Mechanism of Conversion of DDT to DDD by *Aerobacter aerogenes*

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The conversion of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] proceeds by direct reductive dechlorination to DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane] without the intermediate formation of DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene] in still cultures of *Aerobacter aerogenes*. Deuterated DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-2-deuterioethane] was used to determine whether an unsaturated intermediate was formed during the reaction. Retention of the deuterium atom in the product would exclude the possibility of a two-step reaction involving DDE. Under anaerobic conditions, 92% conversion of DDT to DDD was measured in the ether extract. Extraction and recovery studies were conducted on parallel samples using DDT-^{14}C. Sixty-six per cent of the activity was recovered in the ether phase. The product was identified by mass spectroscopy, vapor-phase chromatography, and thin-layer chromatography on silica gel. Mass spectra were obtained by volatilizing the DDD directly from silica gel in the probe of the mass spectrometer. The parent peak occurred at m/e 319, due to the contribution of the species $C_{14}H_9D^{35}Cl_4$, indicating that the deuterium atom originally present at the 2 position in DDT was retained throughout the reaction.

The mechanism by which *p,p'*-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] is converted to *p,p'*-DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane] is of great interest, in view of the extreme resistance of the chlorinated insecticides to breakdown in soil. In a recent communication, soil microorganisms were reported to have degraded DDT (Chacko *et al.*, 1966). Prior to this, Mendel and Walton (1966) showed that *Aerobacter aerogenes* and *Escherichia coli* are capable of effecting the conversion of *p,p'*-DDT to *p,p'*-DDD and other metabolites. *A. aerogenes*, cultured under anaerobic conditions, converted up to 80% of the DDT to DDD (Wedemeyer, 1966). Cell-free preparations from the same experiment effected an average conversion of 70%. Reduced cytochrome oxidase was implicated in the conversion process. In a number of other biological systems, *p,p'*-DDT is converted to *p,p'*-DDD. The literature has been reviewed by Ott and Gunther (1965).

Nonbiological processes also appear to be important in the degradation of DDT. Heparinized chicken blood containing DDT slowly converted the pesticide to DDD and DDE within 12 weeks, even though the samples were sealed and stored at $-20°$ C. (Ecobichon and Saschenbrecker, 1967). As it appears possible that reduced coenzymes, porphyrins, and other metalloproteins could carry out these steps by simple chemical redox reactions, the demonstration that dilute solutions of iron porphyrins are rapidly oxidized in the presence of DDT at room temperature is of considerable interest (Castro, 1964). Whether the conversion process is purely biochemical or chemical cannot yet be clearly stated.

Regardless of the catalyst in this reaction, it seems desirable to understand the mechanism by which this conversion takes place. The appearance of a number of structurally related products from DDT metabolism poses a question. Are DDD and DDE related by a common breakdown pathway, or do they originate by independent processes? Thus far it has been impossible to provide unequivocal evidence, either for or against the theory of independent origins. Deuterated DDT, labeled in the 2-position [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-2-deuterioethane], was employed to determine whether microbial conversion proceeds by dehydrochlorination of DDT and subsequent reduction of DDE or by reductive dechlorination (Figure 1). In the former case, the loss of the deuterium label is to be expected. DDT uniformly labeled in the phenyl rings with ^{14}C was used to examine the quantitative aspects of the extraction and biological conversion. Part of this investigation has been reported (Plimmer *et al.*, 1967).

EXPERIMENTAL

Deuterated DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-2-deuterioethane] was recrystallized from 95% ethanol until the constants agreed with those reported in the literature for DDT (m.p. 108.5–9°). The nuclear magnetic resonance spectrum was consistent with the above structure, since no signal due to the presence of a hydrogen atom at the 2-position was observed. The mass

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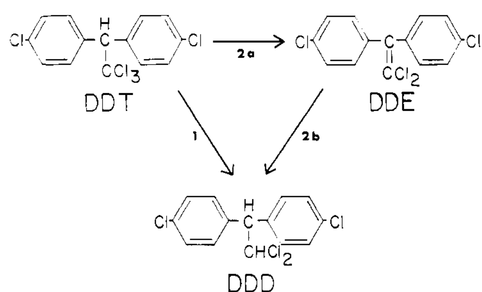

Figure 1. Alternative pathways for conversion of DDT to DDD

Route 1 involves reductive dechlorination
Route 2 involves a two-step reaction with DDE serving as an intermediate product

spectrum indicated that the material contained more than 98% of the deuterated compound. For mechanism studies a stock solution of the deuterated DDT was prepared in acetone at a concentration of 5 mg. per ml. For recovery studies, a stock solution of labeled DDT was prepared containing DDT-^{14}C (uniformly labeled in the aromatic rings = 1.39 μc. or 0.1 mg.) and DDT (4.9 mg.) per ml. of acetone.

Mass spectral studies were performed on a Bendix Model No. 12 mass spectrometer using a direct inlet probe. Gas chromatography was performed on a Wilkens Aerograph Model 204B gas chromatograph with a 5-foot stainless steel column of $^1/_8$-inch i.d. containing 5% SE 30 on Chromosorb W (AW/DCMS) at a column temperature of 212° C. The hydrogen flame detector was used. Carrier gas (N_2) flow rate was 30 ml. per minute.

All determinations of radioactivity were carried out in a Nuclear Chicago Mark I liquid scintillation spectrometer. Aqueous samples (1 ml.) were monitored in a counting solution (15 ml.) composed of 1,4-dioxane (1 liter), 2-ethoxyethanol (100 ml.), PPO (6.0 grams), POPOP (0.3 gram), and naphthalene (90.0 grams). Nonaqueous samples were counted in a solution composed of toluene (1 liter), PPO (5 grams), and POPOP (150 mg.). All counting systems were optimized to 100% efficiency by the channels ratio–external standard method.

A. aerogenes cultures were grown in shake flasks containing 3% Trypticase Soy broth medium (1 liter) maintained at 37° C. Cells in the log phase of growth (after 7 hours) were harvested by centrifugation (at 10,000 G for 10 minutes at 20° C.). The harvested cells were rinsed once and suspended in 0.85*M* NaCl solution (1 liter). To each liter was added 1 ml. of DDT stock solution (5 mg. per ml. in acetone). DDT-^{14}C and deuterium-labeled DDT were used in separate experiments. In a second series of experiments DDE, DDT, and a mixture of DDT and DDE (1 ml. of a solution containing 5.0 mg. per ml. in acetone) were added to three separate volumes (1 liter) of resuspended cells. The initial inoculum was two tenths that of the first set of series of experiments. Incubation was continued in still culture for 86 hours at 37° C.

The culture medium (1-liter volume) was extracted with ether (2 volumes of 300 ml. followed by 2 volumes of 100 ml.). The emulsified cell debris was removed by centrifugation during the course of the extraction. Ether extracts were combined, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. A crude oil was obtained and examined by chromatography.

The crude oil was dissolved in a mixture of methanol and chloroform (2 ml.) and examined by gas chromatography. The retention times under the conditions specified were 6.6 and 8.6 minutes for DDD and DDT, respectively. The quantities of DDT, DDD, and DDE present in the extracts were determined by measuring peak heights and comparing with values obtained by injection of known quantities of authentic materials. Under the experimental conditions described, negligible conversion of DDT to DDD occurred on the gas chromatographic column (Ott and Gunther, 1965). In experiments using deuterated DDT, gas chromatography of the ether extract indicated 92% conversion of DDT to DDD. Column conversion, in the absence of suspended cells, was negligible. Based on recovery studies with labeled DDT, the ether layer contained 2.68 mg. of DDD, which represents 60% of the total calculated conversion of added DDT to DDD.

In the experiments in which DDT and DDE were incorporated together in the medium, 75% of the DDE was recovered unchanged from the ether layer, 50% of the DDT was recovered, and the yield of DDD was 28% of the theoretical value calculated on added DDT. When DDE alone was present, 78% was recovered unchanged.

The identity of the products was established by cochromatography with authentic samples on 20 × 20 cm. glass plates coated with a layer (0.25 mm.) of silica gel G (Merck). The plates were air-dried after the application of the coating. The solvent system used was carbon tetrachloride–cyclohexane (1 to 1, v./v.). Compounds were visualized under the ultraviolet lamp. DDT and DDD were shown to be present in the ether extract of the bacteria incubated with DDT. No DDD was detected in extracts of the bacteria incubated with DDE alone.

By thin-layer chromatography, both DDT (R_f = 0.87) and DDD (R_f = 0.68) were shown to be present in the ether extract of the bacteria on comparing with the mobility of authentic samples. The conversion of DDT to DDD by *A. aerogenes* after 86 hours at 37° C. was confirmed by thin-layer and gas chromatography.

The quantitative aspects of the recovery of DDT and DDD were examined using DDT-^{14}C. The culture and extraction of *A. aerogenes* were repeated using the conditions described previously. Radioactivity in the ether, cell debris, and water fraction was measured in a liquid scintillation counter and found to be 66, 11, and 3%, respectively. The cell debris and aqueous fractions were repeatedly extracted, then acidified and continuously extracted with ether for 3 days in a liquid-liquid extractor. The extracts contained DDT and DDD as determined by thin-layer and gas chromatography. Other metabolites described by Wedemeyer (1966) were not detected. Parallel studies by Braunberg (1967) under a variety of experimental conditions failed to detect compounds other than DDT and DDD in the same cultures of *A. aerogenes* used in our studies.

For mass spectroscopic study, the extract was applied to a thin-layer plate as a narrow streak and allowed to develop. The plate was then divided into strips (*ca.* 2 cm.

wide) parallel to the solvent front. The bands were scraped off the plate separately and each was divided into equal portions. One portion was extracted several times with a small quantity of ether. The filtered ether extract was concentrated and injected into the gas chromatograph. A quantity of the remaining portion of silica was placed in the glass capillary tube directly in the probe of the mass spectrometer. The material which volatilized from the silica in the heated probe afforded good spectral data. Pure DDD was thus obtained and examined.

RESULTS AND DISCUSSION

Bacterial conversion of DDT to DDD has been demonstrated in a number of pure culture studies of a single microbial species (Chacko *et al.*, 1966; Ott and Gunther, 1965; Plimmer *et al.*, 1967) especially with soil isolates (Chacko *et al.*, 1966). The reaction appears to be an anaerobic process in the case of most bacterial species examined (Ott and Gunther, 1965; Plimmer *et al.*, 1967). The optimal cultural conditions required to achieve the most rapid and efficient conversion have not been investigated in this laboratory.

Although the DDT-DDD conversion has been reported in a number of systems, the exact mechanism of the reaction is still obscure. The experiments of Kallman and Andrews (1963) on the conversion of DDT to DDD by yeast demonstrated that DDE is not reduced by this organism. It seemed unlikely that dehydrochlorination occurred. Parallel results were obtained when DDE was incubated with *A. aerogenes*. DDE was not reduced and 78% of the added DDE was recovered in the ethereal extract of the culture medium. The use of deuterium-labeled DDT has enabled us to demonstrate clearly that, in the case of *A. aerogenes*, conversion to DDD occurs with the replacement of chlorine by hydrogen (or hydride ion) and confirms that no unsaturated intermediate is involved. Examination of Figure 1 reveals that the latter route would involve loss of the deuterium atom.

Significant features of the mass spectrum are shown in Table I. The relative abundances of masses greater than *m/e* 200 are shown in Table II. The parent peak for DDD occurs at *m/e* 319 and corresponds to the empirical formula $C_{14}H_9D^{35}Cl_4$. Two isotopes of chlorine of masses 35 and 37 occur in the ratio 100 to 32.7%, respectively. The presence of the isotope of higher mass is responsible for the appearance of peaks at *m/e* 321. The parent peak is of low abundance and appears as a weak signal; consequently, peaks of higher mass than 323 were not observed. Peaks of mass 318 and 320 which would correspond to the empirical formula $C_{14}H_{10}{}^{35}Cl_4$ and $C_{14}H_{10}{}^{35}Cl_3{}^{37}Cl_3$ were absent. Perfluorokerosine, which has a peak at 319, was used as a calibration standard.

Additional evidence is provided by the occurrence of peaks at *m/e* 236 and 238. The peak at 236 arises from the loss of a mass 83 units from the parent peak. This loss corresponds to the fragment $CH^{35}Cl_2$. It is significant that the remaining fragment of empirical formula $C_{13}H_8D^{35}Cl_2$ contains deuterium, indicating that this atom is still retained in the bis-*p*-dichlorophenylmethane fragment. If hydrogen instead of deuterium were present in the fragments discussed, peaks would occur at one mass unit lower than observed.

Table I. Summary of Significant Mass-to-Charge Ratios and Corresponding Formulas for DDD and One of the Principal Fragmentation Products

Ion	Formula	*m/e*
M	$C_{14}H_9D^{35}Cl_4$	319
	$C_{14}H_9D^{35}Cl_3{}^{37}Cl_2$	321
	$C_{14}H_9D^{35}Cl_2{}^{37}Cl_2$	323
M-$CH^{35}Cl_2$	$C_{13}H_8D^{35}Cl_2$	236
	$C_{13}H_8D^{35}Cl^{37}Cl$	238

Table II. DDD (Deuterated) Mass Spectrum

(Relative abundance of ions having *m/e* greater than 200 calculated as percentage height of peak *m/e* = 236)

m/e	% Peak 236	*m/e*	% Peak 236
200	93.4	236	100
201	91.7	238	83.4
202	73.3	272	87.5
205	272	287	100.0
212	185.0	319	86.7
217	146.7	321	53.3
224	70.0	323	61.7
234	70.0		

Further proof of a direct conversion of DDT to DDD is the failure of DDE to produce DDD. According to the approach of Stanier (1947), if DDE were involved in the DDT-DDD conversion, the amount of DDD detected in DDE cultures would have to exceed 28%, or the amount of DDD formed from DDT under identical conditions. As previously stated, no DDD was detected in DDE cultures. A mechanism could be hypothesized in which DDT actually retains the hydrogen (or deuterium) atom as a complex with the double bond in a short-lived dehydrochlorination step; this hydrogen atom could then be involved in the subsequent hydrogenation of DDE to DDD. If this mechanism were operative, simultaneous reduction of DDE to DDD would be expected to occur in the molecular environment responsible for reduction of DDT. In experiments in which DDT and DDE were incubated together, however, the rate of production of DDD did not exceed that from DDT alone and the recovery of DDE was the same as that of DDE incubated alone.

The trichloromethyl moiety appears in many organic pesticides, and in many respects is one of the most difficult groups to degrade from a metabolic standpoint. Under anaerobic conditions, however, DDT is apparently susceptible to comparatively rapid reductive dechlorination by numerous species of microorganisms. The conclusion may therefore be drawn that *A. aerogenes* effects the conversion of DDT to DDD by a process of reductive dechlorination. Experiments were carried out to determine the distribution of radioactivity among the ethereal extract, aqueous layer, and cell debris after the conversion. Except for the addition of a small quantity of ^{14}C-ring-labeled DDT, conditions used were identical to those described for experiments with nonradioactive material. Measurement of the distribution of radioactivity showed that the ethereal extract contained 66%, the cell debris 11%,

and the aqueous layer 3% of the original total added. Thin-layer chromatography indicated that the ethereal layer contained DDT and DDD as the only radioactive materials. Continuous ether extraction of the aqueous layer and cell debris after acidification afforded only DDT and DDD as evidenced by thin-layer chromatography.

Experiments are in progress to attempt to isolate and determine the identity of breakdown products formed during longer periods of incubation.

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