Interaction of DDT and the Gastrointestinal Microflora of the Rat

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Osborne-Mendel rats were maintained on diets containing 100 p.p.m. of *p,p'*-DDT. The microflora of the gastrointestinal tract were not significantly altered by this diet. Pure cultures of bacteria, typically found in the gastrointestinal tract, were grown in the presence of DDT. Most of the genera studied degraded the pesticide to DDD; the exception was the Gram-positive cocci. The negative findings for those organisms may be due to intracellular binding of DDT and its metabolites. These data provide evidence that DDD in the feces of rats fed DDT may largely be the product of microbial rather than mammalian metabolism.

Studies in this laboratory have indicated that the microorganisms of the animal gastrointestinal tract are capable of dechlorinating DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] (Mendel and Walton, 1966). Other investigators have shown the reductive dechlorination of DDT by baker's yeast (*Saccharomyces cerevisiae*) (Kallman and Andrews, 1963); *Proteus vulgaris* (Barker *et al.*, 1965); *Escherichia coli* (Mendel and Walton, 1966; Wedemeyer, 1966, 1967); *Serratia marcescens* (Stevensen, 1965); *Actinomycetes* (Chacko *et al.*, 1966); *Aerobacter aerogenes* (Mendel and Walton, 1966; Wedemeyer, 1966, 1967); *Klebsiella pneumoniae* (Wedemeyer, 1966); and by mixed microflora of the rumen and of lake water (Miskus *et al.*, 1965). The normal microflora of the gastrointestinal tract must be considered a major agent for the formation of *p,p'*-DDD [1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethane] in rats fed *p,p'*-DDT. Lemire and Fredette (1961) have indicated a stimulation in the growth of Gram-negative rods in general by DDT. The object of the work reported here was to determine the effect of DDT in the diet upon the organisms constituting the major microflora of the gastrointestinal tract of the rat and to study the metabolism of DDT by the various genera of bacteria found there.

METHODS

Weanling Osborne-Mendel rats were raised to an age of 7 to 10 weeks (between 200 and 300 grams) on a semisynthetic diet low in chlorinated pesticide residues. The diet consisted of casein (vitamin-free), 20%; sucrose, 65%; vitamin fortification mixture, 1%; Mazola oil, 3%; coconut oil (nonhydrogenated), 7%; and salt mixture (Jones-Foster), 4%. Analysis: 0.0024 p.p.m. *p,p'*-DDT; 0.0008 p.p.m. *o,p'*-DDT.

The diet of the test animals was fortified to contain 100 p.p.m. of *p,p'*-DDT. The pesticide used in these studies was Food and Drug Administration Primary Standard grade *p,p'*-DDT, FDA No. 200. The material melts between 109.0° and 110.9° C. and is certified to be 99.5 ± 0.05% pure. The pesticide was dissolved in a little ethyl ether and mixed with 20 times its weight of Mazola oil; the ether was removed by evaporation and the solution thoroughly mixed into the diet sample.

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Feeding was ad libitum and the daily food intake was not quantitated. The spiked diet seemed to be as well accepted as the control. This was also indicated by the volume of intestinal contents—similar volumes were found in both groups. The animals were each killed (by decapitation) at the same time of day to aid in obtaining approximate uniformity in the volume of the contents of the alimentary tract. The bacterial population of the gastrointestinal tract was determined by the methods of Smith and Crabb (1961). Immediately after death, the entire alimentary tract was removed and ligated to separate the anterior and posterior stomach, seven equal portions of the small intestine, large intestine (the caecum and the colon), and the rectum. The volume of the contents of the two portions of the stomach, segments 1, 3, 5, and 7 of the small intestine, the large intestine, and the feces (contents of the posterior of the rectum) were estimated and diluted to 10 ml. with sterile distilled water. The numbers of the different kinds of bacteria were estimated by colony counts on selective media. Some of the limitations of these methods, including the absence of an efficient selective medium for enumerating bacteroides, have been discussed previously by Smith and Crabb (1961).

The samples were diluted serially with sterile distilled water. Then 0.1 ml. of each dilution was placed on each of the plates of the selective media and distributed uniformly over the surface of the medium with a glass spreader. Duplicate plates of each dilution were made.

Coliform organisms were estimated on MacConkey agar (Difco) incubated for 20 hours. Typically, red colonies were observed; in most animals, however, a portion of the colonies was colorless. These, when isolated and cultured, were slow lactose fermenters of typical coliform morphology. These colonies were included in the coliform count.

Lactobacilli were estimated on Rogosa's S1 agar (Difco), incubated in candle jars at 37° C.

Enterococci were counted on a modification of Barnes thallous acetate–tetrazolium–glucose agar (TITG agar) (Barnes, 1956) modified as follows: To 100 ml. of sterile nutrient agar medium (Difco) were added 5 ml. of sterile 20% glucose solution, 1 ml. of 1% 2,3,5-triphenyl tetrazolium chloride (sterilized by steaming 30 minutes), and 1 ml. of 10% thallous acetate (sterilized by autoclaving at 10 p.s.i. for 15 minutes).

Two types of colonies were observed on the TITG medium. These were characterized by Barnes as white or

pale pink colonies characteristic of group D streptococci of low reducing power—*Streptococcus faecium*, *Streptococcus durans*, *Streptococcus bovis*—and colonies with a deep red center and narrow white periphery characteristic of *Streptococcus faecalis* and its variants *zymogenus* and *liquifaciens*.

Bacteroides were counted on neomycin blood agar. This was prepared from blood agar base (Difco) by the addition of 50 ml. of human blood and 70 mg. of neomycin sulfate per liter. The plates were incubated anaerobically (hydrogen atmosphere) in Torbal anaerobic jars for 48 hours at 37° C. Several colonial types were noted. Included in the bacteroides counts were only colonies of those types which were composed of nonsporing, nonbranching Gram-negative rods or filaments.

Clostridium perfringens (*C. welchii*) was estimated from the number of lecithinase-producing colonies of appropriate appearance on neomycin Nagler agar (Lowbury and Lilly, 1955).

Yeast was estimated from the number of colonies on Sabouraud agar (Difco).

Following these studies of the effect of DDT in the diet upon the microflora of the gastrointestinal tract, a study was made to determine which of the genera found are capable of degrading DDT in vitro. Representative colonies were picked from the selective media. Each colony which was picked for the study of its metabolism was checked as to morphology, and in some instances biochemical character, and was subcultured. Then 10 ml. of trypticase soy broth (Difco) containing 2.50 μg. of *p,p'*-DDT was inoculated and incubated anaerobically for 20 hours at 37° C. The entire contents of the tubes were extracted three times with equal volumes of diethyl ether. The solvent was evaporated and the material redissolved in petroleum ether. After chromatographic cleanup through activated Florisil (Klein *et al.*, 1963), the chlorinated hydrocarbons were determined by gas-liquid chromatography by the method of Klein *et al.* (1963). In those cases where low conversion was found in 20 hours, longer incubations up to 72 hours were carried out.

Cultures showed heavy growth in 20 hours with the exception of all of the lactobacilli and some of the fusiform cultures. In these cases, incubations were continued to 72 hours or until the absorbance at 450 mμ was approximately 1.0 (20-mm. light path).

Several genera other than those isolated from the rat gastrointestinal tract were studied. The strains of bacillus and staphylococcus were isolated as laboratory contaminants. The culture of *Pseudomonas aeruginosa* was a Food and Drug test organism (B491). Two laboratory strains of *Clostridium perfringens* were also tested for ability to metabolize DDT. One of these, New York, was of fecal origin; the other, FDA B-2, was associated with a case of food poisoning.

RESULTS AND DISCUSSION

The average of the colony counts for the six animals (three of each sex) on the control diet and for six animals (three of each sex) on the test diet are given in Table I. Promotion of bacterial growth in the presence of DDT, such as Lemire and Fredette (1961) observed with Gram-negative rods grown on agar plates, was not found in gastrointestinal segments of the rat.

The decision to carry out the DDT degradation studies by pure cultures growing under anaerobic conditions was made after a comparison of aerobic *vs.* anaerobic metabolism of DDT. Parallel inoculations of *E. coli* (K-12) were made in trypticase soy broth containing 0.25 μg. of *p,p'*-DDT per ml. One culture was incubated at 37° C. on a rotary shaker, another at the same temperature in a Torbal anaerobic jar under hydrogen. Aerobically, 98.6% of the recovered pesticide was DDT. Anaerobically, only 32.4% of the material was DDT, the remainder being DDD. The total recovery was 65% in each case.

The percentages of DDT, DDD, and DDE [1,1-dichloro-2, 2-bis (*p*-chlorophenyl) ethylene] in the chlorinated hydrocarbon fraction recovered after incubation of the cultures with DDT are shown in Table II. Control incubations of 2.5 μg. of DDT in 10 ml. of uninoculated trypticase soy broth yielded only unchanged DDT. Degradation of DDT to DDD was found in all of the genera studied except micrococcus. In Table II, the organisms are placed in one of three categories on the basis of the per cent total recovery of chlorinated pesticide. From cultures of organisms in the high recovery category—i.e., the Gram-negative rods of the genera bacterium and pseudomonas—70% or more of the chlorinated pesticide was recovered by ether extraction. Using the same extraction method, only 30% or less of the pesticide was recovered

Table I. Log_{10} Viable Count (Average of Six Animals) per Milliliter of Organ Contents[a]

Organisms	Diet	Stomach		Small Intestine Portion				Large Intestine	Feces
		A	P	1	3	5	7		
E. coli	Control	4.2	3.5	2.6	3.0	3.8	4.8	5.2	5.1
	DDT	4.5	3.7	2.5	3.1	4.5	4.7	6.2	4.6
C. perfringens	Control	N	N	N	N	N	4.0	6.1	5.4
	DDT	N	N	N	N	N	4.1	6.8	6.4
Streptococci	Control	3.8	3.9	3.1	4.1	4.6	5.2	5.6	5.6
	DDT	5.0	4.7	2.8	4.8	5.3	5.1	6.4	6.2
Lactobacilli	Control	6.2	5.6	6.6	6.4	6.8	6.5	6.0	6.1
	DDT	6.9	5.4	4.6	5.7	6.8	7.7	7.8	7.6
Yeasts	Control	6.0	4.6	7.1	6.0	6.6	6.2	5.9	5.5
	DDT	6.7	4.6	3.8	4.3	6.2	7.0	7.5	7.4
Bacteroides	Control	N	N	N	N	N	5.4	8.0	7.3
	DDT	N	N	N	N	N	4.9	6.4	6.6

[a] A = anterior portion of stomach.
P = posterior portion of stomach.
N = none.

Table II. In Vitro Dechlorination of DDT by Microorganisms of Various Genera

	Recovered Residues, % Recovered as			Number of Isolates Tested	Total Chlorinated Hydrocarbon Recovery Class[a]
	DDE	DDD	DDT		
Bacillus	3	13	84	8	M
Clostridium	0	29.2	67.8	11	M
Micrococcus	0	0	100	9	L
Lactobacillus	5.6	6.8	87.5	20	L
Streptococcus	4.9	6.3	88.8	17	L
Bacteroides	5.0	26.5	68.5	9	M
Yeast	0	91.6	8.4	12	M
Coliform	3.0	86.3	10.7	47	H
Pseudomonas	0	15	85	1	H

[a] Total recovery class: H, more than 70% of the DDT which was added was recovered as DDT or metabolites. M, 31–70% recovered. L, 30% or less recovered.
Organisms grown anaerobically in 10 ml. of trypticase soy broth containing 2.5 μg. of *p,p'*-DDT.

Table III. The Effect of Incubation Time on In Vitro Bacterial Dechlorination of *p,p'*-DDT

Organism	Time, Hours	Recovered Chlorinated Hydrocarbon, % Recovered as			
		DDE	DDD	DDT	Other
Bacillus	20	3	13	84	
	48	3.4	80.5	14	2.1
	72	0	83	7.5	8.9
Pseudomonas	20	2.3	5.2	92.5	
	48	7.3	46	46.7	
	72	8.9	56	35.1	
C. perfringens	20	0	55	44.5	
N. Y.	48	4.5	84	4.8	6.7
	72	0	100	0	
C. perfringens	20	0	81	19	
FDA-B-2	48	6.7	50.5	31.8	11.0
	72	0	68.5	17.6	13.7

Organisms grown anaerobically in 10 ml. of trypticase soy broth containing 2.5 μg. of *p,p'*-DDT.

from the cultures of micrococcus, lactobacillus, and streptococcus, which comprise the low recovery category. The genera of organisms in which the lowest percentages of dechlorinated pesticide were observed were also those in which the total recovery was quite low.

The metabolism of DDT by two strains of clostridium, one strain of bacillus (a laboratory isolate), and one strain of pseudomonas in 20, 48, and 72 hours is shown in Table III. The rate of dechlorination varies considerably between the strains studied. The strain of *C. perfringens* of fecal origin (FDA-B2) showed a significantly higher rate than the food poisoning isolate. The slow dechlorination by the bacillus and *Ps. aeruginosa* may be a reflection of their slow anaerobic growth.

Trypticase soy broth containing 0.25 μg. of DDD per ml. was inoculated with *E. coli* (K-12) and incubated for periods of from 1 to 9 days. The cultures were centrifuged after incubation, the supernatant was extracted with ether, and the cells were ground in a mortar with sharp sand and then extracted with ether. Only DDD was recovered. The recovery decreased with the increasing time of incubation as follows: 88% at 1 day, 62% at 2 days, 36% at 5 days, and 16% at 9 days. Low recoveries may be the result of intracellular binding of the pesticide, making it unavailable to extraction by this method or by the formation of metabolites not extractable by ether.

The recovery of chlorinated pesticide residues from cultures of *E. coli* incubated for 20 hours was, in control experiments, in excess of 80% using this extraction procedure. The cause of low recovery obtained with the cocci and lactobacilli is not clear. Further study of the metabolism of DDT by these organisms is being carried out in this laboratory.

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