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EFFECT OF CHLORDECONE ON pH AND TEMPERATURE DEPENDENT SUBSTRATE ACTIVATION KINETICS OF RAT BRAIN SYNAPTOSOMAL ATPases

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Abstract—Chlordecone, a polycyclic chlorinated insecticide known as Kepone, inhibited the activities of $(Na^+–K^+)$ATPase and Mg^{2+}–ATPase in rat brain synaptosomes. Altered pH and specific activity curves for both enzymes demonstrated significant inhibition by chlordecone in buffered acidic, neutral and alkaline pH ranges. Noncompetitive inhibition with respect to activation by ATP in the case of $(Na^+–K^+)$ATPase was indicated by altered V_{max} values with no significant change in K_m values at any pH studied, except at pH 9.5. Mg^{2+}–ATPase was inhibited uncompetitively as evidenced by altered V_{max} and K_m values. The activities of both ATPases were decreased in the presence of chlordecone at higher temperatures. Activation energy (ΔE) values were found to be decreased significantly in the presence of chlordecone at 37°. Arrhenius plots of both ATPases preincubated with chlordecone were found to be nonlinear. In the presence of chlordecone, V_{max} was decreased without significant change in K_m values for $(Na^+–K^+)$ATPase at all temperatures, suggesting a noncompetitive type of inhibition. In the case of Mg^{2+}-ATPase, similar noncompetitive type inhibition was obtained at 27° but not at 32 and 37°. The kinetic data in general suggest that the chlordecone inhibited $(Na^+–K^+)$ATPase noncompetitively and Mg^{2+}-ATPase uncompetitively at all pHs and temperatures studied. The present data suggest that inhibition of $(Na^+–K^+)$ATPase and Mg^{2+}-ATPase, the two membrane-bound enzymes in synaptosomes, by chlordecone is temperature dependent and pH independent.

It is well documented that chlordecone causes a number of untoward effects in higher animals such as decreased reproduction [1, 2], cellular alterations [1, 3], hepatic carcinoma [4, 5], and hepatobiliary dysfunction [6–8]. In addition, numerous biochemical studies with chlordecone showed altered mitochondrial enzymatic activities and energy metabolism in different cells [9–12].

Chlordecone interferes with the cellular energy metabolism by inhibiting ATP synthesis (oligomycin-sensitive Mg^{2+}-ATPase) in mitochondria as well as ATP hydrolysis [$(Na^+–K^+)$ATPase] reactions [13]. Based on these studies it was proposed that the membrane-bound ATPase system might be the prime target for chlordecone toxicity [13–16]. Mg^{2+}-dependent $Na^+–K^+$-stimulated ATPase, a structural component of various tissue cell membranes [17], is shown to be involved in the transport of Na^+ and K^+ across the cellular membranes [18–21]. Several studies suggest that the uptake process of catecholamines in the central nervous system is dependent on $(Na^+–K^+)$ATPase, which may serve as a receptor located at or near the receptor site of the catecholamines [22–25]. The mitochondrial membrane contains an oligomycin-sensitive Mg^{2+}-ATPase which is involved in the terminal step of oxidative phosphorylation resulting in the synthesis of ATP [26]. These two membrane-bound enzymes are inhibited both *in vitro* and *in vivo* by chlordecone in different cell types [13]. Since the kinetic parameters of membrane-bound enzymes often display complex temperature and pH dependence patterns [27], the present study was initiated to study the *in vitro* effects of chlordecone on temperature and pH dependent substrate activation kinetics of $(Na^+–K^+)$ATPase and Mg^{2+}-ATPase in the rat brain synaptosomes in an effort to understand the mechanism of enzyme inhibition by chlordecone.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 175 g each were obtained from the Charles River Breeding Laboratory, Wilmington, MA. All biochemicals used for enzyme assays were obtained from the Sigma Chemical Co., St. Louis, MO. Chlordecone (99% pure) was provided by the U.S.E.P.A. Pesticide Repository.

Rats were decapitated and the brains were quickly removed and placed in ice-cold 0.32 M sucrose solution containing 1.0 mM EDTA and 10 mM imidazole, pH 7.5. Synaptosomes of each brain were prepared by a Ficoll–sucrose gradient procedure [16, 25, 28]. The tissues were homogenized in 9 vol. of sucrose solution with a ground-glass homogenizer. Homogenates were centrifuged at 750 *g* for 10 min, and the pellets were resuspended in sucrose solution and then recentrifuged at 17,000 *g* for 20 min. Pellets were resuspended in 10 ml of sucrose solution and layered on two-step discontinuous Ficoll–sucrose gradients consisting of 13% (w/v) Ficoll in 0.32 M sucrose and 7.5% Ficoll (w/v) in 0.32 M sucrose.

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After centrifugation at 65,000 *g* for 45 min, synaptosomal fractions were obtained at the interfaces of the 7.5 and 13% Ficoll–sucrose layers. Synaptosomal bands were removed, diluted with 9 vol. of sucrose solution, and centrifuged at 17,000 *g* for 10 min. Synaptosomal pellets were resuspended in the appropriate amount of sucrose solution, divided into small aliquots and quick-frozen in liquid nitrogen. Frozen samples were stored at −80°.

Determination of ATPase activities

A coupled enzymatic method was used to determine ATPase activity [24, 29]. The 3-ml standard reaction mixture contained: 3 mM ATP, 3 mM Mg^{2+}, 100 mM Na^+ 20 mM K^+, 135 mM imidazole/HCl buffer (pH 7.5), 0.2 mM NADH, 0.5 mM phosphoenol pyruvate, 9 units of pyruvate kinase, and 12 units of lactic acid dehydrogenase. Synaptosomal preparations (50 μl) with a protein content of 20–30 μg were used. The reaction rate was proportional to the amount of protein used in this study. Absorbance changes in the reaction mixture which were linear were measured at 340 nm over 10 min for calculation of specific activity. Enzyme activity was expressed as micromoles of inorganic phosphate per milligram of protein per hour. The effect of chlordecone was assessed by preincubating the enzyme with chlordecone before the reaction was started with ATP.

Total ATPase activity was measured with Mg^{2+}, Na^+ and K^+ present in the reaction mixture. Mg^{2+}-ATPase was measured in the presence of 1 mM ouabain, a specific inhibitor of (Na^+–K^+)ATPase. Ouabain-sensitive (Na^+–K^+)ATPase was obtained as the difference between total ATPase activity and Mg^{2+}-ATPase activity. Protein was determined by the method of Lowry *et al.* [30] using bovine serum albumin as the standard.

Kinetic analysis

Kinetic analysis of the effects of chlordecone on various substrate activation parameters of rat brain synaptosomal ATPase at various pH and temperature settings was undertaken to determine the nature of the inihibition. Kinetic analyses were performed as described by Ahmed *et al.* [31] and Phillips *et al.* [32]. Variations in chlordecone concentration as well as mean apparent K_m and V_{max} values for independent studies are listed under results or in the figure legends.

Expression of results

Each point on the graphs is the mean ± S.D. of at least four different synaptosomal preparations, and each preparation was assayed three times. Double-reciprocal plots of kinetic data were constructed according to the method of Lineweaver and Burk [33]. Data were subjected to regression analysis and the regression lines plotted for best straight-line fit. Data were also analyzed by Student's *t*-test to assess differences between control and experimental treatments; a value of $P < 0.05$ was considered significant.

RESULTS

Effects of pH on chlordecone inhibition of ATPases

The pH of individual incubation mixtures was varied from 5.5 to 9.5 in imidazole/HCl buffer [31]. A pH optimum of 7.5 was observed for both ATPases (Figs. 1 and 2). Inhibition by chlordecone (both at 1.5×10^{-6} and 3×10^{-6} M) was independent of pH throughout the range studied.

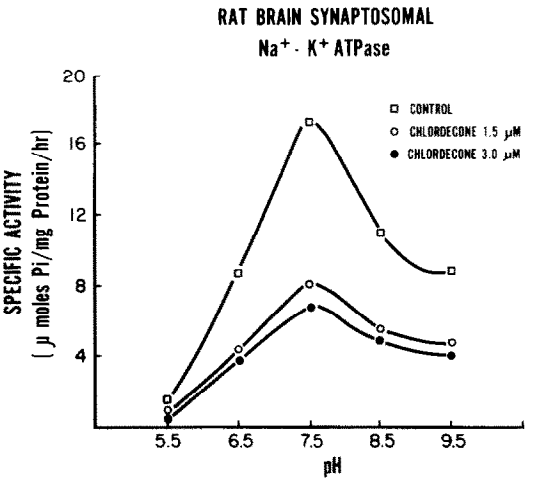

Fig. 1. Effect of pH on inhibition of rat brain synaptosomal (Na^+–K^+)ATPase by chlordecone.

Kinetic analysis

Substrate activation parameters of brain synaptosomal (Na^+–K^+)ATPase and Mg^{2+}-ATPase were examined at pH 6.5, 7.5, 8.5 and 9.5 and at two concentrations of chlordecone (Fig. 3(A–D) and Table 1). Activation of both ATPases at low-affinity nucleotide-directed sites by ATP was assayed by varying the ATP concentration from 0.5 to 3 mM, while maintaining all other assay conditions constant. Double-reciprocal plots of ATP-activated (Na^+–K^+)ATPase showed a decrease in the apparent V_{max} and an increase in apparent K_m at pH of 6.5, 8.5 and 9.5 when compared to optimum pH (7.5). In the presence of chlordecone, similar reductions in apparent V_{max}, without significant changes in apparent K_m (except at pH 9.5), were observed. In the

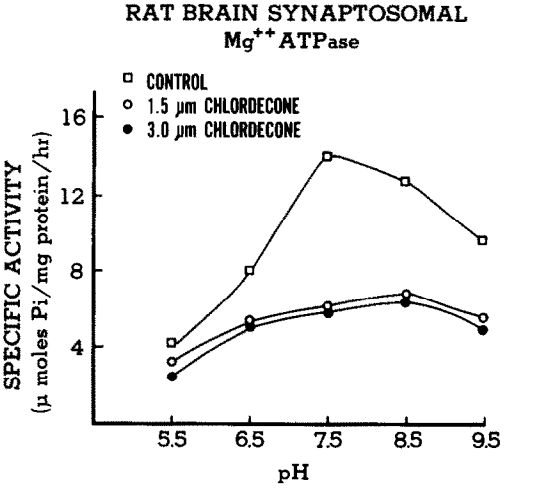

Fig. 2. Effect of pH on inhibition of rat brain synaptosomal Mg^{2+}-ATPase by chlordecone.

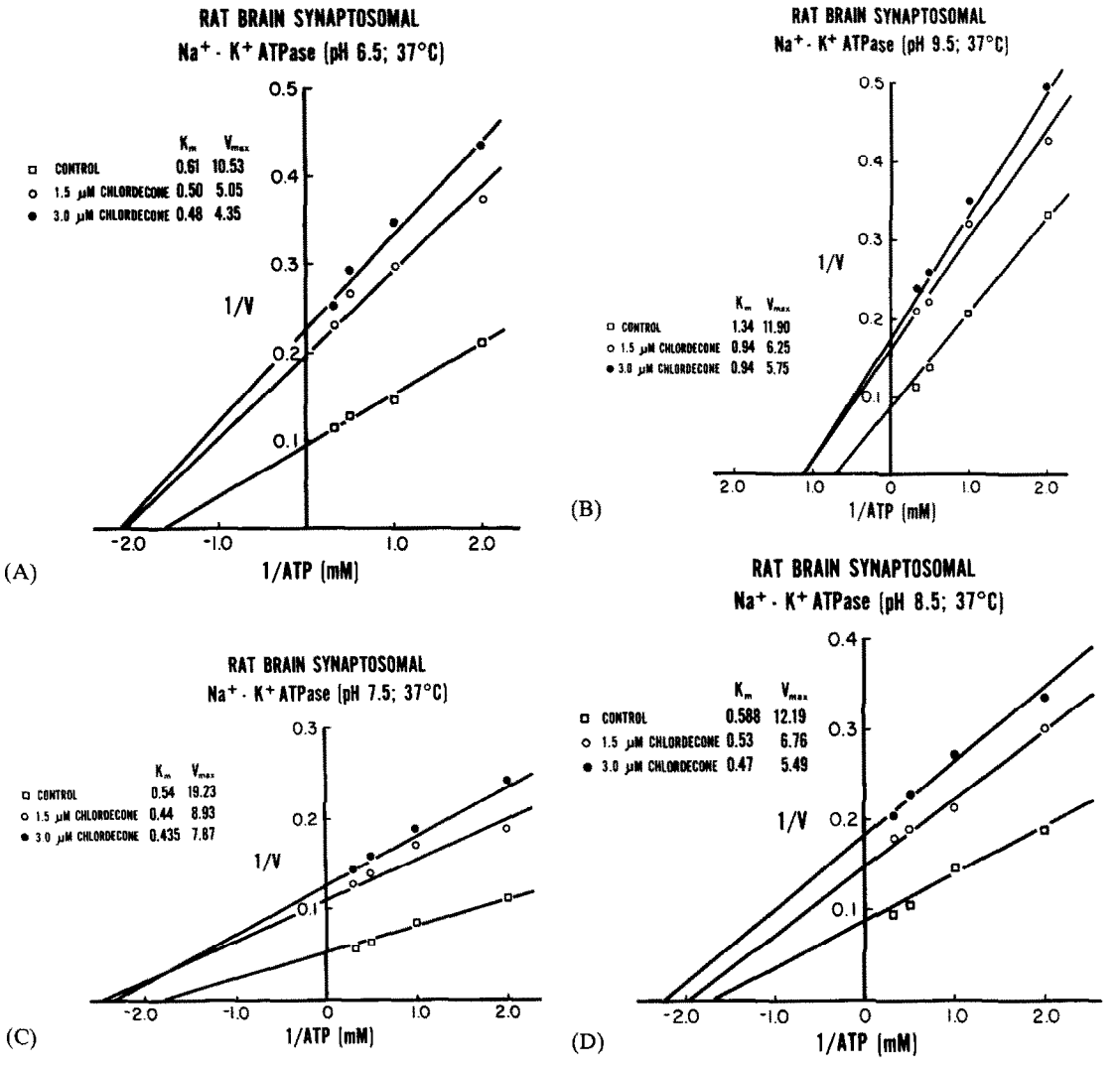

Fig. 3. Double-reciprocal plots of the activation of rat brain synaptosomal (Na^+–K^+)ATPase by ATP at 37° with different pHs in the absence and presence of chlordecone. Key: (A) 6.5 pH; (B) 9.5 pH; (C) 7.5 pH; and (D) 8.5 pH.

case of Mg^{2+}-ATPase, significant decreases in both apparent V_{max} and K_m values were found at each pH studied (Table 1). The decreases in apparent V_{max} in the case of (Na^+–K^+)ATPase and in both V_{max} and K_m in the case of Mg^{2+}-ATPase did not increase in parallel with the increase in concentration of chlordecone from 1.5×10^{-6} M. These results suggested that the effect of chlordecone on (Na^{2+}–K^+)ATPase was independent of substrate ATP (at low-affinity binding sites) and was thus of a classical noncompetitive type, whereas Mg^{2+}-ATPase inhibition was uncompetitive with respect to ATP at each pH studied.

Table 1. *In vitro* effect of chlordecone on substrate activation kinetics of rat brain synaptosomal membrane Mg^{2+}-ATPase at different pH values

	Control		Chlordecone (1.5×10^{-6} M)		Chlordecone (3.0×10^{-6} M)	
pH	K_m (mM)	V_{max}*	K_m (mM)	V_{max}	K_m (mM)	V_{max}
6.5	0.75	10.0	0.48	6.1	0.49	5.8
7.5	0.57	15.6	0.42	8.8	0.22	6.3
8.5	0.77	15.6	0.32	6.9	0.27	6.8
9.5	0.68	12.2	0.37	6.5	0.43	6.4

* V_{max} values are expressed as μmoles of P_i per mg protein per hr.

Effect of temperature on inhibtion of ATPases by chlordecone

The temperature of the individual incubation reaction mixtures was varied from 22 to 37° (Figs. 4A and 5A), and the maximum activities of (Na^+–K^+)ATPase and Mg^{2+}-ATPase were observed at 37°. The inhibition by chlordecone increased with increase in temperature, reaching maximum inhibition at 37°. The inhibition was found to be concentration-dependent at all temperatures. Arrhenius plots (Figs. 4B and 5B) showed that the curves were linear from 22 to 37° which is in

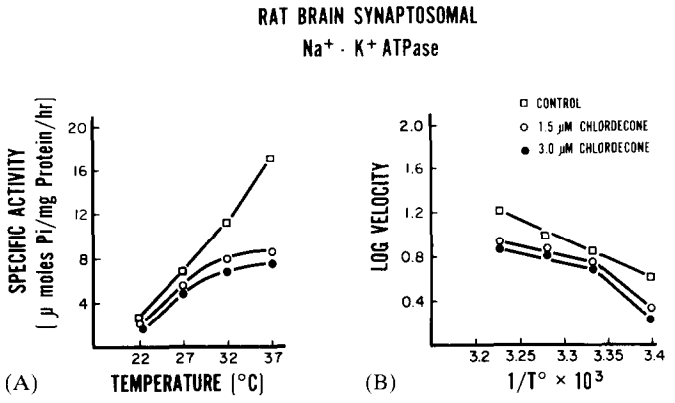

Fig. 4. (A) Effect of temperature on the inhibition of rat brain symaptosomal (Na^+–K^+)ATPase by chlordecone. (B) Arrhenius plots of the estimated V_{max} values for the (Na^+–K^+)ATPase in the absence and presence of chlordecone.

agreement with earlier reports [34, 35]. In the presence of chlordecone, the curves were nonlinear and an inflection was observed at 27° in the case of both enzymes, suggesting temperature-dependent changes in the activation energy. Activation energy values in the presence of chlordecone decreased significantly in the range of 32–37°, but not at lower temperatures, for (Na^+–K^+)ATPase, whereas in the case of Mg^{2+}-ATPase the decrease was also observed at 22–27° (Table 2).

Kinetic analysis

(Na^+–K^+)ATPase. The K_m values of (Na^+–K^+)ATPase were found to decrease with increase in temperature from 27 to 37° in the absence and presence of chlordecone. V_{max} values were found to increase with temperature, and this increase was more pronounced at 37°. In the presence of chlordecone, the K_m values were not altered, whereas V_{max} values were decreased significantly. These results indicate that the chlordecone inhibited (Na^+–K^+)ATPase noncompetitively by decreasing V_{max} without affecting enzyme–substrate affinity (K_m) at all temperatures studied (Figs. 3C and 6A–6B).

Mg^{2+}-ATPase. The results showed no significant change in the K_m values when the temperature was increased from 27 to 32° in the absence and presence of chlordecone. When the temperature was increased from 32 to 37°, the K_m was significantly decreased with and without chlordecone (Table 3). This suggests that the enzyme–substrate affinity was more affected at optimum temperature than at lower temperatures. The V_{max} values were increased with increase in temperature from 27 to 32°, but no such increase was observed at 37° (Table 3). In the presence of chlordecone, V_{max} values were decreased significantly with maximum decrease at 37°. In general, the results indicate that there was significant change in the apparent K_m in the presence of chlordecone at 27° as compared with the control, whereas at 32° and 37° a slight decrease was observed. These results suggest that even though enzyme–substrate affinity was increased in the presence of chlordecone at 32 and 37°, the maximal velocity was decreased significantly at all temperatures. The decreases in

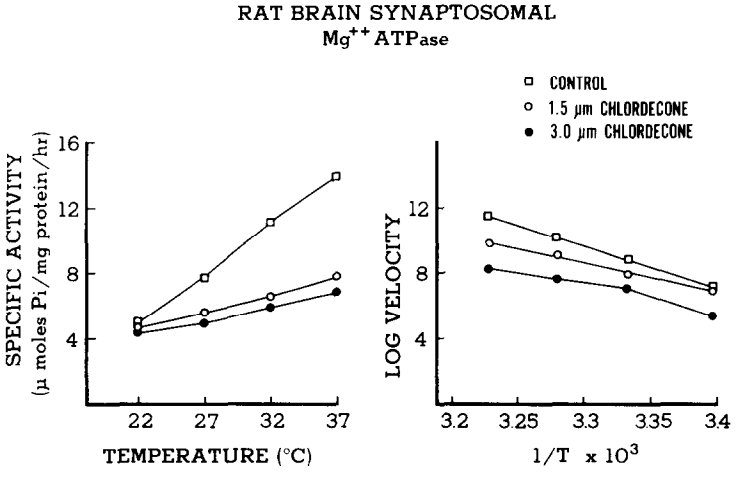

Fig. 5. Left panel: Effect of temperature on the inhibition of rat brain synaptosomal Mg^{2+}-ATPase by chlordecone. Right panel: Arrhenius plots of the estimated V_{max} values for the Mg^{2+}-ATPase in the absence and presence of chlordecone.

Table 2. *In vitro* effects of chlordecone on activation energy (ΔE) values of brain synaptosomal membrane ATPases

	Activation energy* [cal/(mole × 10^3)]					
	(Na^+–K^+)ATPase			Mg^{2+}-ATPase		
Chlordecone concn	22–27°	27–32°	32–37°	22–27°	27–32°	32–37°
0	35.10 ± 4.9	16.87 ± 3.4	16.07 ± 3.8	15.69 ± 4.0	12.98 ± 2.7	8.70 ± 1.9
1.5×10^{-6} M	35.11 ± 5.5	15.45 ± 2.4	2.55 ± 0.9†	5.79 ± 0.9†	11.50 ± 3.0	6.30 ± 2.0
3.0×10^{-6} M	34.10 ± 4.2	15.74 ± 3.1	3.63 ± 1.1†	3.89 ± 1.1†	11.10 ± 2.6	4.69 ± 1.8‡

* Each value is the mean ± S.D. of four determinations.
† Significantly different from controls ($P < 0.001$).
‡ Significantly different from controls ($P < 0.01$).

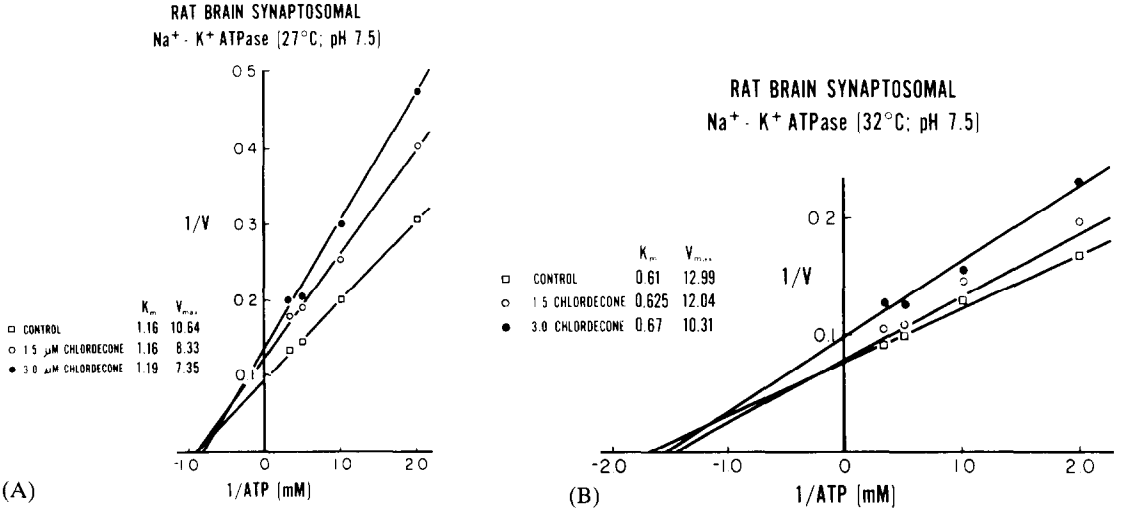

Fig. 6. Double-reciprocal plots of the activation of rat brain synaptosomal (Na^+–K^+)ATPase by ATP at 7.5 pH with different temperatures in the absence and presence of two (micromolar) concentrations of chlordecone. Key: (A) 27° and (B) 32°.

Table 3. *In vitro* effect of chlordecone on substrate activation kinetics of brain synaptosomal Mg^{2+}-ATPase at different temperatures

	Control		Chlordecone (1.5×10^{-6} M)		Chlordecone (3.0×10^{-6} M)	
Temperature	K_m (mM)	V_{max}*	K_m (mM)	V_{max}	K_m (mM)	V_{max}
27°	1.19	11.4	1.06	8.3	1.04	6.8
32°	1.25	15.6	1.00	10.6	0.96	9.1
37°	0.57	15.6	0.42	8.8	0.22	6.3

* V_{max} values are expressed as μmoles of P_i per mg protein per hr.

V_{max} and K_m at 32 and 37° indicate that the inhibition of Mg^{2+}-ATPase by chlordecone was uncompetitive, whereas at 27° the inhibtion was noncompetitive in nature, in which only V_{max} was decreased in the presence of chordecone.

DISCUSSION

The present results indicate that chlordecone is a potent inhibitor of (Na^+–K^+)ATPase and Mg^{2+}-ATPase activities of rat brain synaptosomes. This chlorinated hydrocarbon inactivated these enzymes at low concentration (1.5×10^{-6} M). Similar observations on the inhibition of (Na^+–K^+)ATPase and Mg^{2+}-ATPase activities by a number of chlorinated hydrocarbons, including chlordecone, were reported earlier in fish brain [14], rat tissues [9, 10, 13, 16], and mouse brain [16]. The kinetic studies showed that the chlordecone noncompetitively inhibited synaptosomal (Na^+–K^+)ATPase with respect to acti-

vation of the enzyme by substrate, which is in agreement with an earlier report [13]. Except at pH 9.5, the changes in pH did not show any remarkable effects on K_m and V_{max} values. These results further indicated that the chlordecone decreased the activity of enzyme without affecting its affinity for ATP, suggesting that chlordecone binds to (Na^+–K^+)ATPase at sites not associated with substrate binding. The differential response of the enzyme to chlordecone at pH 9.5 compared to lower pH levels might be due to modification of ionizable moieties such as amino groups. Similar results were reported by Sen *et al.* [36] for (Na^+–K^+)ATPase at pH 9.0 with reference to fluorescein isothiocyanate inhibition.

The results on Mg^{2+}-ATPase showed that the enzyme was uncompetitively inhibited over the pH range studied. A noncompetitive type of inhibition of chlordecone was reported for oligomycin-sensitive ATPase in the mitochondria of rat tissues [13]. The uncompetitive type of inhibition observed in the present study could be due to the differential sensitivity of the oligomycin-sensitive and oligomycin-insensitive components of total Mg^{2+}-ATPase to chlordecone. In this case also, the variation in pH did not affect either apparent K_m or V_{max} of the enzyme. The above data suggested that the inhibition of the membrane-bound synaptosomal ATPase by chlordecone was pH independent.

The temperature variation studies showed that the percent inhibition of (Na^+–K^+)- and Mg^{2+}-ATPases by chlordecone was increased with increase in temperature. Similar findings were reported with K^+-*para* nitrophenyl phosphatase [37]. These results suggest that chlordecone is more inhibitory at high temperatures, unlike DDT, another chlorinated hydrocarbon, which is more inhibitory at lower temperatures [38]. The changes in the slopes of Arrhenius plots of both ATPases in the presence of chlordecone could be due to differential inhibition of the enzymes at different temperatures by chlordecone. Charnock *et al.* [34] reported that the changes in the slopes of Arrhenius plots of (Na^+–K^+)ATPase could be due to the varying temperature dependence of two different steps of the overall reaction, i.e. the Na^+ stimulation of phosphorylation of the phosphorylated enzyme complex and the K^+-induced dephosphorylation of the phosphorylated enzyme complex. The changes in activation energy for (Na^+–K^+)ATPase and Mg^{2+}-ATPase activities over the temperature range of 22 to 37° apparently result from temperature-dependent alteration in K_m [27, 39]. The changes in kinetic parameters at low and high temperatures observed in the present study could be correlated to the temperature-dependent alterations in the mobility of membrane lipids which regulate the membrane-bound ATPase activity [40]. Caldwell and Haug [39] showed that the catalytic components of the Ca^{2+}- and Mg^{2+}-dependent ATPase activities were temperature-dependent up to 34°. The marked decrease in V_{max} values of both the ATPases by chlordecone in the present study suggests that the inhibition of the enzyme activity or alteration in the substrate activation kinetics was temperature dependent.

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