Effect of Nutrient Additions on the Apparent Cometabolism of DDT

Frederic K. Pfaender[1] and Martin Alexander*

1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and 4,4′-dichlorobenzophenone (DBP) were produced in sewage treated with 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT). The addition of glucose enhanced the rate of DDD formation but slowed DBP biosynthesis, and additions of diphenylmethane reduced the rate of formation of both DDD and DBP. The numbers of microorganisms potentially able to cometabolize DDT were high in raw sewage, but their abundance rose markedly as a result of the addition of glucose and diphenylmethane. Many of these microorganisms produced DDD, DDE, and DBP.

The persistent pesticide 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), long thought to be resistant to microbial degradation, has recently been shown to be decomposed extensively by pure cultures of bacteria (Pfaender and Alexander, 1972). Because DDT was not used as a substrate for growth, it was apparently cometabolized by these bacteria. Cometabolism refers to the metabolism of a substance by a microorganism which is unable to use that compound for energy or as a source of any of the elements required for growth (Horvath and Alexander, 1970). Cometabolism may be involved in the biological transformation of many pesticides, such as 2,3,6-trichlorobenzoic acid (Horvath, 1971), dieldrin (Matsumura *et al.*, 1968), hexachlorocyclohexane (Yule *et al.*, 1967), and endrin (Patil *et al.*, 1970).

The identities of the enzymes involved in the cometabolic conversion of DDT to 4,4′-dichlorobenzophenone (DBP) and *p*-chlorophenylacetic acid are unknown, but these enzymes are constitutive and are present in *Hydrogenomonas* sp. when the bacterium is grown in media devoid of the insecticide (Pfaender and Alexander, 1972). Similar or identical constitutive enzymes may be present in a variety of other microorganisms inhabiting natural ecosystems, as suggested by the data of Matsumura *et al.* (1971) and Patil *et al.* (1972). The present study was initiated to obtain additional evidence on the significance of cometabolism in the microbial transformations of DDT in nature.

MATERIALS AND METHODS

One-liter samples of raw sewage were placed in 2-l. Erlenmeyer flasks, half of which were autoclaved for 30 min. DDT and inorganic salts were added to each flask to give final concentrations of 0.005% DDT, 10 n*M* $(NH_4)_2SO_4$, 10 m*M* Na_2HPO_4, 4 m*M* KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.5 mg each of $Ca(NO_3)_2$ and $FeSO_4$ per liter of sewage. To two sterilized and two unsterilized samples, glucose was added to a final concentration of 0.10%, 4.0 ml of diphenylmethane/l. was added to a similar set, while a third set received no further additions. All flasks were incubated without shaking at 30°, and after a thorough mixing of the flasks' contents, 50-ml samples were removed at regular intervals and kept frozen until extraction.

At the same time intervals, the numbers of bacteria in the samples were determined by plate counting on a medium containing 1% glucose, the inorganic salts listed above, and 1.5% agar in filtered sewage. The number of colonies was determined after 7 days incubation at 30°, and then 20 colonies were selected at random from each of the three treatments and at each sampling period. The isolates were grown for 72 hr in the glucose–salts medium, the cells were collected by centrifugation, and they were then resuspended in 10 ml of 0.1 *M* phosphate buffer, pH 7.0. These cells were incubated at 30° under a nitrogen atmosphere for 4 days in the presence of 2.0 μg of DDT.

Samples from the treated or untreated sewage were thawed, acidified to pH 2.0 with concentrated H_3PO_3, and continuously extracted with ethyl ether for 6 hr. The extracts were concentrated in a flash evaporator and esterified by boiling with 10% BCl_3-methanol. The microbial cultures were extracted in a separatory funnel with a 1:1 mixture of *n*-hexane–ethyl ether. All extracts were dried and resuspended in acetone, and the resulting solution was analyzed by gas chromatography as described previously (Pfaender and Alexander, 1972).

RESULTS

The products formed from DDT by microorganisms in sewage were essentially the same as those observed earlier (Pfaender and Alexander, 1972), although the amounts varied with the amendment. 1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and DBP were the principal products formed from DDT in each of the sewage communities, but small amounts of the other compounds previously found were usually present also.

The disappearance of DDT and the formation of several products in unamended and amended sewage are shown in Figure 1. The data are expressed in terms of the quantity of DDT or its metabolites in the 50-ml samples collected. Only DDE, DDD, and DBP formation are given, as these metabolites represented more than 95% of the products generated. The quantities of the various compounds appearing in the sterile controls have been subtracted from the amounts shown; usually no such products were detected, although occasionally traces of DDD and DDE were found. The products present initially were contaminants in the DDT preparation. The recoveries of the 7.0 μmol of DDT present in the 50-ml samples were consistently low, but they increased with time of incubation, probably the result of release of the organically bound chlorinated compounds as organic components of the sewage were degraded.

The data in Figure 1 show that DDD appeared at reasonably rapid rates in unamended sewage. The rate was reduced somewhat when diphenylmethane was added, but it was markedly enhanced in the presence of glucose. DBP was formed slowly in the untreated sewage, and the rate of its appearance was diminished upon the addition of either of the carbon sources.

The addition of glucose to the sewage resulted in a rapid and marked increase in the number of bacteria, while the rise in numbers began later but still was appreciable in samples amended with diphenylmethane (Table

Graduate Field of Microbiology and Laboratory of Soil Microbiology, Cornell University, Ithaca, New York 14850.

[1] Present address: Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27514.

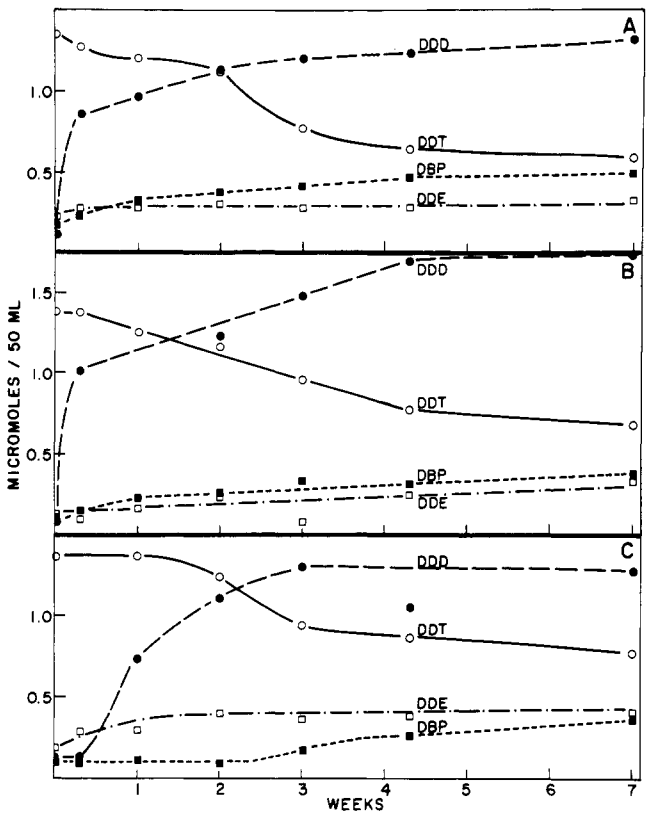

Figure 1. Appearance of DDD, DDE, and DBP in sewage amended with DDT. The sewage also received no additions (A), glucose (B), or diphenylmethane (C).

I). A more modest rise was evident in the untreated raw sewage, presumably resulting from the decomposition of organic compounds present therein.

The percentage of the isolates able to cometabolize DDT was estimated on the basis of the frequency of this activity among the 20 isolates obtained at each sampling period. An isolate was arbitrarily said to be active in cometabolism if it was able to convert at least 10% of the DDT to products. The data in Table I demonstrate that a high percentage of organisms is capable of cometabolizing DDT, even in sewage not provided with sugar or a structural analog of DDT, such as diphenylmethane. It is surprising that the percentage able to cometabolize the insecticide rose as the total bacterial numbers increased, possibly a result of the greater frequency of cometabolizing activity among the faster growing bacteria utilizing the natural or added organic compounds.

The abundance of bacteria which are potentially capable of cometabolizing DDT was obtained by multiplying the percentage abundance of these organisms (from the 20 test isolates) and the total bacterial count of the sewage. It is evident from the counts so obtained that the additions of glucose or diphenylmethane bring about an enormous stimulation in the number of cometabolizing bacteria (Table I). Although the percentage increase of cometabolizers was appreciable even in the unamended sewage, the rise in relative abundance of cometabolizers coupled with the actual increase in cell density is reflected in the marked rise in organisms with the potential for partially modifying the DDT molecule. Nevertheless, it has not yet been established that the abundance of cells actually cometabolizing DDT does in fact increase.

Five distinct patterns of product formation by these organisms were noted. Some accumulated DDD, DDE, and DBP, others DDD and DDE, some DDD and DBP, a few accumulated only DBP, while still others accumulated only DDD. The number of isolates showing each of these patterns in each of the three types of microbial communities is shown in Table II. No marked differences in the pattern of products accumulating among the isolates from the three treatments were evident.

DISCUSSION

No direct means are as yet known for testing the significance of cometabolism as a mechanism for the microbial transformation of pesticides in nature; however, it is usually assumed that this is the mechanism when a process is carried out in nonsterile but not in sterile samples from a natural ecosystem and no organism can be obtained which is able to use the pesticide as a source of one of its essential nutrient elements. In the present study, the observation that the formation of metabolic products from DDT parallels the increase in numbers of bacteria potentially able to cometabolize DDT provides additional circumstantial evidence for the functioning of cometabolism in the microbial transformations of this insecticide. The supposition that the addition to sewage of a biodegradable analog of DDT, such as diphenylmethane, might selectively stimulate cometabolizing heterotrophs, and hence enhance the rate of insecticide biodegradation was not supported by the data.

A stimulation of DDT metabolism by the addition of nutrients has been shown to occur in soil (Ko and Lockwood, 1968; Parr *et al.*, 1970). The enhanced rate of destruction resulting from the addition of glucose or natural carbonaceous materials structurally unrelated to the insecticide probably can be ascribed to the frequency of DDT-cometabolizing cells in natural ecosystems and their stimulation by the additional carbon source. The rate of metabolism would then decline, as observed herein, when the cell density falls because of the disappearance of readily available carbon sources. This hypothesis requires that the enzymes in DDT cometabolism either be constitutive or be induced by noninsecticidal compounds. Evidence that the enzymes are present in cultures grown in the absence of added DDT has already been obtained (Pfaender and Alexander, 1972).

To account for the persistence of DDT in nature in view

Table I. Changes in Bacterial Numbers and in Abundance of Bacteria Cometabolizing DDT in Treated and Untreated Sewage

	Unamended sewage			Glucose-amended sewage			Diphenylmethane-amended sewage		
Days	Total no.[a]	% cometabolizing DDT	No. of DDT cometabolizers[a]	Total no.[a]	% cometabolizing DDT	No. of DDT cometabolizers[a]	Total no.[a]	% cometabolizing DDT	No. of DDT cometabolizers[a]
0	3.8	35	1.3	5.6	40	2.2	5.8	30	1.7
7	11	55	6.1	5000	90	4500	53	60	32
14	120	75	90	580	65	380	2200	70	1500
21	3.8	65	2.5	62	50	31	8100	80	6500
30	0.98	35	0.34	76	35	27	8600	60	5200
42	1.3	20	0.26	6.7	15	1.0	1000	25	250

[a] Number × 10^6/ml of sewage.

Table II. Products Formed from DDT by Isolates from Amended Sewage

Products formed	Number of isolates		
	Sewage	Sewage + glucose	Sewage + diphenylmethane
DDD, DDE, DBP	29[a]	17	31
DDD, DDE	31	35	34
DDD, DBP	2	11	3
DBP	1	0	1
DDD	2	8	0
None	75	69	71

[a] Number represents total number of isolates forming these products from DDT.

of the existence of microorganisms which, in culture at least, can effect its extensive biodegradation, it has been postulated that the insecticide is metabolized by few microorganisms, none of which is capable of utilizing the compound as a carbon or energy source (Pfaender and Alexander, 1972). On the basis of the present data, it seems more likely that DDT persists owing to the fact that cells which can cometabolize the chemical, although sometimes numerous, do not express a high activity. The activity of some of the organisms may not be expressed at all. Furthermore, inasmuch as a species particularly active in cometabolism has no necessary selective advantage in nature, its population density would not increase in response to DDT application.

ACKNOWLEDGMENT

The authors express their thanks to Janice Zelaska and Theresa Ho for able technical assistance.

LITERATURE CITED

Horvath, R. S., *J. Agr. Food Chem.* **19,** 291 (1971).
Horvath, R. S., Alexander, M., *Can. J. Microbiol.* **16,** 1131 (1970).
Ko, W. H., Lockwood, J. L., *Can. J. Microbiol.* **14,** 1069 (1968).
Matsumura, F., Boush, G. M., Tai, A., *Nature (London)* **219,** 965 (1968).
Matsumura, F., Patil, K. C., Boush, G. M., *Nature (London)* **230,** 325 (1971).
Parr, J. F., Willis, G. H., Smith, S., *Soil Sci.* **110,** 306 (1970).
Patil, K. C., Matsumura, F., Boush, G. M., *Appl. Microbiol.* **19,** 879 (1970).
Patil, K. C., Matsumura, F., Boush, G. M., *Environ. Sci. Technol.* **6,** 629 (1972).
Pfaender, F. K., Alexander, M., *J. Agr. Food Chem.* **20,** 842 (1972).
Yule, W. N., Chiba, M., Morley, H., *J. Agr. Food Chem.* **15,** 1000 (1967).

Received for review October 20, 1972. Accepted January 8, 1973. This investigation was supported by Public Health Service Training Grant ES-00098 from the Division of Environmental Health Sciences.

Determination of Carbofuran and 3-Hydroxycarbofuran Residues in Small Fruits

Ian H. Williams* and Marilyn J. Brown

A method is described for determining residues of carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) and its 3-hydroxy metabolite in strawberries, raspberries, blueberries, and cranberries. The extraction method of Cook *et al.* (1969) was modified to include a dichloromethane extraction of the filtered solids. Cleanup was on a silica gel–alumina column with mixtures of dichloromethane–ether and acetone–methanol as eluents. Determination was by gas chromatography (gc) with nitrogen detection using a Coulson conductivity detector. Recoveries of both carbofuran and 3-hydroxycarbofuran from fortified samples averaged greater than 86% over the range 0.2 to 5.0 ppm.

Recent field experiments have demonstrated the efficacy of spray applications of carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) for controlling root weevils and a moth larva in small fruits. These include: the strawberry root weevil, *Brachyrhinus ovatus* (L.), and the bush weevil, *Nemocestes incauptus* (Horn), in strawberries; the bud weevil, *B. singularis* (L.) in raspberries; the black vine weevil, *B. sulcatus* (Fab.), in blueberries; and the black-headed fireworm, *Rhopobota naevana* (Hub.), in cranberries. For further evaluation of this insecticide for use on these crops, analysis of the fruit for residues of both the parent compound and its toxic 3-hydroxy metabolite (2,3-dihydro-3-hydroxy-2,2-dimethyl-7-benzofuranyl methylcarbamate) was required. This paper describes the method developed and recoveries obtained from fortified samples of strawberries, raspberries, blueberries, and cranberries.

Several methods for the determination of carbofuran residues have been published and various crops have been analyzed (Bowman and Beroza, 1967; Butler and McDonough, 1971; Cassil *et al.*, 1969; Cook *et al.*, 1969; Van Middelem *et al.*, 1971). Apparently there has been no previous study of carbofuran in small fruits. Most of the published methods follow similar techniques for extraction and cleanup based on the original method of Cook *et al.* (1969) for corn. Acid hydrolysis is used to release any conjugated 3-hydroxycarbofuran to the aglycone form. This is followed by extraction of the aqueous phase with dichloromethane and column cleanup to remove possible interferences. Final determination is by gc, either directly with nitrogen detection or after conversion to compounds sensitive to electron capture or flame photometric detection. The method to be described employs direct determination with conductivity detection of nitrogen.

For small fruits some modifications were required in the basic method of Cook *et al.* (1969) and some points of interest arose which should have a bearing on methodology for other crops.

Canada Department of Agriculture, Vancouver 8, British Columbia, Canada.

EXPERIMENTAL SECTION

Apparatus. A boiling flask and condenser, as described by Cook *et al.* (1969), were used. The filtration apparatus