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THE ACTION OF CYCLODIENE PESTICIDES ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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Abstract—The effects of a number of chlorinated hydrocarbon pesticides have been studied on isolated rat liver mitochondria. Inhibition of mitochondrial function was found to be dependent upon the ratio of pesticide to protein, and to some extent upon the type of pesticide. Most of the pesticides tested exerted more than one effect depending upon the concentration. At low concentrations (less than 20–25 nmoles/mg protein), State 3 respiration, DNP-activated respiration and valinomycin-induced swelling were inhibited by certain cyclodienes. These results cannot be explained by inhibition of the electron transport chain or uncoupling. They appear to be associated with restrictions of ion movements across the membrane (DNP, valinomycin-K^+ complex, ADP). On increasing the concentration of pesticide, lysis of the membranes and uncoupling can be induced. The electron transport chain can also be inhibited, but only with 5–10 times greater concentrations of pesticide than required for uncoupling. In general, the cyclodiene pesticides are slightly more effective inhibitors than the non-cyclodienes tested.† The effectiveness of the cyclodienes is, however, dependent upon its stereochemical properties. These findings suggest that the cyclodienes may inhibit mitochondria in a common, and, perhaps, specific manner. The specificity and mode of action of cyclodienes was found to be similar to that described earlier (B. D. Nelson and C. Williams, *Agric. fd Chem.* **19**, 339 (1971)), using growing yeast cells as the test system.

In a previous study [1] we demonstrated that certain cyclodiene pesticides inhibit the growth of *Saccharomyces cerevisiae* utilizing non-fermentable sugars as the sole energy source, but had no effect on the growth of cells utilizing only fermentable sugars such as glucose. It was concluded that the cyclodienes acted on *S. cerevisiae* by specifically inhibiting mitochondrial oxidative metabolism. Furthermore, it was shown that the cyclodienes do not act by inducing respiratory deficient mutants as do a wide variety of other chemicals [2, 3], but rather appeared to have a direct action on mitochondrial enzymes. This inhibitory effect was specific only for certain structurally related cyclodienes such as chlordane, heptachlor, heptachlor epoxide, aldrin, and dieldrin, but not for chlorinated hydrocarbons structurally unrelated to the cyclodienes, such as lindane, mirex, DDT and several DDT analogs.

In the present paper we have extended these findings to include the action *in vitro* of cyclodiene pesticides on rat liver mitochondria. The results show that oxidative phosphorylation is inhibited by the cyclodienes, and, furthermore, the structural requirements for inhibition of mitochondrial function are the same as observed for inhibition of yeast cell growth [1]. It is suggested that interference with oxidative phosphorylation may represent one of the mechanisms of cellular toxicity of the cyclodienes.

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† (a) *Cyclodienes*—Chlordane, 1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan; heptachlor, 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene; endosulfan, 1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dimenthanol cyclic sulfite; dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo-exo-1,4:5,8-dimethanolnaphthalene; aldrin, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a- hexahydro-endo-exo- 1,4:5,8- dimethanonaphthalene; endrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8-8a octahydro-*endo-endo* 1,4:5,8-dimethanonaphthalene; isodrin, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a hexahydro-*endo-endo*-1,4:5:8-dimethanonaphthalene. (b) *Non-cyclodienes*—Mirex, dodecachlorooctahydro-1,3,4-methano-2H-cyclobuto[cd]pentatene; lindane, 1,2,3,4,5,6-hexachlorocyclohexane; DDT, 1,1,1-trichloro-2,2 *bis*(*p*-chlorophenyl)ethane.

METHODS

Mitochondria were prepared from rat livers as described earlier [4]. Submitochondrial particles were prepared from heavy liver mitochondria by the sonication procedure of Azzi *et al.* [5]. Mitochondria were suspended at 20 mg protein/ml in 2 mM EDTA, pH 8·5, and sonicated for 1 min in a Branson Sonicator at a power output of 4. The sonicate was centrifuged at 10,000 *g* for 15 min and the resulting supernate was then centrifuged for 30 min at 100,000 *g*. The pellet was washed twice in 0·25 M sucrose.

Respiration was measured polarographically using a Clark oxygen electrode. The standard reaction mixture contained 50 mM Tris–Cl (pH 7·5), 5 mM K_2HPO_4, 5 mM $MgSO_4$ and 100 mM KCl. Oxidative phosphorylation was measured by the incorporation of $^{32}P_i$ into $AT^{32}P_i$. The molybdate extraction procedure described by Pullman [6] was used to separate

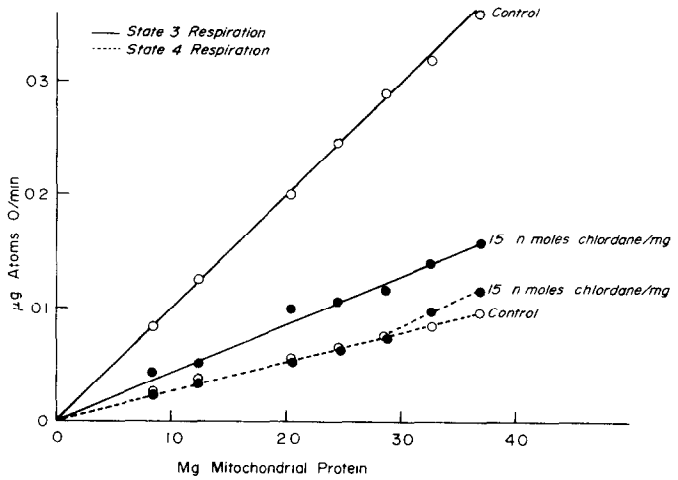

Fig. 1. Chlordane inhibition of mitochondrial function as a function of mitochondrial protein. Mitochondrial protein and chlordane concentrations were increased in parallel so that the molar concentration of chlordane increased while the ratio of chlordane to protein remained constant at 15 nmoles/mg protein. Mitochondria were pre-incubated for 2 min in the presence of both pesticide and substrate prior to initiating ADP-activated (State 3) respiration. The reaction media contained 5 mM succinate, 0·490 μmoles ADP and 2 mg of mitochondrial protein in a total volume of 1·7 ml. Temperature was 25°.

$^{32}P_i$ from $AT^{32}P_i$. Radioactivity was counted using the Cerenkov method as previously described [4].

Pesticides of 99% purity or greater were supplied by Drs. L. Fishbein and J. McKinney of this Institute. Solutions (15 mM) were prepared in dimethylsulfoxide and small volumes of 1–20 μl were added directly to the reaction vessels. Preliminary experiments have shown that dimethylsulfoxide concentrations to 10% (v/v) do not interfere with respiration or phosphorylation.

RESULTS

Inhibition of mitochondrial function by chlorinated hydrocarbon pesticides is dependent upon the ratio of pesticide to mitochondrial protein rather than on the absolute concentration of the pesticide. This is shown in Fig. 1 for chlordane. In this experiment the concentrations of both chlordane and mitochondrial protein were increased proportionally so as to maintain a ratio between them of 15 nmoles chlordane/mg protein. It can be seen that State 3 respiration (ADP-stimulated) is inhibited to the same extent regardless of the absolute concentration of pesticide. The data also show that this concentration of chlordane inhibits State 3 but not State 4 respiration.

The differential effect of chlorinated hydrocarbons on States 3 and 4 are examined more thoroughly in Fig. 2. The figure shows concentration–effect curves for two cyclodienes (chlordane and heptachlor) and a non-cyclodiene (lindane). Several differences are discerned. First, less pesticide is required to inhibit State 3 respiration when cyclodienes are used. State 3 is 50 per cent inhibited by 20–25 nmoles chlordane or heptachlor/mg protein, whereas approx 100 nmoles lindane/mg protein is required for the same affect. Nearly identical concentration–effect curves have been obtained for all the cyclodienes examined (α and β chlordane, aldrin, dieldrin, endosulfan, heptachlor, heptachlor epoxide) with the exception of two: isodrin and endrin. In contrast, all of the noncyclodiene chlorinated hydrocarbons tested gave titration curves identical to that shown for lindane (Fig. 2) regardless of the diversity in structure. These included, in addition to lindane, mirex, DDT and several DDT analogs. These results suggest a common, and perhaps specific, mode of action for the cyclodienes, in spite of the fact that the inhibitory concentrations are only 4-fold lower than for the non-cyclodiene pesticides.

Figure 2 also shows that the pesticides appear to have multiple, concentration-dependent actions on

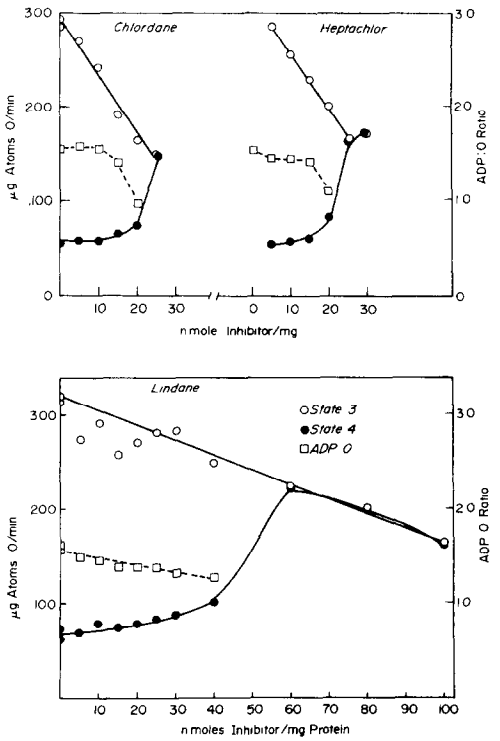

Fig. 2. The effects of various chlorinated hydrocarbon pesticides on oxidative phosphorylation in rat liver mitochondria. Conditions of the experiment are described in Fig. 1 and in Methods.

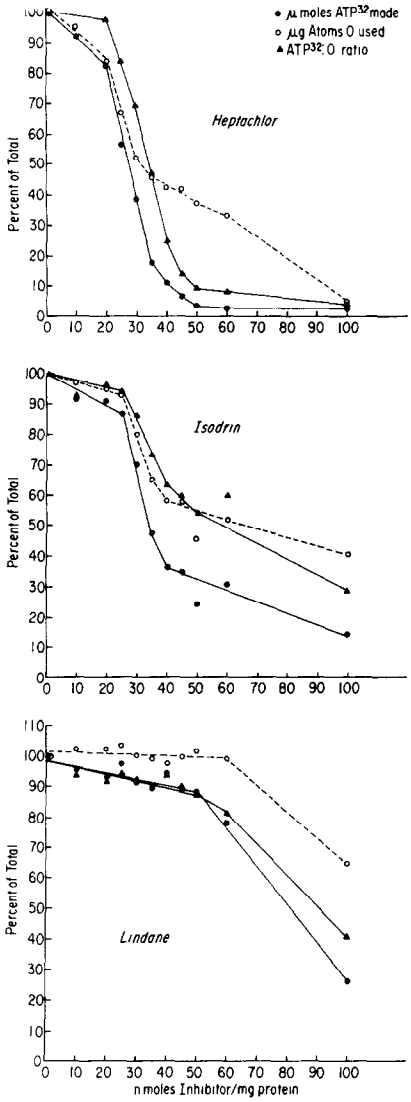

Fig. 3. Inhibition of $AT^{32}P$ synthesis by certain chlorinated hydrocarbon pesticides. Experimental conditions are as described in Methods and Fig. 1 with the exceptions that 100,000 cpm of carrier-free $^{32}P_i$ was included in the reaction media and the ADP concentration was increased to 1·5 mM. Succinate was the substrate. Control values (100%) in the three experiments ranged between 0·134 and 0·143 μg atoms O/min per mg protein, 0·258 to 0·270 μmoles $AT^{32}P$ formed/min per mg protein and an $AT^{32}P$:O ratio of 1·85–2·00.

oxidative phosphorylation. The most sensitive step involves inhibition of State 3 respiration followed by uncoupling of oxidative phosphorylation. The latter is indicated by a decrease in the ADP:O ratio and activation of State 4 respiration. The onset of uncoupling by cyclodienes occurs near 20 nmoles/mg protein, and maximal activation of State 4 is achieved by an additional 10–15 nmoles/mg protein.

Since the ADP:O ratio can not be measured when States 3 and 4 have the same respiration rate (respiratory control index is unity) uncoupling was also measured by the incorporation of $^{32}P_i$ into $AT^{32}P$ (Fig. 3). The figure again shows results obtained from two cyclodienes (heptachlor and isodrin) and a non-cyclodiene (lindane) chlorinated hydrocarbon. The curve obtained with heptachlor was also obtained with the other cyclodienes tested (α and β chlordane, aldrin, dieldrin, endosulfan, heptachlor epoxide), except isodrin (Fig. 3) and endrin (not shown). The titration curve for endrin is similar to that shown for lindane (Fig. 3). Thus, the concentration–effect curves obtained measuring the synthesis of $AT^{32}P$ are in excellent agreement with those shown in Fig. 2. These data support the suggestion that, with the exception of isodrin and endrin, the cyclodienes act similarly. As shown in Fig. 3, uncoupling by isodrin is biphasic, with only 50 per cent uncoupling in the same range of concentrations in which heptachlor gives 100 per cent uncoupling. This biphasicity could be due to a site specific uncoupling by isodrin, or to some unique solubility properties of the pesticide. The fact that isodrin and endrin do not mimic the other cyclodienes might be due to steric differences; the carbon skeleton of isodrin and endrin being *endo-endo* while the other are *endo-exo*.

Inhibition of State 3 respiration by low concentrations of cyclodiene (Figs. 1 and 3) cannot be attributed to direct inhibition of the electron transport chain. The latter requires much higher concentrations of the pesticide (Fig. 4). Titration curves similar to those in Fig. 4 have been obtained using both submitochondrial preparations and frozen-thawed mitochondria. In view of the latter observation, it seems unlikely that the high concentration of chlordane required to inhibit electron transport can be due merely to the relocation of the high affinity binding sites to the inside of submitochondrial particles during sonication [5]. Figure 4 also shows that different sites on the respiratory chain exhibit varying sensitivities to chlordane. In contrast, in coupled mitochondria inhibition curves similar to those shown in Figs. 2 and 3 for chlordane and heptachlor

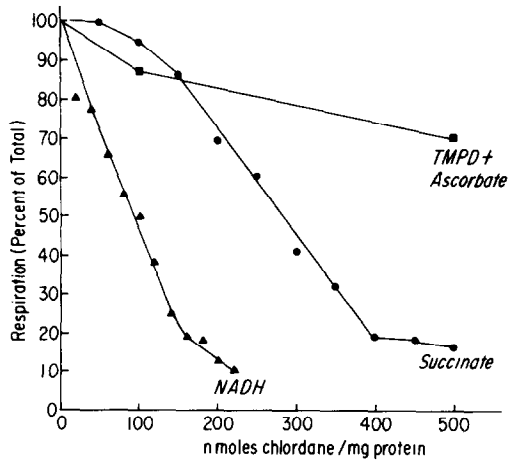

Fig. 4. Chlordane inhibition of electron transport in rat liver sub-mitochondrial particles. Particles were pre-incubated with chlordane for two min before adding substrate. The reaction media contained 50 mM Tris–HCl (pH 7·5), 5 mM $MgSO_4$, 100 mM KCl, 0·4–0·8 mg of protein and either 1 mM NADH, 5 mM succinate or 5 mM ascorbate + 100 μM TMPD in a total vol of 1·7 ml. The temp was 25°. Control respiratory values (μg atoms O/min per mg) were 0·232 for succinate, 0·570 for NADH and 0·800 for ascorbate + TMPD.

Table 1. Effect of chlordane on ATPase activity in frozen-thawed rat liver mitochondria

Chlordane (nmoles/mg protein)	ATPase activity (μmoles ATP split/min per mg)
0	0·256
30	0·290
100	0·325
150	0·235

Mitochondria were preincubated for 3 min with chlordane before initiating ATPase activity by addition of 1·2 mM ATP. The reaction mix contained 50 mM Tris–HCl, pH 7·5, 30 mM KCl, 5 mM $MgSO_4$, 0·75 mM phosphoenolpyruvate, 0·18 mM NADH, 2 μM rotenone, 12·5 μg phosphoenolpyruvate kinase (425 μmoles product/min per mg), 7·5 μg lactic dehydrogenase (670 μmoles product/min per mg) and 0·4 mg mitochondrial protein in a total vol of 2·5 ml. The temp was 30°. Oxidation of NADH was measured at 340 nm.

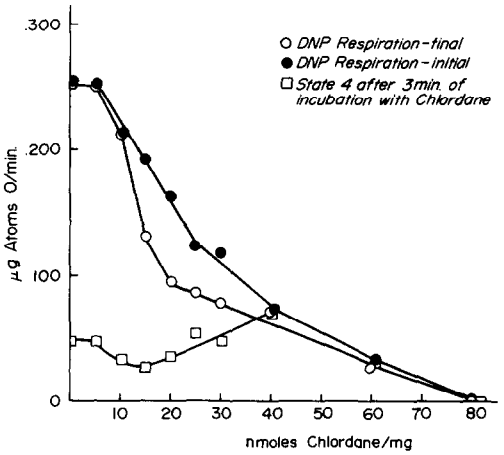

Fig. 5. Chlordane inhibition of DNP-activated respiration in rat liver mitochondria. Mitochondria (2 mg protein) were incubated in the presence of 5 mM succinate and the indicated concentrations of chlordane. After 3 min, 150 μM DNP was added. Respiration rates are given for the initial and final rates obtained after adding DNP. Other conditions are described in Methods.

are obtained using a large variety of substrates (glutamate, β-hydroxybutyrate, succinate or ascorbate-TMPD). These results clearly indicate that low concentrations of cyclodienes inhibit State 3 respiration and synthesis of ATP by a mechanism not involving inhibition of the respiratory chain. The action of chlordane on State 3 respiration is, furthermore, not attributed to an oligomycin-like action [7] of the pesticide since ATPase activity is not inhibited, even at high (150 nmoles/mg protein) concentrations (Table 1).

A surprising finding, however, is that dinitrophenol (DNP) activation of respiration is inhibited by the same concentrations of chlordane as required to prevent State 3 respiration (Fig. 5). Since the respiratory chain *per se* is not inhibited at these concentrations of pesticide (Fig. 4) these data are interpreted to indicate that chlordane may inhibit the movement of DNP across the membrane.

The possibility that low concentrations of chlordane may interfere with respiration by restricting ion movement is further suggested by studies shown in Figs. 6 and 7. Preincubation of mitochondria with varying concentrations of chlordane inhibit valinomycin-induced swelling. Inhibition occurs regardless of the energy source (succinate, glutamate, β-hydroxybutyrate, ascorbate-TMPD or ATP). Inhibition of valinomycin-induced swelling with ATP as the energy source again shows that chlordane does not act on a respiratory chain component at low concentrations. It is also unlikely that chlordane inhibition of valinomycin-induced swelling is due to uncoupling, since

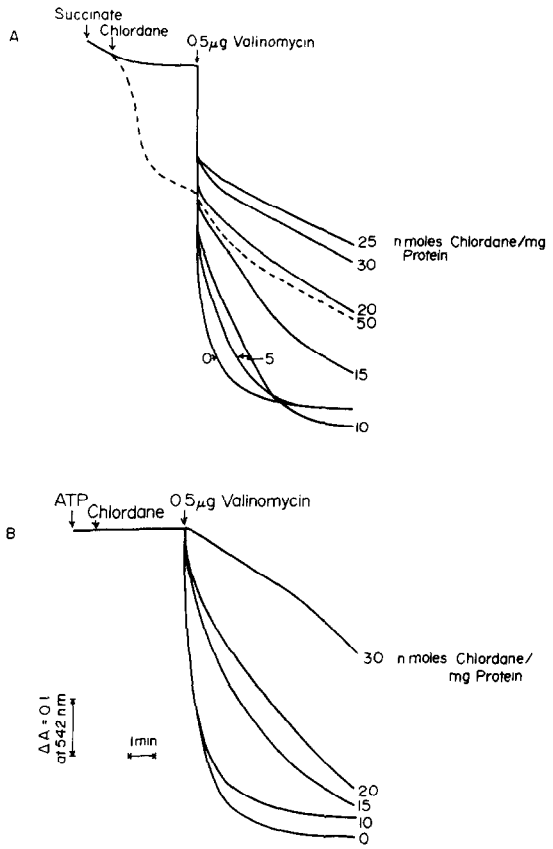

Fig. 6. Inhibition of valinomycin-induced swelling by chlordane. Mitochondria were pre-incubated with chlordane for 3 min before adding valinomycin. The reaction media contained 150 mM KCl, 50 mM Tris–HCl (pH 7·5), 4 μM rotenone, 0·6 mg mitochondrial protein and either 5 mM succinate or 2·5 mM ATP plus 5 mM $MgCl_2$ in a total vol of 3 ml. The temp was 30°.

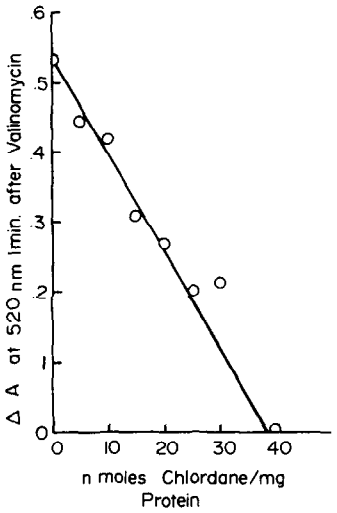

Fig. 7. Inhibition of valinomycin-induced mitochondrial swelling by chlordane. The conditions of the reaction are described in Fig. 6. Succinate was the substrate.

the concentrations of chlordane required (Fig. 7) are slightly less than needed for uncoupling (Figs. 1 and 2). The concentration of chlordane inhibiting valinomycin-induced swelling is also less than those which induce non-energy linked swelling of the mitochondria (Fig. 8). The latter affect of chlordane occurs between 25–40 nmoles chlordane/mg protein, and correlates well with its uncoupling activity (cf. Fig. 8 and Figs. 1 and 2). Thus, the swelling data clearly indicate that the action of chlordane is at least 2-fold: at low concentrations it prevents ADP and DNP activated respiration and valinomycin-induced swelling by a yet undetermined mechanism, which does not appear to involve direct inhibition of the respiratory chain or uncoupling of oxidative phosphorylation. At slightly increased concentrations it produces a lytic effect on the membrane which is accompanied by uncoupling.

DISCUSSION

The action of chlorinated hydrocarbon pesticides on mitochondrial function has been repeatedly studied with widely varying results [8–11]. One reason for the lack of agreement regarding the affects of these pesticides is clearly due to concentration differences. This is emphasized by the present study. Carefully constructed *in vitro* concentration–effect curves reveal that inhibition of oxidative phosphorylation by chlorinated hydrocarbon pesticides is strictly protein (or lipid) dependent. Expression of pesticide in molar concentrations is of little value unless protein concentrations are also stated. This is not surprising in view of the low water solubility of most chlorinated pesticides [12]. Furthermore, small changes in the concentration of pesticide (when expressed relative to protein or lipid) can give strikingly different inhibitory effects on the mitochondria. For example, mitochondria can be uncoupled by an increase of about 10 nmoles pesticide/mg protein.

Concentration–effect curves also provide comparative information on the inhibitory effectiveness of different chlorinated pesticides, as well as on their modes of action. Based on such curves, the pesticides tested fall into two categories containing certain cyclodiene and non-cyclodiene chlorinated hydrocarbons respectively. The difference in the amount of pesticide required for inhibition of State 3 respiration is only 3–4 times greater for the non-cyclodienes than for the cyclodienes. However, in view of the high reproducibility of the concentration–effect curves (the chlordane curve does not very by more than 5 nmoles/mg protein), these differences are quite significant. Thus, we conclude that some specificity of action of the pesticides on mitochondrial function *in vitro* exists. This belief is supported by the observation that the inhibitory effectiveness of the cyclodienes is dependent upon the stereochemical properties of the molecule; the *endo-endo* forms [13] being less effective than *endo-exo*. Similar stereochemical properties were also reported for cyclodiene-mediated inhibition of oxidative metabolism in growing yeast cultures [1]. Whether these differences are due to solubility differences of the pesticide in the lipid phase, or to specific protein binding [14] cannot be decided.

The mechanism of inhibition of mitochondrial function by chlorinated hydrocarbon is of interest. It is clear that they exert more than one inhibitory effect depending upon the concentration in the membrane. The most easily observed effect of the cyclodiene is uncoupling of oxidation and phosphorylation. Complete uncoupling is induced by increasing the concentration of chlordane over a range of approx 10 nmoles/mg protein. This sharp transition suggests a detergent-like action of the pesticide on the mitochondrial membrane. Indeed, uncoupling is associated with lysis of the inner membranes as indicated by non-energy linked swelling (Fig. 8) and electron microscopy (not shown).

A more interesting action of chlordane appears to involve inhibition of State 3 respiration, DNP-activated respiration and valinomycin-induced swelling. These affects cannot be attributed to inhibition of

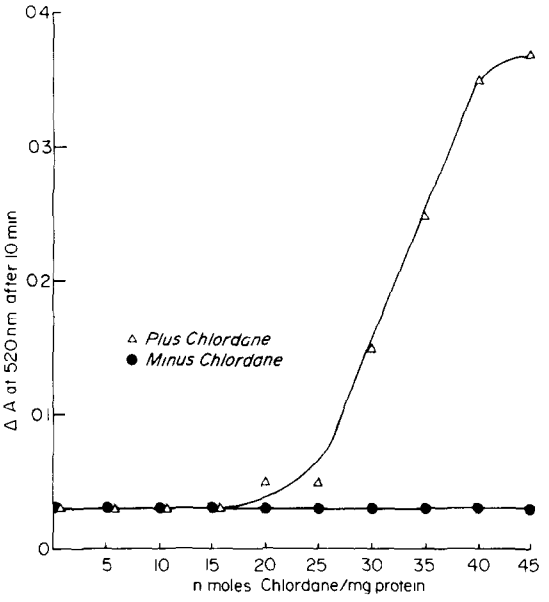

Fig. 8. Chlordane-induced nonenergy linked mitochondrial swelling. The reaction conditions are the same as in Fig. 6 with the exception that exogenous substrates were excluded. Rotenone (4 μM) was added to prevent the oxidation of endogenous substrates.

the electron transport chain or to uncoupling (which would obviously not interfere with DNP-activated respiration). In view of the results, particularly with respect to DNP-activated respiration, we tentatively conclude that low concentrations of chlordane act by restricting ion movements through the membrane. The mechanism by which this is achieved appears, however, to lack specificity. One possible explanation might be that, similarly to cholesterol [15], chlordane alters the fluidity of the lipid bilayer, reducing the mobility of proteins and inhibiting the movement of small mol. wt substances across it [16]. Such a suggestion might also be in keeping with the effects of chlorinated pesticides on the generation of nerve action potentials [17, 18] or the activity of the Na^+-K^+-activated ATPase [19].

In a previous study [1] we reported that selected chlorinated hydrocarbon pesticides inhibited the growth of yeast by inhibiting oxidative metabolism. We pointed out (1) that the action of the pesticide was not the result of a mutation but rather due to a direct action on the mitochondria, (2) that the pesticide did not inhibit the electron transport chain, but rather the phosphorylation reactions and (3) that inhibition was much greater with selected cyclodienes than with the non-cyclodienes tested. Our present results using isolated rat liver mitochondria lead to the same conclusions, particularly with regards to points (2) and (3). Whether these correlations are fortuitous, or imply a general mechanism of action of the cyclodienes is not known. It is, however, subject to experimental testing. Studies correlating the intracellular distribution and concentration of pesticide with the metabolic state of isolated mitochondria should be carried out. Mitochondria isolated from rats fed with dieldrin have been shown to be uncoupled [20], but mitochondrial pesticide concentrations were not determined.

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