Bull. Environ. Contam. Toxicol. (1984) 33:106–113

Environmental Contamination and Toxicology

Effects of Lindane on Liver Mitochondrial Function in the Rat

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A frequently reported manifestation of chlorinated hydrocarbon pesticide toxicity at the biochemical level is the derangement of mitochondrial function. Benzenehexachloride, toxaphene, cyclodiene insecticides (Byczkowski 1976, Fukami 1976; Nelson 1975, Pardini et al. 1971, 1980) and other pesticides and their derivatives or conversion products (Byczkowski 1976, Fukami 1976, Nelson 1975, Pardini et al. 1971, 1980) have been shown to inhibit mitochondrial electron transport systems in vitro. Lindane causes swelling of rat hepatocyte mitochondria in vivo (Khaikyna et al, 1975), and modifies mitochondrial oxidative metabolism in rat liver (Nelson 1975), in fish bluegill (Hiltibran 1974) and in various other living organisms including insects (Fukami 1976 , Zakolodkina 1963).

Many pesticides are known to influence ATPase activity in animal tissues (Koch 1969, Matsumura and Patil 1969). Na^+-K^+ ATPase, involved in the active transport across cell membrane (Schwartz et al. 1972, Skou 1965) , and oligomycin-sensitive Mg^{2+} ATPase involved in oxidative phosphorylation (Byczkowski et al. 1973, Desaiah et al. 1977, Kagawa and Racker 1966, Racker et al 1975) have been shown to be inhibited by a variety of chlorinated hydrocarbon pesticides (Byczkowski 1976, Koch 1969,70, Nelson 1975). In reports by Koch (Koch 1969, 1969,70) lindane decreased ATPase activity in homogenates of tissues from rabbit, chicken, lake trout and cockroach.

Studies in this paper pertain to the in vitro effect of lindane on some aspects of mitochondrial oxidative metabolism in liver.

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MATERIALS AND METHODS

L-Glutamic acid was purchased from E. Merck, Darmstadt, F.R.G. Sodium salts of succinic acid, ATP, ADP, 2,4-dinitrophenol(DNP) and rotenone were from Sigma Chemical Company, St. Louis, Mo, U.S.A. The analytical standard of γ-hexachlorocyclohexane (lindane) was obtained from the U.S. Environmental Protection Agency, Research Triangle Park, NC. All other chemicals and solvents, of analytical grade were obtained from BDH Chemicals Ltd., Poole, U.K.

All the experiments were performed with male albino rats of Wistar strain, weighing about 150 g. The animals were reared on a nutritionally adequate laboratory stock diet.

Animals were killed by decapitation. The livers were quickly removed and chilled in 0.25 M sucrose containing 0.01 M Tris-HCl, pH 7.4 and 0.001 M EDTA. They were weighed and a 10% homogenate made in sucrose-Tris-EDTA medium, using a Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle. After removal of the nuclear fraction (Katyare et al. 1971), mitochondria were isolated by centrifuging the nuclei-free supernatant fluid at 12,000 g (Johnson and Lardy 1967) for 10 min. The resulting mitochondrial pellets were washed once and suspended in sucrose-Tris-EDTA to give a concentration of about 80 mg/ml. All operations were carried out at 4°C.

Respiration rates were measured with a Clark oxygen electrode at 25°C using a Gilson oxygraph model KM (Gilson Medical Electronics, Middleton, Wiscosin, USA). The reaction medium contained, in a total volume of 1.3 ml: 225 mM sucrose, 10 mM potassium phosphate buffer (pH 7.4), 10 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 2 mg of mitochondrial protein (Satav and Katyare 1982). Succinate (5 mM) or glutamate (5 mM) was used as the substrate. Rotenone (2 µg) was present in the medium in which succinate was used as substrate. Small aliquots of ADP (200 nmoles in 10 µl) were added and the rates of respiration in the presence of added ADP (State 3) and after its depletion (State 4) were recorded. ADP/O ratios and the respiratory control index (RCl) were calculated as described previously (Chance and Williams 1956).

ATPase activity was determined in a medium containing 15 mM KCl, 50 mM Tris-HCl (pH 7.3), 5 mM $MgSO_4$, 6 mM ATP, 2 µg rotenone and 1.6 mg of mitochondrial protein in a total volume of 1 ml. Whereever indicated, 50 µM dinitrophenol (DNP) was used. The reaction was started by the addition of mitochondria and carried out at 30°C for 10 min (Byczkowski et al. 1973). At the end of the incubation period , the reaction was terminated by addition of 0.5 ml of chilled (W/V) 10% trichloroacetic acid. Appropriate zero hour controls were run simultaneously. The tubes were immediately chilled and centrifuged. Inorganic phosphate libe-

rated in the supernatant fraction was estimated by the method of Fiske and Subba Row (1925). Protein was determined by the Lowry method (Lowry et al 1951) using crystalline bovine serum albumin as a standard.

Lindane was added in dimethylsulphoxide (DMSO) solution in a volume of 10 µl. The same amount of DMSO was added to controls. Preliminary experiments have shown that DMSO at the low concentrations used does not interfere with respiration, phosphorylation or ATPase activity. Other additions are as indicated in the tables.

RESULTS AND DISCUSSION

The effects of lindane at increasing concentrations on ADP/O ratio and RCI, using succinate or glutamate as substrate in rat liver mitochondria are shown in Tables 1 and 2.

Table 1. Effect of lindane *in vitro* on respiration and oxidative phosphorylation in rat liver mitochondria using succinate as substrate

Lindane (n moles/flask)	ADP/O ratio	Oxygen uptake (m µmoles/min/mg protein)		
		State 3(ADP)	State 4(-ADP)	RCI
0	1.70	60.76	14.70	4.13
25	1.57	68.34	21.56	3.17
50	1.16	66.11	23.52	2.81
100	0.72	63.50	35.28	1.80

Results are averages of values obtained from 4 independent experiments.

With succinate as the respiratory substrate, the mitochondria showed good respiration in the absence of lindane. With 50nmoles and 100 nmoles of lindane in the incubated medium about 30 and 60% of the coupling activity is lost, respectively. This is also reflected in a significant decrease in RCI, indicating loss of mitochondrial integrity. State 3 respiration was unaffected.The insecticide stimulated state 4 respiration 2-3 fold resulting in a decrease in the RCI. The RCI is greatly decreased at all concentrations of the insecticide and is 23% , 32% and 56%, respectively, with 25, 50 and 100 nmoles of lindane. Lindane at higher concentrations (150 nmoles and above) results in total loss of coupling activity.

Table 2: Effect of lindane *in vitro* on respiration and oxidative phosphorylation in rat liver mitochondria using glutamate as substrate

Lindane (nmoles/flask)	ADP/O ratio	Oxygen uptake (m μmoles/min/mg protein) State 3(ADP)	State 4(-ADP)	RCI
0	3.15	22.50	2.13	10.56
25	2.74	27.00	5.00	5.40
50	2.70	22.60	5.00	4.52
100	1.96	23.52	8.84	2.66
125	1.74	21.60	12.24	1.76

Results are averages of values obtained from 4 independent experiments.

Table 2 shows the effect of lindane on respiration and oxidative phosphorylation in rat liver mitochondria with glutamate as substrate. At the lower concentration of lindane (25 and 50 nmoles) , ADP/O ratio decreases by about 14% and RCI by about 45-55%, respectively. At higher concentrations, coupling activity shows a significant decline. As in the case of succinate, state 3 respiration remained unaltered with glutamate at all concentrations of lindane, whereas state 4 respiration increased steadily. Also, RCI, was affected to a greater extent as compared to succinate. In the case of succinate, RCI decreases by 23-56% at the insecticide levels studied (Table 1), whereas a 50-80% decrease was noted with glutamate as substrate (Table 2).

The effect of lindane on ATPase activity of rat liver mitochondria are presented in Table 3. Both Mg^{2+} ATPase and basal ATPase activity(Mg^{2+}omitted in the reaction medium) were assayed. These experiments were carried out on mitochondria with ADP/O ratios about 1.7 to 1.8 with succinate and 3.1 with glutamate as substrates.

The data shows that lindane inhibited both Mg^{2+}ATPase and basal ATPase activity *in vitro* with rat liver mitochondria. Lindane at 100 and 200 nmoles inhibited Mg^{2+}ATPase activity by 46% and 60%, respectively. The inhibition of basal ATPase activity was 33% and 50% at 100 and 200 nmoles, respectively. DNP at 50 μg stimulated the ATPase activity, with or without $Mg^{2+}$and this effect is totally abolished by lindane when added at

Table 3: Effect of lindane on ATPase activity in rat liver mitochondria

Lindane (nmoles)	DNP (50 μM)	ATPase activity (μmoles Pi/mg protein/15 min) with Mg^{2+}	without Mg^{2+}
0	-	0.28	0.22
0	+	2.70	2.50
50	-	0.20	0.20
50	+	0.22	0.20
100	-	0.15	0.15
100	+	0.16	0.16
200	-	0.11	0.12
200	+	0.12	0.12

The experimental conditions are as described in text. Results are averages of values obtained from 4 independent experiments.

50 nmoles are higher. In presence of lindane at any level, there was no difference between the basal and Mg^{2+}ATPase activity levels

Many chlorinated hydrocarbon pesticides have been investigated for their effects on mitochondrial function (Byczkowskii 1976, Fukami 1976, Nelson 1975, Pardini et al. 1971, 1980). The present studies indicate that the ability of rat liver mitochondria to oxidize succinate and glutamate as well as their capability for coupled phosphorylation are significantly influenced by lindane *in vitro*.

The differential effect of lindane on state 3 and state 4 respiration in rat liver mitochondria noticed in the present studies has also been reported by Nelson et al. (1975). However, whereas in the present study, there is no effect on state 3 respiration, as measured with both succinate and glutamate, Nelson (1975) found a steady decline in succinate oxidation with increasing concentrations of lindane. The present results are however, in agreement with observations in beef heart mitochondria (Pardini et al. 1971). Whereas, state 4 respiration showed a steady increase with the concentration of lindane in our study, the earlier report with rat liver mitochondria (Nelson 1975) indicated an initial increase followed by a decrease. At concentration of lindane over 60 μg/mg mitochondrial protein, these investigators found the respiratory rate in both states 3 and 4 were identical. On the basis of their studies with beaf heart mitochondria, Pardini et al (1971) concluded that the NADH-CoQ segment of the respiratory chain may be inhibited by lindane, the finding in the present study that glutamate oxidation in the coupled mitochondria is unaffected points to the lack of an effect on any segment of the electron transfer chain *per se*. It should be pointed out that the concentration of lindane which exhibited the inhibitory effect in the study of Pardini et al (1971) is higher by at least one order of

magnitude from those used in the present study or that of Nelson (1975).

An analysis of the effects of lindane on coupled phosphorylation with succinate or glutamate as substrates, indicates that sites II and III of coupled phosphorylation alone are impaired with little or no effect on site I. This may be inferred from the fact that 100 nmole of lindane decreased coupling by about 60% with succinate as substrate, but only about 37% with glutamate as substrate. The effect of lindane on oxidative phosphorylation is concentration dependent. In an earlier investigation of the response of rat liver mitochondria to lindane, Nelson (1975) reported a similar effect on coupled phosphorylation as assessed by 32P incorporation into ATP. Although no attempt was made to understand the mechanism of action of lindane on oxidative phosphorylation, it is interesting to note that pentachlorophenol, a mammalian metabolite of lindane, is known to bind to mitochondria and act as an uncoupler (Weinbach 1954, Weinbach et al. 1963). It is believed that this metabolite arises essentially by microsomal metabolism and, therefore, it may not be possible to implicate this compound in the effect of lindane on isolated mitochondria.

An important biochemical effect of chlorinated hydrocarbon pesticides is their influence on ATPase systems in mammalian and insect tissues(Byczkowski et al. 1973, Byczkowski 1976, Kagawa and Racker 1966, Koch 1969, 1969/70, Matsumura and Patil 1969, Nelson 1975, Racker et al. 1975, Schwartz et al. 1972 , Skou, 1965). In the present studies, lindane inhibited Mg^{2+} ATPase activity and even more markedly DNP stimulated ATPase in rat liver mitochondria. These effects of lindane on rat liver mitochondrial ATPase have not been reported before. Other studies in the literature on the effects of several other organochlorine pesticides on basal Mg^{2+}ATPase and DNP stimulated ATPase activities of mitochondria, have provided divergent findings(Byczkowski et al. 1973, Byczkowski 1976, Koch 1969, 1969/70, Nelson 1975, Yap et al. 1975). Many investigators have reported an inhibition of mitochondrial ATPase activity in animals administered DDT (Koch 1969, 1969/70, Yap et al. 1975). On the other hand, a stimulation of ATPase activity by DDT and its metabolites, both in vivo and in vitro has also been observed (Byczkowski et al. 1973, Byczkowski 1976). Kepone, another insecticide belonging to this class has also been shown to be a potent inhibitor of oligomycin-sensitive Mg^{2+}ATPase activity in isolated perfused rat liver mitochondria(Desaiah et al. 1977). As observed in the present studies, like oligomycin and kepone, lindane was also inhibitory to DNP-stimulated Mg^{2+}ATPase as well as native Mg^{2+}ATPase. That these effects on ATPase may be relevant to the insecticidal action of these agents is indicated by the strong correlation between certain measurements of neurotoxic effects of organochlorine pesticides and the ATPase inhibition (Yap et al. 1975). In vitro inhibition of fish brain ATPase by cyclodiene insecticides and

related compounds has been shown (Yap et al. 1975).

From these findings on mitochondrial energy generation, it is reasonable to conclude that some of the toxic effects appearing in mammals due to lindane administration may arise from an influence of the compound on the respiratory chain, uncoupling of oxidative phosphorylation and ATPase activity. These effects may be of toxicological significance in animals and may serve as an index in assessing safety of such pesticides to non-target organisms.

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Received December 20, 1983; accepted April 4, 1984