The *in Vitro* Effect of Dieldrin on Respiration of Rat Liver Mitochondria[1] (35352)

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Widespread use of chlorinated pesticides in insect control programs has resulted in residues of these materials throughout the environment and man. At substantial dose levels, the administration of most pesticides to animals results in signs of toxicity (1). The effect of chronic low levels of pesticides on microsomal proliferation, mixed function oxidases and drug metabolism enzymes has been widely studied (2–4), but there are few data on the effects of pesticides on mitochondria. Johnston (5) showed that 1×10^{-5} *M* DDT (2,2-bis(*p*-chlorophenyl) -1,1,1-trichloroethane) inhibited rat heart mitochondrial cytochrome oxidase, but not succinic dehydrogenase. In an unpublished preliminary study, conducted in this laboratory, growing rats were injected intraperitoneally for a 2-month period every other day with either 4 or 20 mg of Dieldrin[2]/kg. After 60 days, isolated liver mitochondria from HEOD-treated rats exhibited poor respiratory control (6) and depressed NADH oxidase activity. The work described below was initiated to uncover the mode of HEOD inhibition of mitochondrial respiration.

Materials and Methods. Male rats (100–150 g) fed a 20% protein laboratory chow diet were used throughout the studies reported below. All rats were fasted overnight to reduce the liver glycogen content and liver mitochondria were prepared as outlined by Johnson and Lardy (7), except that the isolation and resuspension buffer contained 0.3 *M* mannitol, 0.01 *M* Tris, and 1.0 m*M* EDTA (pH 7.4).

These isolated mitochondria were used directly or modified by sonication or by aging. To prepare aged mitochondria, freshly isolated mitochondria were incubated in the isolation buffer for 45 min at 30°. Sonication was achieved by a 45-sec exposure of mitochondria to a microprobe sonifier set at 50 W. Mitochondrial respiration was measured polarographically with a Clark oxygen electrode at 30° (8). The incubation mixture contained 2.6 to 2.9 ml of buffer and 0.1 to 0.4 ml of mitochondrial preparation depending on the specific experiment. Substrates and inhibitors were added to the oxygen electrode with microsyringes during the incubation period. ADP induced mitochondrial oxygen uptake, *i.e.*, state 4 to state 3 transition (6), and 2,4-dinitrophenol (DNP) uncoupling studies were conducted with 3-hydroxybutyric acid (3HBA) and succinic acid as substrates. In all respects, the results with these two substrates were similar and thus only results using succinate will be presented below. NADH, 3HBA, and succinic oxidase were assayed as outlined previously (8, 9). Cytochrome C oxidase (terminal chain electron transfer) was assayed as outlined by Sanadi and Jacobs (10) using ascorbic acid and *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenylene-diamine (TMPD). All substrates and DNP were dissolved in buffer, while HEOD was dissolved in ethanol. The level of ethanol added to mitochondria in the oxygen electrode did not affect respiration. Mitochondrial protein was estimated with a modified Folin-phenol procedure (11).

Results and Discussion. Inhibitors of mitochondrial respiration either interfere at the

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[2] (HEOD) 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4,-endo-5,8-dimethanonaphthalene.

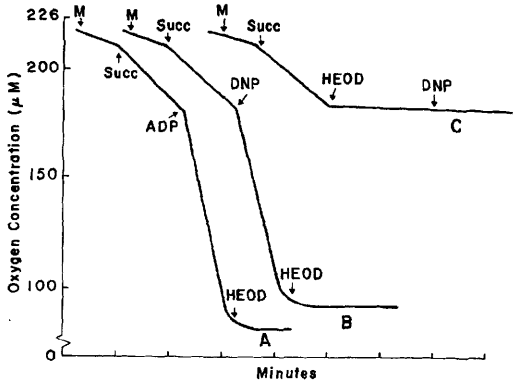

FIG. 1. Effect of HEOD on state 3 respiration and on 2,4-dinitrophenol-released state 4 respiration: Reaction mixture contained 100 m*M* mannitol, 10 m*M* phosphate, 10 m*M* Tris, 5 m*M* $MgCl_2$, and intact mitochondria in a final volume of 3 ml at pH 7.4. The following additions were made to the buffer: M = mitochondria (2.8 mg of protein); Succ = succinate (30 μmoles); ADP (3 μmoles); DNP = 2,4-dinitrophenol (160 mμmoles); HEOD (500 mμmoles).

coupling sites or in the electron transport chain, while uncouplers release controlled respiration by disrupting oxidative phosphorylation. To establish the mode of action of such materials they are usually studied in reference to the well-established DNP uncoupling effect (12). When DNP is added to mitochondria in state 4 (ADP limiting, substrate and O_2 excess) a sudden burst of O_2 uptake is noted since the respiratory control of ADP has been bypassed by uncoupling (12). Addition of an electron transfer inhibitor to mitochondria completely supresses the release of state 4 respiration by DNP. An inhibitor which affects the coupling site beyond the DNP-sensitive site inhibits the ADP-induced transition of state 4 to state 3 respiration; however, subsequent addition of DNP will again release respiration (12). To locate the general site of the HEOD effect, intact coupled mitochondria were used.

The effect of HEOD on electron transfer was studied with sonicated mitochondria. Aged (uncoupled) mitochondria were used to further study HEOD action in uncoupled mitochondria in the absence of DNP. A mitochondrial uncoupler will not modify respiration of aged mitochondria (12). Some inhibitors (or uncouplers) of mitochondrial respiration demonstrate a concentration (*i.e.*, molarity) effect, while others exhibit a specific stoichiometry with mitochondrial protein level. Sonicated and aged mitochondria were used to titrate the HEOD effect on mitochondrial respiration.

Representative results on the effect of HEOD on oxygen uptake of intact coupled mitochondria are shown in Fig. 1. For all results reported, each separate experiment shown in Figs. 1–3 (*i.e.*, a curve) was repeated 2 or 3 times for a given mitochondrial preparation; 4 separate mitochondrial preparations (isolated from different rats on different days) were used for each experiment. The addition of HEOD to mitochondria in state 3 respiration (curve A, Fig. 1) immediately suppressed O_2 uptake. Subsequent additions of DNP (not shown) failed to release respiration. When DNP was added to mitochondria in state 4, subsequent addition of HEOD supressed the DNP-released state 4 respiration (curve B, Fig. 1); alternatively when HEOD was added to mitochondria in state 4, O_2 uptake was inhibited and DNP had no effect (curve C, Fig. 1). DNP additions to aged mitochondria (curve D, Fig. 2) did not cause a further increment in O_2 uptake, however, subsequent addition of HEOD again blocked respiration. Direct HEOD addition to aged mitochondria (curve

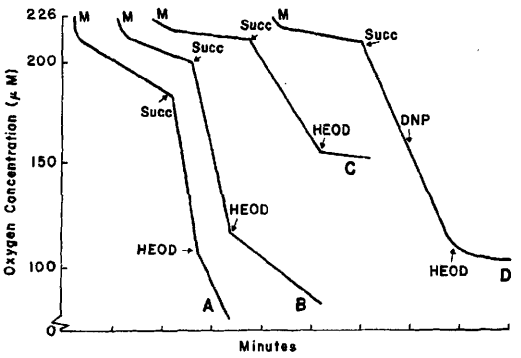

FIG. 2. Effect of mitochondrial protein level on HEOD sensitive mitochondrial respiration: Reaction mixture same as in Fig. 1. The following additions were made to the buffer: (curves A, B, C, and D) M = aged mitochrondria (9, 6, 3, and 3 mg of protein, respectfully); Succ = succinate (30 μmoles); HEOD (500 mμmoles); DNP (160 mμmoles).

TABLE I. Inhibition of Electron Transport in Sonicated Rat Liver Mitochondria by HEOD.[a]

Substrate	Rate of O_2 consumption (mμmoles/mg of protein/min)	HEOD level (mμmoles/mg of protein)	Rate of O_2 consumption after HEOD additions (mμmoles/mg of protein/min)	% of control
$NADH_2$	34.48 ± 2.99	180	10.53 ± 1.78	29.7
3-HBA	19.70 ± 1.40	180	3.56 ± 0.90	18.1
Succinate	39.51 ± 3.55	180	3.42 ± 1.10	9.1
TMPD-ascorbate (cytochrome C oxidase)	47.83 ± 1.16	180	51.22 ± 2.87	107.0

[a] Reaction mixture same as Fig. 1. The following additions were made to the buffer: sonicated mitochondria (2.8 mg of protein), either $NADH_2$ (0.6 mg), or 3-HBA (30 μmoles), or succinate (30 μmoles), or TMPD (0.9 μmoles), and ascorbic acid (4.5 μmoles).

C, Fig. 2) also blocked respiration. These results are consistent with the conclusion that HEOD acted at a site prior to DNP (12), presumably in the electron transport chain.

To further explore the contention that HEOD acts on the mitochondrial electron transport chain and to locate the probable site of electron flow inhibition, the effect of HEOD on $NADH_2$ and 3-HBA oxidase, succinic oxidase, and terminal chain electron flow (ascorbate-TMPD-cytochrome C oxidase, 8) was studied with sonicated mitochondria. The results of this study (4 separate experiments) are shown in Table I. These data showed that when 3-HBA, NAD_2, or succinate were the substrates for electron flow, HEOD markedly inhibited electron transport; however, cytochrome C oxidase was not inhibited by HEOD additions. Further work showed that a 10-fold addition of HEOD to that shown in Table I did not inhibit cytochrome C oxidase. These data show that HEOD inhibited electron flow in or about the cytochrome B site. However, terminal chain electron flow was not affected. These results can be contrasted with those of Johnston (5) who concluded that DDT (another chlorinated hydrocarbon) inhibited cytochrome C oxidase activity in heart mitochrondria. However, the level of DDT necessary to cause this inhibition was similar to the level of HEOD needed to inhibit mitochrondrial electron transport in this study.

The site of mitochondrial electron flow inhibition by HEOD is similar to the site of electron flow inhibition by antimycin (13) and since the antimycin effect was dependent on protein level (13), further experiments were conducted to establish the relationship between HEOD level and mitochondrial protein level on electron flow inhibition. The results for these studies are presented in Figs. 2 and 3. Figure 3 shows that successive additions of HEOD to a constant level of mitochondrial protein caused successive increments of inhibition of oxygen uptake.

When HEOD additions to mitochondria were kept constant, but mitochondrial protein level was varied (Fig. 2), HEOD inhibition was decreased with each added increment of

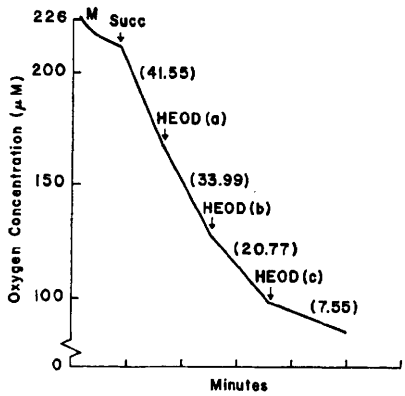

FIG. 3. Effect of level of HEOD (μmoles/mg of protein) on respiration of sonicated rat liver mitochondria: Reaction mixture was same as in Fig. 1. The following additions were made to the buffer: M = sonicated mitochondria (3.6 mg protein); Succ = succinate (30 μmoles); HEOD(a) (150 mμmoles); HEOD(b) (additional 100 mμmoles); HEOD(c) (additional 150 mμmoles to a final of 400 mμmoles). The values in parentheses are the actual O_2 uptake rates (mμmoles of O_2/mg of protein/min).

mitochondrial protein [Fig. 2 (curves A) 9, (B) 6, and C, 3 mg of protein]. Thus, HEOD affected mitochondria in a manner similar to antimycin (13) since the HEOD effect on mitochondria was dependent on protein level.

Concentration of dieldrin (HEOD) in human adipose tissue of 5.2 μmoles/kg (14), and in bovine liver of 3.38–3.9 μmoles/kg (15) have been reported. Since HEOD is primarily concentrated in lipids, a substantial level of this pesticide may be associated with mitochondria as they contain a high level of lipids. It is therefore possible that mitochondrial respiration in various organs of animals and man may be impaired by residual dieldrin (HEOD).

Summary. The *in vitro* effect of dieldrin (HEOD) on respiration of rat liver mitochondria was studied. It was found that HEOD inhibited mitochondrial respiration at a site prior to the DNP-sensitive coupling site. Further work showed that HEOD inhibited electron transport in the mitochondrial electron chain in the cytochrome B area; terminal chain electron transport was not effected by HEOD. The effect of HEOD was dependent upon the level of mitochondrial protein and not on the molar concentration of the pesticide in the reaction mixture.

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