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Anaerobic Metabolism of DDT Analogs by Pigeon Liver Preparations

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The amounts of *p,p'*-DDT and of five other trichloroethane derivatives decreased upon incubation under anaerobic conditions with 12,000*g* × 30 min pigeon liver supernatant fraction. The addition of an exogenous NADPH-generating system sometimes, but not invariably, increased the rate of metabolism. Only one hexane-soluble metabolite was detected in the postincubation reaction medium of each of the six trichloroethane derivatives. After isolation by tlc the six metabolites were shown by mass spectrometry to have molecular weights 34 units less than their parent compounds. Comparison of the isotope patterns in the spectra of each substrate and its metabolite reveals that in each case the metabolism involves the loss of a chlorine atom. From these data it is concluded that several substituted trichloroethanes undergo reductive dechlorination when they are incubated with liver preparations in an atmosphere of nitrogen. Two dichloroethane derivatives, tested in a similar manner, were unchanged and were recovered quantitatively. Mass spectrometric and chromatographic data of reactants and products are recorded.

1,1 - Dichloro - 2,2 - bis(*p* - chlorophenyl) ethane (*p,p'*-DDD) is often present in human body fat (1, 2). It is formed from *p,p'*-DDT by a route which, in *Aerobacter aerogenes* (3), does not involve intermediate production of *p,p'*-DDE. *p,p'*-DDD is also produced from *p,p'*-DDT by rumen fluid microorganisms and, anaerobically, by vertebrate liver preparations (4, 5); in the rat, it is subsequently converted to *p,p'*-DDA (6).

Difficulties associated with the high persistence of *p,p'*-DDT and its two principal metabolic products make it desirable that it should be replaced by a more biodegradable analog. Methoxychlor is a likely candidate because its oxidative metabolism differs from that of *p,p'*-DDT (6) but little is known about the anaerobic metabolism of this and other DDT analogs.

Pigeon liver (7) and rumen microorganisms (8) convert *o,p'*-DDT to *o,p'*-DDD, but the anaerobic metabolism of several DDT analogs by strains of *Aerobacter aerogenes* varies both with the strain and with the substrate (9). In particular, reductive dechlorination of methoxychlor was observed in only one of the nine cultures investigated. The object of the present study was to isolate and identify the principal metabolites formed when six trichloroethane derivatives were incubated under anaerobic conditions with 12,000*g* × 30 min supernatant fluid prepared from the liver of feral pigeons.

MATERIALS AND METHODS

The following substances were used as substrates:

(a) 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane, *p,p'*-DDT

(b) 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane, *p,p'*-DDD

(c) 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl) ethane, *o,p'*-DDT

(d) 1,1,1-trichloro-2,2-bis(*p*-methylphenyl) ethane, dimethyl-DT

(e) 1,1,1-trichloro-2,2-bis(*p*-ethylphenyl) ethane, diethyl-DT

(f) 1,1-dichloro-2,2-bis(*p*-ethylphenyl) ethane, perthane

(g) 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl) ethane, methoxychlor

(h) 1,1,1-trichloro-2,2-bis(*p*-bromophenyl) ethane, dibromo-DT

Diethyl-DT was synthesized by the method of Stephenson and Waters (10). The brown oily product was extracted with hexane. After removing the hexane by evaporation the residue distilled as a straw-colored liquid, bp 224–228°C at 0.5 torr. Purification was by tlc (Kieselgel G/hexane) with glc monitoring, the molecular weight of 340 being confirmed by mass spectrometry.

Dimethyl-DT, dibromo-DT, and methoxychlor were donated by Geigy Ltd., Basel. The remaining substances were purchased; perthane was purified by tlc, the remainder by recrystallization.

The method of preparation of 12,000*g* liver supernatant fluid and the incubation procedure were described earlier (5). Each incubation tube contained 0.8 ml of 12,000*g* liver supernatant fluid (24–36 mg of protein), 0.6 ml of pH 6.8 phosphate buffer, and 0.2 ml of 0.16 *M* KCl. No NADPH-generating system or other cofactors were added unless otherwise stated.

The techniques of clean-up, solvent extraction, and glc analysis were similar to those used by Walker (4), the gas chromatograph being provided with a SE 52 silicone column and an electron-capture detector. That part of the hexane extract not used for glc analysis was concentrated using a stream of nitrogen and the components separated by tlc. The plates were sprayed with 0.5% $AgNO_3$ in 95% alcohol and irradiated with light of wavelength 254 nm.

Mass spectra were obtained using an A.E.I. MS 9 mass spectrometer operated at a resolving power of 2000 and an electron beam energy of 70 eV. The samples were introduced into the ion chamber using the direct-insertion probe at a source temperature of about 150°C. To avoid errors when counting the spectrum through regions of low intensity the molecular weights were confirmed using the peak-matching technique with heptacosafluorotributylamine as the reference compound.

RESULTS

No significant loss by evaporation of any of the eight DDT or DDD analogs occurred from buffered suspensions exposed to a stream of nitrogen for 3 hr in the absence of supernatant fluid. Other tests indicated that metabolism was negligible (0–4%) when the liver supernatant fluid was preheated at 90°C for 20 min before adding substrate and incubating in nitrogen.

Two derivatives of dichloroethane and six of trichloroethane were incubated as substrates with the 12,000*g* supernatant fluid, each substance being used on at least three occasions with replication within each experiment. Routine controls included tubes containing supernatant fluid but no substrate and others harvested without incubation immediately after adding the substrate.

Upon anaerobic incubation with supernatant fluid, the six trichloroethane derivatives disappeared at somewhat similar rates. Figure 1 shows the progress curves, calculated for 35 mg of protein per ml, rate being assumed to be proportional to protein content within the narrow limits (30–45 mg per ml) employed. For each substance, on at least two occasions, the standard incubation mixture was supplemented by addition of an NADPH-generating system. Table 1 shows the percentage of substrate metabolized after 1.5 hr, as assessed from progress curves constructed using data from samples removed after 1, 1.5, and 2 hr.

When the two dichloroethane derivatives, DDD and perthane, were added to the supernatant fluid, the variation in the amounts recovered after 0, 1, 2, 3 hr was well within the sampling error. Secondary metabolism is, therefore, not to be expected when DDT and diethyl-DT are incubated with liver preparations under anaerobic conditions.

When *p,p'*-DDT, *o,p'*-DDT, and di-

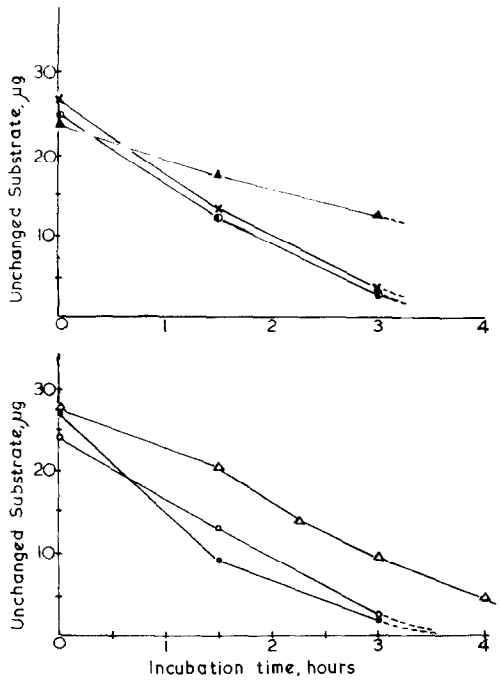

FIG. 1. *Metabolism of DDT analogs when incubated with 12,000g pigeon liver supernatant in anaerobic conditions. Each substance tested on three occasions, 24–28 µg being added in 10 µl ethanol to a mixture of supernatant fluid and phosphate buffer, pH 6.8. Protein content in different tests, 30–45 mg ml^{-1}. Rate of disappearance of substrate calculated for 35 mg protein ml^{-1} and each point is the mean of six results.* ▲——▲, *o,p'-DDT;* ×—— ×, *p,p'-DDT;* ◐——◐, *dibromo-DT;* △——△, *diethyl-DT;* ○——○, *dimethyl-DT;* ●——●, *methoxychlor.*

bromo-DT were added to liver supernatant fluid, the disappearance of the substrate peak was matched by the appearance on glc of another peak, indicating the formation of a substance with a shorter retention time on the column employed. However, three other trichloroethane derivatives (methoxychlor, dimethyl-DT, and diethyl-DT), at dilutions needed to produce peaks of half full-scale deflection, gave inconspicuous metabolite peaks and their metabolism was only readily followed after reactant and product had been separated by tlc.

Relative retention times (*p,p'*-DDT = 100) on the SE 52 silicone column of substances used, or formed, in biological tests are given in Table 2, together with the retention times of some chemically produced DDE analogs. Observed R_f values are recorded, as is the R_f value of the DDT used as a reference standard on each occasion. Methoxychlor moves only 2–4 mm from the starting line when hexane is the moving phase, but using mixtures of hexane and diethyl ether, optimum separation of methoxychlor and its metabolite was reached with 88% hexane and 12% ether (v/v).

Mass spectrometry was used to confirm the identity of all the substrates and their isolated metabolites. The mass spectra of *o,p'*-DDT and its metabolite are shown in

TABLE 1

Effect of an Exogenous NADPH-Generating System on the Anaerobic Metabolism of DDT Analogs[a]

Substrate	Exogenous NADPH system	Metabolism in 90 min per 35 mg protein (as % of amount of substrate added)	
		a	b
o,p'-DDT	−	28	20
	+	45	30
Dimethyl-DT	−	45	52
	+	63	50
Diethyl-DT	−	41	33*
	+	35	47*
Dibromo-DT	−	50	47
	+	79	43
Methoxychlor	−	66	55
	+	78	56

[a] Two experiments a and b, done using each analog; results calculated from four or more within-test observations. Livers from mature male pigeons except for diethyl-DT(*), in which the bird was a mature female. Unsupplemented system: 0.8 ml of 12,000*g* liver supernatant fluid, 0.6 ml of phosphate buffer, pH 6.8, 0.2 ml of 0.16 *M* KCl, 10 µl of alcoholic solution of substrate (2–3 µg/µl). The supplemented system also contained 1.7 mg NADP, 3 mg glucose-6-phosphate, and 1.6 units of glucose-6-phosphate dehydrogenase.

Fig. 2, the simple fragmentation patterns obtained from these compounds being typical of those from all substrates and metabolites examined. For each pair of substances the molecular weight of the metabolite is always 34 mass units (i.e., ^{35}Cl–^{1}H) less than the corresponding substrate.

TABLE 2

GLC and TLC Characteristics of Some Analogs of DDT, DDD, and DDE

Substance	glc Relative retention times; DDT = 100 SE 52 column	tlc R_f values; kieselgel G
Hexane as moving phase		
p,p'-DDT	100	0.54–0.59
p,p'-DDD	79	0.38 (DDT = 0.59)
p,p'-DDE	52.0	—
o,p'-DDT	70.8	0.63 (DDT = 0.56)
o,p'-DDD	56.4	0.35 (DDT = 0.56)
p,p'-dibromo-DT	239	0.54 (DDT = 0.59)
p,p'-dibromo-DD	192	0.34 (DDT = 0.59)
p,p'-dibromo-DE	122	0.69 (DDT = 0.59)
p,p'-dimethyl-DT	45.0	0.39 (DDT = 0.54)
p,p'-dimethyl-DD	32.0[a]	0.23 (DDT = 0.54)
p,p'-dimethyl-DE	25.1	—
p,p'-diethyl-DT	88.4	0.62 (DDT = 0.58)
p,p'-diethyl-DD	64.9[a]	0.49 (DDT = 0.58)
p,p'-methoxychlor	191	0.05 (DDT = 0.57)
12% Diethyl ether in hexane as moving phase		
p,p'-methoxychlor	191	0.41 (DDT = 0.92)
p,p'-methoxy-DD	144[a]	0.35 (DDT = 0.92)
p,p'-methoxy-DE	108	—

[a] For a peak of the same size as that produced by the DDT analogs, approximately 10–15 times the amount is necessary.

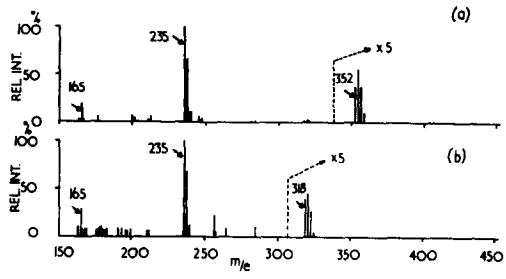

FIG. 2. *Mass spectrum of o, p'-DDT and its metabolic product. The spectra show the principal fragments produced from (a) o, p'-DDT and (b) its metabolic product, above m/e 150. The molecular weight difference of 34 is consistent with reductive dechlorination having occurred. The isotope pattern at m/e 235–237 indicates the presence of two chlorine atoms.*

DISCUSSION

The mass spectra of the trichloroethane derivatives used as substrates show base (i.e., most intense) peaks due to the loss of $—CCl_3$ from the molecular ions. Each isolated metabolite shows the same base peak as its parent substance, indicating the loss of $—CHCl_2$ from its molecular ion. Fragmentation thus occurs in such a way as to leave the aromatic part of the molecule intact and positively charged. In addition, all the compounds examined, with the exception of methoxychlor, yielded fairly strong peaks at *m/e* 165. These peaks are due to the loss of substituents R_1, R_2, from the phenyl rings.

The presence of chlorine or bromine in the molecule produces characteristic isotope patterns in the mass spectra since both exist as more than one stable abundant isotope. The relative intensity of ions containing combinations of several atoms of these isotopes are calculated by substituting the abundance ratios into a binomial expansion. In the molecular ion of *o,p'*-

DDT, for example, the method predicts that the relative intensities of the peaks due to $^{35}Cl_5$; $^{35}Cl_4\ ^{37}Cl$; $^{35}Cl_3\ ^{37}Cl_2$; $^{35}Cl_2\ ^{37}Cl_3$ are in the ratio 3:5:3:1. Ions containing a larger proportion of Cl^{37} are of very low intensity. Figure 2(a) shows that the experimental intensities are in good agreement with the calculated values. Similarly, for the metabolite of *o,p'*-DDT, the relative intensities of isotope peaks in the molecular ion indicate that only four chlorine atoms are present. This confirms the conclusion of French and Jefferies (7), based on chromatographic evidence, that *o,p'*-DDT undergoes reductive dechlorination under the same conditions as does *p,p'*-DDT. The molecular weights of substrate/metabolite pairs, interpreted in the light of these isotope patterns, indicate that all six DDT analogs undergo reductive dechlorination when incubated with liver supernatant fluid under the conditions of these experiments.

From the foregoing results, it appears that metabolism of DDT analogs by avian liver under anerobic conditions presents a simpler pattern than is found using *Aerobacter aerogenes* (9), although one qualification is necessary. For the DDT/DDD system, the only one for which recovery data are available, 30–35% of the DDT disappearing from the 12,000*g* supernatant reaction medium could not be accounted for as DDD. A preliminary study with ^{14}C-DDT suggested that some at least of the nonextracted material is associated with the organic matter removed by the Kieselgel G during cleanup, rather than present as a water-soluble substance which fails to partition into hexane.

The three trichloroethane analogs with alkyl or methoxy groups in the phenyl rings give glc peaks of about the same height as *p,p'*-chlorinated or brominated analogs at comparable concentration. However, Mendel and co-workers (9) observed that methoxy-DD was of low reactivity to electron capture gas chromatography, a property now found to be shared by dimethyl-DD and diethyl-DD. In more complex analytical situations this 10- to 20-fold difference in reactivity between some DDT analogs and their dichloroethane derivatives could cause difficulty.

In the absence of an exogenous NADPH-generating system, the six trichloroethane derivatives are metabolized at rather similar speeds by pigeon liver 12,000*g* supernatants of similar protein content (Fig. 1). On each of the three occasions diethyl-DT and *o,p'*-DDT were investigated, they disappeared more slowly than p,p'-DDT tested simultaneously. In the presence of NADPH-generating system a small but variable increase in metabolism often occurred (Table 1). Similar variation was previously noted for p,p'-DDT itself (5) and possibly indicates individual differences in the level of endogenous cofactors.

There is direct evidence that reductive dechlorination by liver supernatant fluid of *p,p'*-DDT and its diethyl analog occurs rapidly in comparison to any secondary change involving their dichloroethane metabolites. Indirect evidence suggests the same may be true for the remaining compounds, for only one new glc peak and one new tlc spot were visible in samples incubated with liver supernatant fluid for 3 hr.

A better understanding of the metabolism of DDT analogs is essential to enable their environmental persistence to be assessed. The present work shows that, in an *in vitro* system, methoxy-DD is as readily formed as is the highly persistent DDD. It is noteworthy that Kapoor and co-workers (6) did not detect any of it in their *in vivo* studies, possibly because the aerobic *O*-demethylation system attacks both methoxychlor and methoxy-DD. Owing to the existence of this metabolic route the possible environmental advantages of methoxychlor merit reappraisal.

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