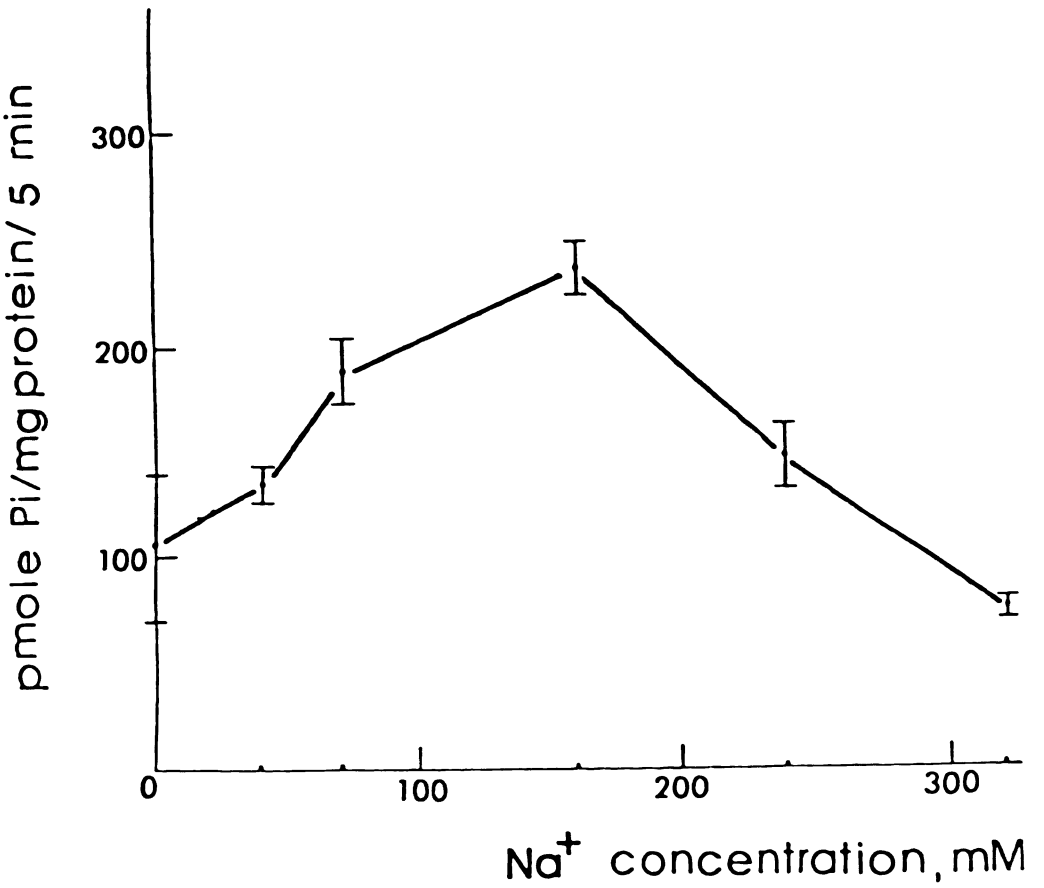

Figure 16. Effect of Na^+ concentration on total Mg^{2+} ATPase activity. Other ionic compositions were: K^+ = 160 mM, Mg^{2+} = 10 mM. ATP concentration was 5.6 x 10^{-8} M and KCN concentration was 2 mM. Experimental variability reported as standard error.

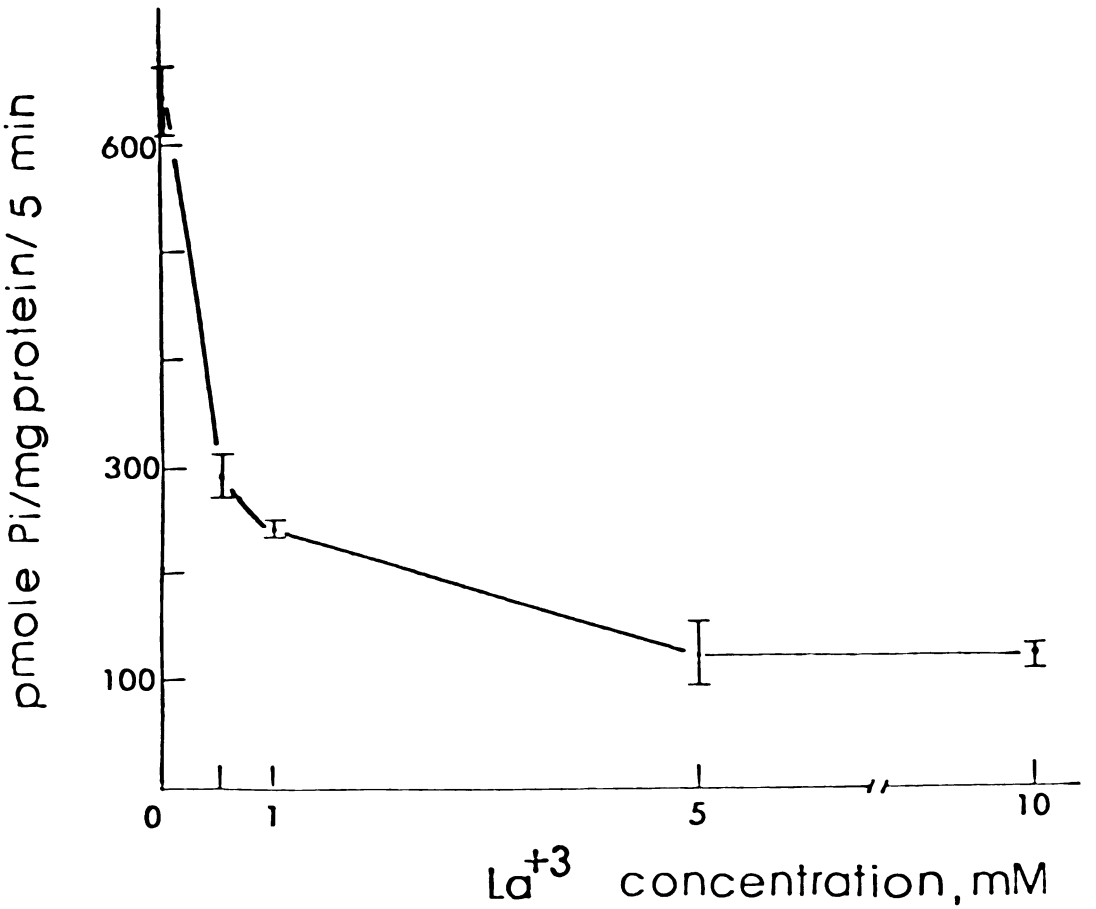

Figure 17. Effect of lanthanum concentration on total Mg^{2+} ATPase activity. Incubation conditions were: Na^{+} = 160 mM, K^{+} = 160 mM, Mg^{2+} = 10 mM, and ATP concentration was 5.6×10^{-8} M. Experimental variability reported as standard error.

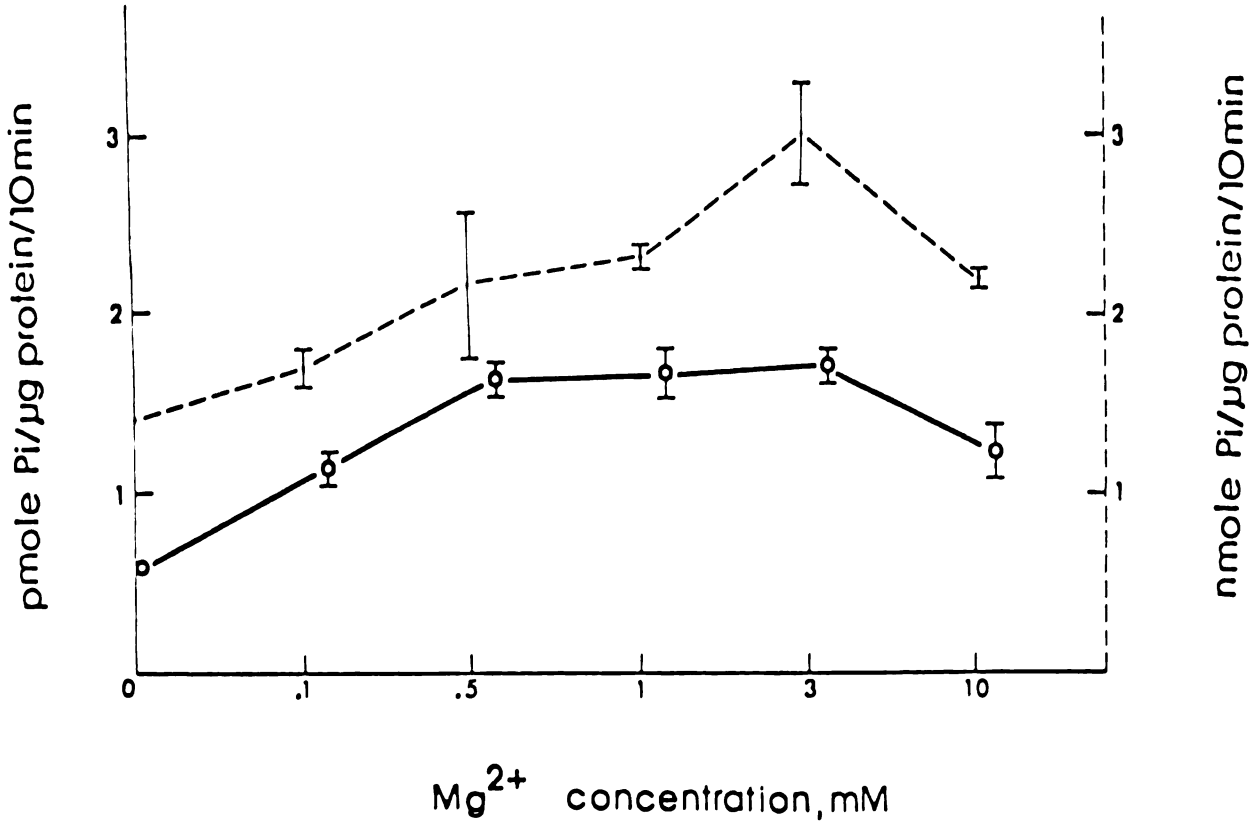

Figure 18. Effect of Mg^{2+} concentration on myosin-type ATPase activity. Other incubation conditions were: K^{+} = 60 mM, ATP = 10^{-3}M (dotted line) or 5.6 x 10^{-8}M (solid line), and EGTA = 0.5 mM. Experimental variability reported as standard error.

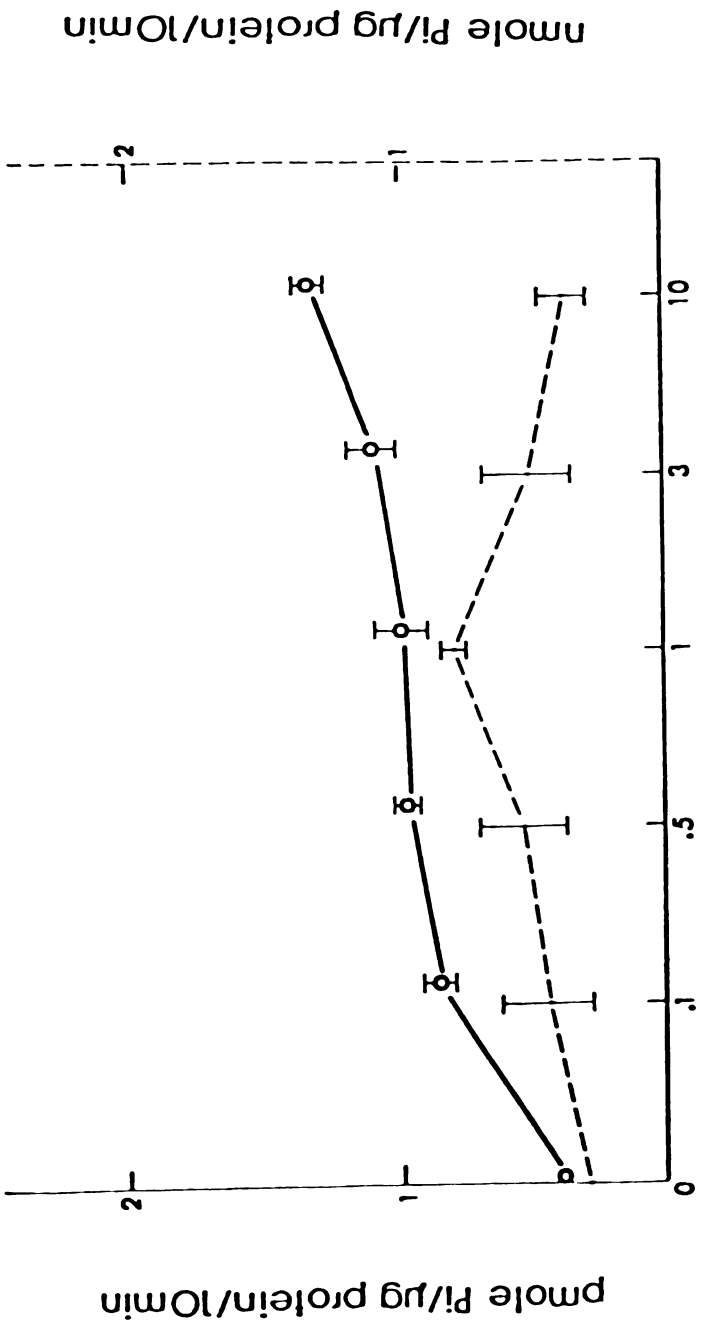

Figure 19. Effect of Ca^{2+} concentration on nerve myosin-type$_3$ ATPase activity. Other incubation conditions were: K^+ = 60 mM, ATP = 10^{-3} (dotted line) or 5.6 x 10^{-6}M (solid line), and EGTA = 0.5 mM. Experimental variability reported as standard error.

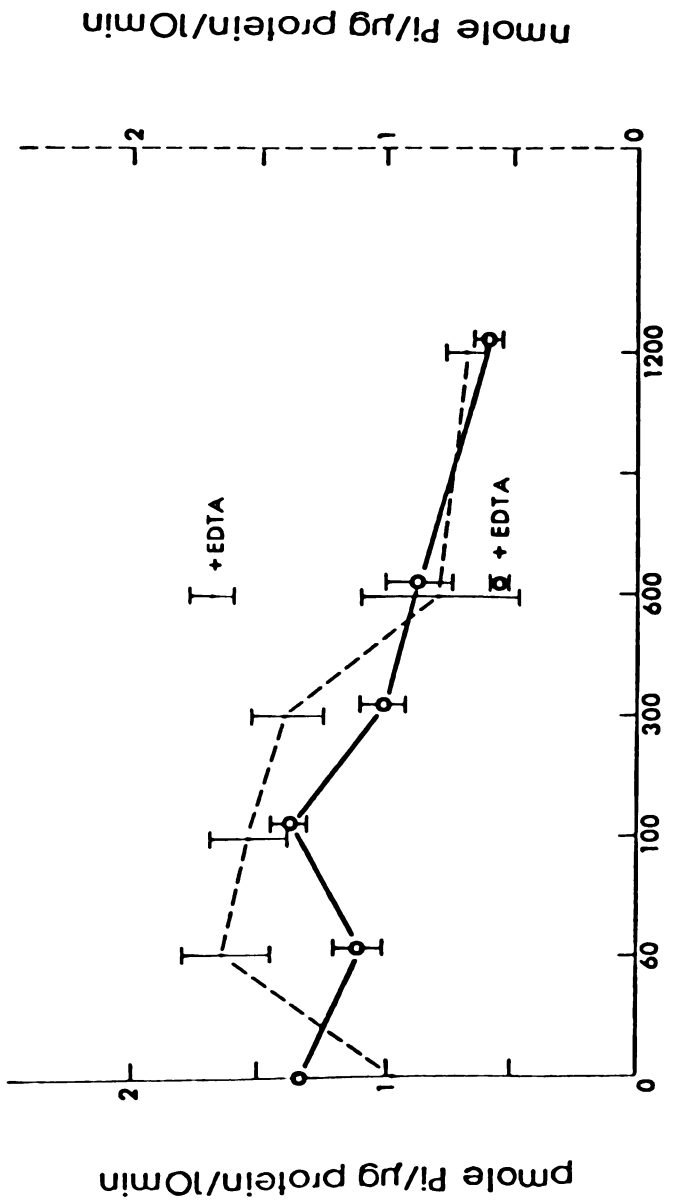

Figure 20. Effect of K^+ concentration on nerve myosin-type ATPase activity. Other incubation conditions were: Mg^{2+} = 1 mM, ATP = 10^{-3}M (dotted line) or 5.6 x 10^{-8}M (solid line), EGTA = 0.5 mM, EDTA = 2.5 mM. Experimental variability reported as standard error.

is no ATPase activity due to heavy neurofilaments (HNF) in the absence of Mg^{2+} at this K^+ concentration but report 50% of optimal response in total HNF activity when assayed in 600 mM K^+ plus 1 mM Mg^{2+}. By utilizing this same assay method with our membrane preparation, it is seen (Figure 20) that addition of 2.5 mM EDTA to the 600 mM K^+ buffer results in a 116% increase in ATPase activity over the level that is seen in the presence of 1 mM Mg^{2+}. The above evidence clearly indicates the presence of a high-titer of brain myosin in our enzyme preparation. As to the neurofilaments of axoplasm, the above data would suggest the possibility of its presence in our preparation because of its overall similarity in ion sensitivity to the data obtained here. However, the specific activity of the neurofilament ATPase in the whole axoplasm is rather low in the order of 2 nmol/min/mg under optimum conditions whereas under these same conditions, the total ATPase activity of our membrane preparation was in the order of 200 nmol/min/mg protein. Therefore, even if it is present in our nerve preparation, its contribution cannot be more than 1% of the total ATPase activity.

DISCUSSION

Since ATP is a common substrate for many enzyme systems, it is most important to design methods to clearly distinguish each enzyme system from others before their functional roles can be disclosed. In the present study, it was decided that such classification is more clearly defined by first establishing a basal level of activity to which one compares any changes which occur when certain selective additions are made to the assay system.

The properties of Na^+-K^+ ATPase have been described by many workers. This enzyme requires a precise ratio of Na^+ and K^+ in addition to Mg^{2+}. Its sensitivity toward cardiac glycosides has also been well documented (e.g., Skou and Hillberg, 1969). Thus, the recognition of this enzyme in the squid axon

preparation has not been difficult (Brinley and Mullins, 1968). On the other hand, the presence of cardiac glycoside insensitive Na^+-K^+ ATPase in the squid nerve had been noticed by other workers (Sjodin, 1974) where as much as 50% of the total Na^+-K^+ stimulated ATPase activity had been found to be insensitive to cardiac clycosides such as ouabain.

In this study, we have also found such an enzyme system. Its properties can be studied either in the presence of 10^{-4}M ouabain and/or at a low ATP concentration. Aside from substrate affinity, the optimum concentrations of Na^+ and K^+ were similar to those found for ouabain sensitive Na^+-K^+ ATPase. The major difference between the two enzyme sytems is their temperature sensitivities (i.e., the ouabain sensitive Na^+-K^+ ATPase having a much higher temperature dependency). While the functional role of ouabain sensitive Na^+-K^+ ATPase is likely to be "Na^+-pumping," that of the ouabain insensitive Na^+-K^+ ATPase remains unclear. Nevertheless, its recognition as a distinct entity is possible by its characteristic stimulatory response to the Na^+ and K^+ ratio over that of Mg^{2+} stimulation alone.

Ca^{2+}-Mg^{2+} ATPase is recognized as the difference in the ATPase activity between the sum of Mg^{2+} stimulated plus Ca^{2+} stimulated activity and the total activity seen in the combined Ca^{2+}-Mg^{2+} buffer. This method of detection is similar to the approach used by Robinson (1976) and Duncan (1976) but corrects also for nonspecific Ca^{2+} stimulation. There are two additional characteristics which aid the recognition of this enzyme; first, its high temperature dependency and second, its relatively low (10^{-5}M) requirement for Ca^{2+}. As judged by the level of activity of the Total Ca^{2+} and Mg^{2+} ATPase system (Figure 8), the enzyme occupies a significant portion of the total Ca^{2+}-stimulated ATPase complex. Its functional role could be construed as a "Ca^{2+}-pump," though its localization in specialized Ca^{2+} sequestering components such as endoplasmic

reticulum is also possible (Henkart et al., 1978). Since this enzyme has a relatively low Km value ($\simeq 10^{-8}$M) for ATP, a direct relationship to the above ATP-induced Ca^{2+} extrusion activity at this stage is uncertain. On the other hand, Baker and Glitsch (1973) observed a drastic decrease in the baseline Ca^{2+} efflux in the squid giant axon by the addition of apyrase which hydrolyzes ATP and thereby removes endogenous ATP. Also close similarities do exist in the amount of Ca^{2+} which is most efficiently extruded by active ATP hydrolysis (DiPolo, 1977) and which optimally activates our enzyme. Moreover, both these phenomena have a strict Mg^{2+} requirement. Thus, it is probable that there is an ATP-dependent Ca^{2+} effluxing mechanism operating even at a low ATP concentration.

Properties of Ca^{2+} ATPase have been described by Lauter et al. (1977) and recently by Matsumura and Ghiasuddin (1979) who have studied the characteristics of such an ATPase in the axonal preparation of the leg nerve fiber of the American lobster, Homarus americanus. The Ca^{2+} requiring ATPase recognized in the squid membrane preparation is similar to that from the lobster in many ways. Particularly important is its requirement of a relatively high concentration of Ca^{2+} (1 mM) and its low sensitivity to temperature (i.e., Q_{10} = 2.23). As judged by the similarity of the Ca^{2+} influence, it is possible that the Ca^{2+}-ATPase which can accept Li^{+} (Baker et al., 1969) in place of Na^{+} (Figure 13) is the same enzyme as Ca^{2+} ATPase. To detect the enzyme, it is necessary to remove Mg^{2+} by adding EDTA from the time of homogenization throughout the entire duration of enzyme preparation. The difference between the basal activity with the buffer composition of Na^{+} (160 mM), K^{+} (160 mM), Tris-HCl (30 mM) and that plus 1 mM of Ca^{2+} is regarded as the criterion for this enzyme. A critical proof is, however, still lacking that it is indeed an ecto-enzyme.

In brief then, the effect of Ca^{2+} on various ATPase systems seems to fall into two groups; those in which Ca^{2+} stimulates at low concentrations, require Mg^{2+}, and are highly temperature sensitive (Duncan, 1976; Robinson, 1976; DiPolo, 1977; Hasselback, 1978), and those that require higher levels of Ca^{+}, are Mg^{2+} independent, and are relatively temperature insensitive (Matsumura and Ghiasuddin, 1979; Rosenblatt et al., 1976; Lauter et al., 1977).

As for the presence of myosin in the squid nerve, See and Metuzals (1976) have described its properties by biochemical and electron microscopic examination. The most characteristic aspects of this ATPase are the inhibitory effects of Mg^{2+} at high (they are tested at 560 mM) K^{+} concentrations and the subsequent stimulation of activity with addition of EDTA. Since such a property is extremely rare among ATPases, it is certainly a useful indicator of the presence of myosin. The specific activity of this ATPase is rather high (i.e., in the order of 100 nmol Pi released/min/mg protein at 560 mM K^{+} + 2 mM EDTA). This value is at least in the same order of magnitude as the one obtained in the current study under a similar experimental condition (K = 600 mM + 2.5 mM EDTA), indicating that the bulk of the ATPase activity under these conditions must be due to this enzyme.

The presence of a high titer of myosin in the membrane preparation is interesting in the light of the recent discovery by Metuzals and Tasaki (1978) that the inner cell surface of the live and perfused giant axon shows a fine network of myosin probably cross-linked by actin. These workers associated the presence of such net-like structures to the excitability of the membrane. Indeed, the removal of the net-like structure by pronase resulted in the loss of excitability. In our preparation, the membranes have never been subjected to high ion concentrations until the time of assaying. Therefore, it is not surprising

that myosin which seems to be tightly bound to the inner membrane surface should be carried into the final preparation.

There is no doubt that the total Mg^{2+} ATPase assay method must have activated many types of Mg^{2+}, Na^{+} and K^{+} stimulated ATPases. It must be mentioned here that the purpose of this particular experiment was not to define a method to detect a specific enzyme system; rather it was to examine the total ATPase behavior which might be of help in the interpretation of some of the data which elecrophysiologists obtain when they study ATP dependent transport of radioactive ions. For instance, the data obtained by this method may be compared to that of de Weer's (1976) who studied ATP dependent, Mg^{2+} extrusion mechanisms. He found the optimum Mg^{2+} extrusion (i.e., hypothetical "Mg^{2+}-pump") occurred at 10 mM Mg^{2+} concentration, the same value as the one obtained here. Moreover, the sensitivities to lanthanum of these two systems were identical. The Mg^{2+}-pump is also dependent on external Na^{+} (Mullins and Brinley, 1975; de Weer, 1976), and most likely to internal K^{+}. The overall similarity of the Mg^{2+}-pump to the total Mg^{2+} ATPase studied here offers a future possibility of finding a specific ATPase responsible for the Mg^{2+}-pump among the Mg^{2+}-stimulated ATPases in the membrane preparation.

Finally, it should be noted that no claim has been made that all these ATPases are located in or on the axonic membrane. That is, the possibility of contribution by other components such as glial cell fragments and axoplasmic material cannot be overlooked. Indeed, Fischer et al. (1970) could not improve the purity of axonic material by further treatment of the membrane preparation (e.g., similar to the current preparation) by a discontinuous sucrose density gradient technique. Thus, the possibility exists that any of these ATPases could belong to some non-axonic component of the preparation. Also, in no cases is it implied that each enzyme system consists of a pure enzyme. The possibility of

the presence of several similarly behaving ATPases, or isozymes, in each system cannot be overruled. Terms such as Ca^{2+}-Mg^{2+} ATPase instead of a more generic Ca^{2+}-Mg^{2+} ATPases system have been applied for the sake of simplicity.

The presence of ion-stimulated ATPase systems in a variety of excitable tissue is well documented, and in the case of Na^{+}-K^{+} stimulated ATPases, much valuable data have been obtained since their discovery (Skou and Hillberg, 1969). However, as for other ATPases, their importance in the regulation of nerve activity has hardly been studied. In this regard, it is interesting to note that so far the only enzymatic systems known to regulate the ionic environment of a cell are ATPases, and, as such, possibilities do exist that some of the ATPases described here play very important nerve functions.

In summary, as reported originally by Fischer et al. (1970) for Dosidicus gigas and confirmed in Loligo pealei, the retinal nerve seems to be the biological material of choice due to its relatively negligible contamination by extracellular membranes and rather high axonal to supportive material ratio. Further support for the usefulness of this preparation has come from Pant et al. (1979) who has demonstrated the actual transport of extracellular ATP across the squid axon. By using the axon-rich membrane preparation, several ATPases present in the retinal nerves of the squid, Lologo pealei, have been recognized. Though it is probable that there are more ATPases remaining in this preparation, the efforts here provide the initial starting material for future characterization. Such information should be of great interest to electrophysiologists and biochemists alike who may eventually use the data to ascertain the functional roles of these enzymes.

CHAPTER II

PYRETHROID INHIBITION OF NEURAL ATPases OF THE SQUID, LOLIGO PEALEI

INTRODUCTION

Generally, it has been accepted that DDT and pyrethroids act directly on the nervous system causing disruption of normal ion permeabilities in the nerve membrane that are implicit in the generation and conduction of nerve impulses (Shanes, 1951; Wang et al., 1972). Narahashi (1976) has determined that it is the Na^+ and to a lesser extent K^+ permeabilities which are principally affected by DDT and pyrethroids. However, other ionic fluxes have also been found to be mandatory for nerve tissue to function properly, particularly that of Ca^{2+} (Frankenhaeuser and Hodgkin, 1957).

It has been previously established (Matsumura and Clark, 1980) that the retinal nerve is a suitable material for future biochemical works concerning the functional roles of various membrane-bound enzymes. This was primarily because the axons of the optic nerve have a rather high axonal to supportive tissue ratio as compared to the more often studied giant axon (Canessa-Fisher et al., 1967). By utilization of this axon-rich membrane preparation, several ATPases were recognized. The present study was undertaken to determine which, if any, are sensitive to inhibition by pyrethroid insecticides.

It must be pointed out that so far the only biochemical systems known to regulate the ionic environment of a cell are ATPases. They are known to function as indispensable enzymes in neurons carrying out a variety of tasks necessary for normal nerve activities. For these reasons, a keen research interest has been focused on the inhibitory role of DDT on ATPase systems.

Brunnert and Matsumura (1969) demonstrated that both DDT and DDE bind proteins of various nerve components of rat brain and subsequently showed ATPase activity to be inhibited by these insecticides (Matsumura and Patil, 1969). In 1971, Matsumura and Narahashi (1971) reported a correlation between the degree of DDT inhibition of ATPases and the electrophysiological symptoms of DDT poisoning.

Schneider (1975) showed that both DDT and allethrin inhibited (Na + K) ATPase but rejected this as a mechanism of DDT or pyrethroid action since the Na^+ pump (ouabain sensitive) enzymes do not participate directly in the action potential. Doherty and Matsumura (1974) likewise reported that DDT inhibited the ^{32}P incorporation from γ-labeled ATP into proteins from lobster nerve but showed also that such a decrease might be related to the inhibition of a ouabain insensitive Na^+-K^+ ATPase (Doherty and Matsumura, 1975). Desaiah, Cutkomp and Koch (1974) and Desaiah et al. (1975) have reported that a mitochondrial (oligomycin-sensitive), Mg^{2+} ATPase is sensitive to DDT and pyrethroids.

In this context of ionic regulation, Matsumura (1972) has reported that brine shrimp, while relatively unaffected by either high levels of sodium chloride or DDT, were quite susceptible to this combination. In a related work using a species of fresh water alga, Bratterton et al. (1972) showed, even in the presence of high levels of sodium chloride, that DDT-related growth inhibition could be reversed by Ca^{2+} addition.

The effect of DDT (or DDE) on egg shell thinning is well documented. Recently, Miller et al. (1976) demonstrated DDE inhibition of a Ca-ATPase (Ca-pump) which is responsible for active transport of this cation from the blood to the developing shell. In domestic fowl, which is resistant to the thinning aspect of DDE poisoning, no inhibition of this Ca-ATPase was found (EPA, 1975).

Similarly, Huddard et al. (1974) reported that DDT caused almost complete inhibition of Ca^{2+} uptake by sarcoplasmic reticulum in vitro.

Matsumura and Ghiasuddin (1979) have found an extremely DDT-sensitive Ca-ATPase in the axonic preparation from the walking leg nerves of the American lobster. On the basis of Ca^{2+} interaction with the excitability of the axonic membrane, these authors proposed a working hypothesis that this Ca-ATPase is responsible for maintaining the surface Ca^{2+} level. Inhibition of this Ca-ATPase by DDT results in a depletion of Ca^{2+} on the outer surface of the axon leading to a lowering of stimulus threshold.

There have been a number of indications that there is a common poisoning mechanisms between DDT and pyrethroids. It is then the goal of this investigation to determine which ionic parameters are significantly affected by pyrethroids in various subcellular fractions of the squid nervous system. By employing the same organism used by electrophysiologists for studying the basic electrical properties of the nerve membrane, it is hoped to provide a logical biochemical link between the mode of action of pyrethroids and other similarly acting neurotoxins and the observed physiological symptoms of poisoning.

MATERIALS AND METHODS

Animals. The North American species of squid, Loligo pealei, used in this work were captured and kept alive until ready for use at the Marine Biological Laboratory at Woods Hole, Massachusetts in the summer months of 1979 and 1980. They were dissected alive and both retinal nerve (axons) and optic lobes were separately collected. Dissection and storage techniques for nerve tissue have been previously reported (Matsumura, 1977).

Preparation of ATPase Fractions. Preparation of retinal microsomes (axonal) was exactly as previously reported by Matsumura and Clark (1980). Synaptosomal preparation from squid optic lobe (i.e., pinched-off synaptic nerve

terminals of afferent sensory fibers) was as reported by Pollard and Pappas (1979). Optic lobes were removed from the squid and homogenized as a 10% (w/v) solution in 1 M sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 mM dithiothreitol (DTT) using a loose fitted glass-glass homogenizer (Ten Broeck). The homogenate was centrifuged at 20,000 g for 60 min at $4^{o}C$. The suspended pellicle was collected by aspiration with a pasteur pipet, and resuspended into 750 mM sucrose and 0.1 mM DTT.

The axoplasm was also examined for its ATPase content. After decapitation, the hindmost giant axon from the stellate ganglion was dissected from the mantle in flowing seawater and cleaned under a dissecting microscope. The axoplasm was carefully extruded using a microroller and pooled in 750 mM sucrose, 1 mM EDTA and 0.1 mM DTT. Preparation of the microsomal fraction was exactly as for the retinal axons reported above. These fractions were used exclusively in all enzymatic assays unless otherwise indicated.

All enzymes were stable to storage at $-14^{o}C$ except Ca^{2+}-Mg^{2+} ATPase which was prepared fresh and used immediately. Protein was determined by the method of Lowry et al. (1951) and all assays were performed at a standard 10 μg protein per assay tube.

ATPase Assay. The tetraethylammonium salt of gamma (γ)-^{32}P-labeled adenosine triphosphate (ATP), purchased from New England Nuclear, Boston, Massachusetts, was utilized as the substrate for all assays and had a starting specific activity of approximately 26.4 mCi/μ mol. Approximately 3×10^{5} dpm were used per assay tube. The extent of ATPase activity was determined as the amount of ATP hydrolysis (i.e., inorganic ^{32}Pi production) during incubation with the enzyme source in selective ionic buffers.

All water utilized in experimentation was first steam distilled and then sequentially passed through organic removal and mixed-bed deionizing systems.

Water quality was assessed by its conductivity which was measured as ppm of NaCl equivalents. At no time was the water conductivity allowed to exceed 0.1 ppm. Assay conditions, ^{32}Pi workup and standard buffer conditions were exactly as previously reported by Matsumura and Clark (1980).

Chemical. Pyrethroid compounds were obtained as gifts from various sources; pyrethrin (I and II) [pyrethrin I, 2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl 2,2-dimethyl-3-(2-methyl-1-propenyl) cyclopropanecarboxylate], [pyrethrin II, 2-methyl-4-oxo-3-(2,4-pentadienyl)-2-cyclopenten-1-yl 3-(3-methoxy-2-methyl-3-oxo-1-propenyl)-2,2-dimethyl-cyclopropanecarboxylate], resmethrin [5-benzyl-3-furylmethyl (±)-cis, trans-chrysanthemate] and phenothrin [3-phenoxybenzyl (±)-cis, trans 2,2-dimethyl-3-(2-methyl-1-propenyl) cyclopropanecarboxylate] from U. S. Environmental Protection Agency, Health Effects Research Laboratory, Environmental Toxicology Division, Research Triangle Park, North Carolina, (+)-trans allethrin [3-allyl-2-methyl-4-oxocyclopent-2-enyl-(+)-trans-chrysanthemate of (±)-allethrolone] from Dr. T. Narahashi, Department of Pharmacology, Northwestern University, Chicago, Illinois, kadethrin [3-phenoxybenzyl(±)-cis-3-(1,2-ene-thiolactone)-2,2-dimethyl-cyclopropanecarboxylate] from Dr. K. Osawa, University of California, Berkeley, California, permethrin [3-phenoxybenzyl(±)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] from FMC, Agricultural Chemicals Division, Middleport, New York, cypermethrin [(±)-α-cyano-3-phenoxybenzyl(±)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] from Shell Bioscience Laboratory, Sittingbourne Research Center, Sittingbourne, Kent, United Kingdom, decamethrin [(S)-α-cyano-3-phenoxybenzyl-cis-(1R,3R)-2,2-dimethyl-3-(2,2-dibromovinyl) cyclopropanecarboxylate] from Dr. L. Ruzo, Division of Entomology and Parasitology, University of California, Berkeley, California and fenvalerate [(S)-α-cyano-3-phenoxybenzyl-2-(4-chlorophenyl) iso-

valerate] from Shell Development Company, Modesto, California. All of the remaining biochemicals were purchased from Sigma Chemicals, St. Louis, Missouri.

RESULTS

Figure 21 illustrates the structures of the nine pyrethroid insecticides selected for this study. They range from the naturally-occurring esters, pyrethrin (I and II), produced by the flower Chrysanthemum cinerariaefolium to one of the more highly synthetic analogues, fenvalerate. The remaining compounds were chosen as examples of major chemical modifications made during the evolution to more environmentally resilient components better suited for agricultural usage.

Survey of ATPase Sensitivity to Allethrin. Because of the similarity of structure to naturally-occurring pyrethrins and the availability of electrophysiological study results, allethrin was chosen as a model pyrethroid by which we gauged the sensitivity of six ATPases recognized in the retinal axon. These were as described earlier by Matsumura and Clark (1980): (1) ouabain sensitive Na^+-K^+ ATPase (Na^+-pump), (2) ouabain insensitive Na^+-K^+ ATPase (maintenance ATPase), (3) total Mg^{2+} ATPase (nonmitochondrial Mg^{2+} ATPase), (4) Ca^{2+} ATPase (surface Ca^{2+}-sequesterer), (5) Ca^{2+}-Mg^{2+} ATPase (Ca^{2+}-pump), and (6) nerve myosin (contractile protein of the inner wall of axolemma).

The effect of allethrin on ouabain sensitive Na^+-K^+ ATPase determined as the difference in ATPase activity in the presence and absence of ouabain at a high ATP (10^{-4}M) concentration is reported in Figure 22. As indicated by its specific activity, the ouabain sensitive Na^+-K^+ ATPase is quite active accounting for some 50% of the total activity in the retinal nerve preparation. However, Na^+ pump enzyme is completely insensitive to allethrin over the concentration range examined. In fact, this enzyme shows an overall stimulatory

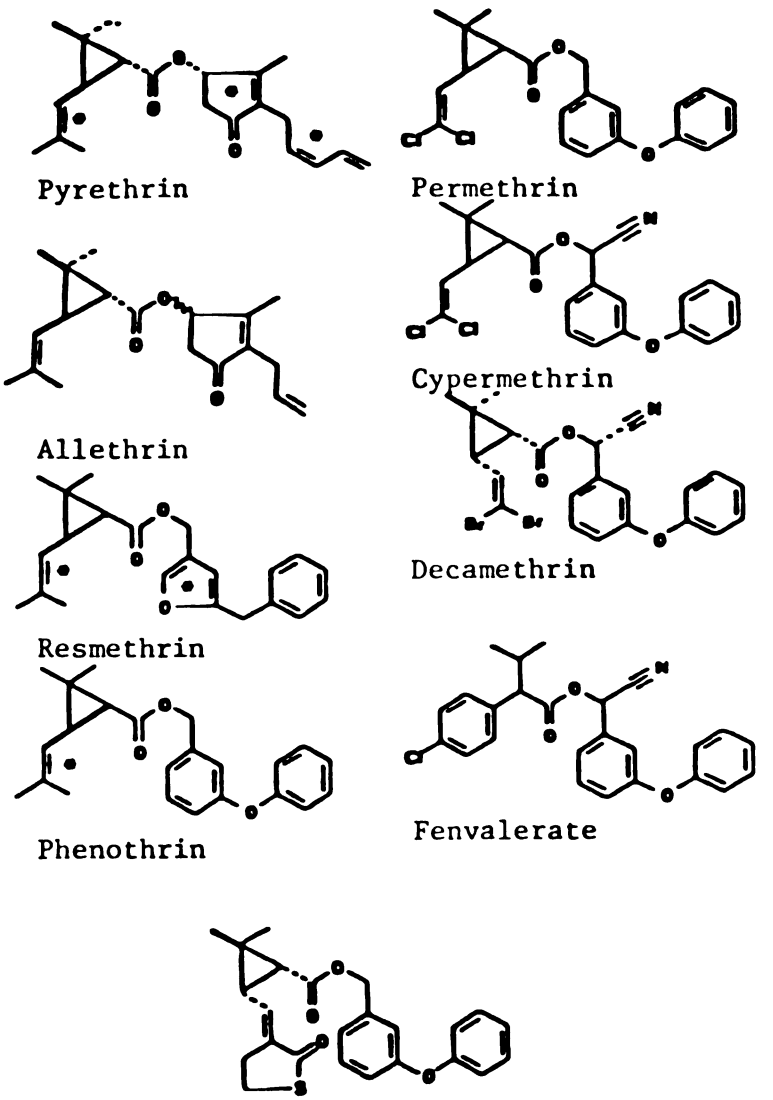

Figure 21. Development of stable pyrethroid esters. Compounds unstable under field conditions are given in the left-hand column. Compounds more stable under field conditions are given in the right-hand column.

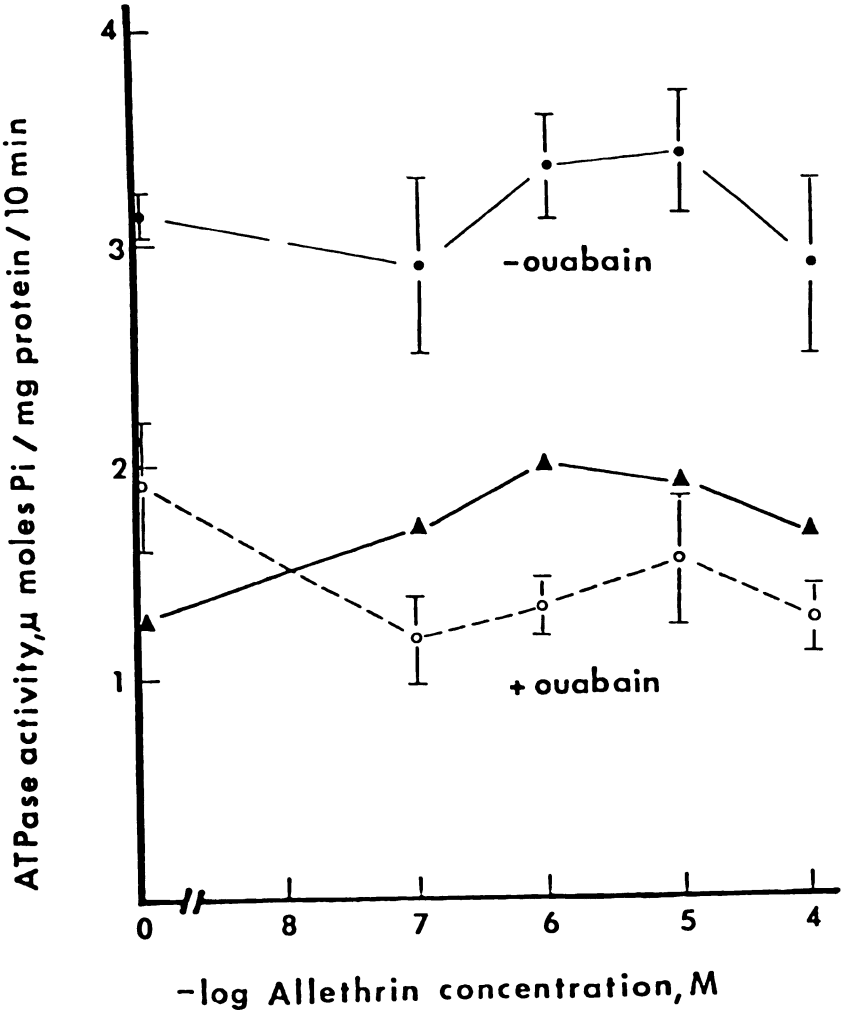

Figure 22. Effect of (+)-trans allethrin on ouabain sensitive Na^+-K^+ ATPase (Na^+ pump) as determined as the difference (▲-▲) between total Na^+-K^+ ATPase activity (-ouabain) and ouabain insensitive Na^+-K^+ ATPase activity (+ ouabain). Enzyme source was the 90,000 g fraction of the squid retinal axon preparation. ATP concentration was 10^{-4}M. Ouabain concentration was 10^{-4}M. Other incubation conditions were: Na^+=160 mM, K^+=40 mM, Mg^{2+}=10 mM in 30 mM Tris-HCl at pH 7.1. Experimental variability is reported as standard error (± S.E.) of mean values of at least two separate experiments.

effect at 10^{-4}M allethrin resulting in a 31.2% increase in activity over the control values (i.e., no allethrin treatment).

Because ouabain, a specific inhibitor of the Na^+ pump, has been shown to cause none of the electrical disturbances characteristic of DDT and pyrethroid poisoning, the Na^+ pump has not been considered as a probable site of action (Matsumura, 1970). This data supports this claim. However, the overall stimulation of Na^+ pump activity may be important in view of the recent work by Narahashi (1979), who reported that DDT and pyrethroids effect Na^+-inactivation mechanisms of the Na^+-K^+gate during action potential generation. An increase in internal Na^+ concentration would be expected to cause increased Na^+ pump activity as it works to remove excess Na^+.

The other of the two ATPases which is activated only at high ATP concentration is nerve myosin. Myosin is recognized as the difference in ATPase activity in the presence and absence of EDTA in 600 mM K^+ at 10^{-3}M ATP as reported by See and Metuzals (1976). As illustrated in Figure 23, nerve myosin is present in relatively high amounts. However, the ATPase activity of nerve myosin is uneffected by allethrin over the concentration range examined. As with the case of ouabain sensitive Na^+-K^+ ATPase, there is a significant increase in myosin activity (21.8%) at high allethrin concentration (10^{-4}M).

Metuzals and Tasaki (1978) have reported that nerve myosin is closely associated with the inner surface of the squid axolemma as a supporting network. Besides these structural considerations, they also believe that myosin may be involved in gating mechanisms during action potential generation in the axon. Our work indicates that although myosin is present in rather high amounts, it is not a likely candidate for the site of action of the pyrethroids. Nevertheless, it still may be implicated in the overall toxicological scheme of pyrethroid

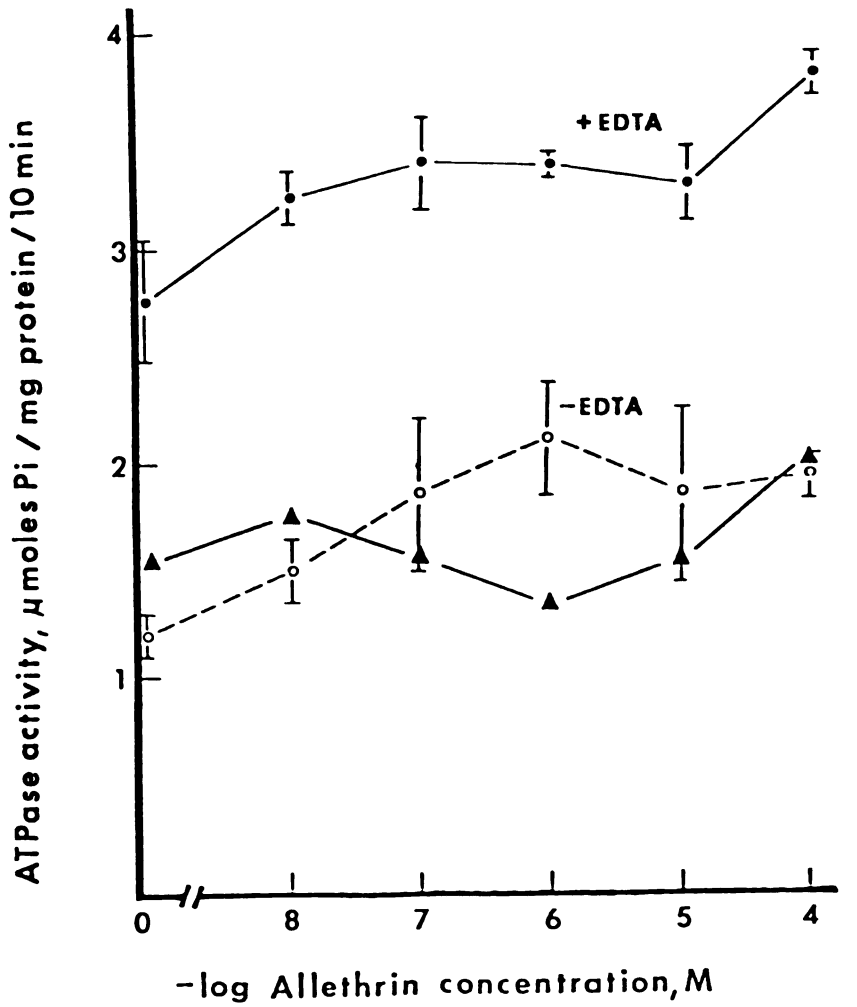

Figure 23. Effect of (+)-trans allethrin on nerve myosin ATPase activity as determined as the difference (▲-▲) between the activity in the presence (+ EDTA) and absence (- EDTA) of EDTA. Enzyme source was the 90,000 g fraction of the squid retinal axon preparation. ATP concentration was 10^{-3} M. Ouabain concentration was 10^{-4}. Other incubation conditions were: K^+=600 mM, Mg^{2+}=1 mM, EDTA=2.5 mM in 30 mM Tris-HCl at pH 7.1. Experimental variability is reported as ± S.E. of mean values of at least two separate experiments.

poisoning due to its apparent requirement of interaction with actin-like proteins during contractility which is Ca^{2+}-regulated.

Figure 24 illustrates the specific activities of the remaining four ATPases identified at a low ATP concentration (5.6×10^{-8}M) in the squid retinal axons. Their sensitivity to allethrin at various concentrations is also reported in Figure 24.

Figure 24-line A shows the ATPase activity of the ouabain insensitive Na^+-K^+ ATPase, an enzyme originally reported in the giant axon by Sjodin (1974), but whose function still remains unclear. Because of its low temperature-sensitivity and stable storage characteristics, it is not believed to be a metabolic pump like the Na^+ pump described above. However, as with the Na^+ pump enzyme, the ouabain insensitive Na^+-K^+ ATPase is relatively uneffected by allethrin, showing only slight inhibition (4.1%) at the highest allethrin concentration examined (10^{-4}M).

The total Mg^{2+} ATPase activity has been included to examine the effect of pyrethroids on nonmitochondrial, Mg^{2+}-stimulated ATPase systems. As shown in Figure 24-line B, this enzyme is relatively active in the axon-rich preparation, but shows only a 25.7% reduction in ATPase activity in the presence of 10^{-4}M allethrin. In this work, Mg^{2+}-stimulated mitochondrial ATPase activity was suppressed by addition of 2 mM CN^-, and therefore, its sensitivity towards allethrin was not determined. In this context, it should be noted that the major Ca^{2+} sequestering mechanism under physiological conditions in both the axon (Henkart et al., 1978) and synaptic nerve terminal (Blaustein et al., 1978) has been determined as endoplasmic reticulum and not the mitochondria.

Figure 24-line C and line D shows the two Ca^{2+}-stimulated ATPases examined for their pyrethroid sensitivity; Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase (Ca^{2+}-pump), respectively. Ca^{2+} ATPase activity was determined as the portion

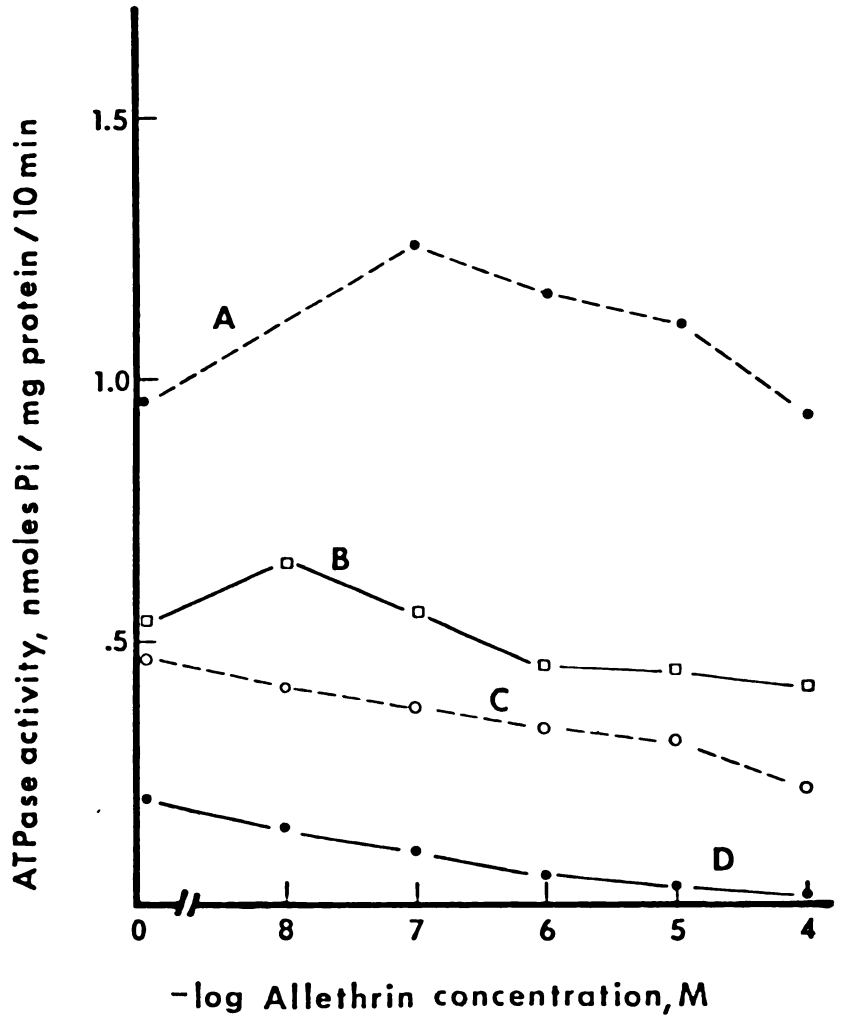

Figure 24. Effects of (+)-trans allethrin on ouabain insensitive Na^+-K^+ ATPase (A), total nonmitochondrial Mg^{2+} ATPase (B), 1 mM-Ca^{2+} ATPase (C), and Ca^{2+}-Mg^{2+} ATPase (D) activities. Enzyme source was the 90,000 g fraction of the squid retinal axon preparation. ATP concentration was 5.6 x 10^{-8}M. Other incubation conditions were: (A)-Na^+=160 mM, K^+=40 mM, Mg^{2+}=10 mM; (B)-Na^+=160 mM, K^+=160 mM, Mg^{2+}=10 mM, KCN=2 mM; (C)-Na^+=160 mM, K^+=160 mM, free Ca^{2+}=1 mM, EDTA=1 mM; (D)-K^+=160 mM, Mg^{2+}=10 mM, free Ca^{2+}=0.01 mM, ethyleneglycol-bis-(-aminoethylether) N,N'-tetraacetic acid (EGTA)=0.5 mM. All buffers were prepared in 30 mM Tris-HCl at pH 7.1. Ouabain concentration was 10^{-4}M. Experimental variability is reported as + S.E. of mean values of at least two separate experiments. Nominal free Ca^{2+} concentrations were established at reported levels by the buffering method described by Portzehl et al. (1964). The specific activities reported for both Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase refer to only Ca^{2+}-stimulated activity.

of activity stimulated in the presence of 1 mM Ca^{2+} over a basal level of activity (i.e., activity in the absence of Ca^{2+}, see Matsumura and Clark, 1980). Ca^{2+}-Mg^{2+} ATPase activity was determined at a relatively lower Ca^{2+} concentration (10^{-5}M) and in the presence of Mg^{2+}. Also, Ca^{2+}-Mg^{2+} ATPase activity is measured only in fresh microsomal preparations whereas Ca^{2+} ATPase is measured only in once-frozen preparations, which completely isolate these two activities due to their separate storage sensitivities. It is interesting to note that the Ca^{2+}-Mg^{2+} ATPase, which is believed to be similar to the Ca^{2+}-pump enzyme reported in nerve tissue and purified from sarcoplasmic reticulum, has the same high temperature-sensitivity of a metabolically active pump, whereas the Ca^{2+} ATPase is very temperature insensitive both in its overall activity and storage characteristics (Matsumura and Clark, 1980). As seen in Figure 24-line C, Ca^{2+} ATPase activity is inhibited over the entire concentration range of allethrin resulting in some 51.3% inhibition at 10^{-4}M allethrin. Ca^{2+}-Mg^{2+} ATPase activity was also found to be very sensitive to allethrin inhibition causing 84.3% reduction in activity at 10^{-4}M. Because of its apparent greater contribution to the total Ca^{2+}-stimulated ATPase activity in the retinal axon (72% of total Ca^{2+} activity, see Figure 24), Ca^{2+} ATPase was examined first in more detail.

The percent inhibition of Ca^{2+} ATPase activity by various pyrethroids, all at a concentration of 10^{-4}M, is illustrated in Figure 25. These compounds are ranked as to their level of inhibition with the most inhibitory on top and the least towards the bottom. The natural ester, pyrethrin, was found to be the most inhibitory causing a 69.3% decrease in Ca^{2+} ATPase activity followed by allethrin and permethrin.

To determine which of the stimulating inorganic ions most affect pyrethroid inhibition, the Na^{+}, K^{+}, and Ca^{2+} concentrations were varied in turn.

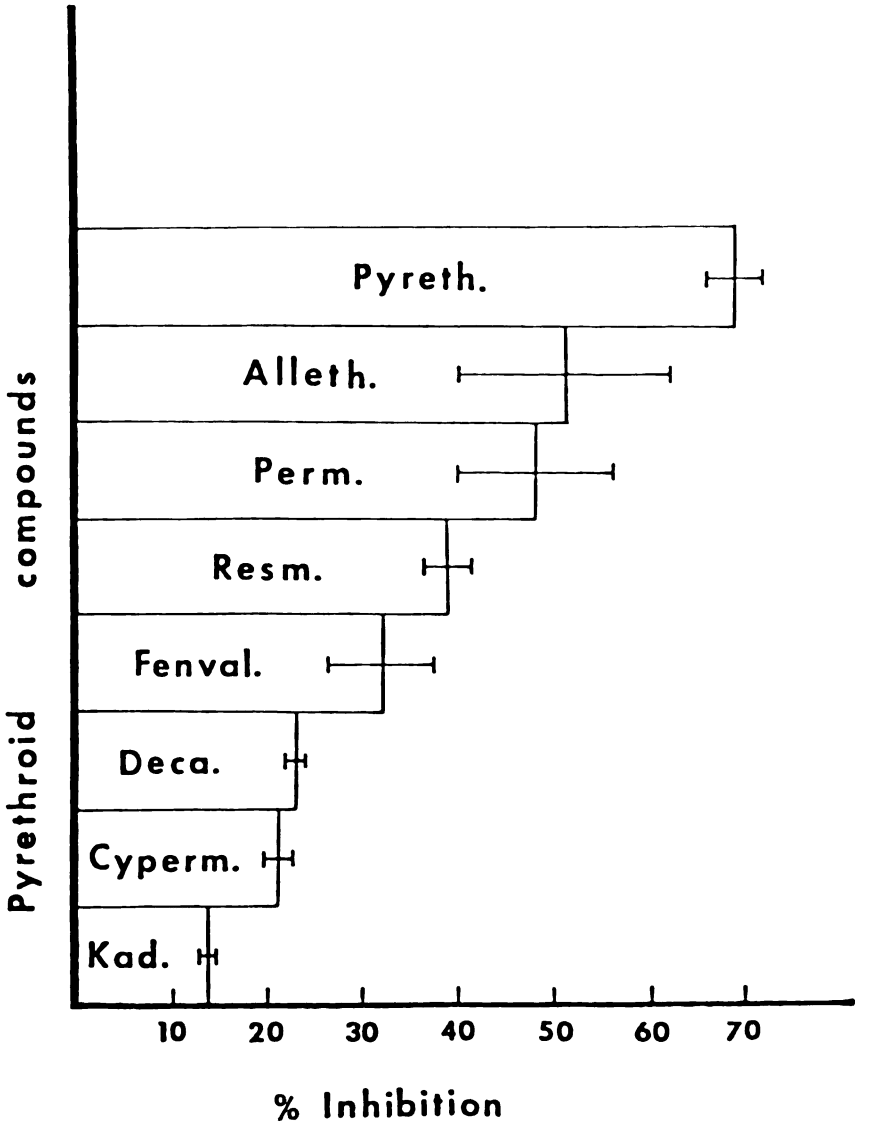

Figure 25. Inhibition of 1 mM-Ca^{2+}ATPase activity by various pyrethroid esters. All pyrethroid concentrations were adjusted to give a final assay concentration of 10^{-4}M. Percent inhibition refers to Ca^{2+}-stimulated activity only. ATP concentration was 5.6 x 10^{-8}M. Ouabain concentration was 10^{-4}M. Other incubation conditions were: Na^{+}=160 mM, K^{+}=160 mM, free Ca^{2+}=1 mM and EDTA=1 mM in 30 mM Tris-HCl at pH 7.1. Experimental variability is reported as $\pm$ S.E. of mean values of at least two separate experiments. Nominal free Ca^{2+} concentration was established at reported levels by the buffering method described by Portzehl et al. (1964).

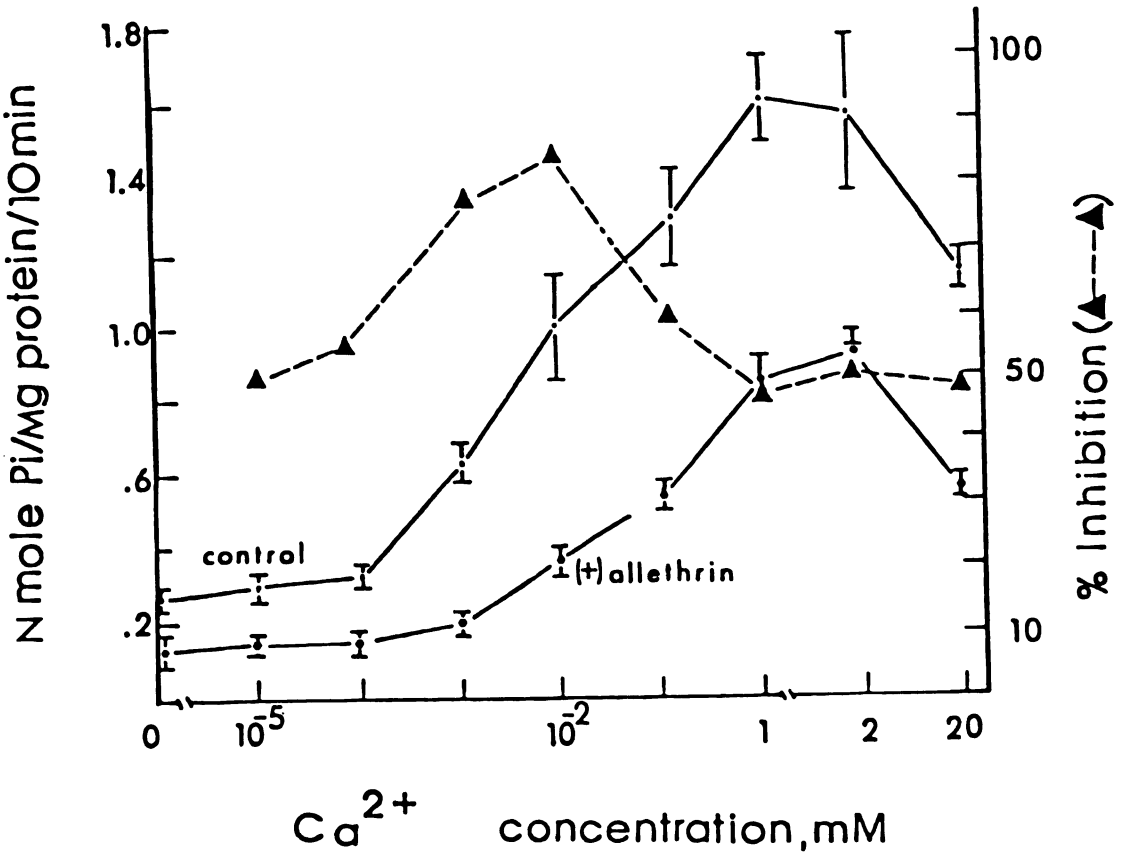

Figure 26. Effect of Ca^{2+} concentration on Ca^{2+} ATPase activity in the presence and absence of 10^{-4}M (+)-trans allethrin. The % inhibition reported refers to % inhibition of Ca^{2+}-stimulated activity only. Enzyme source was the 90,000 g fraction of the squid retinal axon preparation. ATP concentration was 5.6 x 10^{-8}M. Ouabain concentration was 10^{-4}M. Other incubation conditions were: Na^{+}=160 mM, K^{+}=160 mM, and EGTA=1.25 mM, in 30 mM Tris-HCl at pH 7.1. Experimental variability is reported as $\pm$ S.E. of mean values of at least two separate experiments. Nominal free Ca^{2+} concentrations were established at reported levels by the buffering method described by Portzehl et al. (1964).

Na^+ and K^+ ions were found to have a modest effect on allethrin inhibition of Ca^{2+} ATPase activity (Clark and Matsumura, unpublished results), but Ca^{2+} was found to have a significant effect. In Figure 26, the Ca^{2+} concentration has been varied from 10^{-8}M to 20 mM in the presence and absence of 10^{-4}M allethrin. The difference curve (i.e., the difference in values between untreated control and allethrin-treated samples reported as % inhibition) reveals a highly allethrin-sensitive Ca^{2+} ATPase apparent only at low Ca^{2+} concentrations (10^{-6}M). This high affinity Ca^{2+} ATPase was examined for sensitivity toward other pyrethroids.

Although the concentration range of each compound has been expanded (10^{-8} to 10^{-4}M), the same group of pyrethroids reported in Figure 25 was incubated with this high affinity Ca^{2+} enzyme. The results of this experiment are reported in Table 4. The pyrethroids are ranked again as to their inhibitory characteristics with the top compounds most inhibitory, the bottom ones least. Under these conditions, all pyrethroids tested were found to be more efficient inhibitors of this ATPase as compared to the previous experiment (Figure 25) with pyrethrin and allethrin causing almost complete inhibition of Ca^{2+}-stimulated activity. At the same time, it is noteworthy that the order of ranking of the compounds is the same that was reported for the 1 mM-Ca^{2+} ATPase.

Because of the obvious importance of overall Ca^{2+} regulation in the nervous system, the Ca^{2+}-Mg^{2+} ATPase of the retinal axon was also examined. The pattern of pyrethroid inhibition was studied using the same compounds and concentration range as above. The results of this experiment are reported in Table 5. The pyrethroids have been ranked as before. On the whole, these compounds are more inhibitory on Ca^{2+}-Mg^{2+} ATPase than on Ca^{2+} ATPase as judged by their apparent I_{50} values (compare Tables 4 and 5). However, the most

Table 4. Percent Inhibition of a High Affinity,[a] 10^{-6}M-Ca^{2+} ATPase by Various Pyrethroid Esters[b]

Compound	Concentration (M)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Pyrethrin	15.7 ± 15.8	35.0 ± 3.3	45.8 ± 1.6	88.9 ± 8.8	95.6 ± 4.3
Allethrin	13.1 ± 2.3	35.1 ± 2.0	33.7 ± 3.8	58.7 ± 1.3	93.0 ± 1.0
Permethrin	29.6 ± 11.6	39.4 ± 2.7	48.6 ± 3.0	59.4 ± 11.0	60.9 ± 10.5
Resmethrin	+22.5 ± 11.3[c]	+2.4 ± 6.5	26.3 ± 17.4	36.2 ± 6.4	59.4 ± 3.6
Fenvalerate	+25.0 ± 3.6	00.6 ± 1.0	41.9 ± 18.1	62.8 ± 11.7	54.0 ± 0.9
Phenothrin	19.2 ± 1.5	40.1 ± 4.6	50.7 ± 23.7	61.5 ± 8.0	53.8 ± 1.1
Decamethrin	22.5 ± 0.8	23.0 ± 20.7	35.9 ± 12.1	34.1 ± 2.6	51.1 ± 1.9
Cypermethrin	+34.4 ± 0.5	32.8 ± 22.6	45.1 ± 19.0	58.6 ± 13.0	44.4 ± 12.8
Kadethrin	14.0 ± 15.6	20.5 ± 19.6	31.3 ± 9.3	34.7 ± 14.9	43.0 ± 0.4

[a]For high affinity Ca^{2+} ATPase activity, 10^{-6}M free Ca^{2+} concentration adjusted by a Ca EGTA/EDTA buffer, Portzehl et al. (1964).

[b]Data are expressed as percent inhibition of Ca^{2+}-stimulated ATPase activity of samples treated with inhibitor versus controls. Percentages are expressed as the means ± standard error from at least two separate experiments.

[c]+Denotes stimulation of treated samples over control value.

Table 5. Percent Inhibition of Ca^{2+}-Mg^{2+} Stimulated ATPase Activity[a] by Various Pyrethroid Esters[b]

Compound	Concentration (M)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Decamethrin	24.7 ± 3.2	89.2 ± 5.7	90.5 ± 2.8	100	100
Cypermethrin	12.8 ± 5.5	75.8 ± 17.8	100	100	100
Permethrin	10.6 ± 1.2	88.1 ± 14.4	82.0 ± 24.7	100	100
Phenothrin	18.3 ± 12.7	67.9 ± 1.6	72.4 ± 8.7	97.0 ± 1.4	100
Kadethrin	12.2 ± 2.3	54.4 ± 24.1	84.0 ± 8.3	89.2 ± 4.0	100
Fenvalerate	15.1 ± 8.3	41.3 ± 11.2	84.5 ± 2.4	94.4 ± 5.7	97.9 ± 1.0
Pyrethrin	26.0 ± 1.3	50.3 ± 3.9	67.1 ± 5.6	76.4 ± 13.6	84.6 ± 0.4
Resmethrin	10.9 ± 0.8	47.4 ± 8.2	77.8 ± 7.6	79.8 ± 7.8	81.3 ± 4.9
Allethrin	18.9 ± 1.0	48.3 ± 1.6	73.6 ± 5.4	61.9 ± 8.4	82.8 ± 4.2

[a]Free Ca^{2+} concentration was determined by a Ca EGTA buffer system, Portzehl (1964).

[b]Data are expressed as percent inhibition of Ca^{2+} stimulated ATPase activity of samples treated with inhibitor versus controls. Percentages are expressed as the means ± standard error from at least two separate experiments.

obvious difference in the inhibition pattern is that the pyrethroid ranking has undergone a general reversal. That is, with the notable exception of permethrin, which ranked third in both tests, those compounds which were most inhibitory on Ca^{2+} ATPase (e.g., pyrethrin, allethrin, resmethrin) are least inhibitory on Ca^{2+}-Mg^{2+} ATPase activity. Likewise, those compounds least inhibitory on Ca^{2+} ATPase (e.g., decamethrin, cypermethrin, phenothrin) are most inhibitory to Ca^{2+}-Mg^{2+} activity.

To verify this apparent divergence in the site of action between the more synthetic analogues and the natural type configurations, the pinched-off synaptic nerve terminals (synaptosomal preparation of the optic lobe) were examined for the presences of both a well-documented Ca^{2+}-Mg^{2+} ATPase and Ca^{2+} ATPase (Yamaguchi et al., 1979). The inhibitory pattern of pyrethroid action on Ca^{2+} ATPase activity from the optic lobe synaptosomes is given in Table 6. Although the list of compounds has been abbreviated, the pyrethroids used were still ranked as to their level of inhibition as above. Not only are these compounds generally more inhibitory to Ca^{2+} ATPase activity in the synaptosome versus the axon preparation (compare Table 6 at 10^{-4}M to Figure 25 data), but as before the natural ester, pyrethrin, and its analogue, allethrin, are significantly more inhibitory than either permethrin or cypermethrin. This correlates well with the data presented in Table 4. In a similar manner, Table 7 summarizes the inhibitory pattern of these four pyrethroids on Ca^{2+}-Mg^{2+} ATPase in the optic lobe synaptosomes. Again, these compounds are generally more inhibitory to Ca^{2+}-Mg^{2+} activity in the synaptosome than the axon. Also the same reversal of the pattern of inhibition seen in the axon (Table 5) is also reproducible in the synaptic preparation. That is, the phenoxybenzyl compound, permethrin, and its α-cyano analogue, cypermethrin, are significantly more inhibitory than either pyrethrin or allethrin on Ca^{2+}-Mg^{2+} ATPase activity.

Table 6. Percent Inhibition of 1 mM-Ca^{2+} ATPase Activity[a] from Squid Optic Lobe Synaptosomes by Various Pyrethroid Esters[b]

Compound	Concentration (M)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Pyrethrin	+12.1 ± 1.7[c]	9.3 ± 0.2	12.2 ± 1.2	40.8 ± 2.9	74.1 ± 5.0
Allethrin	+22.3 ± 3.7	+3.9 ± 0.1	11.2 ± 0.2	26.8 ± 3.9	70.8 ± 4.3
Permethrin	17.4 ± 0.5	3.4 ± 0.3	13.2 ± 1.3	35.3 ± 2.3	34.3 ± 1.1
Cypermethrin	4.6 ± 0.3	10.9 ± 0.3	22.8 ± 1.9	34.6 ± 3.4	29.1 ± 1.8

[a]Free Ca^{2+} concentration was determined by a_2Ca^{2+} EDTA buffer system in the absence of Mg^{2+}, Portzehl et al. (1964). Specific activity of Ca^{2+} ATPase under experimental conditions was .2728 ± .11 nmoles Pi/mg prot./10 min.

[b]Data are expressed as percent inhibition of Ca^{2+} stimulated ATPase activity of samples treated with inhibitor versus controls. Percentages are expressed as the means ± standard error from at least two separate experiments.

[c]+Denotes stimulation of treated samples over control value.

Table 7. Percent Inhibition of Ca^{2+}-Mg^{2+} ATPase Activity[a] from Squid Optic Lobe Synaptosomes by Various Pyrethroid Esters[b]

Compound	Concentration (M)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Permethrin	+9.4 ± 2.4[c]	68.7 ± 31.7	88.0 ± 4.1	100	100
Cypermethrin	+5.8 ± 1.1	69.5 ± 20.2	75.6 ± 4.7	88.0 ± 4.2	100
Pyrethrin	+34.9 ± 1.0	43.6 ± 10.0	64.9 ± 5.2	86.1 ± 6.1	100
Allethrin	+13.8 ± 0.9	+12.7 ± 2.8	59.7 ± 9.5	70.3 ± 14.3	81.1 ± 10.7

[a] Free Ca^{2+} concentration was determined by a Ca EGTA buffer system, Portzehl et al. (1964). Specific activity of Ca^{2+}-Mg^{2+} ATPase under experimental conditions was 0.1522 ± 0.02 nmoles Pi/mg prot./10 min.

[b] Data are expressed as percent inhibition of Ca^{2+} stimulated ATPase activity of samples treated with inhibitor versus controls. Percentages are expressed as the means ± standard error from at least two separate experiments.

[c] +Denotes stimulation of treated samples over control value.

It should be noted however that in both the axon and synaptosome, Ca^{2+} ATPase is the major Ca^{2+}-stimulated enzyme examined. This ATPase is responsible for approximately 72% of the total Ca^{2+} activity (i.e., the summation of the specific activities of Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase) in the axon and some 64% in the synaptosome (see appropriate footnotes, Tables 6 and 7). However, the total Ca^{2+} activity in the synaptosome is only 66% of that found in the axon. When one compares the specific activity of the Ca^{2+}-Mg^{2+} ATPase in the synaptosome versus the axon, one finds that the synaptosome has 15% less of this enzyme activity than the axon. Yet, when one examines the Ca^{2+} ATPase activity in a similar manner, the synaptosome has 41% less activity than the latter. Therefore, the Ca^{2+}-Mg^{2+} ATPase appears to be relatively more important in overall Ca^{2+} regulation in the synaptosome as compared to the axon.

Since ATP is a common substrate for many biochemical reactions, the axoplasm of the giant axon of the squid was examined to determine what, if any, contamination it might be contributing to our microsomal preparations. It was subsequently found that the axoplasm did indeed contain a modest ATPase activity, some of which was Ca^{2+}-stimulated (Table 8). However, the Ca^{2+} ATPase activity was only 21% of that found in the axon and the Ca^{2+}-Mg^{2+}-stimulated activity was only 17% of the axonal level. Nevertheless, neither of these two Ca^{2+}-stimulated ATPases in the axoplasm showed any sensitivity to allethrin up to concentrations of 10^{-4}M. In fact, allethrin showed consistent increases in activity in both basal and Ca^{2+}-treated samples. Such a response is quite different from that reported in the microsomal fraction of retinal axons and synaptosomes of synaptic nerve terminals from the optic lobe. In both these preparations, allethrin only inhibited Ca^{2+}-stimulated activity and was never seen to effect basal activity.

Table 8. Effect of Allethrin on 1 mM-Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase Activities[a] Found in the Axoplasm of the Giant Axon of Squid, Loligo pealei

ATPase[b]	Allethrin Concentration (M)					
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
	n moles Pi/mg protein/10 min					
Ca^{2+} ATPase						
Basal	.424 ± .14	.646 ± .02	.572 ± .02	.696 ± .08	.677 ± .02	.700 ± N.A.[c]
Total	.519 ± .03	.668 ± .01	.593 ± .01	.737 ± .09	.748 ± .01	.799 ± .09
Ca^{2+} stimulated	.095	.022	.021	.041	.071	.099
Ca^{2+}-Mg^{2+} ATPase						
Basal	.154 ± .02	.173 ± .03	.301 ± .08	.401 ± .02	.366 ± .00	.406 ± .01
Total	.185 ± .07	.169 ± .04	.451 ± .01	.346 ± .06	.379 ± .04	.369 ± .01
Ca^{2+} stimulated	.031	—	.150	—	.013	—

[a]Free Ca^{2+} concentration adjusted by Ca EDTA/EGTA buffer systems, Portzehl et al. (1964).

[b]ATPase activities, basal, total and Ca^{2+}-stimulated (i.e., difference), refer to those activities defined previously by Matsumura and Clark (1980).

[c]N.A. (not available).

As seen in the axoplasm, stimulation by allethrin is quite reminiscent of the data reported for nerve myosin above. It has been previously reported by Matsumura and Clark (1980) that the retinal axon of the squid does indeed contain a high amount of myosin-like ATPase activity. Unfortunately, no attempt was made to solublize myosin-like proteins from the axoplasm as recently reported by Baker and Schlaepfer (1978) prior to collecting the microsomal pellet by ultracentrifugation. Nevertheless, given the response of the axon to pyrethroids as reported above, it is doubtful that the axoplasmic ATPase activity contributes largely to the overall inhibitory behavior of pyrethroids on membrane-bound ATPases. However, experiments to clarify this point need to be done.

DISCUSSION

The presence of ion-stimulated ATPase systems in a variety of excitable tissues is well documented and has been greatly enlarged upon since their discovery (Skou and Hillberg, 1969). Their importance in the regulation of ionic fluxes which control nerve activity is just now being understood. Recent advances in the role played by calcium in the modulation of numerous physiological processes may have provided us with a key to understand these complex mechanisms. This is particularly true in the area of nerve physiology where the importance of calcium regulation has been long implicated as mandatory for proper nerve function.

Since the initial findings of Ringer (1883), calcium has been implicated in the regulatory aspects of properly functioning excitable tissues. In a pioneering work with voltage clamp technique, Frankenhauser and Hodgkin (1957) found that the reduction of external Ca^{2+} on the axon resulted in spontaneous oscillations or repetitive activity, much like the repetitive discharges seen during DDT or pyrethroid poisoning. Increasing the external Ca^{2+} concentration again

stabilizes the axon by raising the threshold and increasing the membrane resistance. Tasaki (1974) has likewise stated that "the existence of a divalent cation (Ca^{2+}) salt in the external medium and its absence in the axon interior is an an indispensable condition for the maintenance of excitability." The fact that the only enzymes known to regulate ions across semipermeable membranes are ATPases and the finding by us that only Ca^{2+}-stimulated ATPases of the squid nervous system appear to be clearly sensitive to pyrethroids makes these enzymes prime suspected targets for these insecticides. In this context, Matsumura and Narahashi (1971) have reported that if Ca^{2+} is added to the bathing solution of a nerve in the initial stages of DDT poisoning, the symptoms are suppressed. If Ca^{2+} is then removed, the symptoms of poisoning reappear. By similar means, Gammon (1980) has also shown that a reduction of calcium in the saline solution causes permethrin-resistant insects (Spodoptera littaralis), which under normal calcium conditions are relatively recalcitrant to permethrin, to mimic the electrical nerve patterns of poisoning seen in permethrin-susceptible insects.

The effect of Ca^{2+} on various ATPase systems appears to fall into two groups; those in which Ca^{2+} stimulation requires Mg^{2+}, and are highly temperature sensitive and those which are Mg^{2+} independent, require Na^{+}, and are relatively temperature insensitive (Matsumura and Clark, 1980).

The Ca^{2+} ATPase of the squid axon shows many of the same characteristics of the ecto-Ca^{2+} ATPase reported by Matsumura and Ghiasuddin (1979). It has the same ion requirements, the same pH optimum, a high ATP affinity, comprises the major Ca^{2+}-stimulated activity in the axon, has the same temperature insensitivity and stable storage characteristics. It may be postulated, therefore, that pyrethrin and closely related compounds such as allethrin act primarily at the level of the axon by disrupting Ca^{2+} ATPase which

is the predominant Ca^{2+} regulating enzyme located there. This is thought to be very similar to the action of DDT. It is likewise of interest that among the pyrethroids tested, only allethrin shows a DDT-like negative temperature correlation in toxicity (Wang et al., 1972; Gammon, 1979). Whether or not the high affinity Ca^{2+} ATPase (10^{-6}M) reported above is identical with the allethrin-sensitive portion of 1 mM-Ca^{2+} ATPase is a matter of speculation at this time. However, it is extremely sensitive to allethrin inhibition and is temperature insensitive in both its activity and storage characteristics.

On the other hand, it must be pointed out that pyrethroids which are more highly modified (e.g., contain phenoxybenzyl, halogens, and α-cyano moieties) are significantly more inhibitory to Ca^{2+}-Mg^{2+} ATPase than to Ca^{2+} ATPase activity. With this in mind, the recent findings by Vijverberg and van den Bercken (1979) are most illuminating. They have shown that both allethrin and permethrin cause repetitive discharges to occur in the frog sciatic nerve. Neither cypermethrin nor decamethrin could mimic this response. However, cypermethrin and decamethrin did cause a frequency-dependent depression of the action potential whereas allethrin and permethrin were incapable of this. Therefore, it seems highly likely that these two groups of pyrethroid esters may have their primary site of action on quite distinctly different components of the nervous system.

In view of such a qualitative divergence, it may be speculated that the greater <u>in vivo</u> toxicity of the halogenated phenoxybenzyl and α-cyano compounds may be due to not only being metabolized less rapidly and being partitioned more efficiently, but also by interfering with both Ca^{2+} ATPase and Ca^{2+}-Mg^{2+} ATPase activities. In this context, it has been shown in the presynaptic region of the nerve that a major function of Ca^{2+}-Mg^{2+} ATPase is to regulate the internal Ca^{2+} level and thereby control the rate of transmitter

release (Blaustein et al., 1978). Inhibition of Ca^{2+}-Mg^{2+} ATPase in this region by insecticide action is expected to cause increased transmitter release and synaptic facilitation (Yamaguchi et al., 1980). In the case of pyrethroid poisoning, any electrical disturbance generated in the axon could be greatly potentiated by increased neurotransmitter release at the synapse. Also, inhibition of Ca^{2+}-Mg^{2+} ATPase activity is expected to gradually block the axonic excitation function. As DiPolo (1978) showed in the squid axon, the bulk of Ca^{2+} extrusion at physiological Ca^{2+} levels and therefore maintenance of Ca^{2+} gradient across the axonic membrane is carried out by an ATPase which is most likely to be identical to the enzyme described previously (Matsumura and Clark, 1980).

In conclusion, membrane-bound ATPase systems of the squid optic nerve and brain provide a sensitive and selective means to study the mode of action of DDT and pyrethroids at the molecular level in nerve membranes. By correlating the inhibition of Ca^{2+}-stimulated ATPases by pyrethroid esters to overall disruption of Ca^{2+} regulation in the nervous system of a species that has been well researched in this respect, one should be able to provide some clue of the mechanism of biochemical and physiological interactions and thereby contribute to the overall understanding of the basis of pyrethroid toxicities.

CHAPTER III

PYRETHROID INHIBITION OF NEURAL ATPases OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

INTRODUCTION

The nervous system constitutes the major site of action for the majority of modern organic insecticides, including DDT and pyrethroids. Narahashi (1976) has established that the basis for the excitation in axons poisoned with DDT and similar-acting compounds, such as allethrin, is the increase in negative after-potential. This increased negative after-potential has been attributed to principally a reduction in the falling phase of the Na^+ current. The steady-state K^+ current is also decreased but to a lesser extent.

Besides these axonal events, pyrethroids have also been reported to affect other aspects of the nervous system with a high degree of affinity, especially sensory structures (Wouters and van den Bercken, 1978). Clements and May (1977) have demonstrated that the repetitive activity recorded in the affarent sensory nerve of the chordotonal organ poisoned with various pyrethroids was eliminated by removal of the organ. The implication is that the pyrethroids principally affected the impulse-generating mechanisms of the sense organ itself and not the affarent nerve. Also, pyrethroids which were most effective in terms of killing in vivo were of the 3-phenoxybenzyl and α-cyano-3-phenoxybenzyl type. Similarly, Orchard (1980) has reported that extremely low levels of decamethrin (10^{-10}M) caused a doubling of electrical activity of the axons of neurosecretory cells from the brains of Rhodnius prolixus.

It is interesting, however, that Matsumura and Narahashi (1971) have also reported that if Ca^{2+} is added to the bathing solution of a nerve in the initial

stages of DDT poisoning, the symptoms are suppressed. The removal of Ca^{2+} once again causes these symptoms of poisoning to reappear. Similarly, permethrin-resistant insects (Spodoptera littaralis), which under normal Ca^{2+} conditions are relatively recalcitrant to permethrin, are caused to mimic the electrical nerve patterns of poisoning seen in permethrin-susceptible insects by the reduction of Ca^{2+} concentration in the saline (Gammon, 1980).

Although Ca^{2+} plays no part in action potential generation per se, Ca^{2+} has long been implicated in the regulatory aspects of properly functioning excitable tissues (Ringer, 1883). Of the three major excitable phenomena which occur in biological systems (the generation of action potentials, the release synaptic transmitter, and muscle contractions), none are successfully initiated by depolarization stimuli alone but become successfully activated only in the combined presence of Ca^{2+} (Katz, 1966). The central role occupied by Ca^{2+} in the modulation of numerous physiological processes may have provided us with the key by which we might understand these complex mechanisms. This is particularly true in the area of nerve physiology.

It is well documented that the nervous system functions by using electrical potentials which are self-generated by selectively segregating inorganic ions across the nerve membrane. The fact that the only enzymes which are known to regulate ions across semipermeable membranes are ATPases and the recent findings that Ca^{2+}-stimulated ATPases appear to be highly sensitive to insecticidal action makes these entities prime suspects for insecticides.

Ghiasuddin and Matsumura (1979) have identified a Ca^{2+}-stimulated ecto-ATPase from the lobster walking leg nerve which is highly sensitive to DDT inhibition. They have characterized the function of this enzyme as one of Ca^{2+} maintenance of surface Ca^{2+} on the nerve membrane. Inhibition of this enzyme could lead to a hypocalcemic state of the nerve membrane resulting in nerve

instability. Clark and Matsumura (1981a) have also reported Ca^{2+}-stimulated ATPases from the squid nervous system which are very sensitive to pyrethroids. Besides a Ca^{2+} ATPase, which appears to be very similar to the ecto-enzyme detailed above, these researchers have also described a Ca^{2+} Mg^{2+} ATPase. Of the pyrethroids examined, the more natural-type configurations (e.g., allethrin) clearly showed a preference towards Ca^{2+} ATPase inhibition whereas the more highly modified pyrethroids (e.g., cypermethrin) mainly inhibited Ca^{2+} Mg^{2+} ATPase. The present study was undertaken to determine whether similar Ca^{2+}-stimulated ATPase activities exist in organisms targeted for control by pesticides, such as insects, and whether there is a correlation of pyrethroid sensitivity from nontargeted to targeted organisms.

MATERIALS AND METHODS

Animals. Adult male cockroaches, Periplaneta americana, were used in this study. Roach brains were obtained and fractionated in the manner described by Telford (1968). After removing the antennae, the roach was decapitated. The whole brain, including the corpus allatum and subesophageal ganglion, was dissected from the head capsule. The brain was cleaned of connective and muscle tissues and placed into cold homogenizing solution consisting of 750 mM sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 3 mM imadazole buffer adjusted to pH 7.3.

Preparation of Insect Brain Fractions. Twenty roach brains, prepared as outlined above, were pooled in 5 ml cold homogenizing solution and homogenized using a teflon-glass homogenizer at 1000 rpm for 3 min. The homogenate was diluted to 250 mM sucrose with cold distilled, deionized water and centrifuged at 1000 g for 10 min at $3^{o}C$. The 1000 g pellet was discarded, and the 1000 g supernatant passed through glass-wool. The filtrate was centrifuged at 20,000 g for 30 min at $3^{o}C$. The 20,000 g supernatant was decanted and recentrifuged at

105,000 g for 120 min at 3°C. The 105,000 g pellet (microsomal tissue fraction) was resuspended into 6 ml of a storage buffer containing 750 mM sucrose, 0.1 mM DTT and 3 mM imadazole buffer at pH 7.3. The microsomal fraction was then placed on ice until used.

The 20,000 g pellet was fractionated further by a discontinuous sucrose gradient (Telford, 1968). After resuspension, the 20,000 g pellet was placed on top of a four-tiered gradient consisting of 1.8 M, 1.5 M, 1.2 M and 1.0 M sucrose. The resulting gradient was centrifuged at 105,000 for 120 min at 3°C. The material at the 1.2 M - 1.5 M interface was collected by aspiration with a pasteur pipet, diluted to 250 mM sucrose with distilled, deionized water and recentrifuged at 20,000 g for 45 min. The 20,000 g fractionated pellet (synaptosomal tissue fraction) was resuspended into 6 ml storage buffer and placed on ice until used.

All enzymes were stable to storage on ice (4°C) except Ca^{2+}-Mg^{2+} ATPase which was prepared fresh and used immediately. Protein was determined by the method of Lowry et al. (1951), and all assays were performed at a standard enzyme concentration, 10 µg protein per assay tube.

Determination of ATPase Activity. The enzymatic hydrolysis of gamma (γ)-^{32}P-labeled adenosine triphosphate (ATP) and the subsequent production of radiolabeled inorganic phosphate (^{32}Pi) was used as an indicator of ATPase activity in all assays.

The ionic compositions of various buffers used to selectively stimulate certain ATPase activities are summarized in Table 9. The determination of the activity of selected ATPases is given in Table 10. The extent of inorganic phosphate (Pi) production from ATP was determined as follows: 0.1 ml of the above nerve preparations were added to 0.9 ml of 30 mM imadazole buffer (pH 7.3) with various ion combinations. (γ-^{32}P) ATP in 10 µl of distilled water was

added and the system thoroughly mixed. The final concentration of ATP was 5.6 x 10^{-8}M. The system was incubated for 10 min at 30°C and the reaction stopped with 0.3 ml of 10% trichloroacetic acid (TCA) at 0°C. One mg of bovine serum albumin (BSA) plus 1.35 mg KH_2PO_4 in 0.1 ml distilled water was added, and after mixing, the system was allowed to equilibrate for 5 min at 25°C. The system was centrifuged at 3000 g for 3 min, and the supernatant quantitatively transferred to a clean assay tube. Approximately 100 mg of activated charcoal was added and the tube thoroughly mixed. The sides of the tube were rinsed with 0.2 ml of ethanol, and after mixing the system was centrifuged at 3000 g for 3 min. A 0.5 ml aliquot was taken for liquid scintillation counting.

The tetraethylammonium salt of (γ-^{32}P) ATP, purchased from New England Nuclear, Boston, Massachusetts, was utilized as the substrate for all assays and had a starting specific activity of 26.4 mCi/μ mol (approximately 3 x 10^5 dpm were used per asay tube). All aqueous solutions were prepared in distilled, deionized water which had also been cleaned of any organic contaminants. Water quality was assessed by its conductivity which was measured in ppm of NaCl equivalents. At no time was the water conductivity allowed to exceed 0.1 ppm.

ATPase Inhibition Studies. All agents to be tested were prepared in buffer or in organic solvents at stock concentrations adjusted to deliver the final assay concentration (1 ml) in a 10 μl aliquot. All agents, unless stated otherwise in the text, were equilibrated with the enzyme-buffer mixture for a preincubation period of 10 min at 25°C prior to the addition of (γ-^{32}P) ATP.

Any influence of an agent on a specific ATPase activity was determined as either an increase or decrease in activity apparent in the treated sample when compared to an untreated control sample. Such changes are reported as either relative ATPase activity (remaining) or percentage (%) of control where

untreated control sample value equals 100%. Whenever organic solvents were utilized, proper solvent controls were included.

Determination of Permethrin Levels and Effects in Various Insect Brain Fractions, In Vivo Studies. In order to determine the amount of permethrin bound to brain tissue in severely poisoned roaches, the following procedure was adopted. Twenty live male roaches were injected intraperitoneally at an LD_{95} dose with radiolabeled ^{14}C-permethrin (1 µg/roach, Chadwick, 1979). Once convulsions were well established, the roaches were sacrificed and brains fractionated exactly as described above. The amount of ^{14}C-permethrin bound to whole brains, microsomal and synaptosomal fractions was determined radiometrically by liquid scintillation counting. Cis-permethrin (^{14}C-carbonyl) was utilized throughout this study in order to minimize metabolic conversion and had a specific activity of 58.2 mCi/mmole. Protein was determined by the method of Lowry et al. (1951). Twenty roach brains contained approximately 15.25 mg protein. Microsomal and synaptosomal fractions from this amount of brain contained 1.02 mg and 420 µg protein, respectively.

In order to determine the relative effects of permethrin binding, a collaborative study was attempted utilizing unlabeled cis-permethrin at the same LD_{95} dose. Roaches were treated exactly as outlined above but now permethrin-containing brain fractions were utilized to measure the specific activities of various Ca^{2+}-stimulated ATPases under standard assay conditions as described in Table 10.

In Vitro Studies. In these experiments, enzyme was prepared from untreated roaches by the methods described previously. An I_{50} dose of (cis)^{14}C-permethrin, calculated from figure 31 for non-mitochondrial Ca^{2+}-Mg^{2+} ATPase (i.e., 3.8×10^{-7}M for microsomes, 2.3×10^{-8}M for synaptosomes), was added during the 10 min preincubation period of a standard ATPase assay. Upon

completion of the 10 min incubation period with unlabeled ATP, the assay was terminated by quick cooling, and the enzyme collected by ultracentrifugation using the method of Matsumura and Hayashi (1969). During these experiments, Tris-ATP was used as a substrate in order not to interfere with ^{14}C-permethrin determination. The amount of ^{14}C-permethrin bound to microsomal and synaptosomal fractions was likewise determined by liquid scintillation radiometry.

Chemicals. The pyrethroid insecticides used in this study were obtained as gifts from various sources; (+) trans allethrin[2] [3-allyl-2-methyl-4-oxocyclopent-2-enyl-(+)-trans-chrysanthemate of (±)-allethrolone] from Dr. T. Narahashi, Department of Pharmacology, Northwestern University, Chicago, IL, permethrin[2] [3-phenoxybenzyl(±)-cis, trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] and [cis, ^{14}C-permethrin (^{14}C-carbonyl)] from FMC, Agricultural Chemicals Division, Middleport, NY, and cypermethrin[2] [(±)-α-cyano-3-phenoxybenzyl(±)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] from Shell Bioscience Laboratory, Sittingbourne Research Center, Sittingbourne, Kent, United Kingdom. The following pharmacological active agents were obtained from various sources; carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP)[3] from Boehringer Mannheim GmbH, West Germany, A23187[3] from Eli Lilly Company, Indianapolis, IN, valinomycin[1] from Aldrich Chemical Company, Milwaukee, WI, Tiapamil[3] from F. Hoffmann-LaRoche Company, A.G., Basal, Switzerland, triflurperazine · 2HCl[2] from Smith, Kline & French Corporation, Carolina, PR., dithiobis(succinimidyl propionate) (DSP)[3] from Tridom Chemical Inc., NY, and sodium orthovanadate[1], Fisher Scientific Company, Pittsburgh, PA. The remaining chemicals were all received from Sigma Chemical Company, St. Louis, MO; dithiotheitol (DTT),[1] ethylenediamine tetraacetic acid (EDTA),[1] imadazole,[1] ethyleneglycol

bis(β aminoethylether)-N,N'-tetraacetic acid (EGTA)[1], tris(hydroxymethyl) amino methane · ATP, (Tris-ATP)[1], ouabain,[1] lanthanium chloride ($LaCl_3$),[1] ruthenium red,[1] colchicine,[2] cytochalasin B,[2] tetrodotoxin (TTX),[1] mersalyl acid,[1] trypsin,[1] tryspin inhibitor (soybean, type 1-S), tetracaine,[1] atractyloside,[2] veratridine,[2] pentylenetetrazole,[1] saponin,[1] oligomycin,[2] sodium azide,[2] and 2,4, dinitrophenol (DNP).[2] The following media were used for solubilizing these agents: [1]H_2O, [2]ethanol, [3]dimethyl sulfoxide (DMSO)

RESULTS

Characterization of Insect Brain ATPases. Ionic modulation of numerous ATPase systems have been reported in a variety of tissues. The method of choice has been to first establish a basal level of activity to which one compares any changes which occur when selective additions are made to the assay system. Table 9 summarizes the buffer compositions used to characterize Ca^{2+}-stimulated ATPase activities in the insect brain. Again, stimulation by specific ions, high ATP affinity and susceptibility to specific inhibitors were used as criteria to identify selective activities.

As previously described by Matsumura and Clark (1980) in the squid system, Ca^{2+}-stimulated ATPases appear to fall into two groups; those in which Ca^{2+} stimulation requires Mg^{2+}, and are highly temperature sensitive (i.e., Ca^{2+}-Mg^{2+} ATPases) and those which are Mg^{2+} independent, requiring Na^{+}, and are relatively temperature insensitive (i.e., Ca^{2+} ATPase). Table 10 clearly shows that similar activities are found in both microsomal and synaptosomal tissue fractions of the insect brain. Furthermore, Total ATPase activity, defined as the activity apparent in the presence of a full ion complement for each buffer solution, is subdivided into Ca^{2+}-stimulated, mitochondrial and nonmitochondrial aspects by the following means; Ca^{2+}-stimulated activity refers to that activity determined as the difference between Total activity (plus Ca^{2+}) and basal

Table 9. Standard Buffer Compositions[a]

ATPase studied	Concentration, mM							
	NaCl	KCl	$MgCl_2$	$CaCl_2$[b]	Imadazole	EGTA	EDTA	Ouabain
Basal Ca^{2+} ATPase	160	160	0	0	30	0	1	0.1
1 mM Ca^{2+} ATPase	160	160	0	1	30	0	1	0.1
50 μM Ca^{2+} ATPase	160	160	0	.05	30	0	1	0.1
0.5 μM Ca^{2+} ATPase	160	160	0	$5x10^{-4}$	30	0	1	0.1
Basal Mg^{2+} ATPase	0	160	10	0	30	0.5	0	0.1
Ca^{2+}-Mg^{2+} ATPase	0	160	10	0.01	30	0.5	0	0.1
(Na) Ca^{2+}-Mg^{2+} ATPase	160	160	10	0.01	30	0.5	0	0.1
(Li) Ca^{2+}-Mg^{2+} ATPase	(Li=160)	160	10	0.01	30	0.5	0	0.1

[a]All buffer solutions were adjusted to pH 7.3 using HCl.

[b]Free Ca^{2+} concentrations were adjusted using CaEDTA or CaEGTA buffer systems at levels previously described by Portzehl et al. (1964).

Table 10. Distribution of ATPase Activities in Microsomal and Synaptosomal Fractions of the Insect Brain

ATPase studied[a]	Preincubations[c]			ATPase activities + S.E.[d]			
	Tissue fraction[b]	Saponin	Mitochondrial poisons	Total[e]	Ca^{2+} stimulated[f]	Mitochondrial[g]	Non-Mitochondrial[h]
				(pmoles Pi/mg protein/10 min)			
Basal Ca^{2+} ATPase	micro	0	0	48 ± 17	0	-	-
1 mM Ca^{2+} ATPase	micro	0	0	332 ± 210	3272 ± 220	-	-
50 µM Ca^{2+} ATPase	micro	0	0	2016 ± 234	1968 ± 232	-	-
0.5 µM Ca^{2+} ATPase	micro	0	0	227 ± 35	179 ± 33	-	-
Basal Ca^{2+} ATPase	syn	0	0	121 ± 3	0	-	-
1 mM Ca^{2+} ATPase	syn	0	0	1804 ± 180	1683 ± 160	-	-
50 µM Ca^{2+} ATPase	syn	0	0	1286 ± 210	1147 ± 240	-	-
0.5 µM Ca^{2+} ATPase	syn	0	0	272 ± 10	151 ± 19	-	-
Basal Mg^{2+} ATPase	micro	0	0	673 ± 25	0	76 ± 13	0
Basal Mg^{2+} ATPase	micro	0	+	597 ± 51	0	0	597 ± 51
Ca^{2+}-Mg^{2+} ATPase	micro	0	0	1053 ± 98	380 ± 42[h]	134 ± 9	0
Ca^{2+}-Mg^{2+} ATPase	micro	0	+	919 ± 84	322 ± 60[h]	0	919 ± 84
(Na^{+}) Ca^{2+}-Mg^{2+} ATPase	micro	0	+	683 ± 42	86 ± 30	-	-
(Li^{+}) Ca^{2+}-Mg^{2+} ATPase	micro	0	+	634 ± 64	37 ± 12	-	-
Basal Mg^{2+} ATPase	syn	0	0	458 ± 25	0	-	-
Basal Mg^{2+} ATPase	syn	+	0	1046 ± 89	0	460 ± 18	0
Basal Mg^{2+} ATPase	syn	+	+	586 ± 53	0	0	586 ± 53
Ca^{2+}-Mg^{2+} ATPase	syn	0	0	429 ± 14	0	-	-
Ca^{2+}-Mg ATPase	syn	+	0	1042 ± 74	0	315 ± 14	0
Ca^{2+}-Mg ATPase	syn	+	+	727 ± 47	141 ± 19[h]	0	727 ± 47

Table 10 (cont'd.)

[a]Refers to enzymatic activity selectively stimulated by ionic buffers described in Table 9.

[b]Micro (microsomal) and syn (synaptosomal), respectively.

[c]Intact synaptosomal tissue fractions were disrupted by pretreatment with saponin at a concentration of 250 μg/ml for 30 min at 4°C. Mitochondrial poisons consist of a mixture of azide, DNP and oligomycin, see text, Results.

[d]Specific activity of each enzyme is reported as the mean ± standard error of at least six separate experiments of two replicates each.

[e]See text, Results.

[f]See text, Results

[g]See text, Results

[h]See text, Results

activity (minus Ca^{2+}), mitochondrial activity refers to the difference in activity in the presence and absence of mitochondrial poisons (i.e., 0.1 mM azide, 0.1 mM DNP and 0.7 μg/ml oligomycin, Blaustein et al., 1978a). Nonmitochondrial activity refers to the activity remaining in the presence of mitochondrial poisons.

As in the case with the squid (Clark and Matsumura, 1981a), Ca^{2+} ATPase activity (Table 10) is consistently higher in the microsomal preparation compared to the synaptosomes. Ca^{2+}-Mg^{2+} ATPase is approximately equal in the two preparations as judged by Total activity. It is also noteworthy that nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity is greatly reduced by the presence of Na^{+}or Li^{+}. A similar phenomena has been reported by Blaustein et al. (1978b) and others with ATP-dependent Ca^{2+} transport mechanisms.

The integrity of the synaptosomal fraction has been judged by using a naturally-occurring surfactant, saponin. Saponin disruption is viewed as a less destructive means of rupturing intact membrane systems than the more often used method of osmotic shock. It is believed that saponin interfers principally with cholesterol-rich membranes (e.g., plasmalemma) rather than with cholesterol-poor membranes (e.g., innermitochondrial membranes or endoplasmic reticulum). It is clearly seen that saponin pretreatment of synaptosomes at 250 μg/ml for 30 min at $4^{o}C$ (Blaustein et al., 1978a) yields not only an increased basal Mg^{2+} activity but that the heretofore absent Ca^{2+}-stimulation, which is apparent in undisrupted microsomal fractions, now becomes evident in the synaptosomes. It is interesting that much of the increased Mg^{2+}-dependent basal ATPase activity of the synaptosome is sensitive to mitochondrial poisons. Also, in microsomal preparations, Ca^{2+} addition to basal Mg^{2+} media yields a slight increase in overall mitochondrial ATPase activity. This Ca^{2+}-stimulated increase is not apparent in the saponin-disrupted synaptosomes.

Mitochondrial and nonmitochondrial ATPase activities from disrupted synaptosomes were also determined using a variety of selective inhibitors. The results of these experiments are outlined in Table 11. Ruthenium red (10^{-8}M) has been implicated as a very specific inhibitor of Ca^{2+} sequestering by mitochondria (Carafoli and Crompton, 1978). Similar levels of ruthenium red were also seen to decrease the apparent mitochondrial Mg^{2+}-ATPase activity of the insect brain, but had no inhibitory effect on nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity. FCCP, an extremely potent uncoupling agent of oxidative phosphorylation, was also inhibitory to mitochondrial activity, but ineffective to nonmitochondrial activity. Atractyloside, an inhibitor of mitochondrial ATP-ADP carrier systems (Lehninger, 1977) selectively inhibited mitochondrial activity by some 80%.

Blaustein et al. (1978a) has reported the proteolytic enzyme, trypsin, can also be used to delineate mitochondrial versus nonmitochondrial activities in disrupted synaptosomes. Proteolytic digestion was accomplished by incubating the saponin-disrupted synaptosomes with trypsin (1 mg/ml) for 5 min at 30°C. Trypsin digestion was terminated by addition of trypsin inhibitor (1.5 mg/ml). Trypsin was also added simultaneously with a trypsin inhibitor as control. External trypsin refers to addition of trypsin prior to saponin disruption. As seen in Table 4, trypsin digestion clearly reduces the nonmitochondrial ATPase activity but leaves the mitochondria unaffected. Trypsin plus trypsin inhibitor had no effect on mitochondria activity but did effectively protect a portion of the nonmitochondrial activity. External trypsin had little effect on either system.

Determination of Pyrethroid Action on ATPases of the Insect Brain. A number of ATPase systems have been shown to be sensitive to insecticide action, particulary to DDT and pyrethroids. Recently, Clark and Matsumura (1981a)

Table 11. Influence of Cellular Inhibitors on Mitochondrial Mg^{2+} ATPase and Nonmitochondrial Ca^{2+}-Mg^{2+} ATPase Activities from Disrupted Synaptosomes[a] of the Insect Brain

Additions	Mitochondrial Mg^{2+} ATPase[b]	Nonmitochondrial Ca^{2+}-Mg^{2+} ATPase[c]
	% of control ± S. E.[d]	
Ruthenium red (10^{-8}M)	51.4 ± 2.7	+ 100[f]
Ruthenium red (10^{-7}M)	6.8 ± 4.2	+ 100[f]
FCCP (10 µg/ml)	23.7 ± 5.0	135.0 ± 24
Atractyloside (50 µM)	20.4 ± 1.6	
Trypsin (1 mg/ml)	116.1 ± 3.8	17.2 ± 1.6
Trypsin (1 mg/ml)+ Trypsin Inhibitor (1.5 mg/ml)[e]	106.9 ± 2.1	58.7 ± 3.1
External Trypsin (1 mg/ml)[e]	101.6 ± 2.8	115.8 ± 6.0

[a]Disruption of intact synaptosomes was accomplished by saponin pretreatment at 250 µg/ml for 30 min at 4°C.

[b]See footnote g, Table 10.

[c]See footnote h, Table 10.

[d]% of control refers to the percentage of activity remaining in the presence of inhibitor compared to untreated sample. Percentages are expressed as the means ± standard error of two separate experiments (four replicates).

[e]Trypsin inhibitor was added simultaneously with trypsin. External trypsin refers to trypsin added prior to saponin disruption.

[f](+) denotes stimulation above untreated control value, see Fig. 34.

have reported that Ca^{2+}-stimulated ATPases of the squid nervous system are highly sensitive to pyrethroids. In order to determine if a similar situation exists in the Ca^{2+}-stimulated ATPases of the insect brain, the microsomal and synaptosomal brain fractions were incubated with and without 10^{-6}M permethrin at various Ca^{2+} concentrations.

Figure 27 summarizes the effect of Ca^{2+} concentration on permethrin inhibition of Ca^{2+} ATPase activity. As judged by the difference curve (i.e., difference between untreated control and treated, respresenting permethrin sensitive enzyme activity), permethrin is consistently more inhibitory at low Ca^{2+} concentrations in both the microsomal and synaptosomal fractions. Comparatively, permethrin is more inhibitory to synaptosomes than on microsomes. The result of a similar experiment on nonmitochondrial Ca^{2+}-Mg^{2+} ATPase is presented in Figure 28. Again, permethrin is more inhibitory at low Ca^{2+} concentrations, and the synaptic preparation is more sensitive to permethrin inhibition than microsomes. Also apparent from a comparison of difference curves (Figure 27 versus Figure 28) is that nonmitochondrial Ca^{2+}-Mg^{2+} ATPase is more inhibited overall than the Ca^{2+} ATPase activity whether the comparison is between microsomal or synaptosomal preparations.

It is also noteworthy that patterns of response of Ca^{2+}-Mg^{2+} ATPase to change in Ca^{2+} concentration (Figure 28) are distinctly different from microsomal and synaptosomal preparations. The microsomal fraction gave a peak of activity at 10^{-5}M Ca^{2+} which is very similar to the situation reported for the squid (Matsumura and Clark, 1980). In the synaptic preparation, this stimulation was not seen.

The role of mitochondria in intracellular calcium homeostasis has been an active area of research recently. Therefore, the role of Ca^{2+} concentration on mitochonrial ATPase activity from disrupted synaptosomes was examined by

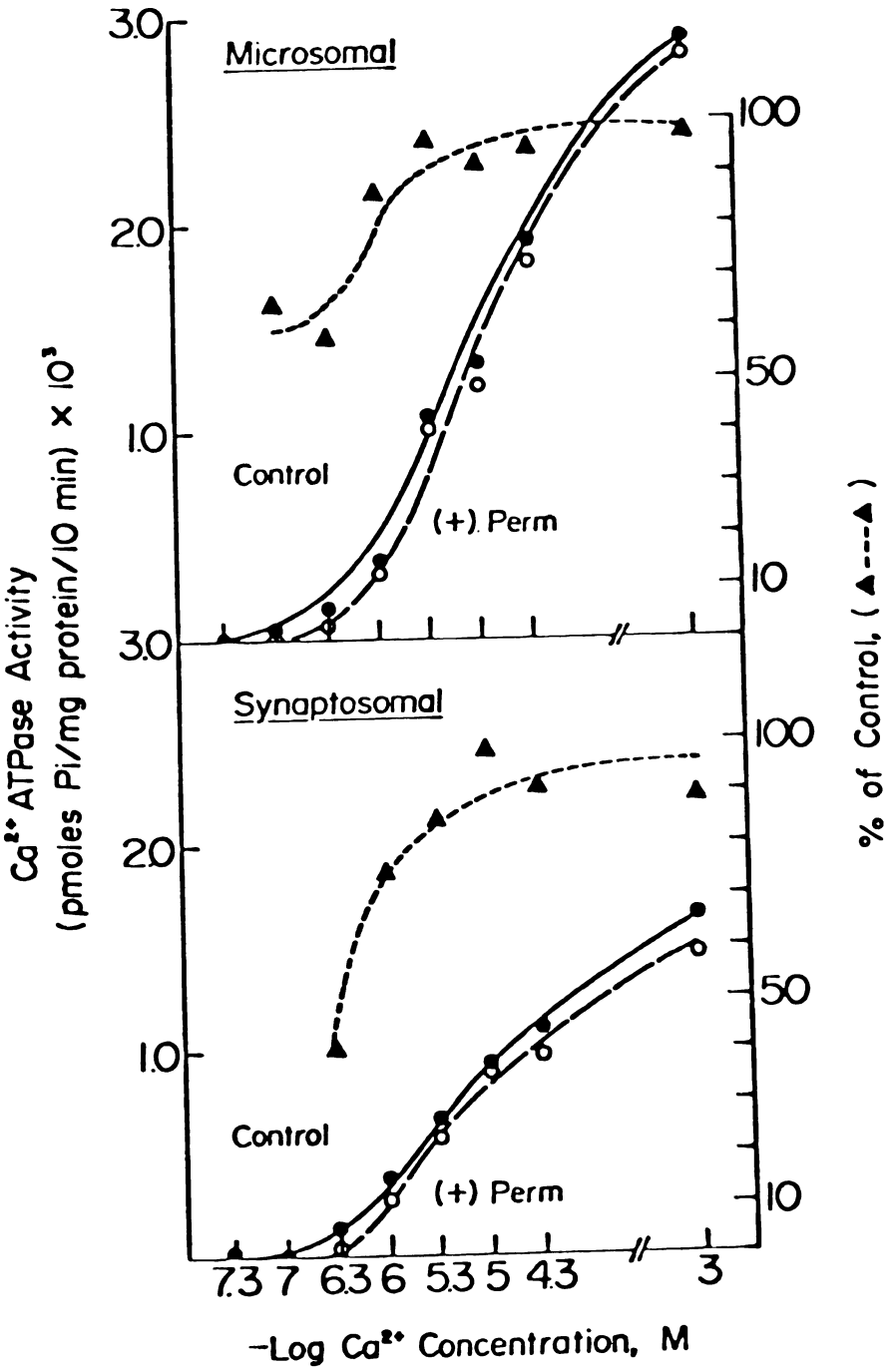

Figure 27. Effect of Ca^{2+} concentration on Ca^{2+} ATPase activity in the presence (○—○) and absence (●—●) of 10^{-6}M permethrin. The difference curve (▲- -▲) is reported as the percentage of activity remaining of an untreated control value which was taken to be equal to 100%. The specific activity of the ATPase refers to Ca^{2+}-stimulated activity of microsomal and intact synaptosomal tissue fractions.

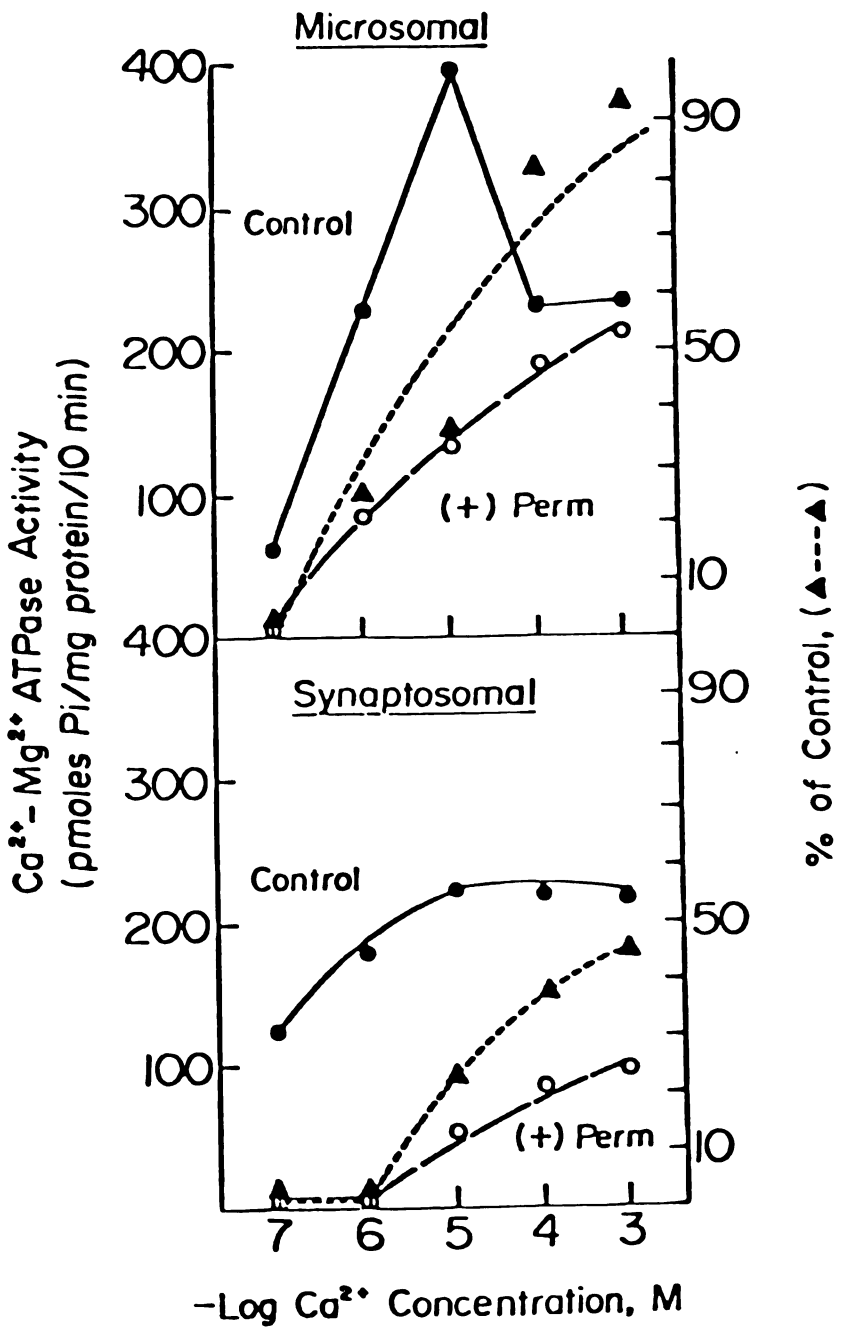

Figure 28. Effect of Ca^{2+} concentration on nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity in the presence (○— —○) and absence (●——●) of 10^{-6} M permethrin. The difference curve (▲- -▲) is reported as the percentage of activity remaining of an untreated control value which was taken to be equal to 100%. The specific activity of the ATPase refers to Ca^{2+}-stimulated activity of microsomal and disrupted synaptosomal tissue fractions.

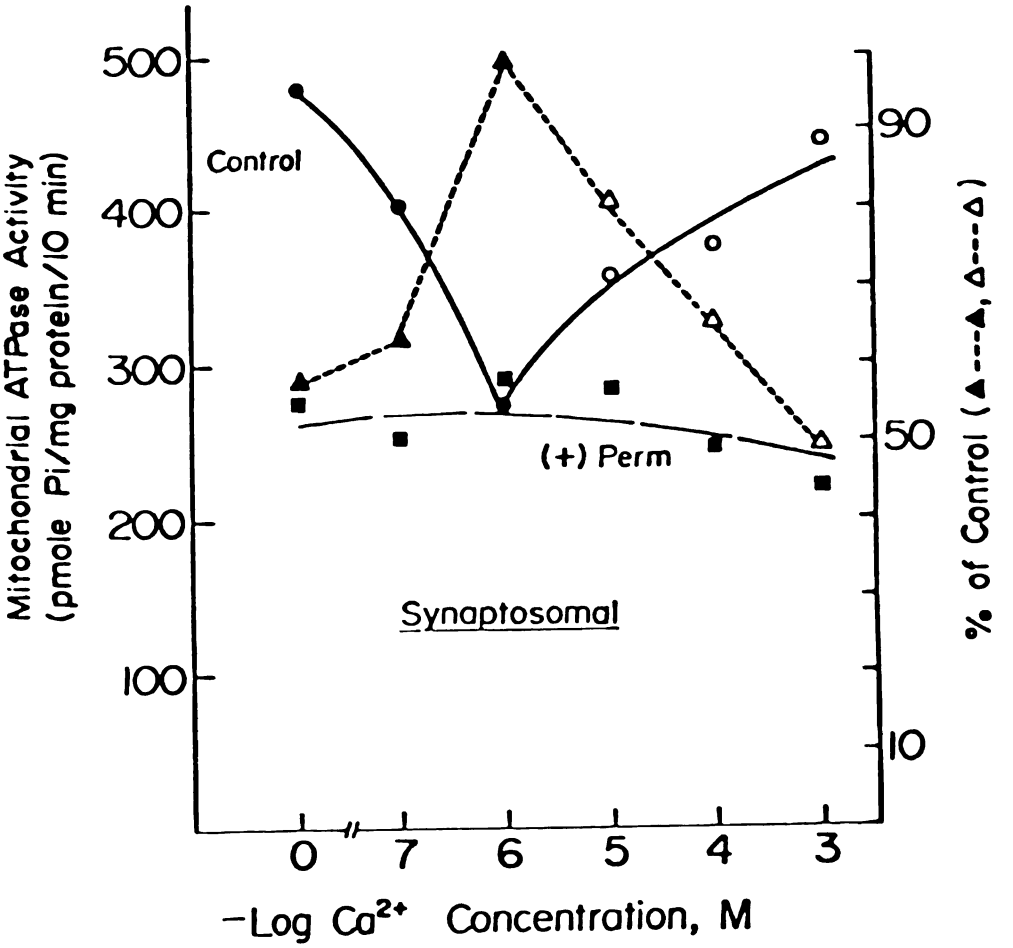

Figure 29. Effects of Ca^{2+} concentration on mitochondrial ATPase activity in the presence (■— —■) and absence (●——○) of 10^{-6}M permethrin. Mitochondrial ATPase activity was determined as that activity sensitive to mitochondrial poisons (see Methods). The difference curve (▲- -△) is reported as the percentage of activity remaining of an untreated control value which was taken to be equal to 100%. The specific activity of the ATPase refers to the activity of the disrupted synaptosomal tissue fraction.

similar means in the presence and absence of 10^{-6}M permethrin. The results are illustrated in Figure 29. In the absence of Ca^{2+} (i.e., basal Mg^{2+} ATPase activity), permethrin caused a 42% reduction in mitochondrial Mg^{2+} ATPase activity. This is similar to the results of Desaiah et al. (1975). However, additions of low levels of Ca^{2+} (10^{-7}-10^{-6}M) caused a decrease in control mitochondrial ATPase activity which was not reflected apparently in the treated samples (i.e., Ca^{2+} inhibition not permethrin inhibition). On further Ca^{2+} addition (10^{-5}-10^{-3}M), there was an observable Ca^{2+}-stimulation of mitochondrial ATPase activity which is sensitive to permethrin inhibition. These results may be of interest because the Ca^{2+}-sequestering aspect of mitochondria has been shown to be inhibitory to the ATP-generating system of mitochondria. Generation of ATP from ADP in the mitochondria has been shown to be related to the F_1 factor (i.e., F_1-ATPase or F_1 synthase) which has been determined as identical to the forward reaction of the oligomycin sensitive Mg^{2+} ATPase activity of the intact mitochondria (Leninger, 1977). Furthermore, it has been shown that the half saturation value for Ca^{2+} uptake by the mitochondria is in the range of 10^{-5}M Ca^{2+} and that at least a proportion of this energy-linked uptake of Ca^{2+} is supported by ATP hydrolysis which is oligomycin sensitive (Malmstrom and Carafoli, 1979). At this point, it may be proper to speculate that the absence of increased ATPase activity in the presence of increasing Ca^{2+} concentration in the synaptosomal fraction (illustrated in Figure 28) may be due to the presence of some endogenous Ca^{2+} buffering system which is capable of stabilizing the free Ca^{2+} concentration at a level lower than the imposed Ca^{2+} load (e.g., $< 10^{-5}$M). Whether or not such buffering is due to mitochondria, calcium binding proteins on some other organelle has not been ascertained at the time.

Nevertheless, both Ca^{2+} ATPase and nonmitochondrial Ca^{2+}-Mg^{2+} ATPase have been shown to have rather high affinities for Ca^{2+} (Figures 27 and 28, respectively) and because Ca^{2+} regulation has been implicated as mandatory for proper nerve function, these ATPases were examined in more detail concerning their sensitivity towards pyrethroids.

Figure 30 illustrates pyrethroid inhibition curves (i.e., allethrin, permethrin and cypermethrin) for Ca^{2+} ATPase at three Ca^{2+} concentrations in microsomal and synaptosomal preparations. Again, it is apparent that not only are these compounds more inhibitory at lower Ca^{2+} concentrations, but that they are also slightly more inhibitory when applied to synaptic preparations. In addition to this, allethrin is seen as consistently more inhibitory to Ca^{2+} ATPase activity than either permethrin or cypermethrin. This increased inhibition attributed to allethrin is apparent irregardless of which tissue fraction is examined.

Similar experiments were performed on Ca^{2+}-Mg^{2+} ATPase and the results are illustrated in Figure 31. The top two graphs represent Total Ca^{2+}-Mg^{2+} activity from both microsomal and synaptosomal fractions. The bottom two graphs represent nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity from the same two fractions, respectively. All three pyrethroids are more inhibitory to the nonmitochondrial activity compared with the total activity regardless of the preparation examined. It is also apparent from Figure 31 that Ca^{2+}-Mg^{2+} ATPase activity is relatively more susceptible to the action of permethrin and cypermethrin than to allethrin. These results are quite similar to those previously reported for the squid (Clark and Matsumura, 1981a) where pyrethrin and its closely related synthetic analog, allethrin, primarily inhibited Ca^{2+} ATPase whereas highly modified pyrethroids such as cypermethrin mainly inhibit Ca^{2+}-Mg^{2+} ATPase.

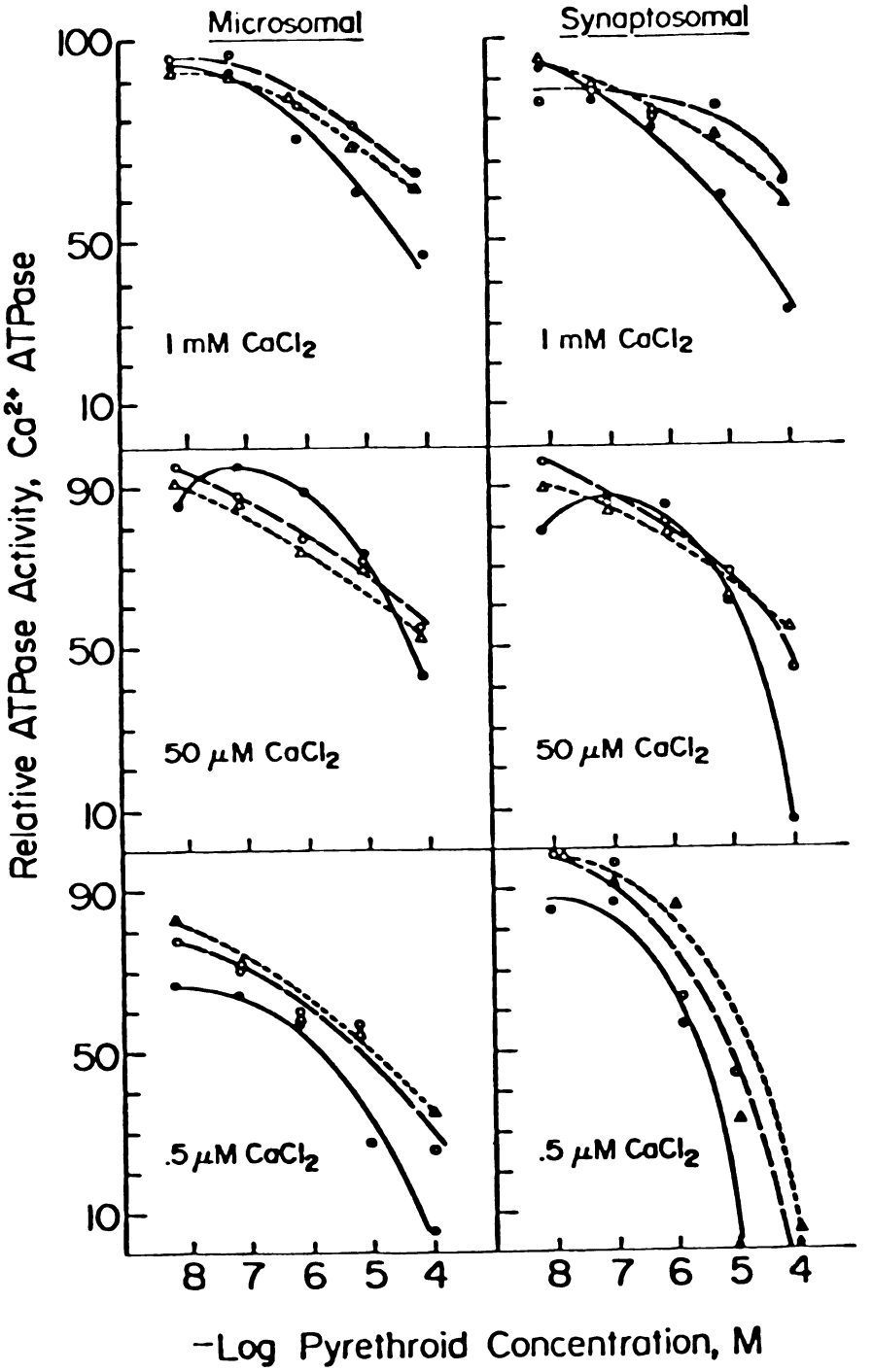

Figure 30. Inhibition of Ca^{2+} ATPase activity by three pyrethroid esters; allethrin (●——●), permethrin (○— —○) and cypermethrin (△- -△). ATPase activity is reported on a relative basis as the percentage of activity remaining of an untreated control value taken as equal to 100% for each Ca^{2+} concentration examined. Ca^{2+}-stimulated activity was examined from microsomal and intact synaptosomal tissue fractions.

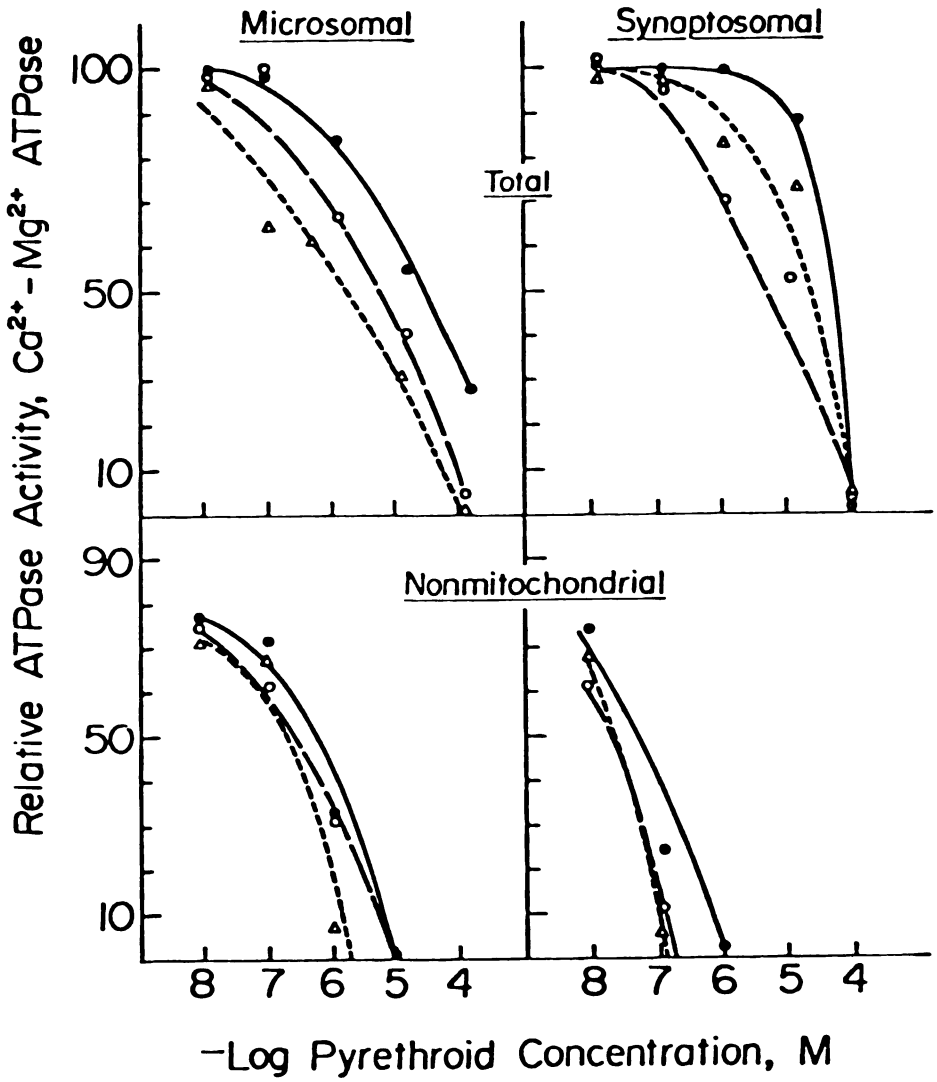

Figure 31. Inhibition of Ca^{2+}-Mg^{2+} ATPase activity by three pyrethroid esters; allethrin (●——●), permethrin (○— —○) and cypermethrin (△- -△). ATPase activity is reported on a relative basis as the percentage of activity remaining of an untreated control value taken as equal to 100%. Ca^{2+}-stimulated activity was utilized from microsomal and disrupted synaptosomal tissue fractions. Total and nonmitochondrial activities are defined in text, see Methods.

In order to determine the relative importance of such in vitro testing, a number of collaborative in vivo and in vitro experiments were performed. The data presented in Table 12 clearly demonstrates that in vivo-administered permethrin results in a reduction of all Ca^{2+}-stimulated ATPase activities when compared to control values. It is also evident that in vivo-administered permethrin is more inhibitory overall to synaptic preparations than to microsomal fractions. Permethrin was relatively more inhibitory to Ca^{2+}-Mg^{2+} ATPase activity than to Ca^{2+} ATPase activity at similar Ca^{2+} concentrations irregardless of the tissue fraction examined. These results are compatible with those reported in vitro.

It was also deemed important to determine relative amounts of permethrin bound to brain fractions of insects severely poisoned with permethrin in vivo and in vitro. This was accomplished radiometrically using ^{14}C-permethrin as an indicator of binding. The results of these experiments are given in Table 13. As judged by ng of ^{14}C-permethrin bound per mg brain protein, the synaptosomes bound the most permethrin, followed by the microsomal fraction and then whole brain. These results correlate well with the data presented in Table 12 in that the ATPase activity of the synaptosomal fraction was consistently more inhibited by permethrin than the microsomal preparation. On the other hand, the in vitro administration of ^{14}C-permethrin was more difficult to interpret.

It was found that at an I_{50} dose of ^{14}C-permethrin administered in vitro, more permethrin was bound to either of the tissue fraction examined than what had been reported previously in vivo. This is apparent even though the lower amount of permethrin bound in vivo gave a relatively greater amount of inhibition (see nonmitochondrial Ca^{2+}-Mg^{2+} ATPase data, Table 12). This discrepancy may have arisen due to the possibility of a greater amount of nonspecific binding in the more highly disorganized in vitro preparation.

Table 12. Effects of In Vivo[a] Administered Permethrin on Microsomal and Synaptosomal ATPase Activities of the Insect Brain

ATPase studied	Tissue fraction	Ca^{2+}-stimulated ATPase activity ± S.E.[b] Control	Treated	Percent of Control[c]
(Ca^{2+} concentration)		(pmole Pi/mg protein/10 min)		
Ca^{2+} ATPase				
1 mM	Microsomal	3272 ± 220	2558 ± 32	78.2
50 µM	Microsomal	1968 ± 232	1533 ± 36	77.9
0.5 µM	Microsomal	179 ± 33	92 ± 22	51.4
1 mM	Synaptosomal	1683 ± 160	1113 ± 16	67.1
50 µM	Synaptosomal	1147 ± 240	754 ± 50	65.8
0.5 µM	Synaptosomal	151 ± 19	0 ± 0	0
Ca^{2+}-Mg^{2+} ATPase[d]				
10 µM	Microsomal	322 ± 60	114 ± 9	35.3
10 µM	Synaptosomal	141 ± 19	25 ± 30	17.9

[a]For in vivo studies, a LD_{95} dose of permethrin was injected intraperitoneally. Once convulsions were established, the roaches were sacrificed and brains fractionated as previously described (see Methods). Specific activity of various enzymes was determined under standard assay conditions, see Table 9.

[b]Specific activities of enzymes are expressed as the means ± standard error (S.E.) from two separate experiments for treated samples and from eight separate experiments for untreated control samples.

[c]Data are expressed as percentage of Ca^{2+}-stimulated ATPase activity remaining in samples treated with permethrin versus untreated control.

[d]Data was determined on nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity only.

Table 13. Binding of ^{14}C-Permethrin to Insect Brain Fractions under *In Vivo* and *In Vitro* Conditions

Tissue fraction	*In vivo*[a]	*In vitro*[b]
	(ng permethrin/mg protein)[c]	
Whole brain	1.97[d]	—
Microsomal	2.44	5.81
Synaptosomal	2.86	3.56

[a]Experimental conditions were exactly as described in footnote a, Table 12 except ^{14}C-permethrin was utilized.

[b]For *in vitro* studies, enzyme was prepared from untreated roaches. An I_{50} dose of ^{14}C-permethrin was added during a standard ATPase assay, see Methods.

[c]Data are expressed as the amount of permethrin bound to the total tissue fraction (20 insect brain equivalents) adjusted to a milligram protein level. Twenty insect brains contained approximately 15.25 mg protein.

[d]Given the wet weight of a brain as 2.06 mg, the apparent cis-permethrin concentration in the brain of a roach showing severe poisoning symptoms is estimated at approximately 729 ppb.

Nonetheless, the amount of ^{14}C-permethrin bound in vitro was of the same magnitude as that determined by in vivo means and may reflect an identicalness in the systems.

Influence of Agents Modifying Ionic Fluxes Across Biomembranes. It is well documented that the nerve membrane maintains an electrical potential across itself by selectively segregating certain inorganic ions. These are principally Na^+ and K^+ions. However, much of the function of excitability is most likely regulated by Ca^{2+} ions. Therefore, attempts were made to determine what effect certain modifiers of ionic flux might have on Ca^{2+}-stimulated ATPases in the insect brain.

The results summarized in Table 14 show that in the case of insect brain ATPases, many of the agents examined produced only marginal effects to be of practical use under the experimental condition. However, there are some notable exceptions. Treatment with the K^+ ionophore, valinomycin, resulted in substantial inhibition of all preparations examined particularly to nonmitochondrial Ca^{2+}-Mg^{2+} ATPase from the synaptic preparation. Pentylenetetrazole (PTZ) has been shown to cause increased bursting activity in the ganglionic nerve cells and has been attributed to the release of intracellular stores of Ca^{2+} (Sugaya and Onozuka, 1978). PTZ also showed a high degree of specificity in its action by inhibiting again the nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity of the synaptosome. Mersalyl acid was quite inhibitory to all fractions examined and particularly to nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity. The local anesthetic, tetracaine, has been reported to cause a large decrease in ATP-dependent Ca^{2+} uptake by nonmitochondrial entities of the synaptosome (Blaustein et al., 1978a). A similar decrease in nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity is now reported in both microsomal and synaptosomal fractions in the insect brain by tetracaine action.

Table 14. Influence of Agents Modifying Na^+, K^+, or Ca^{2+} Flux on Ca^{2+}-stimulated ATPase Activities[a] from Microsomal and Synaptosomal Fractions of the Insect Brain

Additions	1 mM-Ca^{2+} ATPase		Nonmitochondrial Ca^{2+}-Mg ATPase[b]	
	Microsomal	Synaptosomal	Microsomal	Synaptosomal
(Concentration)	% of control ± S.E.[c]			
Na^+ flux modifiers				
veratridine (200 µM)	90.4 ± 1.7	78.8 ± 4.6	88.3 ± 6.8	73.6 ± 0.8
ouabain (.1 mM)	105 ± 7.1	98.2 ± 3.0	111 ± 10.7	101 ± 2.5
TTX (5 nM)	94.9 ± 2.5	85.8 ± 9.4	81.7 ± 1.9	96.6 ± 6.5
K^+ flux modifiers				
valinomycin (50 µM)	55.1 ± 7.0	47.0 ± 0.1	63.4 ± 1.0	15.9 ± 1.0
Ca^{2+} flux modifiers				
A23187 (10 µM)	78.2 ± 2.3	71.0 ± 8.7	109 ± 4.6	-
PTZ (50 mM)	91.7 ± 1.0	81.9 ± 7.0	90.1 ± 1.5	0.4 ± 8.5
Tiapamil (10 µM)	98.6 ± 2.6	86.2 ± 0.2	78.0 ± 3.4	91.9 ± 6.3
TFP (10 µM)	83.2 ± 7.1	65.6 ± 1.5	64.7 ± 2.9	114 ± 8.4
DSP (10 µM)	95.4 ± 0.7	79.2 ± 0.1	67.8 ± 2.1	79.0 ± 2.1
Tetracaine (10 mM)			20.4 ± 2.5	32.6 ± 0.6
ATPase inhibitors				
Mersalyl acid (50 µM)	41.5 ± 2.1	53.2 ± 9.8	0.0	32.1 ± 1.0
Colchicine (10 µM)	94.8 ± 0.4	84.9 ± 2.7	77.0 ± 2.5	95.8 ± 7.1
Cytochalasin B (10 µM)	101 ± 1.9	79.7 ± 2.0	67.3 ± 2.4	113 ± 3.7

[a] Ca^{2+}-stimulated ATPase activity refers to activity described as in footnote f of Table 10.

[b] See footnote h of Table 10.

[c] % of control refers to the percentage of Ca^{2+}-stimulated activity remaining in the presence of an added agent when compared to an untreated control. Percentages are expressed as means ± standard error of two separate experiments (four replicates).

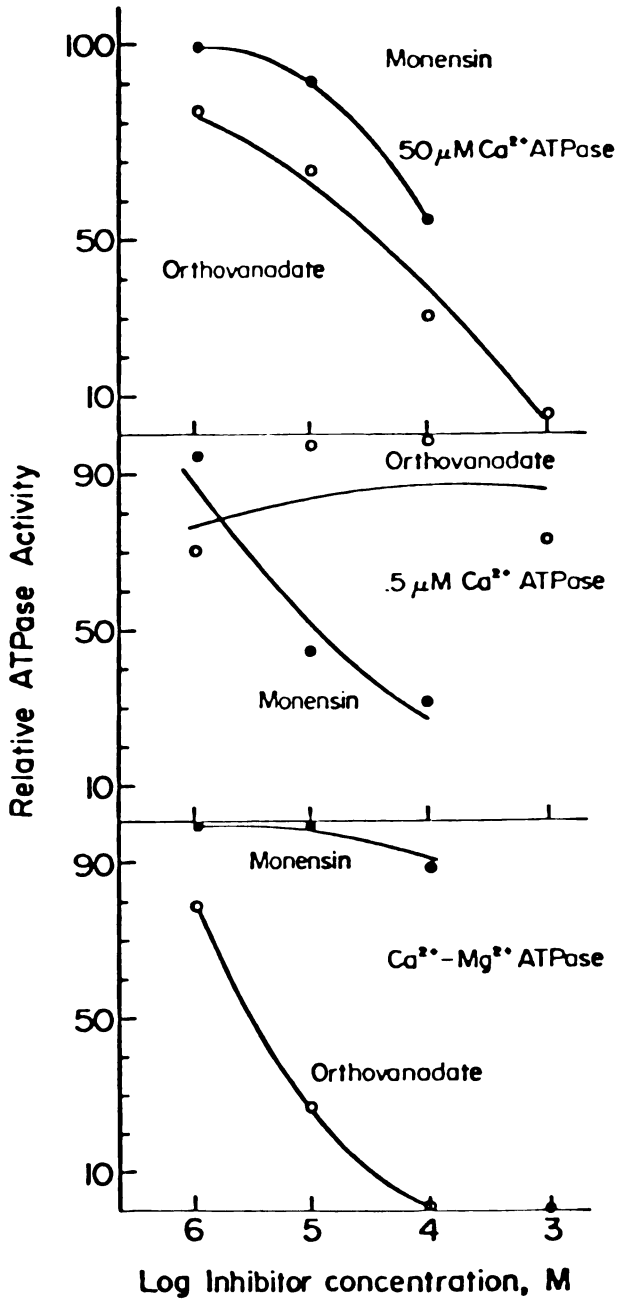

Figure 32. Inhibition of Ca^{2+}-stimulated ATPases by monensin (●——●) and orthovanadate (○——○). ATPase activity is reported on a relative basis as the percentage of activity remaining of an untreated control value taken as equal to 100% for each enzyme system examined. Ca^{2+}-stimulated activity of microsomal tissue fractions was examined. Ca^{2+}-Mg^{2+} ATPase activity refers only to nonmitochondrial activity.

Figure 32 illustrates the effects of two additional flux modifiers on ATPases. These are monensin, a Na^+ ionophore, and orthovanadate, an anionic inhibitor of ATP-dependent Ca^{2+} uptake (Gill et al., 1981). It is apparent from this figure that monensin is increasingly inhibitory to Ca^{2+} ATPase activity as the Ca^{2+} concentration is reduced from 50 μM to 0.5 μM. Over the concentration examined, monensin was ineffective to nonmitochondrial Ca^{2+}-Mg^{2+} activity. On the other hand, orthovanadate was particularly inhibitory to Ca^{2+}-Mg^{2+} ATPase activity. Although orthovanadate also inhibited Ca^{2+} ATPase to some extent at the higher Ca^{2+} concentration (50 μM), by reducing the Ca^{2+} concentration to 0.5 μM this inhibitory response could be eliminated. These results may be important in the assessment of possible mechanisms attributed to ATPase action. Gill et al. (1981) has determined that monensin is a very selective inhibitor of Na^+-Ca^{2+} exchange in membrane vesicles from guinea pig synaptosomes where orthovanadate was principally inhibitory to ATP-dependent Ca^{2+} uptake into these membranes.

The hexavalent dye, ruthenium red, has been shown to inhibit not only Ca^{2+}-stimulated ATPase activity, but also Ca^{2+} transport. At very low concentrations (10^{-8}M), ruthenium red is effective in reducing Ca^{2+} uptake by mitochondria (Carafoli and Crompton, 1978). More recently, however, a number of reports have indicated other cellular aspects which are sensitive to ruthenium red. Watson et al. (1971) found that the Ca^{2+}-pump of the red blood cell was inhibited by ruthenium red ($I_{50}=10^{-5}$M). Rahamimoff and Alnaes (1973) concluded that the main action of ruthenium red (10^{-5}M) on synaptic transmission was at the presynaptic nerve terminal where it decreased the number of quanta of transmitter liberated by the nerve impulse. Ruthenium red had the additional effect of also increasing the frequency of miniature end plate potentials. As represented in Figure 33, ruthenium red reduces Ca^{2+} ATPase

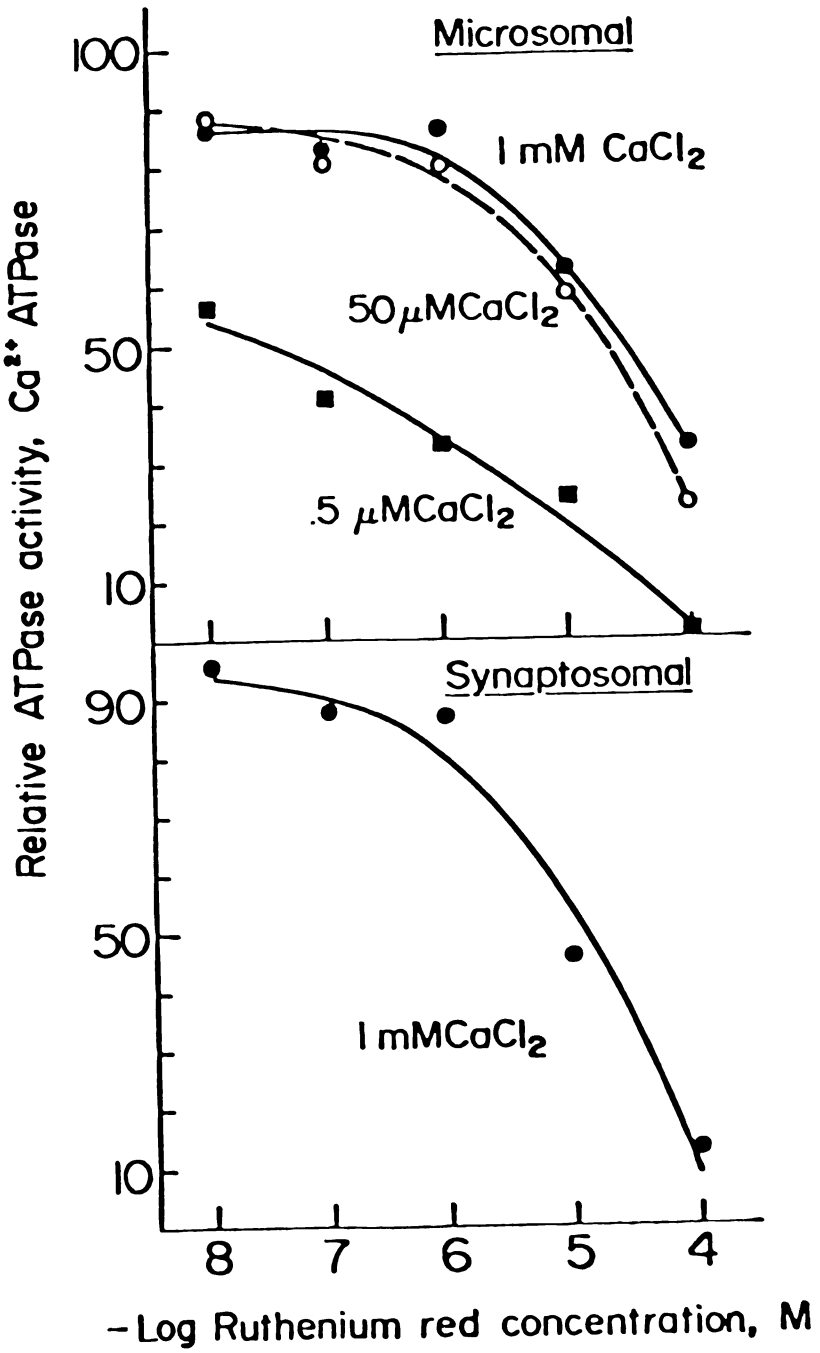

Figure 33. Effect of ruthenium red concentration on Ca^{2+} ATPase activity at three concentrations of Ca^{2+}; 1 mM (●——●), 50 μM (○– – ○) and 0.5 μM (■——■). ATPase activity is reported on a relative basis as the percentage of activity remaining of an untreated control value taken as equal to 100% for each Ca^{2+} concentration examined. Only Ca^{2+}-stimulated activity was examined for ruthenium red sensitivity in both microsomal and intact synaptosomal tissue fractions.

activity of the insect brain in both microsomal and synaptosomal fractions, particularly at concentrations at or above 10^{-5}M. Ruthenium red produced a greater efffect at lower Ca^{2+} concentrations, and was noticeably more inhibitory when applied to synaptic preparations.

Nonmitochondrial Ca^{2+}-Mg^{2+} ATPase was exmained in a similar manner, and the results are illustrated in Figure 34. Again, Ca^{2+}-Mg^{2+} ATPase activity in both brain fractions was significantly reduced by ruthenium red concentrations of 10^{-5}M or greater. It should be noted, however, that in the synaptic fractions, Ca^{2+}-Mg^{2+} ATPase activity was actually stimulated by low levels of ruthenium red (10^{-8}-10^{-6}M).

The rare-earth element, lanthanum (La^{3+}), has been shown also to inhibit both Ca^{2+} transport and Ca^{2+}-stimulated ATPase activity (van Breemen and de Weer, 1970; Baker, 1978; Matsumura and Clark, 1980). This situation was examined using microsomal brain fractions and the findings of these experiments are reported in Figure 35.

From these results, it is apparent that lanthanum is inhibitory to Ca^{2+}-stimulated ATPases only at high lanthanum concentrations (10^{-3}M) which collaborates with other reports (Baker, 1978; Matsumura and Clark, 1980). However, at very low Ca^{2+} concentrations (0.5 μM), Ca^{2+} ATPase activity is greatly enhanced by 10^{-4}M lanthanum. This finding is very reminiscent to that of Baker and McNaughton (1978), who observed a transient rise in Ca^{2+} efflux from the giant axon of the squid only when exposed to Ca^{2+}-free sea water in the presence of 0.3 mM La^{3+}. As in the case with ruthenium red (Figure 34), nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity is increasingly stimulated by low La^{3+} concentrations up to 10^{-4}M. However, ruthenium red stimulation was only apparent in synaptosomal preparations whereas La^{3+} stimulation occurred in the microsomal fraction.

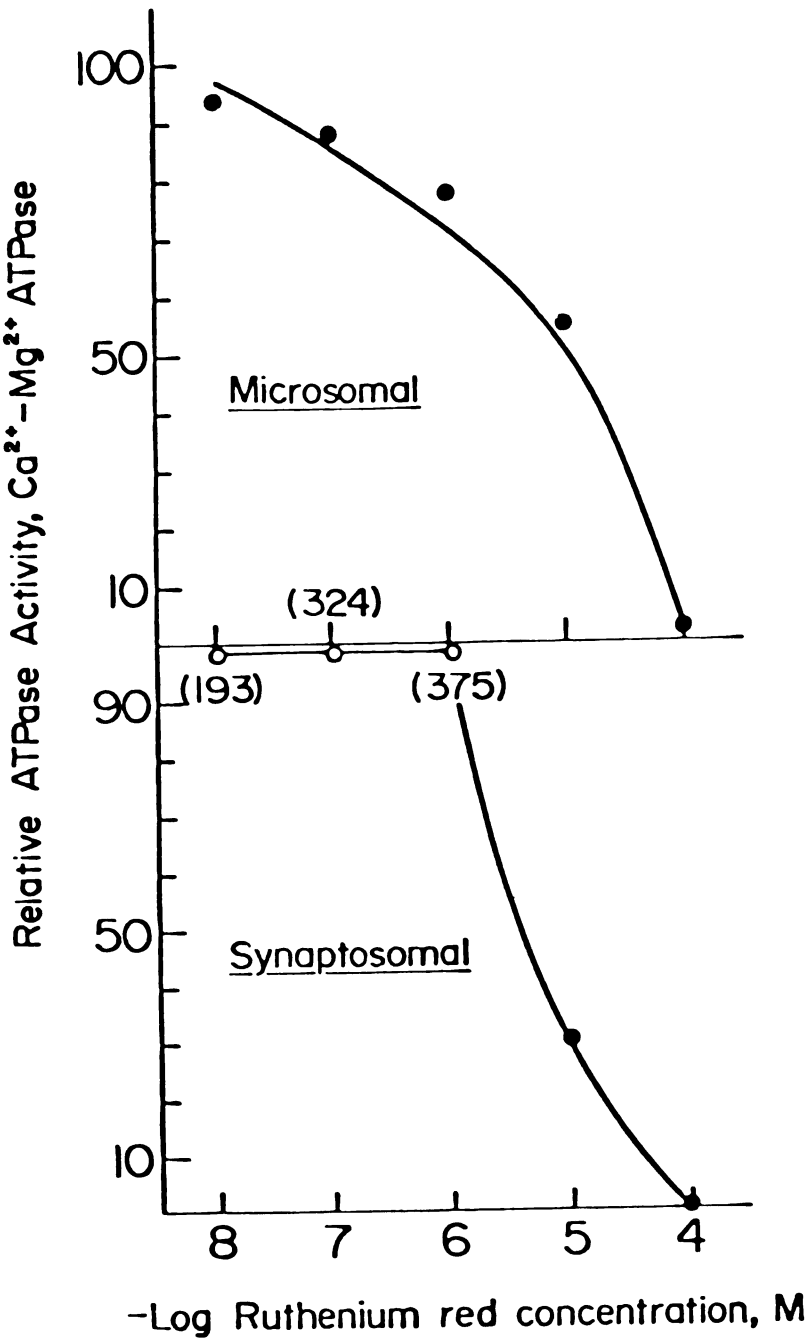

Figure 34. Effect of ruthenium red concentration on nonmitochondrial Ca^{2+}-Mg^{2+} ATPase activity. ATPase activity is reported on a relative basis as percentage of activity remaining of an untreated control value taken as equal to 100%. Only Ca^{2+}-stimulated activity was examined for ruthenium red sensitivity in both microsomal and disrupted synaptosomal tissue fractions. Open circles (O) indicate stimulation of sample value above untreated control. The number in parentheses indicate the percentage of stimulation.

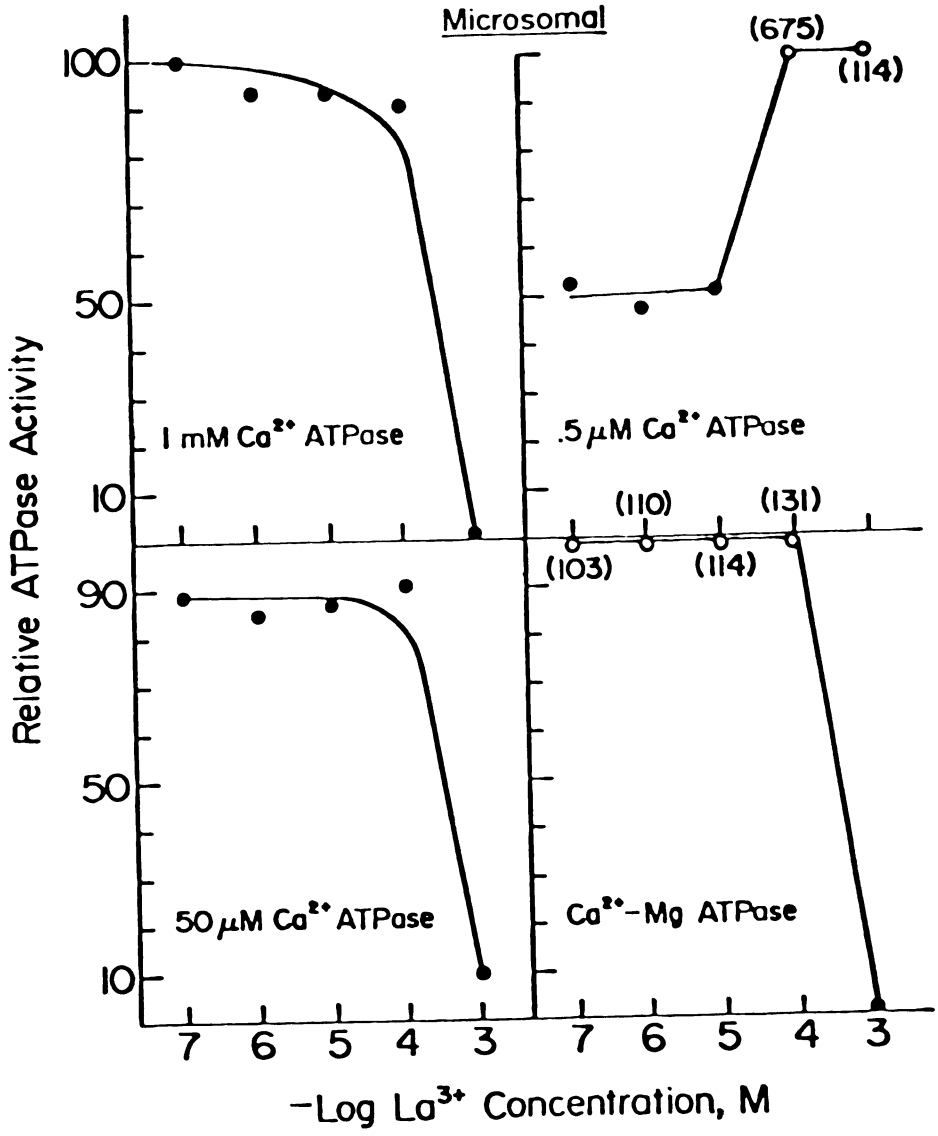

Figure 35. Effect of La^{3+} concentration on Ca^{2+}-stimulated ATPase activity. ATPase activity is reported on a relative basis as the percentage of activity remaining on untreated control value taken as equal to 100% for each enzyme system and Ca^{2+} concentration examined. Only Ca^{2+}-stimulated activity was examined for La^{3+} sensitivity in both microsomal tissue fractions. Open circles (O) indicate stimulation of sample value above untreated control. The numbers in parentheses indicate the percentage of stimulation. Ca^{2+}-Mg^{2+} ATPase activity reported is nonmitochondrial.

DISCUSSION

As previously reported in the squid (Clark and Matsumura, 1981a) and now verified in the American cockroach, pyrethroid insecticides are clearly inhibitory to Ca^{2+}-stimulated ATPases. In both systems, allethrin, a closely related synthetic analog of pyrethrin, primarily inhibited Ca^{2+} ATPase, whereas highly modified pyrethroids such as cypermethrin mainly inhibited Ca^{2+}-Mg^{2+} ATPase. Permethrin behaved as an intermediate between these two extremes in this regard. The insect is also similar to the squid with respect to the site of sensitivity of Ca^{2+}-stimulated ATPases towards pyrethroid inhibition. In both organisms, Ca^{2+}-Mg^{2+} ATPase appears more sensitive to pyrethroid action than Ca^{2+} ATPase although both are clearly inhibited. In the cockroach, the synaptic fraction is more inhibited by pyrethroid action than the microsomal preparation with nonmitochondrial Ca^{2+}-Mg^{2+} ATPase of the synaptosomes being the most sensitive ATPase examined.

This may be particularly important in that it has been shown in the presynaptic region of the nerve that a major function of Ca^{2+}-Mg^{2+} ATPase is to regulate the internal Ca^{2+} level and thereby control the rate of transmitter release (Rahamimoff et al., 1976). Inhibition of Ca^{2+}-Mg^{2+} ATPase in this region by insecticide action is expected to cause increased levels of ionized Ca^{2+} which would promote increased transmitter release leading to synaptic facilitation (Yamaguchi et al., 1980). Presynaptic facilitation of the nerve is an established event occurring during pyrethroid poisoning (Wouters and van den Bercken, 1978).

These in vitro findings have been substantiated by in vivo experiments where permethrin was administered to live roaches. All ATPase activities which were examined were reduced by this treatment, especially nonmitochondrial Ca^{2+}-Mg^{2+} ATPase of the synaptosome. This is the first reported incidence of

in vivo inhibition of Ca^{2+}-stimulated ATPases by pyrethroid insecticides in roaches which clearly showed symptoms of pyrethroid toxicity. More importantly, this in vivo inhibition occurred in the presence of a similar amount of insecticide which was seen to cause a substantial amount of inhibition in vitro.

The synaptosomal fraction of the insect brain also facilitated preliminary attempts to assign probable cellular locations for these enzymes. Ca^{2+} ATPase was found to be active to similar extents in both intact synaptosomal and microsomal preparations. Its characteristics such as stability to cold storage, temperature insensitivity and interaction with various inhibitory agents without membrane disruption indicates an external location for this ATPase on, or an easy access from the outside through, the plasma membrane.

The absence of Ca^{2+} stimulation of Ca^{2+}-Mg^{2+} ATPase of the intact synaptosome and its appearance upon saponin-disruption indicate that this enzyme is probably internalized and most likely associated with a cholesterol-poor membrane system such as the endoplasmic reticulum. Its insensitivity to many mitochondrial poisons and its selective inhibition by trypsin digestion clearly delineates it as nonmitochondrial. This Ca^{2+}-Mg^{2+} ATPase has been shown to be rather unstable, highly temperature sensitive and inhibited by the presence of Na^{+}, Li^{+} and tetracaine. Such a difference in localization indicates that these Ca^{2+}-stimulated ATPases are independently involved in the homeostasis of Ca^{2+} within the nerve cell.

It has been generally accepted that ionized Ca^{2+} within the cell is maintained at submicromolar levels but probably above 10^{-7}M (Baker, 1978; Blaustein et al., 1978b; DiPolo, 1978). This means that the cell maintains a Ca^{2+} gradient of approximately 10^{4}-fold across its plasma membrane. This arrangement of millimolar quantities of Ca^{2+} on the outside and submicromolar quantities on the inside has been shown to be an absolute necessity for

excitability (Tasaki, 1974). Too little Ca^{2+} on the outside or too much Ca^{2+} on the inside results in nerve instability and loss of nerve function. In order to establish and maintain such a Ca^{2+} gradient, the nerve cell must have evolved very efficient Ca^{2+} transport mechanisms.

Because of the apparent dependence on external Na^{+} for Ca^{2+} extrusion, it was first postulated that most of the energy necessary for Ca^{2+} extrusion was provided by the transmembrane Na^{+} electrochemical gradient (Blaustein and Hodgkins, 1969). At micromolar concentrations of intracellular Ca^{2+} and above, this finding is for the most part valid. However, as Ca^{2+} is removed from the cell to approximate physiological conditions, this Na^{+}-Ca^{2+} exchange mechanism becomes progressively more dependent on endogenous ATP (Blaustein and Santiago, 1977). At physiological levels of Ca^{2+} in the giant axon of the squid (0.02-0.06 μM), no Na^{+}-dependent Ca^{2+} efflux was observed without ATP. In addition to this, the majority (50-60%) of Ca^{2+} efflux under these conditions appeared to be due to the action of a Ca^{2+}-pump driven by ATP (DiPolo, 1978). Such an arrangement is obviously suitable for long-term maintenance for low internal Ca^{2+} concentrations but what happens during periods of electrical depolarization when the potential barrier across the nerve is collapsed and ions, including Ca^{2+}, run down their respective concentration gradients?

Whether or not the influx of Ca^{2+} which occurs during nerve cell depolarization outstrips the cell effluxing mechanism is not known at this time. However, it has been shown that during such influx, a number of intracellular organelles apparently accumulate Ca^{2+} very rapidly and only to give it back up during periods of rest (Blaustein et al., 1978b). The most important of these intracellular Ca^{2+} sequestering entities are; mitochondria, Ca^{2+}-binding proteins (CaBP), and nonmitochondrial Ca^{2+} sequestering membrane systems which are probably endoplasmic reticular in origin.

The apparent half-saturation constant for Ca^{2+} uptake has been approximated at 10^{-5}M for mitochondria. Because of this, mitochondria have been characterized as rather large capacity but low affinity Ca^{2+}-sequestering sites (Carafoli and Crompton, 1978). Rahaminoff et al. (1976) reported that mitochondria were capable of reducing external Ca^{2+} concentrations to approximately 10^{-7}M in vitro but this required a relatively long period of time. Also, the mechanisms of Ca^{2+} sequestration by the mitochondria have been shown to be mutually inhibitory to oxidative phosphorylation reactions which principally occur there. For these reasons, mitochondria are not considered to be the major regulator of Ca^{2+} under physiological conditions.

The alternative situation is apparently true for Ca^{2+}-binding proteins such as calmodulin (Baker and Schlaepfer, 1975). They have reported that a number of axoplasmic proteins of the giant axon of the squid bind Ca^{2+} with high affinity but calculated that only 5-10% of the Ca^{2+} load in the axoplasm could exist in this fashion before the capacity of the system was reached. Therefore, it is not likely that Ca^{2+}-binding proteins could serve as a suitable intracellular Ca^{2+} buffering system with the necessary capacity.

Brinley et al. (1977) have reported that 93-95% of an imposed Ca^{2+} load introduced to the giant axon of the squid was buffered by processes insensitive to the oxidative uncoupling agent, FCCP. The indication was that only 6% of a physiological amount of Ca^{2+} was stored in mitochondria with the remainder stored nonmitochondrially. Henkart (1975) first suggested the endoplasmic reticulum as a possible site of Ca^{2+} sequestering when it was found that the endoplasmic reticulum of the axoplasm of the giant axon of the squid swelled in the presence of Ca^{2+}. Further studies (Henkart et al., 1978) verified this initial finding by showing a Ca^{2+}-oxalate precipitant in the endoplasmic reticulum of Ca^{2+}-loaded axons. By these means, it was concluded that the endoplasmic

reticulum has approximately four to five times more volume than mitochondria which may be available for Ca^{2+}-sequestration.

Blaustein et al. (1978a) also reported a very similar situation in the presynaptic nerve terminals (synaptosomes) from rat brain. They concluded that this nonmitochondrial Ca^{2+}-sequestering system (presumably endoplasmic reticulum) is ATP-dependent in a fashion much the same as Ca^{2+} pump mechanisms of the sarcoplasmic reticulum. Furthermore, they have determined the half-saturation constant for Ca^{2+} uptake to be approximately 0.35 μM and a half-saturation constant for ATP to be approximately 10 μM. Therefore, it seems apparent that this high affinity system could very well control Ca^{2+} at very low concentration levels. They summarize the physiological importance of this system as primarily functioning in the buffering of Ca^{2+} that enters the nerve terminals at low stimulation frequencies.

From these statements, a generalized pattern of Ca^{2+} flux can be deduced. Upon depolarization, Ca^{2+} passively enters the nerve cell increasing ionized Ca^{2+} concentration. Due to the time constraints necessary for proper nerve impulse integration and the availability of sequestering sites versus effluxing sites, it is believed that the large proportion of the ionized Ca^{2+} is bound or sequestered internally in the manner described above. This would rapidly reestablish internal Ca^{2+} at resting levels (e.g. < 0.5 μM). As the free Ca^{2+} concentration is reduced, these Ca^{2+} stores are slowly released and distributed back to the plasma membrane where they are removed from the cell by effluxing mechanisms discussed above. Failure of either Ca^{2+}-sequestering or effluxing mechanisms would result similarly in increased ionized Ca^{2+} levels within the cell. The consequence of such an event would be eventual depolarization and conduction blockage in the axon and initially synaptic facilitation followed by depolarization and transmission blockage in the presynaptic nerve terminal

(synaptosomes). The scenario outlined here is very similar to those effects observed during pyrethroid poisoning.

Early electrophysiological results showed that pyrethrin poisoning progressively caused increased spontaneous nerve activity, repetitive discharges and eventual blockage of nerve axonal conduction (Schallek and Wiersma, 1948). The synthetic pyrethroid, allethrin, also acted in much the same manner, causing a supression of the action potential amplitude, an elevated resting potential and eventual blockage of electrical conduction (Narahashi, 1965). Such results are clearly similar to those obtained from hypocalcemic nerve preparations.

Burt and Goodchild (1971) first recognized that pyrethroids may also have an action on the synaptic region of the nerve by showing a high degree of sensitivity to the ganglionic proportions of the nerve cord. Clements and May (1977) and van der Bercken et al. (1973) both have implicated sensory organs as sites of action of pyrethroids and have concluded that the repetitive sensory activity seen during poisoning was apparently associated with the impulse-generating processes in the afferent nerve terminals. Implicit in these findings is that synaptic facilitation may have been involved in sensory organ hyperexcitability.

At this point, it is tempting to draw some correlations between the Ca^{2+}-stimulated ATPases (i.e., Ca^{2+} and Ca^{2+}-Mg^{2+} ATPase) which are clearly sensitive to pyrethroid action and the Ca^{2+} transport mechanisms which control Ca^{2+} regulation in the nerve cells and hence their excitability. In nerve cells which are undergoing low stimulation frequencies, the major Ca^{2+} transporting mechanisms are thought to be ATP-dependent Na^{+}-Ca^{2+} exchange in the plasma membrane and nonmitochondrial ATP-dependent Ca^{2+} uptake (i.e., Ca^{2+}-pump) of the endoplasmic reticulum (Blaustein and Santiago, 1977; Blaustein et al., 1978b).

Na^+-Ca^{2+} exchange has been characterized by the following phenomena; external Na^+ is required, Li^+ is a poor substitute but not apparently inhibitory, Mg^{2+} has no effect, electrical depolarization or depolarization by K^+ inhibited the process, Lanthanum (1 mM) is inhibitory and tetrodotoxin (TTX) and Ca^{2+} antagonists such as D600 have no effect (Baker, 1978). The exchange is not highly temperature sensitive with a Q_{10} of 2.7 (Baker et al., 1969) and ATP acts as a catalyst or modulator but not apparently as an energy source (i.e., not a metabolic pump, Blaustein and Santiago, 1977 and Mullins, 1977). The Na^+ ionophore, monensin, selectively inhibits this process (Gill et al., 1981). All of these phenomena have been also reported for Ca^{2+} ATPase activity or are in accordance with these findings (Matsumura and Clark, 1980; Clark and Matsumura, 1981b).

Nonmitochondrial ATP-dependent Ca^{2+} uptake (i.e., intraterminal Ca^{2+} storage system) has also been characterized (Blaustein et al., 1978b; Blaustein et al., 1978a; Gill et al., 1978). The following are those characteristics which are in common with Ca^{2+}-Mg^{2+} ATPase; a high Ca^{2+} affinity, the presence of Mg^{2+} is required, Na^+ and Li^+ are inhibitory, both are insensitive to mitochondrial poisons and low concentrations of ruthenium red (10^{-8}M), both systems are exposed by saponin-pretreatment of intact terminals but saponin is not itself inhibitory, the activity of each is inhibited by trypsin digestion, tetracaine, mersalyl and orthvanadate (Matsumura and Clark, 1980; Clark and Matsumura, 1981b).

Such correlation is interesting but speculative at best and is not presented as evidence of the identicalness of the two systems (e.g., Ca^{2+} ATPase = Na^+-Ca^{2+} exchange and Ca^{2+}-Mg^{2+} ATPase = Ca^{2+} pump of the endoplasmic reticulum). To verify this relationship, pyrethroid inhibition will have to be demonstrated on ATP-dependent Na^+-Ca^{2+} exchange and ATP-dependent Ca^{2+}

uptake mechanisms. Nevertheless, the above data clearly supports the need for such investigations.

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