Mutagenicity Studies of Dioxin and Related Compounds with the *Salmonella arabinose* Resistant Assay System*

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Abstract

Components of the herbicide Agent Orange—2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and their esters in addition to the contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)—and related chemicals were tested for mutagenicity using the *Salmonella* arabinose resistant assay system. The preincubation test using strain SV50 was performed both with and without metabolic activation. No mutagenic activity was observed for any of the chemicals tested.

INTRODUCTION

Agent Orange was the herbicide used extensively during the Vietnam conflict in the 1960s as a defoliant. It was formulated as a 50:50 mixture of butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). In addition, it contained free 2,4-D and 2,4,5-T, di- and trichlorophenol, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hughes *et al.*, 1975; Young *et al.*, 1978). TCDD was generated as an unintended contaminant in the manufacture of 2,4,5-T.

Although there is no current exposure to Agent Orange, there was widespread exposure of Vietnamese and American military personnel

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and civilians to Agent Orange in Vietnam. Also, some of the components of Agent Orange are in use today. 2,4-D is currently used by home gardeners, and both 2,4-D and 2,4,5-T are used as industrial herbicides. TCDD was present as a contaminant of commercially used products (International Agency for Research on Cancer, 1977), and was also found in contaminated soil in some areas (McConnell *et al.*, 1984). Because of the past exposure to Agent Orange and 2,4,5-T, and current exposure of chemical workers, farm workers, and home gardeners to 2,4-D preparations, these compounds were tested for mutagenicity.

Mortelmans *et al.* (1984) tested 11 Agent Orange related compounds in the Ames *Salmonella* microsomal assay using preincubation. No mutagenic activity was observed. This study was conducted to test the mutagenic activity of 2,4,-D, 2,4-D n-butyl ester, 2,4,5-T, 2,4,5-T *n*-butyl and isobutyl esters, and TCDD in addition to home gardening formulations of 2,4-D isooctyl ester and 2,4,5-T isooctyl ester with the preincubation test of another bacterial assay, the Salmonella/arabinose-resistant (Ara^r) assay system. In a study by Xu *et al.* (1984) it was found that the Salmonella Ara^r forward mutation assay system had similar mutagenic sensitivity to known mutagenic compounds when compared to the Ames assay. In addition, certain compounds exhibited a stronger mutagenic response than in the Ames assay and a few compounds produced a mutagenic response where none had been evident in the Ames assay. It was also found when comparing the plate incorporation to the preincubation test that the latter, while never less sensitive than plate incorporation, was occasionally more sensitive in its mutagenic response.

METHODS

Test Chemicals

All test compounds were obtained from Radian Corporation (Austin, TX). Chemical stocks were freshly prepared for each experiment using dimethylsulfoxide (DMSO) as the solvent.

Tester Strain

In the Ara^r assay system, the arabinose-sensitive (Ara^s) strain SV50 of *Salmonella typhimurium* was employed for measuring mutation from Ara^s to Ara^r. The cell concentration was determined with a Spectronic 20 colorimeter (Bausch & Lomb) and adjusted to 1.5×10^7 cells/mL in distilled water prior to use.

Media

Soft agar for SV50 contained 0.6% Bacto-Difco agar and 0.5% NaCl. Before use, 0.3 mL of 20% L-arabinose and 0.1 mg of glucose were added to 2 mL of molten soft agar. The glucose was omitted when S9 activation was used. M9 medium was used as bottom agar; it contained 1.5% Bacto-Difco agar, 1.5% supplement stock (2 mg tryptophan/mL, 2 mg threonine/mL, and 1 mg uracil/mL). 1.5% glycerol stock (20% glycerol), and 10% M9 salt stock (10 g NH_4Cl, 60 g Na_2HPO_4, 30 g KH_2PO_4, 50 g NaCl, and 2.05 g $MgSO_4 \cdot 7H_2O$ per liter of distilled water). Medium for overnight culture contained the same components as those of M9 medium except agar. In addition, 0.1 mL of ampicillin stock (5 mg/mL) was added to 25 mL of the overnight culture medium.

S9 Mix

Male Sprague-Dawley CD rats or male Syrian hamsters were injected intraperitoneally with Aroclor 1254 in corn oil (500 mg/kg body weight) 5 days before they were killed. The preparation of liver S9 homogenate and S9 mix (10% of liver S9) followed the procedure described by Ames *et al.* (1975).

Mutagenicity Assay

The preincubation test was performed according to the method of Xu *et al.* (1984), with modifications. To describe this test in brief: 0.1 mL of the test compound was added to a 17 × 100 mm plastic tube containing 0.1 mL of an overnight culture of SV50, and 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) or S9 mix. Each mixture in a tube was incubated at 37°C for 30 min with rotation in a roller drum. Thereafter 2 mL of soft agar was added to each tube. After mixing, the soft agar mix was overlayed onto an M9 agar plate (petri plate containing 16–18 mL M9 medium). The plates were incubated at 37°C for 3 days, at which time colonies were counted using an Artek counter (model 880).

Each compound was tested using triplicate plates at 5–7 dose concentrations separated by half-log intervals. Ten milligrams per plate was used as a high concentration for each compound, except for TCDD which, because of the limited amount available, was tested at 1 mg/plate. Each compound was assayed in 2 independent experiments. Controls were routinely included in each experiment. 2-Aminoanthracene (2AA) was used as a positive control for *Salmonella* responsiveness and S9 activity.

A compound was defined as mutagenic when the average number of mutants obtained was two-fold or greater than the solvent control and showed a dose-response relationship.

RESULTS

Table I presents the test results for each compound. The values shown are averages for the 2 tests of each compound (a total of 6 plates). A minimum of 5 dose levels at semilog intervals were tested for each compound even though not all values are shown. No mutagenic responses to the test compounds were observed under any of the assay conditions. The results were similar in both the original and repeat experiments. Also, with the use of activation, neither hamster nor rat S9 induced any mutagenic response. Dose responses, even without doubling, were not observed in any of the compounds. However, in all compounds except for TCDD (which was tested at lower concentrations), variable toxicity was evident at the higher dose levels of 3333 and 10000 μg/plate. All solvent controls with DMSO gave the expected results. Furthermore, 2AA, the positive control, responded strongly to both hamster and rat S9 activation.

TABLE I
Test results of Agent Orange components and related chemicals in the *Salmonella*/Ara^r assay system

	Ara^r mutants/plate[a]		
μg/plate	NA[b]	Rat S9[b]	Hamster S9[b]
	2,4-Dichlorophenoxyacetic acid (2,4-D)		
0.0[c]	52 ± 5.4	121 ± 8.4	147 ± 14.4
33.0	44 ± 3.8	118 ± 14.9	152 ± 14.4
333.0	47 ± 4.4	139 ± 5.2	153 ± 15.3
3333.0	8 ± 2.3	83 ± 5.3	75 ± 5.0
POS[d]	—	789 ± 54.1	949 ± 53.0
	2,4-D, Isooctyl ester (Ded-weed, LV-69)		
0.0	69 ± 15.4	187 ± 31.7	216 ± 24.5
33.0	55 ± 10.0	227 ± 38.2	257 ± 56.4
333.0	31 ± 6.2	223 ± 39.9	255 ± 64.6
3333.0	8 ± 3.3	60 ± 26.9	48 ± 21.9
POS	—	717 ± 72.0	616 ± 14.9
	2,4-D, *n*-butyl ester		
0.0	52 ± 6.3	162 ± 18.6	167 ± 21.4
0.33	44 ± 2.5		
3.3	39 ± 3.4		
33.0	13 ± 5.4		

TABLE I (*Continued*)

μg/plate	Ara^r mutants/plate[a]		
	NA[b]	Rat S9[b]	Hamster S9[b]
100.0		154 ± 13.2	142 ± 9.1
1000.0		120 ± 8.5	115 ± 19.9
10000.0		84 ± 8.7	85 ± 12.9
POS	—	586 ± 32.1	638 ± 25.6
	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)		
0.0	66 ± 3.6	135 ± 17.2	179 ± 14.4
10.0	50 ± 4.9	126 ± 13.9	187 ± 13.9
100.0	47 ± 8.5	134 ± 2.7	176 ± 6.6
1000.0	30 ± 5.2	139 ± 11.5	179 ± 14.2
POS	—	589 ± 19.4	677 ± 34.9
	2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)		
0.0	54 ± 1.7	134 ± 11.8	148 ± 14.6
33.0	39 ± 4.7	102 ± 15.9	139 ± 8.8
333.0	40 ± 4.1	103 ± 6.2	117 ± 6.8
3333.0	0 ± 0.0	35 ± 1.8	32 ± 1.2
POS	—	652 ± 79.9	706 ± 91.4
	2,4,5-T, isobutyl ester		
0.0	74 ± 12.8	185 ± 32.8	236 ± 37.7
100.0	54 ± 11.2	189 ± 33.9	238 ± 42.2
1000.0	59 ± 19.2	132 ± 18.4	185 ± 15.4
10000.0	45 ± 4.7	94 ± 19.1	87 ± 16.1
POS	—	803 ± 64.8	826 ± 97.0
	2,4,5-T, isooctyl ester (Ded-weed LV-4T)		
0.0	37 ± 8.2	109 ± 14.5	104 ± 12.2
100.0	46 ± 9.4	157 ± 68.3	152 ± 86.5
1000.0	27 ± 8.7	132 ± 33.4	108 ± 34.3
10000.0	13 ± 3.0	59 ± 13.1	48 ± 11.1
POS	—	877 ± 6.5	1180 ± 17.3
	2,4,5-T, *n*-butyl ester		
0.0	73 ± 12.8	185 ± 32.8	217 ± 27.7
100.0	50 ± 7.2	189 ± 39.4	253 ± 43.4
1000.0	37 ± 10.6	163 ± 29.7	218 ± 44.5
10000.0	32 ± 10.6	89 ± 14.9	83 ± 13.3
POS	—	803 ± 64.8	826 ± 97.0

[a]Results are mean values of two experiments plated in triplicate ± SE (standard error).

[b]NA, not activated: Rat S9, Aroclor 1254-induced rat liver S9: Hamster S9, Aroclor 1254-induced Syrian hamster liver S9.

[c]Negative control used was 0.1 mL of DMSO per plate.

[d]Positive control used was 2.5 μg of 2AA per plate.

DISCUSSION

There is concern about the extensive use of phenoxyacetic acid herbicides such as 2,4-D, and the recent use of 2,4,5-T and its contaminants. TCDD is of particular interest due to its carcinogenicity and extreme toxicity. The manufacture of 2,4,5-T is now prohibited and the use of existing supplies is strictly limited (Environmental Protection Agency 1983); however, 2,4-D and its esters are still in large-scale use. Epidemiological studies on the carcinogenicity of the compounds other than TCDD are often contradictory and inconclusive due to limited exposure levels, small sample size, and/or design of the studies (Hoar, *et al.* 1986; Coggon and Acheson, 1982), but recent studies are trying to correct these shortcomings. However, in rodent studies TCDD has been shown to be carcinogenic (Kociba *et al.*, 1978; National Toxicology Program 1982a, b), and other studies suggest a link between human exposure to TCDD and carcinogenicity (Hoar, *et al.* 1986; Hardell, 1979; Hardell *et al.*, 1981; Hardell, 1981; and Hardell *et al.*, 1982). Because of this, National Institute for Occupational Safety and Health recommends that TCDD be considered a potential occupational carcinogen and that human exposure in occupational settings be strictly controlled (NIOSH, 1984).

The results of this study show no mutagenic activity for any of the 8 compounds tested. Similar results have been obtained by Mortelmans *et al.* (1984) in the Ames *Salmonella* assay in strains TA100, TA1535, TA1537, and TA98 with hamster and rat S9 activation and without activation. Other studies with 2,4-D (Moriya *et al.*, 1983; Seiler, 1978), 2,4,5-T (Shirasu *et al.*, 1976), and TCDD (Geiger and Neal, 1981) in the Ames assay agree with these results. However, studies by Seiler (1973) and Hussain *et al.* (1972) show a positive response of TCDD in *Salmonella* strain TA1532 (equivalent to TA1537) without metabolic activation.

For the majority of the chemicals and most of the studies with TCDD the genotoxicity results were negative. Due to the fact that the Ames assay detects base-pair substitutions and frame-shift mutations through the use of different strains, and that the Ara^r assay, using strain SV50, detects mutations of both types, as well as small deletions, it seems likely that the compounds tested do not cause point mutations. However, the possibility suggested by Mortelmans *et al.* (1984) that the solubility of TCDD may prohibit the test chemical from diffusing into the agar must not be ruled out.

Mutagenic studies involving chromosomal damage as a measure of the potential genetic hazard have been reported. 2,4-D has been shown to significantly increase sister chromatid exchange (SCE) frequencies

in human peripheral lymphocytes (Turkula and Jalal, 1985; Korte and Jalal, 1982) and in cultured Chinese hamster ovary cells (Tomkins *et al.*, 1980), and to cause increases in chromosomal aberrations in human peripheral lymphocytes (Korte and Jalal, 1982). However, a study by Linnainmaa and Vainio (1983) showed that 2,4-D did not increase the frequency of SCE in human lymphocytes when the workers wore protective clothing. Also, 2,4-D, 2,4,5-T, and TCDD failed to increase SCE levels in mouse bone marrow in a study by Lamb *et al.*(1981). In addition, Meyne *et al.* (1985) showed that TCDD caused no increase in SCE, chromosomal aberrations, or micronuclei in mouse bone marrow at dose levels that caused liver damage to the animals. In light of these conflicting findings in the comparison of the mutagenicity and clastogenicity of 2,4-D, 2,4,5-T, and TCDD, and the relative absence of data on the derivatives of these chemicals, it seems important that further studies with mammalian systems be conducted to clarify the differences.

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