Metabolism of ^{14}C-DDT by Ovine Rumen Fluid *in vitro*

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An *in vitro* study of ^{14}C-DDT metabolism by ovine (*Ovis aries*) rumen fluid was conducted to determine the effect of nutritional regimen, concentrate, and roughage. Rumen samples were obtained using a stomach tube. Half of the samples were boiled and half were not. All were incubated at 39° C with ^{14}C-DDT for 0, 4, 8, and 12 hr. Following incubation the samples were extracted, concentrated, thin-layer chromatographed, and assayed by scintillation counting. Concentrate diets were more efficient ($P < 0.01$) in affecting the degradation of ^{14}C-DDT than were the roughage diets. The rate of degradation was noted to increase ($P < 0.01$) with incubation time. ^{14}C-DDD and ^{14}C-DDE were identified as ^{14}C-DDT metabolites. Another metabolite with ^{14}C activity was observed and tentatively identified as DDMU. The pathway of DDT metabolism to DDMU was essentially *via* DDD ($P < 0.01$) rather than DDE.

It is well established that *p,p'*-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] metabolizes to *p,p'*-DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene] in the body and is stored in the adipose tissues primarily in this form (Dale and Quinby, 1963). The subsequent metabolism of DDE appears to proceed rather slowly, if at all (Peterson and Robison, 1964). Several reports (Miskus *et al.*, 1965; Fries *et al.*, 1969; Kutches and Church, 1971) have noted the conversion of DDT to DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane] by bovine rumen fluid. Mendel and Walton (1966) obtained evidence that this same reductive dechlorination process takes place in the gastrointestinal tract of monogastric animals (rats) and results from bacterial action. Bacterial populations, at least in ruminants, are greatly influenced by the kind of nutritional regimen fed the animal (Hungate, 1966).

Despite these and other studies on various aspects of DDT degradation, the Mrak report (1969) stressed the urgent need for expanded research on understanding the mechanism of metabolism with special emphasis on delineating specific influences (*i.e.*, nutritional) on body responses. Previous work in our laboratory (Wilson *et al.*, 1970) established genetic differences in body responses of ruminant animals fed similar diets. It was the purpose of this study to examine the response of genetically similar ruminants fed dissimilar nutritional regimens.

MATERIALS AND METHODS

Sampling. Eight mature (approximately 11 months of age and 41 kg live weight) castrate male (wether) animals (*Ovis aries*) from similar genetic and nutritional backgrounds were used. Twenty-five days prior to sampling these animals were randomly divided into two lots of four animals each. One lot was fed an all roughage (alfalfa hay) diet, and the other was fed an all concentrate (primarily corn with small amounts of oats and barley) regimen. The animals also received the necessary vitamin and mineral supplements as well as free access to water. Following this nutritional treatment, rumen fluid samples (100 ml) were obtained from each animal 2 hr after feeding. These were obtained by a stomach tube and the fluid was filtered through four layers of cheesecloth to remove the large particles. The 100-ml individual samples were pooled by nutritional group, concentrate, and roughage. Each composite sample was then split into two 200-ml portions, and one portion was heated to boiling (100° C) to inactivate the rumen microorganisms.

Incubation. Four 40-ml aliquots of each boiled and regular nutritional group (concentrate and roughage) sample were placed in separate stoppered flasks under CO_2. To each of these 16 samples was added 1.5 μCi of ^{14}C-DDT in hexane. No additional substrate was added. The samples were mixed and incubated at 39° C.

Extraction. Immediately after mixing (0 hr), and at 4, 8, and 12 hr incubation, the 40-ml sample representing each treatment group (boiled concentrate, boiled roughage, regular concentrate, regular roughage) was extracted twice with 20 ml of *n*-hexane in a separatory funnel using alternating 1 min shaking and settling times. During the second extraction, 3 ml of methanol were added to assist in breaking the emulsion and in the solubilization of the more polar compounds. Following the second extraction, the lower layer was drawn off into a glass beaker, and the upper layer remaining in the separatory funnel was reextracted with 10 ml of *n*-hexane. Again, the lower layer was drawn off in the same beaker. The contents of the funnel were transferred to a Kuderna–Danish concentrator apparatus mounted on a steam bath. The material in each beaker was then reextracted three times using the method just described, except only 2 ml of methanol were used in the second extraction. Again, the contents remaining in the funnel were transferred to the appropriate K–D concentrator. This combined extract was then evaporated to 2 ml and transferred to a sample vial.

Separation. Four 20-λ spots per sample were chromatographed on 8-in. plates coated with a 250-μ layer of aluminum oxide as described by Corneliussen (1969). The plates were then developed in a solvent system of 2% (v/v) acetone in *n*-heptane. After development for the prescribed 10 cm, the plates were dried and sprayed with a $AgNO_3$ solution (0.1 g of $AgNO_3$ in 1 ml of H_2O, add 20 ml of 2-phenoxyethanol, dilute to 200 ml with acetone, add a very small drop of 30% H_2O_2 and mix). Following another drying period, the plates were exposed to an ultraviolet light source for not less than 15 min. To assure better visualization, each sample vial was spiked with a cold standard of DDT and metabolites before plating. Nonradioactive standards were cochromatographed with the samples.

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Table I. Overall Effect of Nutritional Regimen and Incubation Time on the Metabolism of ^{14}C-DDT by Ovine Rumen Fluid Table of Means (in percent)

Treatment	DDT	DDD	DDE	DDMU[a]
Nutritional regimen[b]	**	**	**	*
Concentrate	84.1	4.8	10.6	0.5
Roughage	94.7	0.8	4.2	0.3
Incubation time[c]	**	**	**	**
0 hr	100.0	0.0	0.0	0.0
4 hr	94.5	3.3	2.0	0.2
8 hr	87.2	2.9	9.3	0.5
12 hr	86.5	2.2	11.0	0.4

[a] Tentative identification (see text). [b] Pooled means of all 4, 8, and 12-hr samples. [c] Pooled means of all concentrate and roughage samples. Any two means not connected by the same solid line are significantly different from each other. *P <0.05; ** P <0.01.

Table II. Effect of Incubation Time within Nutritional Regimen on the Metabolism of ^{14}C-DDT by Ovine Rumen Fluid Table of Means (in percent)

Treatment	DDT	DDD	DDE	DDMU[a]
Concentrate[b]	**	**	**	**
0 hr	100.0	0.0	0.0	0.0
4 hr	91.5	5.3	3.2	0.0
8 hr	80.4	5.4	13.3	0.8
12 hr	80.4	3.6	15.3	0.7
Roughage[b]	**	**	**	**
0 hr	100.0	0.0[c]	0.0	0.0[d]
4 hr	97.6	1.2	0.8	0.4
8 hr	94.0	0.4	5.3	0.3
12 hr	92.6	0.7	6.6	0.1

[a] Tentative identification (see text). [b] Any two means not connected by the same solid line are significantly (P <0.05) different from each other. [c] 0 hr sample same as 8 and 12 hr but different (P <0.05) from 4 hr; 4 hr sample same as 12 hr but different from 0 and 8 hr. [d] 0 hr sample same as 8 and 12 hr but different (P <0.05) from 4 hr; 4 hr sample same as 8 and 12 hr but different from 0 hr. *P <0.05; **P <0.01.

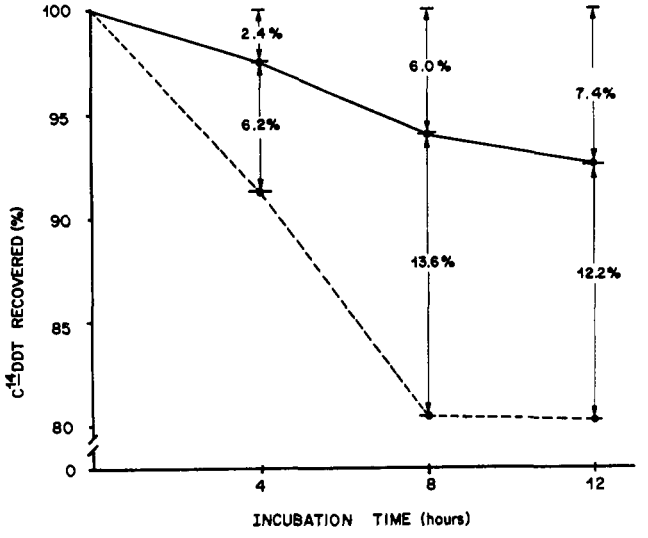

Figure 1. Metabolism of ^{14}C-DDT by ovine rumen fluid samples (concentrate, – – – –; roughage, ——) incubated at 39° C

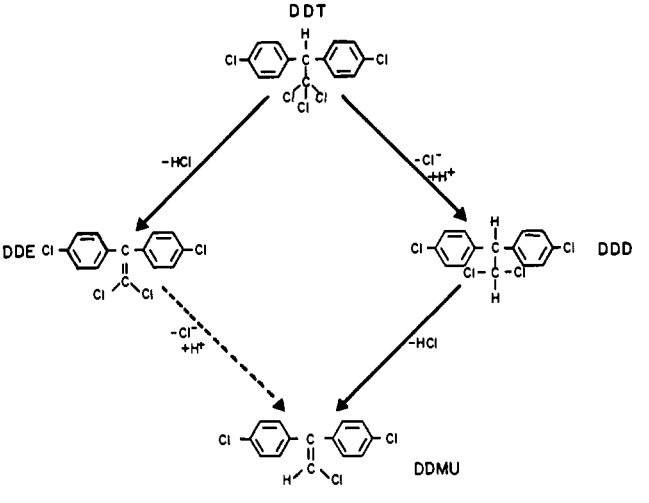

Figure 2. Pathways of DDT metabolism. Established pathways are indicated by solid lines whereas tentative pathways are indicated by broken lines

Radioassay. Once visualized, the spots were identified, scraped, and placed in individual labeled vials containing 15 ml of scintillation solution (4% Packard Soluene 100 in toluene). Radioassay of ^{14}C activity was by a Packard Tri-Carb Model 3375 liquid scintillation spectrometer using 5-min counts and three cycles.

Statistical Analyses. After correcting for background and quenching, the data were analyzed by the analysis of variance (Harvey, 1960) using an IBM 360/67 Computing System. Nutritional regimen and incubation time were the main effects. Between mean differences from group to group were determined by Duncan's multiple range test as modified by Steel and Torrie (1960). Partial correlation coefficients were calculated on an intra type–time variance basis (Harvey, 1960).

RESULTS AND DISCUSSION

All of the spots visualized on the thin-layer plates were positively identified except one with an R_f value of 0.95. This spot has been tentatively identified as DDMU [1-chloro-2,2-bis(*p*-chlorophenyl)ethylene, from the abbreviation of the generic dichloro diphenyl monochloro unsaturated derivative of DDD] based on this R_f as well as on the kinetics of the reactions and its position in the metabolic pathway (Datta, 1970).

Data demonstrating the overall effect of nutritional regimen and incubation time on the metabolism of ^{14}C-DDT by the rumen fluid samples are presented in Table I. These data represent the nonboiled or regular samples. Regardless of treatment (regimen or time) none of the boiled samples were observed to degrade ^{14}C-DDT and, therefore, this information was omitted from the table. This agrees with the results of Miskus *et al.* (1965) and suggests that the inactivation of rumen microorganisms was complete.

Nutritional regimen has been shown to influence significantly the degradation of DDT to its metabolites. Significantly (P <0.01) more of the ^{14}C-DDT was degraded by the rumen fluid obtained from animals fed the concentrate as compared to the roughage diet. This is indicated by the lesser amount of ^{14}C-DDT and the greater amount of ^{14}C-DDD, ^{14}C-DDE, and ^{14}C-DDMU recovered after at least 4 hr of incubation time.

The amount of time the samples were incubated at 39° C also affected (P <0.01) the amounts of ^{14}C-DDT and its metabolites recovered. Generally, the proportion of ^{14}C-DDT recovered decreased progressively with time. This supports the previously reported observations (Miskus *et al.*, 1965) on a similar effect but with bovine rumen fluid. Although the

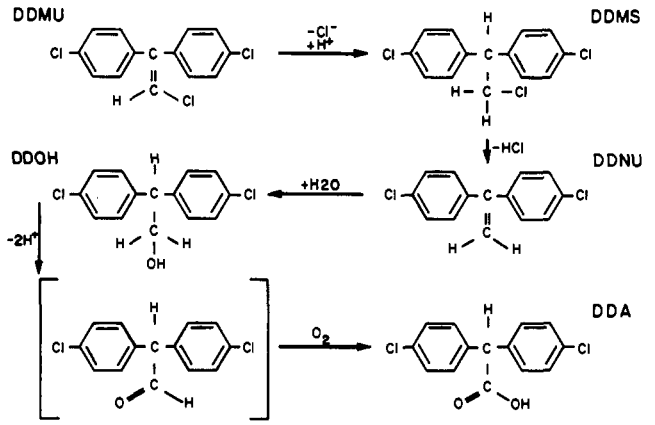

Figure 3. Metabolic pathway involved in the degradation of DDMU to its metabolites

concentration changes in ^{14}C-DDD with incubation time followed a pattern similar to that of ^{14}C-DDT, the amounts of ^{14}C-DDE and ^{14}C-DDMU increased ($P < 0.01$) progressively with time. These data suggest the metabolism of ^{14}C-DDT can be attributed to the microbial activity of the rumen contents. Although working with monogastrics, Mendel and Walton (1966) noted the microflora of the gastrointestinal tract were capable of degrading DDT to DDD and other metabolites. Specifically, they implicated the coliforms–gram-negative bacteria. A preliminary microscopic examination of the microbial population in the fresh (0 hr) samples showed the concentrate sample contained two times as many bacteria and five times as many protozoa as the roughage sample.

A more detailed account of the effect of incubation time within each nutritional regimen is presented in Table II. These data show that the total degradation of ^{14}C-DDT by concentrate samples was generally 2.5 to 3.5 times greater than for those obtained from roughage-fed animals, depending upon the incubation time. A progressively decreasing differential rate of metabolism with increasing time was noted. Figure 1 illustrates that the degradation of ^{14}C-DDT in the concentrate samples reaches a maximum after 8 hr of incubation when approximately 20% of the DDT has been metabolized. Contrasting these results, the roughage samples did not show stabilization even after 12 hr. These latter observations support the *in vitro* bovine rumen studies of Fries *et al.* (1969) and Kutches and Church (1971). Using a buffered medium and ground filter paper as a substrate, Fries *et al.* (1969) noted a linear degradation of ^{14}C-DDT up to 18 hr. Up to 24 hr linear degradation was observed by Kutches and Church (1971), using whole rumen liquor samples from roughage-fed cattle. However, the rate of DDT disappearance, up to 12 hr, noted by Fries *et al.* (1969) was generally 6.5 times faster than reported here and 2.5 times faster than reported by Kutches and Church (1971).

The two pathways of DDT metabolism to DDMU are depicted in Figure 2. The route *via* DDD is generally established (Peterson and Robison, 1964) while that *via* DDE has only recently been suggested (Datta, 1970). In this study, the significant correlation between ^{14}C-DDD and ^{14}C-DDMU ($r = 0.52$, $P < 0.01$) as compared with the nonsignificant correlation between ^{14}C-DDE and ^{14}C-DDMU ($r = 0.21$, n.s.) supports the former rather than the latter route of DDT metabolism. DDMU should not be regarded as an end product of DDT metabolism since it can be further degraded as suggested by Datta (1970) and illustrated in Figure 3.

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