In Vitro Inhibition of Rat Brain ATPase, pNPPase, and ATP-$^{32}P_i$ Exchange by Chlorinated-Diphenyl Ethanes and Cyclodiene Insecticides

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Recent reports have demonstrated that organochlorine insecticides inhibit vertebrate and invertebrate ATPase both in vitro and in vivo. However, the results of these studies are not in complete agreement as to the primary site of inhibitory action. $Na^+ - K^+$ ATPase activity from several sources was reported to be highly sensitive to p,p' DDT and its analogs, and was considered to be the primary site of action for these chemicals (NARAHASHI and HAAS 1967; MATSUMURA et al. 1969; DAVIS and WEDEMEYER 1971; JANICKI and KINTER 1971; CAMPBELL et al. 1964; DOHERTY and MATSUMURA 1975). In other studies, KOCH (1969, 1969/70), KOCH et al. (1969, 1971), CUTKOMP et al. (1971a, 1971b) and DESAIAH et al. (1974a) described $Na^+ - K^+$ ATPase sensitivity to DDT, but also reported that mitochondrial oligomycin-sensitive Mg^{++} ATPase was much more sensitive to diphenyl ethane chemicals than $Na^+ - K^+$ ATPase. Cyclodiene insecticides have been reported to inhibit both $Na^+ - K^+$ and Mg^{++} ATPase activities (KOCH et al. 1969; CHU and CUTKOMP 1971; CUTKOMP et al. 1971b; DESAIAH and KOCH 1975a, 1975b; YAP et al. 1975), but in general, were more effective than DDT as inhibitors of $Na^+ - K^+$ ATPase activity.

Because of the conflicting reports on the action of chlorinated diphenyl insecticides on $Na^+ - K^+$ ATPase and the apparent differences in physiological response to diphenyl and cyclodiene compounds on different types of tissues, this study was initiated to determine the effects of both groups of insecticides on a single species. Experiments were conducted to determine the effects of representative chlorinated diphenyl and cyclodiene compounds on rat brain $Na^+ - K^+$ ATPase, pNPPase (a model for the K^+ mediated portion of the overall $Na^+ - K^+$ ATPase reaction; AHMED and JUDAH 1964), and ATP-$^{32}P_i$ exchange (a measure of oxidative phosphorylation comparable with oligomycin sensitive Mg^{++} ATPase; RACKER 1965).

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METHODS AND MATERIALS

Male Sprague-Dawley rats weighing 200-250 g were used as a source of brain tissue. The microsomal membrane fraction containing the Na^+ - K^+ simulated ATPase activity was isolated as described by AHMED and JUDAH (1964). The enzyme preparation was suspended in a medium of 250 mM sucrose, 10 mM imidazole, and 1mM versine (SIV), pH 7.4, and stored in small lots at -20° C. Rat brain mitochondria for the ATP-$^{32}P_i$ exchange were prepared by homogenizing a single rat brain in SIV to make a 10 percent brain homogenate. The homogenate was then centrifuged for 10 min at 8,000 x g. After the resulting pellet was washed twice in a volume of SIV equal to that of the original homogenate, the washings were combined and recentrifuged for 10 min at 8,000 x g. The resulting pellet was resuspended in SIV as a 10 percent homogenate and used immediately.

The determinations of ATP phosphohydrolase (Na^+ - K^+ ATPase) specific activity was made by the discontinuous inorganic phosphate estimation method of FISKE and SUBBAROW (1925), as reported by AHMED et al. (1971). The reaction mixture for this assay contained 30 mM Tris-HCl (pH 7.45 at 37° C), 3 mM ATP, 110 mM NaCl, 3 mM $MgCl_2$, and 10 mM KCl in a final volume of 2 ml. Samples of the enzyme preparation were diluted to contain 10-12 ug protein per reaction. Incubation time was 15 min at 37° C. Absorbance of samples was measured spectrophotometrically at 660 nm.

The method of AHMED and JUDAH (1964) was used to determine the specific activity of para-nitrophenyl-phosphatase (p-NPPase). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.45 at 37° C), 3 mM $MgCl_2$, 3 mM para-nitrophenylphosphate, and 20 mM KCl in a final volume of 2 ml. Samples of the enzyme preparation were diluted to contain 25-30 ug protein per reaction. Incubation time was 20 min at 37° C. Absorbance of samples was measured spectrophotometrically at 400 nm.

Determinations for ATP-$^{32}P_i$ exchange activity were conducted according to the method of DAWKINS et al. (1959), modified to incorporate the orthophosphate precipitation method of SUGINO and MIYOSHI (1964). The reaction mixture consisted of 2.5 mM ATP, 5 mM $MgCl_2$, 37.5 mM KCl, 100 mM tris-HCl (pH 7.59 at 20° C), and 10 μM of KH_2PO_4 and $^{32}P_i$ (with specific activity of 173.3 cpm/nM inorganic phosphate) in a 2-ml reaction volume. The incubation time was

15 min at 24° C. After ATP extraction, a 1-ml sample of the reaction mixture was diluted in 15 ml Aquasol® [1/] and counted for 10 min in a liquid scintillation counter.

Protein determinations for each enzyme preparation were made according to the method of LOWRY et al. (1951).

The pesticides used in this study were analytical grade gas-chromatographic standards provided by Dr. L.K. CUTKOMP: Chlordane (1,2,4,5,6,7,8,8-octachloro-2, 3,3a,4,7,7a-hexahydro-4,7-methanoindane); p,p'- and o,p-DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane and 1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl) ethane]; TDE [1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene]; endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1, 4,4a,5,6,7,8,8a-octahydro-1,4-endo,endo-5,8-dimethano-napthalene); dieldrin (1,2,3,4,10,10-hexachloro-exo-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo,exo-5, 8-dimethanonapthalene); heptachlor (1,4,5,6,7,8, 8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene); heptachlor epoxide (1,4,5,6,7,8,8-heptachloro-6, 7-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindene); dicofol (Kelthane®) [1/] [1,1-bis(p-chlorophenyl)-2,2,2-trichloro-ethanol]; and Kepone® (decachloro-octahydro-1,3,4-metheno-2H-cyclobuta(cd)pentalen-2-one).

All insecticides were dissolved in ethanol and 2 µl of the required concentration were added to a rapidly stirring reaction mixture with a microsyringe. Ethanol had no effect on ATPase or pNPPase activities at the amounts used.

RESULTS AND DISCUSSION

Table 1 represents the in vitro effects of selected chlorinated diphenyl and cyclodiene insecticides on the specific activities of rat brain $Na^+ - K^+$ ATPase, K^+ stimulated pNNPase and ATP-$^{32}P_i$ exchange. Inhibition of rat brain $Na^+ - K^+$ ATPase by the chlorinated insecticides varied considerably with the molecular structure. This enzyme was most sensitive to chlordane, heptachlor, and Kepone, indicating that these compounds may disrupt membrane sodium-potassium transport, and therefore influence nerve action through this mechanism. Inhibition of rat brain $Na^+ - K^+$ ATPase

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1/ Reference to trade names does not imply Government endorsement.

Table 1. *In vitro* inhibition of rat brain ATPase, pNPPase, and ATP-$^{32}P_i$ exchange by chlorinated diphenyl and cyclodiene insecticides.

Chemical[1/]	% Inhibition of Na^+-K^+ ATPase ± SEM	% Inhibition of pNPPase ± SEM	I_{50} Concentration ATP-$^{32}P_i$ exchange (uM)
Chlordane	82.0 ± 0.5[2/]	33.1 ± 6.9[2/]	20.0[2/]
Kelthane	81.1 ± 1.1	66.9 ± 2.4	8.2
Kepone	76.6 ± 0.6	51.3 ± 4.4	13.0
Heptachlor	72.1 ± 3.3	28.3 ± 2.1	60.0
TDE	51.2 ± 0.8	26.6 ± 2.7	18.0
p,p'-DDT	41.6 ± 0.8	19.9 ± 3.5	---[3/]
o,p'-DDT	40.4 ± 4.4	11.2 ± 1.9	2.0
Heptachlor eposide	21.3 ± 5.6	19.2 ± 0.1	44.0
Endrin	18.7 ± 3.7	14.2 ± 1.8	20.0
Dieldrin	18.7 ± 3.7	10.6 ± 1.9	32.0
Untreated specific activity	138.9 ± 4.9 $umP_i mg^{-1} hr^{-1}$	10.8 ± 0.7 $umpNPP mg^{-1} hr^{-1}$	

1/ For ATPase and pNPPase inhibition studies all chemicals tested at 2×10^{-5}M.

2/ All values based on three observations each, except for Kelthane, TDE, and p,p'-DDT (Na^+-K^+, pNPPase inhibition studies, four observations each).

3/ Not tested.

by the diphenyls, with the exception of dicofol, was comparable with that previously reported by AKERA et al. (1971). The epoxy cyclodienes had little or no effect on rat brain $Na^+ - K^+$ ATPase. The reason for the lower in vitro inhibition by the epoxy compounds was not clear. Two possible explanations are that different modes of action exist for the epoxy and non-epoxy cyclodienes or that under in vitro conditions there was insufficient dispersal of the more insoluble expoxide form.

Once the effect of each compound on the overall ATPase reaction was established, it was possible to consider only the K^+ mediated components in accordance with the following reaction scheme:

$$\text{Enzyme} \xrightarrow[\text{ATP}]{Na^+,\ Mg^{++},} \text{1st Phospho-enzyme} \xrightarrow{Mg^{++},} \text{2nd Phospho-enzyme} \xrightarrow{K^+} \text{Enzyme} + \text{Inorganic phosphate}$$

AHMED and JUDAH (1964) reported K^+ stimulated pNPPase to be a model for the K^+ dependent portion of the $Na^+ - K^+$ ATPase reaction; therefore, pesticide inhibition of pNPPase should correlate with inhibition of the K^+ mediated portion of the overall $Na^+ - K^+$ ATPase reaction. Significance of pNPPase inhibition was determined by single classification analysis of variance, ANOVA; (SOKAL and ROHLF 1969). The preliminary ANOVA indicated a significant difference between insecticide-treated enzymes and the controls ($P<0.001$). The homogenity of the means in the ANOVA were further tested by the sum of squares simultaneous test procedure of GABRIEL (1964). Dicofol and Kepone were significantly ($P<0.001$) more inhibitory than the other insecticides tested. The reason for this disparity in inhibition between these two insecticides and the others was not entirely clear. However, dicofol and reduced Kepone (DESAIAH and KOCH 1975b) were the only insecticides with an available hydroxyl group, which may have increased the binding affinity for these compounds at the receptor site. These results indicate that the observed differences in the overall $Na^+ - K^+$ ATPase reaction were probably due to differences in the ability of the insecticides to inhibit the Na^+ mediated site rather than the K^+ mediated site.

The dosage response I_{50} values for the ATP-$^{32}P_i$ exchange in table 1 were calculated from three insecticide concentrations by probit analysis (FINNEY 1957). These ATP-$^{32}P_i$ exchange data suggested that DDT was a more powerful inhibitor of oxidative

phosphorylation than were the cyclodiene type compounds. Although the ATP-$^{32}P_i$ exchange was not as sensitive as the oligomycin-sensitive Mg^{++} ATPase method used by KOCH et al. (1971, 1972), CUTKOMP et al. (1971a, 1971b, 1972), DESAIAH et al. (1974a, 1974b), and YAP et al. (1972), the results were similar, in that DDT was also the most powerful inhibitor of oxidative phosphorylation. Also, other reports by HILTON and O'BRIEN (1970), ELA et al. (1970), KACEW et al. (1972), and KACEW and SINGHAL (1973) have demonstrated a reduction in mitochondrial oxidative phosphorylation as a result of a DDT treatment. This observation, the inhibition of oxidative phosphorylation by DDT, and the subsequent depletion of ATP could be partly responsible for the classical tremors and electro-physiological responses associated with DDT intoxication in insects.

In general, the results of this study indicated that the cyclodiene type insecticides tested were more inhibitory of Na^+ - K^+ ATPase, whereas the diphenyl compounds were more inhibitory of the ATP-$^{32}P_i$ exchange and oxidative phosphorylation. Only two of the insecticides, dicofol and Kepone, significantly inhibited pNPPase indicating that the Na^+ catalyzed portion of the overall ATPase reaction is probably the most sensitive site for the action of organochlorine insecticides.

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