Pesticidal Chemicals Affecting Some Energy-Linked Functions of Rat Liver Mitochondria *In Vitro*[1]

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Among the several hundred chemical agents which interfere with mitochondrial functions, a number of pesticidal chemicals are known to exert their toxic action primarily by interference with energy production at the mitochondrial level (CASIDA, 1973). However, the central role which mitochondria play in energy production and other important physiological processes such as neurofunction and protein synthesis (LEHNINGER, 1970), active ion transport (LEHNINGER et al., 1967), drug biotransformation (CINTI et al., 1972) and physiological thermogenesis (CHRISTIANSEN et al., 1969), make these organelles a possible locus of secondary action for many other pesticidal chemicals. Obviously, alteration of any of these mitochondrial functions on an acute or chronic basis could have profound cellular implications. Heightened concern over the potential health hazards of environmental use of agricultural chemicals has prompted us to survey the effects of a series of pesticides, representing a wide spectrum of chemical structures and pesticidal activities, on some energy-linked mitochondrial functions.

The primary purpose of this communication is to report and discuss the effect of these pesticidal chemicals on rat liver mitochondria in vitro.

Experimental

Rat liver mitochondria were isolated by conventional methods (KATYARE et al., 1971). Oxygen uptake was measured polarographically at 30° using a Clark Oxygen Electrode. Conditions of the assay have been described previously (ABO-KHATWA and HOLLINGWORTH, 1973). In most instances pesticides of analytical grade (generally > 95% purity) were used as received, dissolved in ethanol (or other appropriate solvent as indicated), and 5µl was added to the mitochondrial suspension after obtaining one state 4/state 3/state 4 respiration cycle (CHANCE and WILLIAMS, 1955). Succinate was used as substrate with 1.2 mg mitochondrial protein per ml of reaction medium (2.5 ml final volume).

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Four energy-linked mitochondrial functions were assayed: a) the ADP/O ratio, measuring the efficiency of respiratory chain phosphorylation, b) state 4 respiration rate (i.e. rate of O_2 uptake in the absence of phosphate acceptor), c) state 3 respiration rate (i.e. rate of O_2 uptake in the presence of phosphate acceptor), and, d) respiratory control index (RCI) (i.e. the ratio of succinate oxidation in the presence of ADP to the rate obtained after the acceptor is exhausted. Mitochondrial protein was determined colorimetrically (GORNALL et al., 1949) using bovine serum albumin as a standard. Common names of pesticides are taken from KENAGA and ALLISON (1969) and JOHNSON (1972). Compounds lacking common names are characterized in Table 1.

Results and Discussion

Table 2 summarizes the results with 47 pesticidal chemicals tested for effects on energy-linked functions of rat liver mitochondria in vitro. Thirteen pesticides were found to be inactive, 3 were effective only at 10^{-3}M, 14 showed effects at 10^{-4}M, 8 showed effects at 10^{-5}, 8 at 10^{-6} and one as low as 10^{-7}M.

With the exception of dicofol which acted as an uncoupling agent of respiratory chain phosphorylation, all chlorinated hydrocarbons tested show inhibition of the O_2 uptake of both state 3 and state 4 respiration but only at a relatively high concentration (10^{-4}M). Dieldrin was about 10 times more potent in this regard and caused a significant decrease in respiratory control. Similar observations of the inhibitory action of chlorinated hydrocarbons on mitochondrial metabolism have been reported on mitochondria isolated from rat liver with dieldrin at concentrations > 10^{-5}M (BERGEN, 1971), beef heart with 11 chlorinated hydrocarbons at concentrations > 10^{-4}M (PARDINI et al., 1971) and rat brain with endrin at 10^{-3}M (KANDA et al., 1968). Furthermore a variety of chlorinated hydrocarbon pesticides have been reported to inhibit oligomycin-sensitive mitochondrial Mg^{2+}-ATPase (CUTKOMP et al., 1971 and 1972), an enzyme system believed to play a central role in energy conservation mechanisms (FESSENDEN-RADEN and RACKER, 1971). CUTKOMP et al. (1971) suggested that by blocking ATP production some chlorinated hydrocarbons could cause deleterious effects on biochemical processes requiring energy such as nerve function. The role of such effects in the overall neurotoxic action of chlorinated hydrocarbons remains to be established. In the case of soluble enzymes at least, inhibition at high concentrations by very lipid soluble compounds such as the chlorinated hydrocarbons may be due to non-specific effects (JACKSON and GARDNER, 1971).

Table 2 also shows that half of the fourteen organophosphates had no significant effect on energy-linked mitochondrial functions even at 10^{-3}M. The type of action produced by the remaining organophosphates was contingent upon the chemical nature of each pesticide. For example, the polychlorinated organophosphates, Dowco 214, chlorpyrifos and Gardona at 10^{-4}M exhibited significant

TABLE 1

Trade and Chemical Names of Some Pesticides Tested

Trade Name	Chemical Name
	Organophosphates
Dowco 214	O,O-dimethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
Gardona	2-chloro-1-(2,4,5-trichlorophenyl) vinyl dimethyl phosphate
Iodofenphos Ethyl	O-(2,5-dichloro-4-iodophenyl) O,O-diethyl phosphorothioate
Paraoxon	Diethyl p-nitrophenyl phosphate
Zytron	O-2,4-dichlorophenyl O-methyl isopropylphosphoramidothioate
	Other pesticides
Aramite	2-(p-tert-butylphenoxy) - 1-methylethyl-2-chloroethyl sulfite
Banamite	Benzoyl chloride (2,4,6-trichlorophenyl) hydrazone
MON - 0858	3-(p-chlorophenyl)-4,5-dichlorosalicylanilide
Juvenile Hormone Mimic D (Stauffer)	6-epoxy-3,7-dimethyl-trans, 2-octen-1 (4-ethylphenol) ether
Morestan	6-methyl-2,3-quinoxaline-dithiol cyclic-S,S-dithiocarbonate
TH-6040	1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea
2,4,5-TP	2-(2,4,5-trichlorophenoxy) propionic acid
U-36,059	1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazopenta-1,4-diene

inhibition of both state 3 respiration rate and respiratory control. Similar actions were observed at a higher concentration (10^{-3}M) with the pesticide diazinon. On the other hand, malathion, Zytron and methidathion (Supracide) acted as uncouplers of respiratory chain phosphorylation. They elicited "loose coupling" marked by reduction of the respiratory control index (RCI) which was due more to increased state 4 respiration rate than to decreased state 3. Although the ADP/O ratio in some cases remained unchanged, higher concentrations of Zytron or methidathion (not shown in Table 2) significantly decreased it. No similar studies have been reported on the effect of organophosphate pesticides on energy-linked mitochondrial functions, but chemical and morphological alterations of mitochondria have been observed with some organophosphates in vivo. Malathion-fed rats showed mitochondrial damage manifested by a decrease in the amount of hexosamine and loss of both nitrogen and mucopolysaccaride from liver mitochondria (FELAND and SMITH, 1972), and parathion induced morphological alterations, particularly to the cristae of mitochondria isolated from the thoracic ganglia of poisoned houseflies (RAMADE and RIVIERE, 1971).

Although the importance of acetylcholinesterase inhibition by organophosphate pesticides has been well investigated, possible adverse effects from the phenolic aromatic moieties released by organophosphate degradation have not been studied satisfactorily. It is conceivable, however, that in some cases phenolic metabolites are released which are capable of altering mitochondrial function. This possibility has been investigated and the results are shown in Table 2. The phenolic hydrolysis products of Iodofenphos ethyl i.e. 2,5-dichloro-4-iodophenol, of chlorpyrifos and Dowco 214 i.e. 3,5,6-trichloro-2-pyridinol and of parathion i.e. p-nitrophenol were active as uncouplers of respiratory chain phosphorylation. Their effectiveness decreased in the order given. The dichloroiodophenol was a particularly effective uncoupling agent and the possibility that its release contributes to intoxication in insects deserves further study. MATSUDA and FUKAMI (1972) showed that phenolic metabolites of parathion and fenitrothion also acted as uncouplers of cockroach muscle mitochondria. Similarly, HEIDKER and PARDINI (1972), reported that azinphosmethyl and several breakdown products inhibited the oxidation of NADH but not succinate by beef heat mitochondria.

Table 2 also shows that the two carbamate pesticides aldicarb and carbaryl had no significant effect on mitochondrial energy-linked functions at concentrations as high as 10^{-3}M. These findings are in accordance with HEIDKER and PARDINI (1972) who reported no significant effect of carbaryl on mitochondrial electron transport. However, dihydroxynaphthalene, a photodegradative product of carbaryl, was a potent inhibitor of mitochondrial enzymes.

TABLE 2

Comparison of the Effects of Pesticides on Four Parameters of Rat-Liver Mitochondria *In Vitro*.

Pesticide	-Log active concn. (M)[a]	General type of effect[b]
Chlorinated hydrocarbons		
chlorobenzilate	4	Inhibitory[2,3]
dicofol	4	Uncoupling[1]
dieldrin	5	Inhibitory[3,4,5]
heptachlor	4	Inhibitory[2,3,4]
methoxychlor	4	Inhibitory[2,3]
Organophosphates (O-P)		
azinophosmethyl	Inactive[c]	
chlorpyrifos	4	Inhibitory[3,4,5]
diazinon	3	Inhibitory[2,3]
dichlorvos	Inactive[d]	
Dowco 214	3	Inhibitory[2,3,5]
Gardona	4	Inhibitory[2,3]
Iodofenphos ethyl	Inactive[d]	
malathion	4	Uncoupling[2,4,5]
methidathion	5	Uncoupling[2,4]
paraoxon	Inactive[d]	
parathion	Inactive[d]	
ronnel	Inactive[d]	
trichlorfon	Inactive[d]	
Zytron	5	Uncoupling[2,3,4]
O-P phenolic metabolites		
2,5-dichloro-4-iodophenol	6	Uncoupling[1,2,4,5]
4-nitrophenol	4	Uncoupling[1]
3,5,6-trichloro-2-pyridinol	5	Uncoupling
Carbamates		
aldicarb	Inactive[d]	
carbaryl	Inactive[c]	

TABLE 2 (CON'T)

Phenylureas		
diuron	4	Uncoupling[2,4,5]
linuron	6	Uncoupling[2,4]
propanil	5	Uncoupling[2,4]
TH-6040	6	Uncoupling[2,4]
Phenoxyacids		
2,4-D	4	Uncoupling[2,4]
2,4,5-T	4	Uncoupling[2,4]
2,4,5-TP	5	Uncoupling[2,4,5]
Formamidines		
chlordimeform	6	Uncoupling[2,4]
U-36,059	6	Uncoupling[2,4]
2,4-Dinitrophenols		
binapacryl	6	Uncoupling[2,4,5]
2,4-dinitrophenol	5	Uncoupling[2,4,5]
Other Pesticides		
allethrin	Inactive[d]	
Aramite	Inactive[d]	
Banamite	6	Uncoupling[2,4]
dodine	4	Inhibitory[1]
fentin acetate[f]	6	Inhibitory[2,3,5]
JH Mimic D	Inactive[d]	
MON-0858[e]	7	Uncoupling[2,4,5]
Morestan[g]	5	Uncoupling[2,4]
Nicotine	Inactive[d]	
piperonly butoxide	3	Inhibitory[1]
tetradifon	4	Inhibitory[1]
trifluralin	4	Inhibitory[3,4]

[a]Minimal concentration (-log M) which produces at least 30% adverse effects on: [1]all four mitochondrial parameters, [2]reduction of RCI, [3]reduction of state 3 respiration rate, [4]state 4 respiration rate reduction (inhibition) or stimulation (uncoupling), and [5]reduction of ADP/O ratio

[b]According to RACKER (1965), uncoupling is characterized by diminution of ADP/O ratio and RCI, and stimulation of state 4 respiration rate with no significant change in state 3 respiration rate. Inhibition refers to diminution of state 3 or state 4 rates of oxygen uptake.

[c,d]Insignificant effect at concentrations of 10^{-4}M and 10^{-3}M respectively.

[e,f,g]Pesticides dissolved in dimethyl sulfoxide, distilled water and N,N-dimethyl formamide, respectively. No significant adverse effects on mitochondria were observed with these solvents alone.

The phenylurea herbicides linuron, propanil and diuron as well as the phenoxyacid herbicides 2,4,5-TP, 2,4,5-T and 2,4-D exhibited uncoupling activity at relatively low concentrations (10^{-6} to 10^{-4}M). They stimulated the state 4 respiration rate without significant change of state 3 rate. Similar uncoupling behavior has been obtained with phenoxyacid herbicides against plant (MATLIB and KIRKWOOD, 1972) and rat liver (BRODY, 1952) mitochondria. The novel insecticide TH-6040 which is chemically related to the phenylureas is believed to interfere with the process of cuticle deposition (VAN DAALEN et al., 1972). This compound also produced marked uncoupling activity at a low concentration (10^{-6}M). At a concentration of 2 X 10^{-5}M TH-6040 produced 30% and 75% decrease in ADP/O ratio and RCI respectively and a 120% increase in state 4 respiration rate. Whether this impairment of mitochondrial respiratory chain phosphorylation is related to its mode of action has yet to be determined.

Another novel group of acaricide-insecticides introduced recently are the formamidines, such as chlordimeform and U-36,059. Both pesticides are known to act slowly on mites and insects with no significant signs of cholinesterase inhibition (DITTRICH, 1966; Upjohn technical bulletin, 1972). In this study both compounds showed uncoupling activity at low concentration (10^{-6}M). However, at a higher concentration such as 10^{-4}M (not shown in the text), the two compounds behaved differently. Chlordimeform in no case produced an inhibitory effect on O_2 uptake (ABO-KHATWA and HOLLINGWORTH, 1973) but U-36,059 at this concentration produced almost total block of mitochondrial respiration with no subsequent response to added ADP or 2,4-dinitrophenol. This action is typical of many other uncoupling agents (VAN DAM and SLATER, 1967). The mode of action of such formamidines is still under investigation.

The acaricidal ester binapacryl, which is believed to exert its uncoupling activity after hydrolysis, is 10 times as potent as 2,4-dinitrophenol. It has been suggested that the addition of the bulky 2-sec-butyl group increases the liposolubility of the dinitrophenol hence increasing its activity (CASIDA, 1973).

The specific acaricide Banamite (a phenylhydrazone derivative) and the pesticide MON-0858 (a salicylanilide derivative), both containing an acidic NH group, were among the most potent uncouplers tested. The action of salicylanilides and phenyl hydrazones as uncouplers of respiratory chain phosphorylation is well documented (WILLIAMSON and METCALF, 1967; HEYTLER, 1963). The acaricide Morestan, for which no mode of action is yet known, also exhibited uncoupling activity at a relatively low concentration (10^{-5}M).

Table 2 also shows that the fungicide fentin acetate is a potent inhibitor of energy-linked mitochondrial functions. Such effects are well known (ALDRIDGE and STREET, 1964).

Aside from the aforementioned uncoupling actions, others were found to elicit inhibitory effect at higher concentrations (10^{-4}, 10^{-3}M). These pesticides were: the fungicide dodine (a guanidine derivative), the synergist piperonyl butoxide, the herbicide trifluralin, and the acaricide tetradifon. Similar results on the inhibitory action of dodine (SYROWATKA, 1970), tetradifon (BUSTAMANTE and PEDERSEN, 1973), and piperonyl butoxide (NELSON et al., 1971) have been reported.

Although there is good evidence that these biochemical changes in energy-linked functions in insects and mammals initiated by some of the more potent of the aforementioned pesticides contribute to their mechanism of toxic action, the safe and efficient use of these pesticides depend on understanding not only their primary mode of action, but also their secondary toxic effects as well (CASIDA, 1973). In their study of pesticides acting as uncouplers and inhibitors of oxidative phosphorylation *in vivo* and *in vitro*, ILIVICKY and CASIDA (1969) showed that some substituted dinitrophenols, benzimidazoles, salicylanilides, phenyl hydrazones were potent uncoupling agents of mouse brain and liver mitochondia *in vivo*. Although 2,4-dinitrophenol, chlordimeform and Morestan were given in lethal doses, no uncoupling activity was observed in mitochondria isolated from poisoned mice. A possible explanation of such findings was offered recently (ABO-KHATWA and HOLLINGWORTH, 1973).

In this study 36% of the pesticides tested had significant deleterious effects on mitochondrial functions at 10^{-5}M or less *in vitro*. The fact that such a significant fraction of pesticides have reasonably potent action on mitochondria underlines the need for continuing study of the mitochondrion as a site of attack by pesticides.

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