Metabolism of *o,p'*- and *p,p'*-DDT by Rumen Microorganisms

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An in vitro fermentation system which provides satisfactory values for reactions such as cellulose digestion was used to study the fate of *o,p'*- and *p,p'*-DDT. The major product of ^{14}C-labeled *p,p'*-DDT metabolism was *p,p'*-DDD, but about 11% of the ^{14}C was in an unidentified polar product associated with the microbial and substrate residues. The *p,p'*-DDT disappeared at the rate of 12% per hour and the products were formed at similar rates. The metabolism of *o,p'*-DDT was identical with respect to rate of disappearance and amount of *o,p'*-DDD formed.

Significant concentrations of DDD occur in milk when DDT is present in the cows' diet. A number of anaerobic microbial systems convert DDT to DDD (Guenzi and Beard, 1967; Johnson *et al.*, 1967). Mendel and Walton (1966) provided evidence that the gastrointestinal microorganisms are the major source of this conversion in the rat. These observations, together with the report of Miskus *et al.* (1965) that DDD was the only product formed from DDT in stagnating rumen fluid, suggest that the rumen would be an important site of this conversion in cows.

Studies of microbial metabolism have usually employed *p,p'*-DDT. The biological behavior of *o,p'*-DDT, which constitutes approximately 15 to 20% of technical grade DDT, differs from *p,p'*-DDT in a number of respects including body retention, estrogenic activity, and induction of liver enzymes (Bitman *et al.*, 1968; Durham, 1967). Possibly, the two isomers of DDT are metabolized differently by microorganisms.

Experiments determined the rates and end-products of *p,p'*-DDT degradation by rumen microorganisms using an in vitro system satisfactory for studying reactions such as cellulose digestion and to compare *o,p'*-DDT degradation with *p,p'*-DDT degradation. Some of the preliminary work on *p,p'*-DDT has been reported (Fries, 1968).

METHODS AND MATERIALS

In Vitro Fermentation System. The system used in these studies was a modification of the Tilley and Terry (1963) method. The fermentations were carried out in 125-ml. Erlenmeyer flasks in a shaking water bath at 40° C. The main features of the system include the use of 0.5 gram of substrate; a relatively large volume (40 ml.) of bicarbonate buffer adequate to maintain the pH level within the usual limits for digestion; an inoculum of microorganisms supplied as strained rumen fluid (10 ml.); a mineral mixture simulating the composition of saliva; a nitrogen source; and continuous CO_2-gassing to maintain anaerobic conditions. The major modifications included some changes in the mineral mixtures and the provision for the continuous CO_2-gassing. A description of the detailed procedure has been prepared by Van Soest and Goering (1968).

In these studies, the substrate consisted of ground filter paper (Whatman No. 42) impregnated with the appropriate pesticide. In experiments with *p,p'*-DDT, 0.5 μCi (25 μg.) of the uniformly ring-labeled ^{14}C compound was used. Similarly, 25 μg. of the unlabeled compounds was used in the experiments with *o,p'*-DDT. Inoculum for all of the work was obtained from a fistulated cow maintained on orchardgrass hay. The fermentations were carried out for varying periods of time up to 48 hours.

Fractionation of ^{14}C from *p,p'*-DDT. The recovery of the ^{14}C activity in *p,p'*-DDT and related compounds was based on a method (Bertuzzi *et al.*, 1967) for extracting pesticides from samples of low fat content. Acetonitrile was added to the samples to provide the recommended 65-to-35 acetonitrile-water ratio. Samples were centrifuged and the liquid phase was decanted. Extraction was repeated twice by resuspending the residue in acetonitrile-water mixture. The acetonitrile-water extracts were combined and made up to a suitable volume and the ^{14}C recovery was determined. An aliquot was taken, diluted with water, and extracted four times with petroleum ether. The petroleum ether extracts were combined, filtered through anhydrous sodium sulfate, and the recovery of ^{14}C was determined. At the concentrations of pesticides used, further clean up of this extract was not necessary for gas liquid chromatography (GLC) and thin-layer chromatography (TLC) determinations.

In the studies with ^{14}C *p,p'*-DDT, extraction by this means yielded lower recoveries of ^{14}C as the fermentation time increased. This suggested a retention of ^{14}C in the residue which was composed largely of undigested filter paper and microbial cells. Archer and Crosby (1967) found that after several solvent extractions of alfalfa hay, additional DDT and related chlorinated hydrocarbons could be obtained by alkaline extraction. To examine this possible explanation for the incomplete recoveries, the residue was hydrolyzed for 1 hour at 80° C. with 20% alcoholic KOH solution. After diluting the hydrolysate to a suitable volume, ^{14}C recovery was determined and an aliquot was extracted with petroleum ether for GLC, TLC, and ^{14}C recovery.

Pesticide Detection and Determination. A GLC method was used to determine the concentrations of pesticides in the petroleum ether extracts. A F&M model 810 instrument equipped with an electron capture detector was used. The 4-foot × 1/4-inch O.D. glass column was packed with 3.8% silicon gum rubber on 80- to 100-mesh Chromosorb W. A 95% argon–5% methane mixture was used as both carrier and purge gas. The column temperature was 185° C.

The TLC method was used to separate the ^{14}C-labeled compounds from the petroleum ether extracts and to confirm the identification of compounds determined by GLC. Aluminum oxide G absorbent, heptane solvent, and silver nitrate incorporated in the layer for visualization were used (AOAC, 1966). The volume of extract spotted was equivalent to 1% of the original sample. Parallel standards containing *p,p'*-DDT (R_f 0.45), *p,p'*-DDD (R_f 0.25), and *p,p'*-DDE (R_f 0.61) were also spotted. After visualization, the areas with the same R_f's as the standards were recovered from the plate.

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Table I. Recovery of ^{14}C in Major Fractions from Fermentation of *p,p'*-DDT for 0 and 48 Hours

Fraction	Mean[a] Recovery, % 0 hr.	48 hr.
Acetonitrile-water	97.9	82.4
Petroleum ether	98.2	79.4
Unextracted	...	3.0
Residue	0.6	10.9
Petroleum ether	0.2	3.6
Unextracted	0.4	7.3
Total recovered	98.5	93.3

[a] Two replicates.

The activity was eluted from the aluminum oxide with petroleum ether and the ^{14}C was determined. In addition, the areas having lesser, intermediate, and greater R_f's than the known compounds were also recovered from the plate and eluted with petroleum ether to determine if they contained levels of ^{14}C above background. Since the results from GLC and TLC were in complete agreement there was no indication of interfering substances in the extracts which would cause a different R_f for a compound in the sample than for that compound in the standard.

The ^{14}C was determined by liquid scintillation counting using the channels ratio method of quench correction.

RESULTS

Recovery of ^{14}C from *p,p'*-DDT Fermentation. The recovery of ^{14}C in each of the major fractions was determined for all of the fermentation times studied. However, only the results from 0 and 48 hours are presented (Table I). These points represent the extremes in both times studied and the distribution and recovery of ^{14}C. The intermediate fermentation times yielded distributions and recoveries between these extremes.

The recovery of ^{14}C was essentially complete through the acetonitrile-water and petroleum ether extraction steps at 0 hour (Table I). The TLC separation of the petroleum ether extract yielded only *p,p'*-DDT.

After a 48-hour fermentation, the recovery of ^{14}C in the major fractions differed from the 0 hour results in several respects (Table I). A smaller but still major portion of the activity was recovered in the acetonitrile-water fraction. The extraction with petroleum ether was nearly complete and yielded only *p,p'*-DDD with a small quantity of *p,p'*-DDT on TLC and GLC separation.

The alkaline dehalogenation treatment was applied to the residue remaining after the acetonitrile extraction. Approximately 11% of the original activity was extracted at 48 hours compared with less than 1% at 0 hours. The identity of this fraction has not been determined. After treatment with alcoholic KOH, it could not be extracted to a significant extent with petroleum ether. Neither *p,p'*-DDE nor the dechlorination product of *p,p'*-DDD was found with GLC and TLC of the petroleum ether phase or TLC of the aqueous phase. This rules out the possibility of incomplete extraction of *p,p'*-DDT or *p,p'*-DDD with the original use of acetonitrite and provides evidence for another route of *p,p'*-DDT metabolism by microorganisms. With TLC the ^{14}C activity remained near the origin suggesting that the material is polar.

Total recovery of activity was only 93.3% at 48 hours compared to 98.5% at 0 hours. The trend toward lower recovery with increasing fermentation time was present in all our work. Volatile products from microbial degradation of DDT have not been found by workers (Guenzi and Beard, 1967) who have attempted to detect them. However, DDT and its analogues will codistill with water vapor (Bowman *et al.*, 1959). A small flow of CO_2 was maintained throughout fermentation system and codistillation probably accounts for the less than complete recovery of ^{14}C as the fermentation time increased.

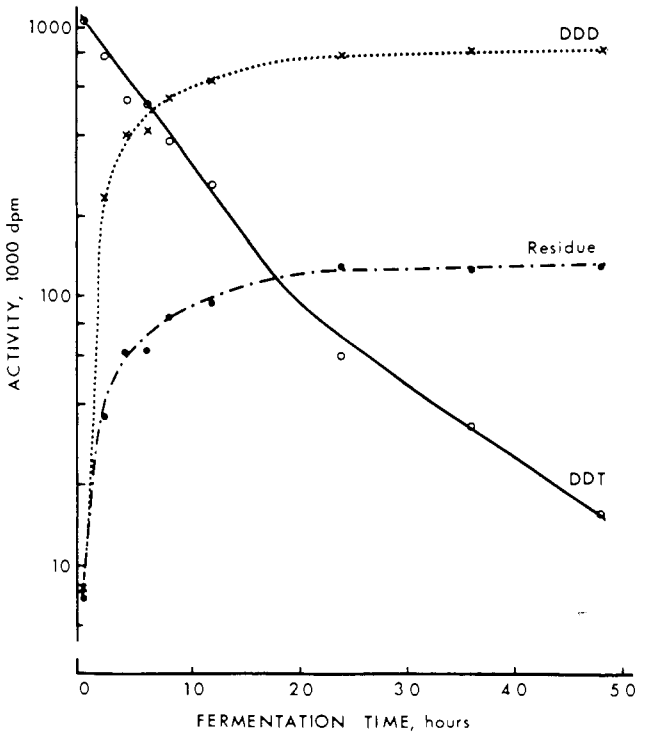

Figure 1. ^{14}C activity of the DDT, DDD, and residue fractions from the fermentation of *p,p'*-DDT. Each point is a mean of two replicates

Rate of *p,p'*-DDT Conversion. A series of fermentations was carried out over a time span from 0 to 48 hours. As shown above, essentially all of the ^{14}C was recovered as *p,p'*-DDT, *p,p'*-DDD, or the residue fraction. When the recoveries of these components are plotted on a logarithmic scale (Figure 1), the disappearance of *p,p'*-DDT was a straight line during the first 18 to 24 hours, indicating a first-order rate. The curves show no evidence of a significant lag phase. Fermentation times considerably less than our 2-hour intervals would be required to demonstrate the presence of a lag phase.

The rate of DDT disappearance slowed after the first 18 to 24 hours. This may reflect the greater analytical errors at low concentrations or the inhibition of fermentation due to substrate exhaustion and accumulation end-products such as volatile fatty acids.

The rate of DDT disappearance during the first 24 hours was approximately 12% of the amount remaining per hour. This corresponds to a half-time of 5.7 hours. The values agree well with our previous report (Fries, 1968) of these parameters.

The ^{14}C from DDT was reduced to DDD and recovered in the residue at first-order rates which were identical to the rate of DDT disappearance. The ratio of activity in DDD-residue was about 6 to 1 at all stages of the fermentation. The results indicate that DDT is metabolized through two pathways, and that DDD and the activity in the residue are end products of these two pathways in this system. The percentage of activity recovered as DDD was higher at all comparable times than was found by Miskus *et al.* (1965) with

Table II. Recovery of Product and Unmetabolized Substrate after 12-Hour Fermentation of 25 μg. of *o,p'*-DDT and *p,p'*-DDT

Substrate	Mean[a] Recovery, μg. DDD[a]	DDT[b]	Total[c]
p,p'-DDT	16.4 ± 1.2[d]	2.8 ± 1.1	21.0 ± 0.3
o,p'-DDT	15.2 ± 0.9	2.9 ± 1.4	20.0 ± 1.2

[a] Four replicates.
[b] *p,p'*- cr *o,p'*- as appropriate.
[c] DDD corrected to the equivalent weight DDT.
[d] Std. dev.

stagnating rumen fluid. This probably reflects the more favorable conditions for continued microbial activity maintained in our system.

Metabolism of *o,p'*-DDT. The work with *o,p'*-DDT was carried out with the unlabeled compound because ^{14}C-labeled *o,p'*-DDT was not available. As a result, only the compounds which could be detected by GLC were determined. A time course study indicated that the pattern of *o,p'*-DDT disappearance and *o,p'*-DDD formation was similar to the pattern for the *p,p'* isomers (Figure 1). To determine if the rates were identical, four replicates of each *o,p'*-DDT and unlabeled *p,p'*-DDT were fermented for 12 hours, a time at which conversion would still be occurring and at which all compounds could be determined with reasonable precision. The results (Table II) indicate that there are no differences between the two isomers in either the quantity of DDT disappearance or of DDD formation. This finding is in marked constrast to the differences that occur in other biological systems (Bitman *et al.*, 1968; Durham, 1967). The 84% total recovery from *p,p'*-DDT in this experiment cannot be compared to the 93.3% total recovery of ^{14}C at 48 hours (Table I). The total recovery of ^{14}C includes the ^{14}C present in the residue which is not *p,p'*-DDT or *p,p'*-DDD. The comparable 48-hour value is 79.4% of the ^{14}C in the petroleum ether extract which is the fraction used for GLC determinations of DDT and DDD.

The lack of the ^{14}C label prevented the evaluation of the possible incorporation of *o,p'*-DDT into the residue fraction. However, since the *o,p'* and *p,p'* isomers were similar in the characteristics that could be determined it may be reasonable to assume similarity in this regard.

DISCUSSION

These studies demonstrate that rumen microorganisms are very effective in converting both DDT isomers to the corresponding DDD isomers. The extent to which the rates found in vitro can be applied in vivo is a matter of conjecture. Cows fed *p,p'*-DDT have ratios of DDD to DDT in the milk from as low as 1.4 to 1 to as high as 5.2 to 1. Dietary factors which influence the rumen retention time or the nature of the fermentation may be important in affecting these differences.

Whiting *et al.* (1968) demonstrated the conversion of DDT to DDD by the perfused bovine liver. While the relative importance of the two conversion sites is not known, the rapid microbial conversion and the location of the two sites suggests that the rumen would be more important.

The apparent formation of a polar product is in agreement with studies in other microorganisms (Guenzi and Beard, 1967; Johnson *et al.*, 1967). The possible significance of this product is impossible to assess. While it may account for 15% of the original DDT in 48-hour in vitro fermentation, there may be less of it formed in vivo with the apparently short retention time of DDT in the rumen. If formed in large amounts in vivo it would probably be excreted via the urine or feces and not be significantly accumulated in the animal body.

The conversion of the DDT isomers to the DDD isomers has several important implications. The toxicity of the DDD isomers are less than the corresponding DDT isomers and any conversion would presumably be beneficial to the animal and the consuming public. This may be especially important in the case of *o,p'*-DDT which exhibits estrogenic activity under some conditions (Bitman *et al.*, 1968). The possible importance of the estrogenic activity under conditions of environmental contamination has not been established. If it is important the rumen organisms would provide a significant detoxification mechanism since *o,p'*-DDD does not exhibit estrogenic activity.

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