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In vivo micronucleus assays on 2,4-dichlorophenoxyacetic acid and its derivatives

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Abstract

The potential for 2,4-D and seven of its salts and esters to induce cytogenetic abnormalities in mammalian cells in vivo was investigated in the mouse bone marrow micronucleus test. All the test materials were administered to male and female mice by oral gavage and the frequencies of micronucleated polychromatic erythrocytes (MN-PCE) in the bone marrow were determined at intervals of 24, 48 and 72 h following dosing. There were no significant increases in the incidence of MN-PCE in the treated mice at any of the bone marrow sampling times. These results are consistent with the reported lack of in vitro genetic toxicity for these materials in various in vitro genotoxicity assays as well as the absence of carcinogenic potential for 2,4-D in both mice and rats. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) and its derivatives are widely used as herbicides and are generally regarded as having low potential for mammalian toxicity [1]. 2,4-D has been shown not to induce pre-neoplastic or neoplastic lesions in rodents and dogs [2,3]. A number of publications dealing with the genotoxicity of 2,4-D appeared in the literature and these studies were reviewed by Munro et al. [1]. In order to generate a comprehensive genotoxicity database on 2,4-D and its various derivatives, members of the Industry Task Force II on 2,4-D Research Data (TFII) have performed additional studies and these studies have been discussed in the previous papers [4,5].

This paper presents the results of eight in vivo mouse micronucleus studies on 2,4-D and its salt and ester derivatives. All studies were conducted in accordance with Good Laboratory Practice regulations and applicable toxicology guidelines for pesticide testing [6–11] and included extensive chemical characterizations of the test substance. All of the studies were performed by either Hazleton Laboratories America or the laboratories of Dow Chemical. The results from these studies are compared to previously published in vivo genotoxicity assays.

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2. Materials and methods

2.1. Test material

The test samples used in these studies were obtained as follows: 2,4-D acid (CAS No. 94-75-1), 2,4-D dimethylamine salt (2,4-D DMA; CAS No. 20-08-39-1), and 2,4-D 2-ethylhexyl ester (2,4-D EHE; CAS No. 1928-43-4) from the Industry Task Force II on 2,4-D Research Data; 2,4-D diethanolamine salt (2,4-D DEA; CAS No. 5742-19-8) from PBI/Gordon; 2,4-D isopropylamine (2,4-D IPA; CAS No. 5742-17-6), 2,4-D triisopropanolamine (2,4-D TIPA; CAS No. 32341-80-3) and 2,4-D 2-butoxyethyl ester (2,4-D BEE; CAS No. 1929-73-3) from DowElanco; and 2,4-D isopropyl ester (2,4-D IPE; CAS No. 94-11-1) from the California Citrus Quality Council.

All dose levels presented in the tables are expressed as active ingredient (a.i.) of the test material formulations based on the following purities: 96.1% for the 2,4-D acid; 73.8% 2,4-D DEA; 66.2% 2,4-D DMA; 50.1% 2,4-D IPA; 70.9% 2,4-D TIPA; 95.6% 2,4-D BEE; 98% 2,4-D EHE; and 97.1% 2,4-D IPE. For comparison purposes the following 2,4-D acid equivalents (a.e.) for the formulations are given: 49.99% 2,4-D DEA; 54.97% 2,4-D DMA; 39.53% 2,4-D IPA; 38.70% 2,4-D TIPA; 65.77% 2,4-D BEE; 64.98% 2,4-D EHE; and 81.55% 2,4-D IPE. The test samples were either dissolved in corn oil, or sterile deionized water, and utilized in the in vivo test system. The concentrations of the test materials in the dosing solutions were confirmed by appropriate analytical methods (HPLC or GC).

2.2. In vivo mouse micronucleus assays

In the bone marrow micronucleus test, 10–11-week-old CD-1 mice (Charles River, Wilmington, MA) were used in the Dow Chemical Studies and ICR (Harlan Sprague–Dawley, Frederick, MD) mice were used in the Covance Laboratories studies. Five mice/sex/group and 3 dose levels plus appropriate vehicle and positive control groups were utilized in each study. Doses for each compound were selected based on the mortality results in rangefinding studies (3 or 5 mice/sex/group). Dose levels in the definitive studies were as follow: 2,4-D acid — 40, 130, and 400 mg/kg; 2,4-D DEA — 44.3, 148, and 443 mg/kg; 2,4-D DMA — 39.7, 132, and 397 mg/kg; 2,4-D EHE — 50, 167, and 500 mg/kg; 2,4-D IPE — 100, 200, and 400 mg/kg; 2,4-D IPA — 37.6, 126, and 376 mg/kg; and 2,4-D TIPA — 54.2, 180, and 542 mg/kg.

Mice were allowed to acclimate for at least 6 days prior to study initiation and temperature and humidity were maintained at approximately 70°F and 50%, respectively. They were maintained on a 12-h light/12-h dark cycle and commercial diet (Purina Certified Laboratory Chow® #5002) and water were available ad libitum.

2,4-D acid, 2,4-D BEE, 2,4-D EHE, and 2,4-D IPE were mixed in corn oil and administered by a single oral gavage in aliquots of 10 ml/kg body weight. 2,4-D DEA, 2,4-D DMA, 2,4-D IPA, and 2,4-D TIPA were mixed in sterile deionized water and also administered by a single oral gavage in aliquots of 10 ml/kg body weight. The appropriate negative controls (10 ml/kg of either corn oil or sterile deionized water) were used in the studies and cyclophosphamide (either 80 or 100 mg/kg of CP served as the positive control).

Bone marrow samples were collected at various intervals after treatment by removing the adhering soft tissue and epiphyses of both femurs and flushing, or aspirating, the marrow into a centrifuge tube utilizing a volume of fetal calf serum. Following centrifugation to pellet the tissue, most of the supernatant was drawn off, the cells were resuspended, and the suspension spread on slides and air-dried. The slides were then fixed, stained in either May–Gruenwald/Giemsa or Wright–Giemsa, and 1000 polychromatic erythrocytes (PCE) were evaluated from each animal for the incidence of micronucleated polychromatic erythrocytes (MN-PCE) [12]. Treatment induced perturbation in bone marrow erythropoiesis was ascertained by determining the relative proportion of PCE to normochromatic erythrocytes (NCE).

2.3. Data analysis

2.3.1. 2,4-D BEE, 2,4-D IPA, and 2,4-D TIPA

The raw data on the counts of MN-PCE for each animal was first transformed by adding 1 to each count and then taking the natural log of the adjusted number. The transformed MN-PCE data was ana-

Table 1
Pattern of mortality from rangefinding studies

Dose (mg/kg body weight)[a]	Sex	No. of dead	Dose (mg/kg body weight)[a]	Sex	No. of dead
2,4-D Acid[b]			2,4-D DEA[b]		
300	M	0/3	295	M	0/3
	F	0/3		F	0/3
675	M	2/3	590	M	1/3
	F	2/3		F	2/3
1050	M	3/3	738	M	3/3
	F	3/3		F	2/3
1425	M	3/3	886	M	3/3
	F	3/3		F	2/3
1800	M	3/3	1033	M	3/3
	F	3/3		F	2/3
2,4-D DMA[b]			2,4-D IPA[c]		
265	M	0/3	250	M	0/5
	F	0/3		F	0/5
530	M	1/3	500	M	1/5
	F	0/3		F	1/5
794	M	3/3	1000	M	5/5
	F	2/3		F	5/5
1060	M	3/3			
	F	3/3			
1324	M	3/3			
	F	3/3			
2,4-D TIPA[c]			2,4-D BEE[c]		
355	M	0/5	250	M	0/5
	F	0/5		F	0/5
710	M	0/5	500	M	3/5
	F	2/5		F	5/5
1420	M	4/5	1000	M	5/5
	F	4/5		F	5/5
			2000	M	5/5
				F	5/5
2,4-D EHE[b]			2,4-D IPE[b]		
400	M	0/3	250	M	0/3
	F	0/3		F	0/3
800	M	3/3	400	M	0/3
	F	3/3		F	0/3
1200	M	2/3	475	M	0/3
	F	3/3		F	0/3
1600	M	3/3	531	M	3/3
	F	2/3		F	2/3
2000	M	3/3	812	M	3/3
	F	3/3		F	3/3

[a] Based on active ingredient content.
[b] Study conducted by Covance Laboratories.
[c] Study conducted by the laboratories of Dow Chemical.

lyzed by a three-way analysis of variance (sex, dose, and time) assuming the three-way interaction was zero. From these initial analyses, the two-way interactions was reviewed for significance. Depending on these reviews, the data was analyzed by either one-, two- or three-way analysis of variance looking at main effects only. Pairwise comparisons of treated vs. control groups was done, if necessary, by a *t*-test with Bonferroni correction for multiple comparisons. The alpha level at which all the tests were conducted was 0.01.

2.3.2. *All other derivatives*

Analysis of the data was performed using an analysis of variance on the square root arcsine transformation which was performed on the proportion of cells with micronuclei per animal followed by Tukey's Studentized range test (HSD) with adjustments for multiple comparison. Analysis were performed separately for each harvest time and sex combination, and also at each harvest time for the sexes combined.

3. Results

In the micronucleus tests on the 2,4-D derivatives, survival of the treated animals was used to define the maximum tolerated dose (MTD, Table 1). Doses of 675 mg/kg or greater of 2,4-D acid were found to be excessively toxic ($\geq$ 67% mortality); therefore, a top dose of 400 mg/kg was chosen. In the case of

Table 2
Results from the in vivo micronucleus test from Covance Laboratories

Treatment (mg/kg)[a]	Sex	N[b]	2,4-D Acid					
			24-h sacrifice		48-h sacrifice		72-h sacrifice	
			% MN-PCE[c]	PCE:NCE[d]	% MN-PCE	PCE:NCE	% MN-PCE	PCE:NCE
Negative control	M	5	0.06 ± 0.04[e]	0.48 ± 0.03	–	–	–	–
10 ml/kg corn oil	F	5	0.06 ± 0.04	0.64 ± 0.07	–	–	–	–
Positive control	M	5	2.94 ± 0.90*	0.48 ± 0.08	–	–	–	–
80 mg/kg CP	F	5	0.96 ± 0.18*	0.70 ± 0.09	–	–	–	–
40	M	5	0.04 ± 0.02	0.59 ± 0.06	0.00 ± 0.00	0.67 ± 0.05	0.00 ± 0.00	0.68 ± 0.08
	F	5	0.06 ± 0.04	0.80 ± 0.12	0.10 ± 0.08	0.64 ± 0.06	0.06 ± 0.02	0.75 ± 0.07
133	M	5	0.06 ± 0.04	0.43 ± 0.03	0.02 ± 0.02	0.47 ± 0.09	0.02 ± 0.02	0.74 ± 0.08
	F	5	0.22 ± 0.15	0.66 ± 0.10	0.02 ± 0.02	0.56 ± 0.06	0.00 ± 0.00	0.73 ± 0.17
400	M	5	0.02 ± 0.02	0.37 ± 0.05	0.04 ± 0.02	0.34 ± 0.04	0.02 ± 0.02	0.32 ± 0.05
	F	5	0.06 ± 0.04	0.82 ± 0.11	0.04 ± 0.02	0.66 ± 0.10	0.08 ± 0.04	0.68 ± 0.17
			2,4-D DEA					
Negative control	M	5	0.04 ± 0.04	0.43 ± 0.05	–	–	–	–
10 ml/kg water	F	5	0.04 ± 0.02	0.95 ± 0.18	–	–	–	–
Positive control	M	5	1.84 ± 0.27*	0.44 ± 0.06	–	–	–	–
80 mg/kg CP	F	5	1.94 ± 0.53*	1.06 ± 0.11	–	–	–	–
44.3	M	5	0.06 ± 0.02	0.30 ± 0.04	0.00 ± 0.00	0.46 ± 0.14	0.08 ± 0.05	0.36 ± 0.05
	F	5	0.10 ± 0.06	0.63 ± 0.08	0.06 ± 0.04	0.62 ± 0.06	0.06 ± 0.02	0.64 ± 0.11
148	M	5	0.08 ± 0.04	0.25 ± 0.05	0.02 ± 0.02	0.29 ± 0.04	0.08 ± 0.04	0.35 ± 0.08
	F	5	0.10 ± 0.03	1.12 ± 0.13	0.06 ± 0.04	0.60 ± 0.12	0.00 ± 0.00	0.86 ± 0.13
443	M	5	0.02 ± 0.02	0.35 ± 0.05	0.02 ± 0.02	0.30 ± 0.04	0.04 ± 0.02	0.29 ± 0.05
	F	5	0.12 ± 0.04	0.66 ± 0.04	0.08 ± 0.05	0.46 ± 0.07	0.00 ± 0.00	0.63 ± 0.06
			2,4-D DMA					
Negative control	M	5	0.06 ± 0.04	0.45 ± 0.07	–	–	–	–
10 ml/kg water	F	5	0.06 ± 0.06	0.84 ± 0.07	–	–	–	–
Positive control	M	5	1.48 ± 0.35*	0.57 ± 0.06	–	–	–	–
80 mg/kg CP	F	5	2.12 ± 0.36*	0.87 ± 0.15	–	–	–	–
39.7	M	5	0.04 ± 0.02	0.51 ± 0.03	0.10 ± 0.03	0.72 ± 0.05	0.12 ± 0.07	0.57 ± 0.07
	F	5	0.02 ± 0.02	0.77 ± 0.08	0.06 ± 0.06	0.73 ± 0.17	0.08 ± 0.04	0.75 ± 0.13
132	M	5	0.06 ± 0.04	0.47 ± 0.08	0.12 ± 0.05	0.62 ± 0.12	0.06 ± 0.06	0.79 ± 0.17
	F	5	0.08 ± 0.06	0.80 ± 0.13	0.00 ± 0.00	0.66 ± 0.12	0.04 ± 0.04	0.70 ± 0.08
397	M	5	0.12 ± 0.06	0.33 ± 0.05	0.02 ± 0.02	0.55 ± 0.06	0.18 ± 0.06	0.48 ± 0.03
	F	5	0.04 ± 0.02	0.75 ± 0.10	0.06 ± 0.04	0.47 ± 0.09	0.08 ± 0.04	0.38 ± 0.13

Table 2 (continued)

Treatment (mg/kg)[a]	Sex	N[b]	2,4-D EHE					
			24-h sacrifice		48-h sacrifice		72-h sacrifice	
			% MN-PCE[c]	PCE:NCE[d]	% MN-PCE	PCE:NCE	% MN-PCE	PCE:NCE
Negative control	M	5	0.08 ± 0.05	0.65 ± 0.07	–	–	–	–
10 ml/kg corn oil	F	5	0.06 ± 0.04	0.52 ± 0.03	–	–	–	–
Positive control	M	5	4.50 ± 0.66*	0.97 ± 0.18	–	–	–	–
80 mg/kg CP	F	5	1.92 ± 0.34*	0.75 ± 0.14	–	–	–	–
50	M	5	0.08 ± 0.06	0.54 ± 0.07	0.12 ± 0.04	0.50 ± 0.05	0.14 ± 0.02	0.45 ± 0.08
	F	5	0.10 ± 0.05	0.67 ± 0.09	0.10 ± 0.04	0.69 ± 0.10	0.02 ± 0.02	0.52 ± 0.04
167	M	5	0.10 ± 0.03	0.54 ± 0.04	0.08 ± 0.05	0.49 ± 0.16	0.06 ± 0.04	0.55 ± 0.10
	F	5	0.06 ± 0.02	0.74 ± 0.06	0.02 ± 0.02	0.61 ± 0.08	0.14 ± 0.02	0.70 ± 0.04
500	M	5	0.12 ± 0.05	0.61 ± 0.05	0.10 ± 0.05	0.67 ± 0.09	0.10 ± 0.05	0.69 ± 0.07
	F	5	0.12 ± 0.04	0.70 ± 0.07	0.12 ± 0.05	0.45 ± 0.03	0.16 ± 0.02	0.49 ± 0.06
			2,4-D IPE					
Negative control	M	5	0.08 ± 0.04	0.52 ± 0.07	–	–	–	–
10 ml/kg corn oil	F	5	0.08 ± 0.02	0.51 ± 0.04	–	–	–	–
Positive control	M	5	4.78 ± 0.85*	0.53 ± 0.11	–	–	–	–
80 mg/kg CP	F	5	2.24 ± 0.46*	0.50 ± 0.09	–	–	–	–
100	M	5	0.10 ± 0.03	0.55 ± 0.09	0.08 ± 0.04	0.37 ± 0.03	0.02 ± 0.02	0.71 ± 0.15
	F	5	0.02 ± 0.02	0.47 ± 0.03	0.04 ± 0.02	0.56 ± 0.03	0.06 ± 0.02	0.73 ± 0.08
200	M	5	0.08 ± 0.05	0.63 ± 0.08	0.00 ± 0.00	0.46 ± 0.05	0.04 ± 0.02	0.76 ± 0.06
	F	5	0.02 ± 0.02	0.47 ± 0.02	0.10 ± 0.03	0.64 ± 0.04	0.08 ± 0.04	0.56 ± 0.04
400	M	5	0.10 ± 0.03	0.43 ± 0.03	0.10 ± 0.05	0.44 ± 0.04	0.12 ± 0.06	0.46 ± 0.04
	F	5	0.20 ± 0.07	0.53 ± 0.07	0.06 ± 0.02	0.53 ± 0.06	0.08 ± 0.02	0.44 ± 0.06

– = Not done.

* Significantly greater than the corresponding vehicle control, $p < 0.05$.

[a] Based on active ingredient content.

[b] Number of mice; 1000 PCE were examined from each animal.

[c] Percent micronucleated cells based on the total polychromatic cells present in the scored optic field.

[d] Ratio of polychromatic (PCE) to normochromatic (NCE) cells, based upon the number of NCE in the optical fields containing 1000 PCE.

[e] Data are means and standard deviations.

2,4-D DEA, doses of 590 mg/kg and above induced ≥ 33% mortality and a top dose of 443 mg/kg was used in the definitive study. For 2,4-D DMA, a top dose of 397 mg/kg was chosen based on mortality at doses of 530 mg/kg and above. Doses of 800 mg/kg or greater of 2,4-D EHE were also excessively toxic (≥ 67% mortality); therefore, a top dose of 500 mg/kg was selected. The MTD used for 2,4-D IPE of 400 mg/kg was based on mortality seen at dose levels of 531 mg/kg and above in the rangefinding study. In the rangefinding test with 2,4-D BEE, dose levels of 500 mg/kg and above were found to be excessively toxic (≥ 60% mortality), while no deaths were observed at 250 mg/kg. From these data, the MTD for 2,4-D BEE was estimated to be 375 mg/kg. The MTDs used for 2,4-D IPA and 2,4-D TIPA, 376 and 542 mg/kg, respectively, were based on mortality at a dose level of 500 mg/kg of 2,4-D IPA and mortality seen at a dose level of 710 mg/kg of 2,4-D TIPA. The MTDs estimated for 2,4-D and its various derivatives were remarkably similar.

Results of the mouse bone marrow micronucleus test for evaluating the potential of 2,4-D acid, and its

Table 3
Results from the in vivo micronucleus test from the laboratories of Dow Chemical

Treatment (mg/kg)[a]	Sex	N[b]	2,4-D IPA					
			24-h sacrifice		48-h sacrifice		72-h sacrifice	
			MN-PCE[c]	% PCE[d]	MN-PCE	% PCE	MN-PCE	% PCE
Negative control	M	5	0.04 ± 0.05[e]	54.0 ± 3.6	0.04 ± 0.05	55.1 ± 12.5	0.06 ± 0.09	54.8 ± 4.6
80 ml/kg water	F	5	0.00 ± 0.00	55.4 ± 9.5	0.06 ± 0.09	59.0 ± 4.3	0.06 ± 0.05	58.9 ± 7.0
Positive control	M	5	3.24 ± 1.59*	49.3 ± 1.8	–	–	–	–
120 mg/kg CP	F	5	3.42 ± 1.28*	52.8 ± 3.6	–	–	–	–
37.6	M	5	0.02 ± 0.04	54.1 ± 5.3	0.10 ± 0.14	58.9 ± 4.4	0.08 ± 0.11	55.5 ± 5.9
	F	5	0.04 ± 0.05	59.3 ± 3.4	0.02 ± 0.04	55.5 ± 9.4	0.08 ± 0.08	65.7 ± 5.0
126	M	5	0.02 ± 0.04	54.3 ± 6.5	0.06 ± 0.05	53.9 ± 6.1	0.06 ± 0.05	57.2 ± 4.5
	F	5	0.00 ± 0.00	58.9 ± 4.1	0.08 ± 0.11	60.4 ± 5.9	0.08 ± 0.08	66.0 ± 1.9
376	M	5	0.04 ± 0.05	53.5 ± 1.9	0.02 ± 0.04	47.3 ± 6.2	0.04 ± 0.05	62.3 ± 3.0
	F	5	0.04 ± 0.05	56.4 ± 4.2	0.08 ± 0.08	52.5 ± 8.4	0.06 ± 0.13	55.3 ± 14.8
			2,4-D TIPA					
Negative control	M	5	0.06 ± 0.09	53.7 ± 9.1	0.08 ± 0.13	60.1 ± 3.8	0.04 ± 0.05	59.5 ± 7.4
10 ml/kg water	F	5	0.08 ± 0.08	58.0 ± 8.3	0.10 ± 0.10	62.6 ± 8.5	0.10 ± 0.10	60.5 ± 7.6
Positive control	M	5	3.88 ± 1.27*	36.2 ± 7.8*	–	–	–	–
120 mg/kg CP	F	5	4.20 ± 0.70*	54.8 ± 4.4*	–	–	–	–
54.2	M	5	0.14 ± 0.21	53.9 ± 9.2	0.08 ± 0.08	53.7 ± 10.9	0.08 ± 0.08	54.6 ± 6.9
	F	5	0.14 ± 0.17	62.8 ± 7.7	0.10 ± 0.17	57.7 ± 4.4	0.16 ± 0.05	62.2 ± 3.0
180	M	5	0.10 ± 0.12	60.5 ± 6.9	0.10 ± 0.10	61.4 ± 6.3	0.12 ± 0.08	58.7 ± 6.2
	F	5	0.06 ± 0.05	61.3 ± 5.4	0.12 ± 0.13	60.4 ± 5.2	0.12 ± 0.08	66.3 ± 3.3
542	M	5	0.06 ± 0.05	55.1 ± 4.7	0.06 ± 0.05	43.8 ± 8.9	0.15 ± 0.13	51.1 ± 21.7
	F	5	0.28 ± 0.22	57.9 ± 7.5	0.10 ± 0.10	54.0 ± 7.7	0.08 ± 0.10	62.5 ± 8.6
			2,4-D BEE					
Negative control	M	5	0.00 ± 0.00	62.2 ± 5.8	0.06 ± 0.09	55.5 ± 7.2	0.10 ± 0.07	65.6 ± 7.1
10 ml/kg corn oil	F	5	0.08 ± 0.11	61.7 ± 5.1	0.00 ± 0.00	63.2 ± 8.2	0.04 ± 0.05	59.9 ± 6.5
Positive control	M	5	4.46 ± 1.56*	46.9 ± 6.0	–	–	–	–
120 mg/kg CP	F	5	3.96 ± 1.05*	54.6 ± 7.3*	–	–	–	–
37.5	M	5	0.02 ± 0.04	54.8 ± 3.9	0.06 ± 0.05	53.9 ± 8.3	0.10 ± 0.07	64.4 ± 4.6
	F	5	0.12 ± 0.11	59.7 ± 6.8	0.06 ± 0.05	65.8 ± 4.0	0.02 ± 0.04	55.7 ± 8.3
125	M	5	0.12 ± 0.13	57.5 ± 3.7	0.10 ± 0.14	56.2 ± 6.1	0.06 ± 0.09	64.1 ± 8.6
	F	5	0.10 ± 0.10	60.8 ± 7.3	0.10 ± 0.12	58.0 ± 3.0	0.06 ± 0.05	63.1 ± 8.5
375	M	5	0.22 ± 0.18	59.1 ± 6.0	0.06 ± 0.09	58.5 ± 7.3	0.18 ± 0.30	65.0 ± 7.6
	F	5	0.08 ± 0.13	58.6 ± 4.4	0.08 ± 0.13	63.8 ± 7.0	0.06 ± 0.13	63.8 ± 5.5

– = Not done.
* Significantly greater than the corresponding vehicle control, $p < 0.05$.
[a] Based on active ingredient content.
[b] Number of mice: 1000 PCE were examined from each animal.
[c] Percent of micronucleated cells based on the total polychromatic cells present in the scored optic field.
[d] PCE × 100/(PCE + NCE).
[e] Data are means and standard deviations.

derivatives, to induce cytogenetic damage in vivo are shown in Tables 2 and 3. There were no significant increases in the frequency of micronucleated polychromatic erythrocytes (MN-PCE) for any of the test materials at any dose level.

4. Discussion

While numerous amine salts and esters of 2,4-D are in commercial use, it has been demonstrated that in biological systems 2,4-D acid is released from these derivatives by enzymatic and non-enzymatic processes. Previous investigations have documented that the acute and subchronic mammalian toxicity of these derivatives are similar when the doses were expressed in acid equivalents [3,13–15] and the remarkable similarity of the maximum tolerated doses estimated in this study for the various derivatives further substantiates the above observation.

Recently, the lack of in vitro genotoxicity of 2,4-D acid, and its salts and esters, has been confirmed in the Ames test, CHO/HGPRT assay, UDS assay and chromosomal aberrations in rat lymphocytes [4,5]. Results from the mouse bone marrow micronucleus tests reported here provide further evidence for the lack of genotoxic potential for 2,4-D in an in vivo test system. Since 2,4-D, as well as its salts and esters, are rapidly and extensively absorbed following oral dosing [16], the systemic availability and the target organ (i.e., bone marrow) exposure was well assured in these studies.

Contrary to these findings, Adhikari and Grover [17] claimed that intraperitoneal administration of 2,4-D (source and purity not specified; dose levels of 17.5, 35, and 70 mg/kg) induced significant increases in bone marrow chromosome aberrations in the rat. However, the statistical conclusion in their study was based upon a small sample size (2 and 5 rats in the control and treated, respectively). Furthermore, the highest aberrant cell frequency observed in the treated rats (6.66%), although significantly different from the concurrent negative control value (1.8%), was similar to another control value reported in the same publication (i.e., 4.5%). Hence, the biological significance of the statistical finding in the above study is of questionable consequence.

The only other in vivo studies are in *Drosophila melanogaster*. The evidence for mutagenic potential of 2,4-D in *D. melanogaster* is inconsistent. Experiments in which the test material was administered to adult flies generally gave negative results for both chromosome and gene mutation end points (e.g., see Refs. [18,19]) where as larval feeding experiments tended to give weak increases in somatic and germ cell mutations (e.g., see Refs. [20,21]). For example, Zimmering et al. [18] did not detect mutagenic activity when they tested a coded sample of 2,4-D (purity > 99%) by adult feeding and injection. Interestingly, Kale et al. [21] reported that all 9 commercial formulations of herbicides tested in their larval feeding experiments, including 2,4-D, induced sex-linked recessive lethal mutations in pre-meiotic germ cells. Kale et al. [21] did not report concurrent negative control data for these experiments. Instead, the authors used a pooled control data base to compare to the treated samples. Furthermore, Kale et al. [21] did not report the actual composition of the test material except stating that it was a 'complex commercial mixture'. The authors reported that 2,4-D was 'a potent mutagen' based upon a mutation frequency of approximately 0.23% in the treated vs. a pooled control value of 0.06%. It is noteworthy that it is not uncommon to have individual runs of experiments with control values of up to 0.3% in the same *Drosophila* strains used by Kale et al. (Canton-S males and Basc females; see Ref. [18]). Hence, the increases observed in the larval feeding study with a complex formulation of 2,4-D could be described as weak, at best.

In conclusion, the findings of the majority of in vitro mutagenicity assays have been negative. The in vivo studies reported here clearly demonstrate the lack of cytogenetic damage. Therefore, based on a weight-of-evidence approach, it may be concluded that 2,4-D is not genotoxic in mammalian systems in vivo. This conclusion is also supported by the absence of any oncogenic responses in rats and mice fed for a lifetime at maximally tolerated dose levels [2].

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References

[1] I.C. Munro, G.L. Carlo, J.C. Orr, K.G. Sund, R.M. Wilson, E. Kennepohl, B.S. Lynch, M. Jablinske, N.L. Lee, A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D, J. Am. Coll. Toxicol. 11 (1992) 559–664.

[2] J.M. Charles, D.M. Bond, T.K. Jeffries, B.L. Yano, W.T. Stott, K.A. Johnson, H.C. Cunny, R.D. Wilson, J.S. Bus, Chronic dietary toxicity/oncogenicity studies on 2,4-dichlorophenoxyacetic acid in rodents, Fundam. Appl. Toxicol. 33 (1996) 166–172.

[3] J.M. Charles, D.W. Dalgard, H.C. Cunny, R.D. Wilson, J.S. Bus, Comparative subchronic and chronic dietary toxicity studies on 2,4-dichlorophenoxy-acetic acid, amine and ester in the dog, Fundam. Appl. Toxicol. 29 (1996) 78–85.

[4] J.M. Charles, H.C. Cunny, R.D. Wilson, J.S. Bus, T.E. Lawlor, M.A. Cifone, M. Fellows, B. Gollapudi, Ames Assays and Unscheduled DNA Synthesis Assays on 2,4-dichlorophenoxyacetic Acid and Its Derivatives, Mutat. Res. 444 (1999) 207–216, (this issue).

[5] B.B. Gollapudi, J.M. Charles, V.A. Linscombe, S.J. Day, J.S. Bus, Evaluation of the Genotoxicity of 2,4-Dichlorophenoxyacetic Acid and Its Derivatives in Mammalian Cell Cultures, Mutat. Res. 444 (1999) 217–225, (this issue).

[6] Environmental Protection Agency-Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Series 82-1, 83-1, and 83-2. U.S. Department of Commerce, National Technical Information Service PB86-108958, Revised edn., November, 1984.

[7] Environmental Protection Agency-Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Series 81, 82, and 83, 1991, Department of Commerce, National Technical Information Service PB91-154617.

[8] Environmental Protection Agency-Federal Insecticide, Fungicide and Rodenticide Act; Good Laboratory Practice Standard. Federal Register. 40 CFR Part 160, July 1, 1990 edn.

[9] Ministry of Agriculture, Forestry and Fisheries. Agricultural Production Bureau, Japan. Good Laboratory Practice Standards, 59 NohSan No. 3850, January, 1985. In: Agricultural Chemical Laws and Regulations, Japan, (II). Society of Agricultural Chemical Industry, Tokyo, Japan, 1985, pp. 56–71.

[10] European Economic Community. Good Laboratory Practice. Official Journal of the European Communities, L15, January 17, 1987.

[11] European Economic Community. Methods for the determination of toxicity. Official Journal of the European Communities, L133, May 20, 1988.

[12] W. Schmid, The micronucleus test for cytogenetic analysis, in: A. Hollaender (Ed.), Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4, Plenum, New York, NY, 1976, pp. 31–53.

[13] S.J. Gorzinski, R.J. Kociba, R.A. Campbell, F.A. Smith, R.J. Nolan, D.L. Eisenbrandt, Acute, pharmacokinetic, and subchronic toxicological studies of 2,4-dichlorophenoxyacetic acid, Fundam. Appl. Toxicol. 9 (1987) 423–435.

[14] B.L. Yano, G.E. Schultze, J.R. Szabo, R.A. Corley, P.F. Cosse, L.H. Billings, Subchronic dietary toxicity of 2,4-D acid, 2,4-D amine salts and 2,4-D esters in rats, The Toxicologist 12 (1992) 123.

[15] J.M. Charles, H.C. Cunny, R.D. Wilson, J.S. Bus, Comparative subchronic dietary toxicity studies on 2,4-dichlorophenoxyacetic acid, amine and ester in rats, Fundam. Appl. Toxicol. 33 (1996) 161–165.

[16] S.W. Frantz, B.E. Kropscott, Pharmacokinetic evaluation of a single oral administration of the 2-ethylhexyl (isooctyl) ester of 2,4-D to Fischer 344 rats, J. Occup. Med. Tech. 2 (1993) 75–85.

[17] N. Adhikari, I.S. Grover, Genotoxic effects of some systemic pesticides: in vivo chromosomal aberrations in bone marrow cells in rats, Environ. Mol. Mutagen. 12 (2) (1988) 235–242.

[18] S. Zimmering, J.M. Mason, R. Valencia, R.C. Woodruff, Chemical mutagenesis testing in *Drosophila*: II. Results of 20 coded compounds tested for the National Toxicology Program, Environ. Mutagen. 7 (1985) 87–100.

[19] R.C. Woodruff, J.P. Phillips, D. Irwin, Pesticide-induced complete and partial chromosome loss in screens with repair-defective females of *Drosophila melanogaster*, Environ. Mutagen. 5 (1983) 835–846.

[20] U. Graf, F.E. Wurgler, The somatic white-ivory eye spot test does not detect the same spectrum genotoxic events as the wing somatic mutation and recombination test in *Drosophila melanogaster*, Environ. Mol. Mutagen. 27 (1996) 219–226.

[21] P.G. Kale, B.T. Petty Jr, S. Walker, J.B. Ford, N. Dehkordi, S. Tarasia, B.O. Tasie, R. Kale, Y.R. Sohni, Mutagenicity testing of nine herbicides and pesticides currently used in agriculture, Environ. Mol. Mutagen. 25 (1995) 148–153.