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EFFECTS OF DICHLORODIPHENYLTRICHLOROETHANE AND ITS ANALOGUES ON RAT LIVER MITOCHONDRIA

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Abstract—The effects of dichlorodiphenyltrichloroethane (DDT) and sixteen analogues on respiration, swelling and latent ATPase of rat liver mitochondria were examined systematically. The compounds tested could be divided into four groups: DDT-type, DDE-type, kelthane-type and others by substituent groups on the ethane bridge of bis(*p*-chlorophenyl) ethane. Most compounds tested were shown to inhibit State 3 respiration. A linear relation was observed between the logarithms of the concentrations giving half-inhibition of State 3 respiration and the logarithms of the partition coefficients of the tested compounds. Four compounds of the kelthane type and chlorobenzilate stimulated State 4 respiration to the level of dinitrophenol-stimulated respiration. The compounds that have a hydroxy group on the ethane bridge rapidly induced mitochondrial swelling, but DDT-type and DDE-type compounds induced swelling when the suspension contained 0.15 M KCl and 5 mM Tris–HCl. Latent ATPase of mitochondria was stimulated to different maximum levels by each of the tested compounds except DDA. The oligomycin-sensitive ATPase of submitochondria was inhibitied by a series of kelthane-type compounds.

Several works concerning the interaction of dichlorodiphenyltrichloroethane (DDT) and its analogues with mitochondria have been published. Parker [1] showed that DDT affects both oxygen consumption and energy production in mitochondria. Byczkowski [2] and Byczkowski *et al.* [3] also investigated the effects of four DDT-type compounds on respiration and ATPase activity of rat liver mitochondria. However, the structure–activity relationships between chlorinated hydrocarbon pesticides and mitochondrial function have not been studied systematically using a number of DDT analogues.

In view of the very low solubilities of chlorinated hydrocarbon pesticides in water, some interaction of these compounds with membrane lipoprotein of mitochondria seems to occur as suggested by Haque *et al.* [4], who observed interaction of lecithin with eight DDT-type compounds. In fact, a structure–activity relation was shown between the solubilities of DDT analogues and their biodegradabilities in fish [5]. Abernathy *et al.* [6] also reported a structure–activity relationship for the induction of microsomal enzymes by DDT analogues in the mouse.

Therefore, to understand the interactions of chlorinated hydrocarbon pesticides with mitochondria, it is necessary to determine certain indexes of hydrophobicity for tested compounds. Partition coefficients of water-insoluble compounds such as DDT analogues are difficult to determine, but can be approximated by high pressure liquid chromatography (h.p.l.c.) [7].

The purpose of the present study was to determine systematically the potencies of DDT and sixteen of its analogues (see Table 1) in affecting respiration, swelling and ATPase activity of rat liver mitochondria and to clarify the structure–activity relations of chlorinated hydrocarbon pesticides.

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MATERIALS AND METHODS

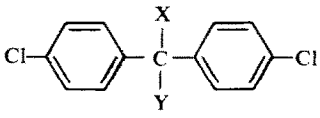
Rat liver mitochondria were prepared by the method of Schneider [8] using male rats of the Wistar strain, weighing 200–300 g. These mitochondria (35 mg/ml) were suspended in 0.25 M sucrose solution as a stock sample. Submitochondria were prepared by sonic disruption of intact mitochondria at 0° for 5 min at 10 KHz using a Kubota KMS-100 sonicater. Protein concentration was determined by the method of Lowry *et al.* [9] using crystalline bovine serum albumin.

Respiration rates were measured at 25° polarographically with a Clark-type oxygen electrode purchased from Beckman. The reaction vessel with an electrode was modified from that of Estabrook [10], and the vessel volume was 5.0 ml. The reaction mixture contained 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 5 mM potassium phosphate (pH 7.4), 10 mM KCl, 5 mM $MgCl_2$ and 0.1 mM EDTA.

Oxygen uptake was recorded after the addition of 50 μl of mitochondrial suspension (1.2 mg of mitochondrial protein). State 4 or 3 respiration [11] was initiated by the addition of 5 mM sodium succinate or 0.8 mM ADP + 5 mM sodium succinate respectively.

Mitochondrial swelling was measured by the method of Lehninger and Remmert [12]. The composition of the incubation mixture besides mitochondria (0.3 mg/ml) is given in the figure legends. The light scattering of the incubation mixture (total volume 2.5 ml) was measured at 520 nm with a Hitachi 200–10 spectrophotometer.

Table 1. Stuctures of substituents, molecular weights and partition coefficients (P) for the compounds tested



Type	Abbreviated or common name	Substituent at X	Substituent at Y	Molecular weight	Log P
DDT	DDT	—H	—CCl_3	354.5	3.22
	DDD	—H	—$CHCl_2$	320.0	2.97
	DDMS	—H	—CH_2Cl	285.6	2.86
	K-3926	—H	—CH_3	251.2	2.96
DDE	DDE		=CCl_2	318.0	3.21
	DDMU		=$CHCl$	283.6	3.08
	DME		=CH_2	249.1	2.96
Kelthane	Kelthane	—OH	—CCl_3	370.5	2.83
	FW-152	—OH	—$CHCl_2$	336.0	2.57
	DCMC	—OH	—CH_2Cl	301.6	2.48
	DMC	—OH	—CH_3	267.2	2.25
Others	DDM	—H	—H	237.1	2.47
	DCBH	—H	—OH	253.1	2.09
	DDA	—H	—COOH	281.1	
	DDDH	—H	—CH_2OH	267.2	2.02
	DCBP		=O	251.1	2.43
	Chlorobenzilate	—OH	—$COOC_2H_5$	325.2	2.44

ATPase activity was measured by the determination of liberated inorganic phosphate according to Fiske and SubbaRow [13]. The reaction mixture (final volume 1.5 ml) contained 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.4), 5 mM $MgCl_2$ and 0.4 mg of mitochondrial or 0.3 mg of submitochondrial protein. The reaction was started by the addition of 5 mM ATP to the mixture. In a control experiment, the same volume of ethanol was added instead of an ethanol solution of DDT analogues. After routine incubation for 15 min at 25°, the reaction was stopped by the addition of 1 ml of 3 N perchloric acid, and then the filtered solution was used for inorganic phosphate analysis.

The structures of substituents and the molecular weights of DDT and its analogues tested are listed in Table 1. On the basis of the kinds of substituent groups on the ethane bridge, these compounds were classified into four groups: DDT-type, DDE-type, kelthane-type, and others. FW-152 [14], K-3926 [15], DMC [15], DDMS [16], DDMU [17], DME [17] and DDDH [18] were synthesized in this laboratory according to the respective methods reported previously. DCMC was prepared in the same manner as FW-152. The other nine compounds were provided by Wako Chemicals (Tokyo, Japan). All the compounds tested were dissolved in ethanol and were found to be of more than 99% pure grade by gas chromatography and h.p.l.c. The ethanol solution of the test compounds was injected with a microsyringe into the reaction mixture. ATP disodium salt, ADP disodium salt and oligomycin were obtained from Boehringer-Mannheim (W. Germany). All other commercial reagents were of analytical grade.

Determination of lipophilic indexes, correlated to partition coefficients, was performed on a Hitachi model 638 liquid chromatograph equipped with a u.v. detector set at 254 nm. The stationary phase was nucleosil $10C_{18}$ (Macherey-Nagel Co., Düren, W. Germany) packed into a 25-cm stainless steel column, 4 mm i.d. The mobile phase was 55, 65 and 75% CH_3CN in H_2O. Flow rate was 1.5 ml/min. Samples for injection were dissolved in CH_3CN at concentrations of 0.1 to 0.5 mg/ml, and 2 μl of the sample solution was injected into the column.

With the reversed-phase high pressure liquid chromatographic method [7] developed for the determination of partition coefficients, the lipophilic index, log k', can be defined as follows:

$$\log k' = \log [(t_R - t_0)/t_0] \quad (1)$$

where t_R is the retention time of a retained peak and t_0 is the retention time of an unretained peak. Log k' values were obtained from their t_R values by the application of equation 1, where the retention time of formamide was used for the t_0 value.

Using known values for $P_{octanol}$ [19], a linear relation between log $k'_{(0\%)}$ and log $P_{octanol}$ was obtained as shown in Fig. 1, yielding the following equation.

$$\log P_{octanol} = 0.813 \log k'_{(0\%)} + 0.20 \quad (2)$$

For this equation N = 7, $r = 0.988$, and $s = 0.056$, where log $k'_{(0\%)}$, N, r and s were log k' values extrapolated to 0% CH_3CN, the number of com-

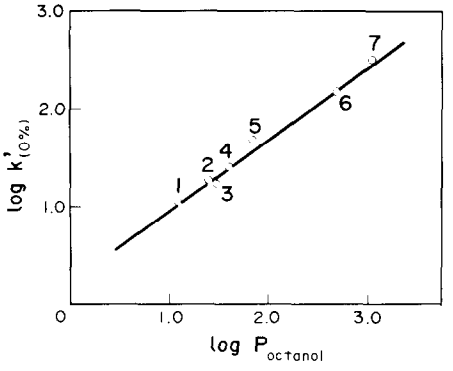


Fig. 1. Plots of log $k'_{(0\%)}$ against log $P_{octanol}$. Log $P_{octanol}$ values were taken from Ref. 18. Experimental conditions are described in Materials and Methods. Key: (1) naphthalene, (2) toluene, (3) nitrobenzene, (4) acetophenone, (5) phenol, (6) coumarin, and (7) benzyl alcohol.

pounds, the correlation coefficient and the standard deviation respectively. The values of log P of the compounds tested were calculated from the above equation and are listed in Table 1. Each value is based upon triplicate measurements which gave statistically identical values.

RESULTS

Inhibition of State 3 respiration. The effects of DDT and its analogues were examined on State 3 respiration of rat liver mitochondria with succinate as a substrate in the presence of ADP. Inhibition of State 3 respiration depended upon the concentration of the tested compound as shown in Fig. 2. DDT-type and DDE-type compounds inhibited strongly State 3 respiration decreasing its rate below that of State 4 respiration. The extent of inhibition appeared to depend upon the chlorine content at the α-position of the ethane moiety. The amounts of DDT and DDE that gave half-inhibition were 0.049 ± 0.011 and 0.062 ± 0.010 μmole/mg of protein respectively. A series of kelthane-type compounds having a hydroxy group on the β-position also inhibited State 3 respiration, and the extent of inhibition increased with the number of chlorine atoms at the α-position. The compounds of the fourth group were poor inhibitors as compared with these three groups. DCBP, DDA and DDDH did not affect State 3 respiration, even if the concentration was 1 mM (4.2 μmoles/mg of protein).

Figure 3 shows a logarithmic plot of the partition coefficients of the tested compounds against the molar concentrations necessary for half-inhibition in State 3 respiration. Using the method of least squares, we tentatively formulated the relationship by the following equation.

$$\log 1/C = 0.82 \log P + 2.21 \qquad (3)$$

For this equation N = 42, $r = 0.940$, and $s = 0.103$, where N was the number of points used in regression, r was the correlation coefficient and s was the standard deviation. This indicates that there was a hydrophobic interaction of these compounds with mitochondria in State 3 respiration.

Stimulation of State 4 respiration. The compounds tested, except for DDT, DDD and DDE, stimulated State 4 respiration (in the absence of ADP) to the 2,4-dinitrophenol (DNP)-stimulated level, as shown

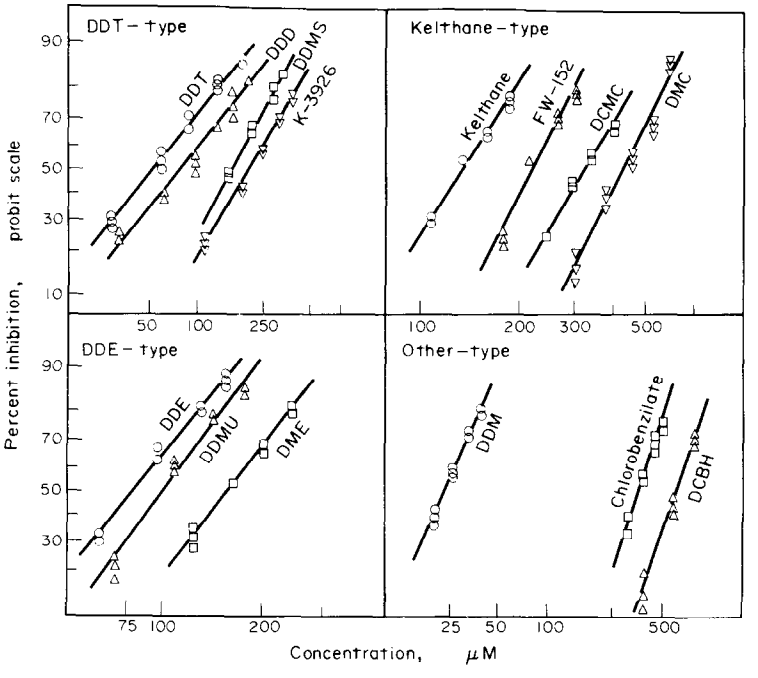


Fig. 2. Effects of DDT and its analogues on State 3 respiration of intact mitochondria in the presence of ADP and succinate. The standard assay system was described in Materials and Methods.

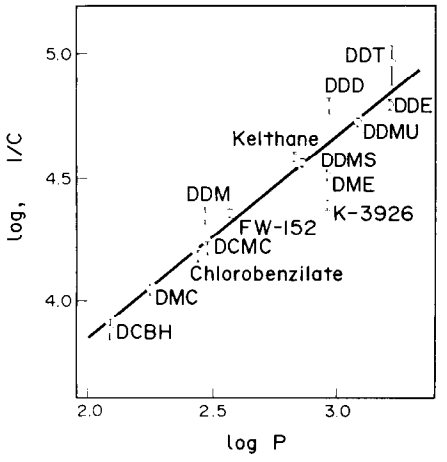


Fig. 3. Relationship between partition coefficients of the compounds tested and their concentrations (C) giving half-inhibition for State 3 respiration. The concentrations giving half-inhibition were obtained from the curves of Fig. 2.

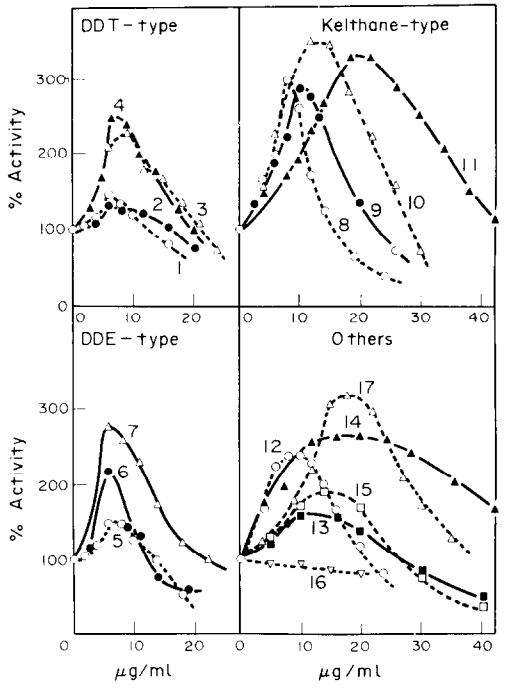


Fig. 4. Effects of DDT and its analogues on State 4 respiration rate of mitochondria in the presence of succinate. Experimental conditions were as described in Fig. 1 except that ADP was not present. DDT-type compounds: (1) DDT, (2) DDD, (3) DDMS, and (4) K-3926; DDE-type compounds: (5) DDE, (6) DDMU, and (7) DME; kelthane-type compounds: (8) kelthane, (9) FW-152, (10) DCMC, and (11) DMC; other compounds: (12) DDM, (13) DCBH, (14) DDA, (15) DDDH, (16) DCBP, and (17) chlorobenzilate respectively. Percent activities were calculated from polarographic traces.

in Fig. 4. Under the conditions employed, 5 μM DNP stimulated State 4 respiration about 3.5-fold. DDT, DDD and DDE revealed only about 1.5-fold stimulation. However, as the concentration of the tested compound exceeded the concentration that gave maximum stimulation, the once stimulated respiration was inhibited, just as State 3 respiration was inhibited by these compounds. Therefore, it seemed that the chlorinated hydrocarbon pesticides used in this experiment had two kinds of effect, a stimulatory effect on State 4 respiration and an inhibitory effect on State 3 respiration or on once-stimulated State 4 respiration.

When a series of kelthane-type compounds was used, the concentrations required for maximum stimulation of State 4 respiration increased with a decrease in the number of chlorine atoms at the α-position. On the other hand, when DDT-type and DDE-type compounds were used as well, the extent of maximum stimulation depended upon the number of chlorine atoms at the α-position, but the concentration for maximum stimulation was almost independent of the chlorine content at the α-position. Chlorobenzilate and DDA had a tendency similar to that of the kelthane-type compounds, and so the extent of stimulation almost reached the DNP-stimulated level. The stimulatory effects of DDM, DDDH and DCBH were similar to those of the DDT-type and DDE-type compounds. DCBH had no effect on either State 4 or State 3 respiration.

DDT, DDE and some other compounds strongly inhibited DNP-stimulated respiration. This inhibition by these compounds was similar to the inhibition of State 3 respiration, and the concentration of DDT giving half-inhibition in the DNP-stimulated respiration was about 0.05 μmole/mg of mitochondrial protein.

Induction of mitochondrial swelling. By measuring the decrease in absorbance at 520 nm for 20 min after the addition of the test compounds, mitochondrial swelling induced by these compounds was examined in three kinds of solutions. In a solution containing 0.15 M KCl and 5 mM Tris–HCl (pH 7.4), all of the compounds tested except DDA induced mitochondrial swelling. Figure 5a shows the dependence of swelling upon the concentration of DDT, DDE or kelthane in the solution. From the curves of swelling as a function of the concentrations of tested compounds, a concentration necessary for half-maximum swelling (S_{50}) was determined. Of the tested compounds, kelthane exhibited the greatest swelling activity. The S_{50} value of kelthane was 0.54 μmole/mg of mitochondrial protein and, under the same conditions, that of myristic acid, which is a representative swelling agent, was 26 μmoles/mg of mitochondrial protein [20].

Logarithms of the S_{50} values were plotted as a function of the partition coefficients of the tested compounds, and the following equation was derived by the method of least squares,

$$\log 1/S_{50} = 0.373 \log P + 2.96 \qquad (4)$$

for N = 16, $r = 0.662$, and $s = 0.307$: It appeared that there was no distinct correlation, as reflected by the poor correlation coefficient.

We examined whether mitochondrial swelling

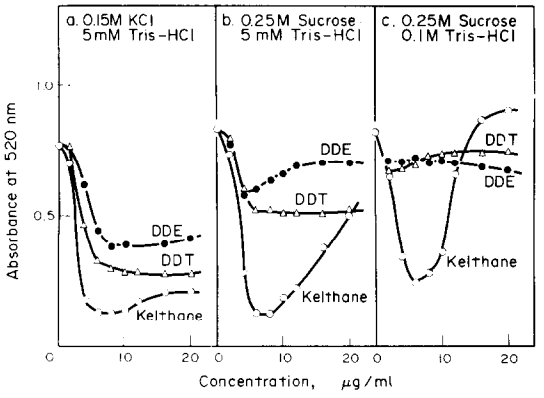


Fig. 5. Effects of the composition of solutions on mitochondrial swelling in the presence of DDT, DDE or kelthane. Absorbance at 520 nm was measured 20 min after incubation with each compound at concentrations indicated on the abscissa. The solution compositions are shown in panels a, b and c.

induced by the test compounds occurs in a solution containing 0.25 M sucrose and 5 mM Tris–HCl (pH 7.4). Both DDT-type and DDE-type compounds no longer induced mitochondrial swelling in this medium, (Fig. 5b), whereas a series of kelthane-type compounds, DCBH, DDDH and chlorobenzilate induced mitochondrial swelling in this solution.

In a test system containing 0.25 M sucrose, the swelling induced by kelthane markedly depended upon its concentration. Figure 5b shows that maximum swelling of mitochondria occurred at 16.2 μM kelthane and that above 16 μM turbidity increased again. A similar induction curve of swelling by kelthane was also observed in a solution containing 0.25 M sucrose and 0.1 M Tris–HCl (pH 7.4) (Fig. 5c). However, it was not clear whether the restoration in turbidity reflected recontraction of mitochondria at high concentrations of kelthane. The details of this phenomenon remain to be elucidated in the future by electron microscopic observation.

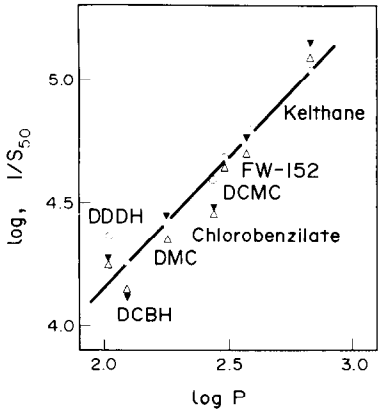


Fig. 6. Logarithmic plot of the S_{50} values against the partition coefficients of the selected compounds. S_{50} is the concentration that gave half-maximal swelling. The solution compositions were: (○) 0.15 M KCl and 5 mM Tris–HCl (pH 7.4), (△) 0.25 M sucrose and 5 mM Tris–HCl, and (▼) 0.25 M sucrose and 0.1 M Tris–HCl. Swelling was measured as the decrease of absorbance at 520 nm 20 min after incubation at 25°. Examples are shown in Fig. 5 (panels a, b, and c).

The other kelthane-type compounds, DCBH, DDDH and chlorobenzilate also caused mitochondrial swelling in a solution containing 0.25 M sucrose and 0.1 M Tris–HCl. DDT-type and DDE-type compounds caused only weak swelling in a solution containing 0.25 M sucrose and 5 mM (Fig. 5b) or 0.1 M (Fig. 5c) Tris–HCl. Compounds capable of causing mitochondrial swelling in these three kinds of solutions have a hydroxy group on the ethane bridge.

Figure 6 shows the relation between the logarithms of S_{50} values and the partition coefficients of seven compounds. A linear relation was observed as shown in the following equation,

$$\log 1/S_{50} = 1.06 \log P + 2.02 \qquad (5)$$

for $N = 21$, $r = 0.951$, and $s = 0.91$: This would indicate that the hydroxy group is required for mitochondrial swelling by chlorinated hydrocarbon pesticides and also that the extent of swelling is dependent upon their lipophilic properties quantified as log P.

Stimulation of latent ATPase of mitochondria. Table 2 shows that every compound except DCBH and DDA stimulated the activity of latent ATPase of intact mitochondria in a solution of 0.25 M sucrose and 0.05 M Tris–HCl. The DDT-type and DDE-type compounds were effective stimulators of latent ATPase, and the stimulation was 2.2- to 3.3-fold of latent ATPase activity. DDM and DDDH increased by about 3.5-fold, and kelthane-type compounds, DCBP and chlorobenzilate by 2- to 2.7-fold, but the activity of ATPase decreased again at higher concentrations in the cases of kelthane-type and DDE-type compounds.

Table 2. Stimulatory effects of DDT analogues on latent ATPase of intact mitochondria

Compounds	ATPase activity* [μmoles $P_i \cdot hr^{-1} \cdot (mg\ protein)^{-1}$]		
	4 μg/ml	8 μg/ml	20 μg/ml
Ethanol	5.2 ± 0.4	5.5 ± 0.9	6.3 ± 0.5
DDT	15.9 ± 1.7	14.9 ± 0.8	17.8 ± 0.8
DDD	12.7 ± 1.6	13.5 ± 0.3	14.5 ± 2.1
DDMS	13.1 ± 1.7	12.4 ± 1.0	11.6 ± 1.6
K-3926	16.9 ± 1.1	13.5 ± 0.6	13.4 ± 1.6
DDE	15.6 ± 0.4	12.7 ± 0.4	8.7 ± 0.8
DDMU	15.8 ± 0.6	12.8 ± 0.4	8.2 ± 1.2
DME	17.2 ± 0.9	12.7 ± 0.4	8.3 ± 1.0
Kelthane	12.6 ± 0.1	11.7 ± 1.5	5.7 ± 0.4
FW-152	11.8 ± 1.0	16.7 ± 1.4	9.9 ± 0.7
DCMC	8.4 ± 0.7	16.2 ± 0.6	13.2 ± 1.1
DMC	8.2 ± 0.8	11.7 ± 1.2	16.4 ± 0.1
DDM	18.7 ± 0.4	19.7 ± 0.3	12.4 ± 1.1
DCBH	7.7 ± 0.3	7.5 ± 0.4	17.3 ± 1.3
DDA	6.1 ± 0.3	5.9 ± 0.5	6.6 ± 0.3
DDDH	8.4 ± 0.6	12.2 ± 0.4	20.1 ± 1.0
DCBP	6.1 ± 0.6	7.5 ± 1.0	17.0 ± 2.0
Chlorobenzilate	7.4 ± 1.1	15.4 ± 0.7	15.6 ± 0.5

* Each value is based upon triplicate determinations.

Table 3. Effects of oligomycin on DDDH- or DDM-stimulated ATPase of mitochondria and submitochondria

Incubation	ATPase activity [μmoles $P_i \cdot hr^{-1} \cdot (mg\ protein)^{-1}$]
Mitochondria (control)	4.9
+ DDDH (10 μg/ml)	18.7
+ DDDH (10 μg/ml) + oligomycin (10 μg)	1.3
+ DDM (6.7 μg/ml)	19.3
+ DDM (6.7 μg/ml) + oligomycin (10 μg)	3.3
+ Oligomycin (10 μg)	1.1
+ 2,4-Dinitrophenol (5 μM)	7.2
Submitochondria	19.0
+ Oligomycin (10 μg)	1.8

Table 3 shows that DDM- or DDDH-stimulated ATPase was oligomycin-sensitive. When the mitochondrial suspension was centrifuged after treatment with these compounds, the resulting supernatant fraction had no ATPase activity. Therefore, it was considered that the ATPase was still bound to the mitochondrial membrane fraction. DNP was a poor stimulator of latent ATPase in this system containing 0.25 M sucrose and 0.05 M Tris–HCl. Submitochondrial particles obtained by ultrasonic disruption had the same ATPase activity as that stimulated by DDM or DDDH.

Effect on oligomycin-sensitive ATPase of sonicated submitochondrial particles. The effects of tested compounds on the ATPase activity of sonicated mitochondrial particles, which was oligomycin-sensitive, were examined (Table 4). At a concentration of 30 μg/ml, kelthane, FW-152 and DDE-type compounds inhibited ATPase activity, the inhibition being 29.4 to 50.1%. This agreed with the fact that the latent ATPase of intact mitochondria was stimulated only slightly at high concentrations of these compounds as shown in Table 2. Kelthane was the most effective inhibitor for the oligomycin-sensitive ATPase of mitochondria, and the amount giving half-inhibition was 0.25 μmole/mg of mitochondrial protein. DDDH, which was the most potent stimulator of ATPase, showed only 13.2% inhibition at 30 μg/ml. The DDT-type compounds showed partial inhibition for the oligomycin-sensitive ATPase of rat liver mitochondria. DDA and chlorobenzilate had no, or little, inhibitory effect. However, it was difficult to determine the relation between I_{50} and log P because of the requirement for a considerably high concentration for half-inhibition.

Table 4. Effects of DDT analogues on the oligomycin-sensitive ATPase of sonicated submitochondria

Compounds (30 μg/ml)	% inhibition*
DDT	29.4 ± 5.8
DDD	24.5 ± 6.5
DDMS	23.8 ± 4.8
K-3926	32.3 ± 5.9
DDE	32.4 ± 5.0
DDMU	29.4 ±5.3
DME	31.3 ± 9.4
Kelthane	57.9 ± 6.7
FW-152	48.7 ± 1.2
DCMC	32.5 ± 4.3
DMC	30.5 ± 2.1
DDM	21.0 ± 2.6
DCBH	12.4 ± 4.0
DDA	1.7 ± 5.4
DDDH	13.2 ± 2.6
DCBP	11.4 ± 4.0
Chlorobenzilate	3.5 ± 6.6
Oligomycin	90.7 ± 1.6

* ATPase activity of untreated submitochondria was 19.5 ± 0.7 μmoles $P_i \cdot hr^{-1} \cdot (mg\ protein)^{-1}$. Each value was based upon triplicate determinations.

DISCUSSION

In correlating their chemical structures with the effects of the chlorinated hydrocarbon pesticides on mitochondrial functions, our interest has been centred on the hydrophobicity of the compounds. In order to deal systematically with the biological effects of such chemicals, quantitative treatment was necessary. In the present report, we have employed log P values to quantify the hydrophobicity, where P is the apparent partition coefficient determined in this work.

It was demonstrated that DDT and its analogues inhibited State 3 respiration of rat liver mitochondria. We have found that the inhibitory effect of the compounds can be expressed in the following equation.

$$\log 1/C = a \log P + b \qquad (6)$$

As a result, a structure–activity correlation was observed between DDT analogues and inhibition of State 3 respiration. The correlation coefficient, $r = 0.940$, would indicate that the inhibition of State 3 respiration was caused by non-specific interaction and was dependent simply upon the amount of apolar fraction.

Coats *et al.* [21], plotting insect toxicity against van der Waals volume for several series of compounds, suggested that steric factors are primarily important to the toxicity of DDT-type compounds. We attempted to correlate I_{50} values of DDT analogues with van der Waals volume of the compounds, but there was no linear relationship between them.

With respect to the interaction of hydrophobic chemicals with membrane, Hansch and Glave [22] found a similar structure-activity relationship between membrane-perturbing agents and hemolysis. By using fifteen hydrophobic compounds, they found that the slope (a in Equation 6) is 0.93 ± 0.17. There is slightly more fluctuation in our results. On the other hand, the report by Lien *et al.* [23] on the toxicity with gram-negative organisms resembles our results. Therefore, the two phenomena—the toxicity on organisms and the inhibition of mitochondrial respiration—appear to occur similarly by means of hydrophobic interaction.

Our results demonstrate that DDT and its analogues have an uncoupling activity that results in stimulation of State 4 respiration. It has been generally accepted that the uncoupling activity of compounds such as nitrophenols is associated with their lipophilic character as well as their ionizing character. Because the compounds employed in this experiment do not have an ionizing group, it seems that the observed uncoupling activity depends upon the hydrophobic property of molecules. Fujita [24] has suggested that the uncoupling activity is linearly associated with the ability to bond hydrophobically onto a proteinous surface of mitochondrial membrane on the basis of the results of Weinbach and Garbus [25] and Hansch *et al.* [26].

It has been already demonstrated by Johnstone [27] that DDT at a concentration of 10^{-4} M inhibits succinoxidase activity *in vitro*. DNP-stimulated respiration in the presence of succinate and succinate oxidation by submitochondrial particles were strongly inhibited by the compounds tested in this work, with a few exceptions. Consequently, the major effect of the DDT analogues would be associated with inhibition in the electron transport system. A possible explanation for the uncoupling activity of these compounds would be that the membrane structure is destroyed owing to the lipophilic interaction of chlorinated hydrocarbon pesticides with membrane so that its permeability to protons or other ions increases.

The fact that the DDT analogues except DDA induced mitochondrial swelling might not be attributable merely to the lipophilicity defined as partition coefficient because only a poor correlation was observed between log S_{50} and log P. It has been reported that hydrophobic substances such as fatty acids [20] and phthalate esters [28] induce mitochondrial swelling in a 0.15 M KCl solution containing 5 mM Tris–HCl. Correlations were found between the chain lengths of fatty acids or phthalate esters and their abilities to induce swelling. The maximum swelling occurred at C_{14} of the alkyl chain for fatty acids and at C_4 for phthalate esters. The concentrations of dibutyl phthalate [28] and myristic acid [20] giving half-maximum swelling were 13 and 26 μmoles/mg of mitochondrial protein respectively. Therefore, the amount of 0.54 μmole/mg in the case of kelthane is remarkable.

In a 0.25 M sucrose solution DDT-type and DDE- type compounds had no induction effect on mitochondrial swelling. Only seven compounds, all of which have a hydroxy group on the ethane bridge caused mitochondrial swelling which appeared to depend upon their lipophilicity. There was a good linear relation between log S_{50} and log P, which was demonstrated by a value of 0.951 for the correlation coefficient (Fig. 5). Therefore, for chlorinated hydrocarbon pesticides to cause mitochondrial swelling, at least in a solution containing 0.25 M sucrose, it seems necessary for the compounds to have a hydroxy group to acquire a detergent-like property, as suggested by Pressman and Lardy [29].

With respect to the interaction of chlorinated hydrocarbon pesticides with the ATPase system from various sources, most data have been confined to the inhibition of ATPase activity *in vitro* or *in vivo*. However, Byczkowski *et al.* [3] showed that DDT and its metabolites stimulated ATPase activity in intact mitochondria of rat liver, and that DDA was the most effective stimulator among four compounds tested, although these compounds had no effect on the ATPase activity of sonicated mitochondria. Their results with regard to the stimulation of ATPase agree with our present results that ATPase activity is stimulated by various tested compounds at low concentrations. But our following findings are apparently different: (1) ATPase activity was inhibited at higher concentrations; (2) ATPase of the submitochondrial particles was also inhibited; and (3) no effect of DDA on ATPase activity was observed. This discrepancy might be attributable to the difference in the reaction mixture; 0.25 M sucrose and 50 mM Tris–HCl were included in our case, and 15 mM KCl and 50 mM Tris–HCl were used in the work of Byczkowski *et al.* [3].

Our results show that DDM and DDDH are the most effective stimulators. From the data shown in Table 2, it can be said that the stimulatory effect of DDT analogues on ATPase activity in rat mitochondria is independent of their lipophilic characters.

Cutkomp *et al.* [30] reported that high single doses of DDT, DDE and dicofol (kelthane) caused over 30% increases in oligomycin-sensitive ATPase of cockroach muscle in *in vivo* experiments. However, many lines of evidence for *in vitro* inhibition of oligomycin-sensitive mitochondrial ATPase by DDT and its analogues have been shown in fish brain [31–33] and insect tissue [34, 35]. According to these studies, the effect of DDT on this enzyme system is several times greater than that of DDE, kelthane or other DDT analogues. Furthermore, DDT inhibited oligomycin-sensitive ATPase more strongly at cooler temperatures [36, 37]. This fact disagrees with our results that kelthane was the most potent inhibitor for oligomycin-sensitive ATPase of sonicated mitochondria. This may have arisen from the difference between these animal species in sensitivity of the mitochondria to chlorinated hydrocarbon pesticides. In fact, the oligomycin-sensitive ATPases from various preparations of several sources appear to be quite different in their sensitivities to DDT, depending upon temperature [36–38].

In earlier studies, Tzagoloff *et al.* [39] reported that binding of a hydrophilic component (F_1) having ATPase activity to a hydrophobic component (F_0) increases sensitivity of the activity to oligomycin and several other inhibitors. Patil and Koch [40] suggested that DDT acts on F_1-ATPase of pig heart

mitochondria in association with one or more membrane components, and also that OSCP (oligomycin sensitive-conferring protein) and phospholipid are essential for DDT sensitivity. It has also been shown by Soper and Pederson [41] that a detergent-solubilized membrane preparation was more sensitive to oligomycin and other inhibitors than was the intact mitochondrial preparation. Consequently, a likely explanation for the effects of DDT analogues on mitochondrial ATPase may be that mild disruption of mitochondrial membrane stimulates ATPase activity and that subsequent heavy disruption inhibits it.

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