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Developmental Toxicity Studies in Rats and Rabbits on 2,4-Dichlorophenoxyacetic Acid and Its Forms

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The potential for 2,4-D and its salts and esters to induce developmental toxicity was investigated in rats (8 studies) and rabbits (7 studies). Maternal toxicity associated with exposure was dependent on the dose level expressed as 2,4-D acid equivalents. The severity of the maternal effect was correlated to the 2,4-D acid-equivalent dose, with increasing dose levels that exceeded renal clearance causing increasingly more severe maternal effects. In both species, maternal body weight effects began to be manifested at dose levels of 30 mg 2,4-D acid equivalent/kg/day. At higher dose levels (50–75 mg/kg/day in rats and 75–90 mg/kg/day in rabbits), body weights and feed consumption were more severely affected. At dose levels ≥ 90 mg/kg/day in rats, clinical signs of toxicity (ataxia, muscular stiffness, and decreased motor activity) and mortality were noted. The no-observed-adverse-effect level (NOAEL) for maternal toxicity in both species across the family of 2,4-D salts and esters was approximately 10 mg/kg/day. Significantly decreased fetal body weights and increased fetal variations were seen in rats only at maternally toxic dose levels in excess of 90 mg/kg/day acid equivalent. At maternally toxic doses in rabbits, embryonal and fetal development were essentially unaffected. There were no effect on maternal reproductive measures such as litter size, resorption rates, or fetal body weights, and there was no evidence of teratogenic activity. In summary, equivalent toxicity of the salts and esters is consistent with rapid and complete metabolic conversion to 2,4-D acid. No adverse fetal effects were noted at dose levels that did not also produce evidence of maternal toxicity or exceed renal clearance of 2,4-D indicating that the developing rat and rabbit fetus were not uniquely sensitive to 2,4-D and its forms.

Key Words: **2,4-dichlorophenoxyacetic acid (2,4-D); developmental toxicity; rats; rabbits; herbicide.**

2,4-Dichlorophenoxyacetic acid (2,4-D) and its various salts and esters are herbicides which are widely used to control broadleaf and woody plants in a variety of applications. It can be formulated as a variety of amine salts, which are more water-soluble than the acid, and ester derivatives, which are readily dissolved in an organic solvent. However, of all these different forms of 2,4-D, two forms, dimethyl-amine salt (DMA) and 2-ethylhexyl ester (EHE), account for approximately 90–95% of the global use of 2,4-D, and the remaining forms make up less than 1–2% of the market each (2,4-D Industry Task Force; personal communication).

A number of studies assessing the developmental toxicity of 2,4-D in mice, hamsters, rats and rabbits were previously reviewed by Munro *et al*. (1992), who concluded that the developmental effects that may be attributable to 2,4-D only occurred at doses that were either maternally toxic or exceeded renal clearance. All these different forms of 2,4-D are rapidly transformed to 2,4-D acid; the amine via rapid dissociation (of the order of 1–2 min) and the ester via hydrolysis. An extensive toxicology database is available for 2,4-D, which includes studies ranging from acute to chronic administrations in multiple species. 2,4-D has been shown to be non-mutagenic (Charles *et al*., 1999a,b; Gollapudi *et al*., 1999), non-neurotoxic (Mattsson *et al*., 1997), non-carcinogenic (Charles *et al*., 1996a), and of moderate toxicity after subchronic and chronic administration (Charles *et al*., 1996a,b,c). Members of the Industry Task Force II on 2,4-D Research Data (TFII) have performed 8 studies in rats and 7 rabbit studies, using the various salt and ester derivatives of 2,4-D. These studies generate a comprehensive developmental toxicity data base on 2,4-D acid.

All studies were conducted in accordance with Good Laboratory Practice regulations and applicable toxicology guidelines for pesticide testing (EEC, 1987, 1988; MAFF, 1985; U.S. EPA, 1984, 1991; U. S. EPA-FIFRA, 1990) and included extensive chemical characterizations of the test substance. The individual studies included herein were performed at the following laboratories: Argus Research Laboratories, Inc., WIL Research, Springborn Life Sciences, Bio/dynamics, Inc., or the laboratories of The Dow Chemical Company.

MATERIALS AND METHODS

Test chemicals and treatments. The test samples used in these studies were obtained as follows: 2,4-D acid (CAS No. 94–75–7), 2,4-D dimethyl-amine salt (2,4-D DMA; CAS No. 2008–39–1), 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

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Research Data; 2,4-D diethanolamine salt (2,4-D DEA; CAS No. 5742–19–8) from PBI/Gordon Corp.; 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 Dow AgroSciences (formerly DowElanco); and 2,4-D isopropyl ester (2,4-D IPE; CAS No. 94–11–1) from Amvac Chemical Corp.

All dose levels presented in the tables are expressed as active ingredient (ai) of the test material formulations based on the following concentrations. Technical grade active ingredients: 2,4-D acid (97.5%); 2,4-D BEE (95.6%); 2,4-D EHE (95.0%); and 2,4-D IPE (97.1%). The following aqueous based manufacturing concentrates were utilized: 2,4-D DEA (73.1%); 2,4-D DMA (66.2%); 2,4-D IPA (50.2%); and 2,4-D TIPA (72.2%). For comparison purposes, the 2,4-D acid equivalent (ae) dose levels for the formulations are also given in the tables, based on the following concentrations: 2,4-D DEA (49.99%); 2,4-D DMA (55.5%); 2,4-D IPA (39.53%); 2,4-D TIPA (38.70%); 2,4-D BEE (65.77%); 2,4-D EHE (63.25%); and 2,4-D IPE (81.55%). The test samples were dissolved in the vehicles corn oil, sterile deionized water, or aqueous methylcellulose. The concentrations of the test materials in the dosing solutions were confirmed by validated analytical methods (HPLC or GC).

Groups of 25–35 bred female rats (depending on the study) were assigned to each test group and dose levels for these studies were selected based upon the results of range-finding studies in pregnant rats. Test materials were administered once daily by gavage at a dose volume of from 4 to 10 ml/kg on gestation days (GD) 6 to15. Dose volumes within a given study were held constant across dose levels. All test materials were administered at the following ai and ae dose levels in mg/kg/day: 2,4-D acid (8, 25, and 75 ai); 2,4-D DEA (15, 75, and 150 ai; 10.2, 50.8, 101.6 ae); 2,4-D DMA (15, 60.2 and 120.4 ai; 12.5, 50, and 100 ae); 2,4-D IPA (22, 65, and 190 ai; 17, 51 and 150 ae); 2,4-D TIPA (32.5, 100, and 325 ai; 17, 51 and 175 ae); 2,4-D BEE (25, 75 and 185 ai; 17, 51 and 125 ae); 2,4-D EHE (15.1, 45.2, and 135.7 ai; 10, 30, and 90 ae); 2,4-D IPE (12.3, 36.9, and 123 ai; 10, 30, and 100 ae). Separate control groups for each compound were administered the appropriate vehicle in a similar manner. All dose levels discussed in this paper will use the 2,4-D acid-equivalent values.

Groups of 18–24 inseminated adult female New Zealand white rabbits were administered the test materials by oral gavage once daily on either GD 6 to 18 or 7 to 19, such that a dose volume of 1–10 ml/kg body weight yielded the appropriate dose. As with rats, dose volumes within a given study were held constant across dose levels. All test materials were administered at the following ai and ae dose levels in mg/kg/day: 2,4-D acid (10, 30, and 90 ai); 2,4-D DEA (15, 30, and 60 ai; 10.2, 20.3, 40.6 ae); 2,4-D DMA (12, 36.1, and 108.4 ai; 10, 30, and 90 ae); 2,4-D IPA (13, 38 and 95 ai; 10, 30 and 75 ae); 2,4-D TIPA (19, 56 and 140 a.i; 10, 30 and 75 ae); 2,4-D BEE (15, 45 and 110 ai; 10, 30 and 75 ae); and 2,4-D EHE (15.1, 45.2, and 113.1 ai; 10, 30, and 75 ae). Separate control groups were administered the appropriate vehicle in a similar manner.

Laboratory animals and care. Adult CD (Sprague-Dawley derived) rats were used in all studies with the exception of the 2,4-D acid study, where Fischer 344 rats were utilized. All rats were obtained from Charles River Laboratories (Portage, MI or Raleigh, NC) and allowed to acclimate to laboratory conditions for approximately 2 weeks. Animals were housed individually, except during mating (one male and one female/cage) in stainless steel suspended cages with wire-mesh floors in climate-controlled rooms at 65–78^0 F, 20–70% relative humidity, and a 12-h light:dark photocycle. Feed (Purina Certified Rodent Chow® No. 5002) and tap water delivered by an automatic watering system were available *ad libitum*. Adult, virgin females were bred overnight with adult males of the same strain. Vaginal smears were taken early in the morning following cohabitation and females were considered to have mated if sperm and/or a vaginal plug were observed. The day on which evidence of mating was observed was defined as GD 0. Females were randomly assigned to test groups in such a way as to most nearly equalize the GD-0 mean group body weights and individually identified.

Stock supplies of New Zealand White rabbits were obtained from Hazleton Research Products, Inc., Denver, PA. Upon receipt in the laboratory, all animals were examined for health status by a veterinarian and acclimated to laboratory conditions for at least 2 weeks. The animal rooms of the facility were designed to maintain environmental conditions with respect to temperature, relative humidity, airflow and lighting conditions, and were regulated for rabbits. Adult females approximately 5 to 7.5 months of age were artificially inseminated with fresh semen collected from bucks of the same strain, and the day of insemination considered Day 0 of gestation. Following insemination, rabbits were given an intravenous injection of 20–100 international units of human chorionic gonadotropin. Rabbits were randomized by body weight into the various groups using a computer-generated procedure. Animals were housed individually in cages with wire floors and were maintained on Certified Laboratory Rabbit Chow No. 5322, Purina Mills, Inc., St. Louis, MO. Municipal tap water was available *ad libitum*.

Maternal and fetal observations. All animals were observed twice daily for signs of treatment-related effects. For rats, maternal body weights were recorded either on GD 0, 6, and 9; or 10, 12, and 15; or 16, and 20; on GD 0 and 20 and daily from 6 to 16; or GD 0 and daily from 6 to 20. For rabbits, body weights were recorded on GD 0, either daily or every third day during the dosing period, and on days 20, 24, 28, and/or 29 of gestation. Dose volumes were adjusted based on the most recent individual body weights. Feed consumption was measured only for rats (except in the 2,4-D acid study) on GD 0 to 6, and at 1–5 day intervals thereafter, depending on the individual study. Complete gross postmortem examinations were performed on all females including those dying spontaneously or sacrificed in a moribund condition. All surviving animals were euthanized on GD 20 (rats), or GD 28 or 29 (rabbits), using either CO_2, T-61 (American Hoechst Corporation, Somerville, NJ) or Beuthanasia®-D. In the rat studies, kidney and liver weights were recorded for each female killed on GD 20 of gestation as well as for those females sacrificed in a moribund condition in the 2,4-D IPA, 2,4-D TIPA, and 2,4-D BEE studies. In order to characterize the potential hemolytic activity of 2,4-D BEE resulting from metabolism of the herbicide ester to 2-butoxyethanol and its known rodent hemolytic metabolite, 2-butoxyacetic acid (Domaradzki *et al.*, 1993; Gingell *et al.*, 1994), blood was collected on GD 20 under light anesthesia, from rats given 2,4-D BEE, and hemoglobin concentration and hematocrit, and erythrocyte (RBC), platelet, and total leukocyte counts were conducted on these samples. Stained blood smears were also prepared from these animals and examined for erythrocyte morphology and differential leukocyte and reticulocyte counts.

At cesarean section, the weight of the gravid uterus, and the number of corpora lutea, and the number and position of implantations, resorptions, and live or dead fetuses were recorded. Uteri with no visible implantations were stained with a 10% solution of ammonium sulfide (Kopf *et al*., 1964), and examined for evidence of early resorptions. Each fetus was individually identified, weighed, sexed, and given a gross examination for external malformations/variations, to include observation for palatal defects. The fetuses in each litter were euthanized. Approximately one-half of the rat fetuses in each litter (alternating fetuses within the litter) were evaluated for visceral malformations/variations (Staples, 1974). The heads of rat fetuses selected for visceral examination were removed, placed in Bouin's fixative, and subsequently sectioned and examined for craniofacial defects (Wilson, 1965). The remaining rat fetuses in each litter were eviscerated, processed, and the ossified skeletal structures stained using alizarin red-S (Crary, 1962; Dawson, 1926). In the rabbit studies, all fetuses were euthanized and examined by dissection under a low-power stereomicroscope for evidence of visceral alterations (Staples 1974; van Julsingha and Bennett, 1977). This also included a fresh examination of the brain. All fetuses were then preserved in alcohol, eviscerated, cleared and stained with alizarin red-S (Dawson, 1926; modification of method of Staples and Schnell, 1964) and examined for skeletal alterations.

Statistical analyses. The studies described herein were conducted at a number of different laboratories, each using a slightly different statistical package. As a result, the descriptions of the statistical methods used for these data focus on the general procedures without, for the most part, reference to specific methodology used with individual studies.

Maternal body weight, maternal body-weight gain, gravid uterine weight,

feed consumption data, litter averages for percent male fetuses, fetal body weights, number of fetuses, litter size, number of corpora lutea, number of implantations, fetal ossification sites, and percent fetal alterations were analyzed in one of the following manners. A Bartlett's Test of homogeneity of variances (Sokal and Rohlf, 1969) was run first. If the Bartlett's test was not significant, an analysis of variance (ANOVA) (Snedecor and Cochran, 1967b) was performed, followed by a Dunnett's test (Dunnett, 1955) if the ANOVA was significant. If the Bartlett's test was significant, a Kruskal-Wallis test (Hollander and Wolfe, 1973) was performed. This was followed by either the Wilcoxon rank-sum test or Dunn's method of multiple comparisons (Dunn, 1964) if the Kruskal-Wallis test was significant.

Pre-implantation loss, post-implantation loss, dead fetuses, and resorptions were analyzed by one of the following: Mann-Whitney U test (Gad, 1978), a censored Wilcoxon test (Haseman and Hoel, 1974) with Bonferroni's correction (Miller, 1966), or a parametric or nonparametric analysis of variance (depending on the outcome of the Bartlett's test), followed respectively by the Dunnett's test or either the Wilcoxon rank-sum test or Dunn's method of multiple comparisons.

Maternal and fetal incidence data were analyzed using the variance test for homogeneity of the binomial distribution (Snedecor and Cochran, 1967a). Fisher's exact test was used to analyze the incidence and number of fetal malformations and variations (SAS, 1985) and pregnancy rate. Fetal sex ratios were analyzed using the Chi-square test (Siegel, 1956a) or a binomial distribution test.

In the studies with 2,4-D acid, 2,4-D DMA and 2,4-D EHE, where the incidence of ties was greater than 75%, the Fisher exact test (Siegel, 1956b) was used instead of the Kruskal-Wallis test. For the rat studies on 2,4-D IPA, 2,4-TIPA, and 2,4-D BEE, standard regression analysis with a trend test was performed with parametric data, while nonparametric data were analyzed using Jonckheere's test for monotonic trend, and all ratios were arc-sine transformed before evaluation.

The significance level in all studies was set at alpha = 0.05, except for the Bartlett's test in some studies which was set at alpha = 0.01.

RESULTS AND DISCUSSION

Maternal Parameters

The results obtained in these rat and rabbit developmental toxicity studies indicate no potential for developmental toxicity with 2,4-D or its salts or esters at dose levels that do not cause maternal toxicity. Effects on fetal development in rats with the various 2,4-D salts and esters were observed only at dose levels that also elicited maternal toxicity. Irrespective of whether the pregnant female was exposed to 2,4-D in the acid form, or as a salt or ester, the toxicity associated with exposure was dependent on the dose level of the acid moiety. Salient results are presented in Table 1 for the rat studies and in Table 2 for the rabbit studies. All levels discussed below are in terms of 2,4-D ae.

In general, maternal body-weight effects in rats began to be manifested at dose levels of 30-mg/kg/day ae. At this dose level, body-weight gain was significantly depressed during the first 3 days of treatment (GD 6 to 9) with 2,4-D EHE, and weight gain over the entire treatment period (GD 6 to 16) was depressed 6.4% with 2,4-D EHE and 18.9% with 2,4-D IPE. With higher dose levels (50–75 mg/kg/day ae), feed consumption (data not shown) and maternal body weights were more severely affected. At dose levels of $\geq$ 90-mg/kg/day ae, clinical signs of toxicity (ataxia, muscular stiffness, and decreased motor activity) were reported with 2,4-D DEA, 2,4-D DMA, 2,4-D IPA, and 2,4-D BEE, and mortality began to be evident. These clinical findings are consistent with those reported in an acute neurotoxicity study in which slight transient alterations in gait and coordination and decreased motor activity were noted at 250 mg/kg as well as very slight and transient locomotor effects at 75 mg/kg (Mattsson *et al*., 1997). At a dose level of 2,4-D TIPA equivalent to 175 mg/kg/day ae, 13% maternal mortality was observed, which exceeded the maximum tolerated dose (MTD) as defined by the U.S. EPA (Farber, 1987).

Consistent with potential formation of a 2-butoxyethanol-derived hemolytic metabolite of 2,4-D BEE (Domaradzki *et al.*, 1993), measurement of hematologic parameters in these animals revealed a slight (4.4%), but statistically significant, decrease in red-blood-cell count and an increase in reticulocyte count at 125 mg/kg/day. Absolute and relative liver and kidney weights of the dams were unaffected by exposure to any of the dose levels tested with 2,4-D IPA, 2,4-D TIPA, or 2,4-D BEE.

The no-observed-adverse-effect level (NOAEL) in rats for maternal toxicity across the family of 2,4-D salts and esters, following daily gavage (bolus) administration, ranged from 8 to 17 mg/kg/day ae. This is similar to the dietary no-observed-effect level (NOEL) of 15 mg/kg/day from 90-day subchronic dietary studies in rats and mice (Charles *et al*., 1996b; Gorzinski *et al*., 1987; Munro *et al*., 1992).

In rabbits, dose-related clinical signs of toxicity were consistently seen with 2,4-D, and its salts and esters at doses at or above 30 mg/kg/day ae. Slight, transient decreases in maternal body weight gain were seen with an acid equivalent dose of 30 mg/kg/day (2,4-D TIPA). With higher acid equivalent doses (75–90 mg/kg/day), more severe maternal effects were noted. Clinical signs of toxicity (myotonia, ataxia, and impaired/lost righting reflex) became evident, accompanied by maternal body-weight losses, and in some cases, significant morbidity and/or mortality (2,4-D DMA, 2,4-D IPA, 2,4-D TIPA, and 2,4-D BEE) was observed. In the case of 2,4-D DMA and 2,4-D BEE, the most severely affected animals died during the dosing period. As a result, weight-gain data, calculated only for those animals alive throughout treatment, were not reflective of the toxicity observed in high-dose groups.

Developmental Parameters

In rats, the following reproductive parameters were unaffected by treatment: litter sizes, resorption rates, and fetal sex ratios. The effects noted in the developing rat fetus were generally confined to the highest dose levels tested with each analog. Significantly decreased fetal-body weights were seen with 2,4-D DEA, 2,4-D DMA, 2,4-D TIPA, 2,4-D EHE, and 2,4-D IPE but only at the highest-dose levels tested with each compound, which were in excess of 90 mg/kg/day ae. The most severe effects on fetal weights were noted with 2,4-D IPE at 100 mg/kg/day ae (-11.1% relative to controls) and 2,4-D TIPA at 175 mg/kg/day ae (-17.8%), dose levels that were also

TABLE 1
2,4-D Salts and Ester Teratology Maternal, Reproductive, and Fetal Parameters in Rats

	2,4-D acid[a]				2,4-D DEA[b]				2,4-D DMA[c]			
Dose (mg/kg/day)	0	8	25	75	0	15	75	150	0	15	60.2	120.4
2,4-D acid equivalent (mg/kg/day)	0	8	25	75	0	10.2	50.8	101.6	0	12.5	50	100
Maternal parameters												
No. bred	35	35	35	35	25	25	25	25	25	25	25	25
No. of deaths	0	0	0	0	0	0	0	0	0	0	0	0
Maternal body weight gain (GD 6–16)	19	18	20	15	58 ± 6.4	57 ± 14.8	47 ± 15.8*	50 ± 12.7	75.3 ± 0.3	72.5 ± 9.8	68.7 ± 7.2*	65.4 ± 10.2**
Reproductive parameters												
No. litters	27	29	27	26	23	24	22	23	22	24	25	25
% Implantations resorbed	13.2 (40/302)	3.5 (10/286)	5.9 (17/286)	8.1 (22/272)	10.5 (38/363)	4.4 (17/385)	4.9 (17/347)	6.6 (23/350)	4.7 (16/385)	3.7 (15/403)	5.8 (26/451)	5.4 (25/461)
% Litters with resorptions	34.5 (10/29)	31.0 (9/29)	28.6 (8/28)	37.0 (10/27)	70.8 (17/24)	45.8 (11/24)	54.5 (12/22)	56.5 (13/23)	42.8 (9/22)	37.5 (9/24)	48.0 (12/25)	64.0 (16/25)
Fetal body weight (g)	3.0 ± 0.1	3.0 ± 0.2	3.1 ± 0.1	3.1 ± 0.3	3.7 ± 0.3	3.6 ± 0.3	3.6 ± 0.2	3.4 ± 0.4**	3.5 ± 0.2	3.7 ± 0.3	3.5 ± 0.2	3.3 ± 0.2**
Fetal parameters												
External examination	262 (27)[d]	276 (29)	269 (27)	250 (26)	325 (23)	368 (24)	330 (22)	327 (23)	352 (21)	388 (24)	425 (25)	436 (25)
Visceral examination	131 (27)	140 (28)	136 (27)	123 (25)	160 (23)	185 (24)	165 (22)	165 (23)	171 (21)	189 (24)	205 (25)	210 (25)
Skeletal examination	131 (26)	136 (29)	133 (27)	127 (26)	165 (23)	183 (24)	165 (21)	162 (23)	181 (21)	199 (24)	220 (25)	226 (25)
Skeletal anomalies												
Reduced ossification of skull	0	0	0	0	9 (5)	3 (3)	24 (14)*	25 (12)	0	0	0	0
Ribs												
Bent	0	0	0	0	0	0	17 (6)*	8 (4)	0	0	0	0
Wavy†	0	0	0	0	0	0	0	0	0	0	2 (1)	5 (4)
14th full rib(s)	0	0	0	0	0	0	0	1 (1)	0	0	0	0
Extra (cervical)†	0	0	1 (1)	4 (4)	1 (1)	1 (1)	2 (2)	12 (7)*	1 (1)	1 (1)	2 (2)	1 (1)
Extra lumbar (rudimentary)	0	1 (1)	0	4 (3)	5 (4)	10 (6)	11 (9)	28 (15)*	0	0	0	0
Sternebrae												
Incomplete and unossified	0	0	0	0	0	0	0	0	2 (1)	1 (1)	4 (2)	9 (7)
Total external malformations	2 (2)	2 (2)	0	4 (4)	1 (1)	0	0	1 (1)	0	0	1 (1)	0
Total visceral malformations	0	1 (1)	0	0	0	0	0	1 (1)	1 (1)	0	0	0
Total skeletal malformations	1 (1)	0	1 (1)	2 (2)	0	0	0	2 (2)	1 (1)	2 (2)	8 (5)	14 (10)

	2,4-D IPA[e]				2,4-D TIPA[e]				2,4-D BEE[e]			
Dose (mg/kg/day)	0	22	65	190	0	32.5	100	325	0	25	75	185
2,4-D acid equivalent (mg/kg/day)	0	17	51	150	0	17	51	175	0	17	51	125
Maternal parameters												
No. bred	30	30	30	30	30	30	30	30	30	30	30	30
No. deaths	0	0	0	1	0	0	0	4	0	0	0	0
Maternal body weight gain (GD 6–16)	53 ± 10	55 ± 10	51 ± 10	48 ± 11	50 ± 11	45 ± 9	44 ± 8	29 ± 14	64 ± 12	60 ± 11	62 ± 9	56 ± 9
Reproductive parameters												
No. litters	29	27	28	23	30	27	28	23	26	27	30	27
% Implantations resorbed	5.7 (23/403)	4.7 (18/407)	7.4 (27/409)	9.4 (22/361)	5.3 (24/446)	6.0 (22/406)	7.0 (29/441)	16.8 (62/334)	8.0 (26/388)	5.0 (20/399)	4.3 (20/451)	5.4 (22/397)
% Litters with resorptions	52 (15/29)	48 (13/27)	68 (19/28)	67 (16/23)	50 (15/30)	56 (15/27)	64 (18/28)	58 (14/23)	62 (16/26)	48 (13/27)	43 (13/30)	56 (15/27)
Fetal body weight (g)	3.51 ± 0.21	3.54 ± 0.23	3.45 ± 0.23	3.37 ± 0.25	3.42 ± 0.23	3.52 ± 0.20	3.45 ± 0.40	2.81 ± 0.55*	3.53 ± 0.24	3.66 ± 0.19	3.62 ± 0.23	3.44 ± 0.23
Fetal parameters												
External examination	380 (29)	365 (27)	364 (28)	302 (23)	422 (30)	352 (27)	382 (28)	272 (23)	362 (26)	379 (27)	431 (30)	375 (27)
Visceral examination	198 (29)	191 (27)	189 (28)	157 (23)	219 (30)	183 (27)	199 (28)	144 (23)	188 (26)	196 (27)	226 (30)	195 (27)
Skeletal examination	182 (29)	174 (27)	176 (28)	148 (23)	203 (30)	170 (27)	183 (28)	131 (23)	174 (25)	183 (27)	205 (30)	181 (27)
Skeletal												
Ribs												
Wavy†	1 (1)	1 (1)	0	0	0	0	5 (4)	5 (4)	0	0	1 (1)	0
Fused†	0	0	1 (1)	0	0	0	0	5 (4)	0	0	0	0
Extra (cervical)†	0	0	0	1 (1)	0	0	0	3 (1)	0	0	1 (1)	0
Extra (lumbar) (rudimentary)	7 (5)	5 (4)	8 (7)	15 (6)	3 (2)	4 (4)	7 (6)	11 (6)	18 (10)	16 (10)	42 (21)	45 (18)
Missing†	0	0	0	0	0	0	0	0	0	0	0	1 (1)
Thickened	2 (2)	2 (1)	1 (1)	0	0	0	5 (4)	12 (7)	0	2 (1)	4 (4)	3 (2)
Total external malformations	0	0	1 (1)	3 (2)	0	2 (2)	0	5 (5)*	0	1 (1)	0	1 (1)
Total visceral malformations	3 (1)	1 (1)	1 (1)	1 (1)	0	1 (1)	0	7 (6)*	0	2 (2)	1 (1)	3 (3)
Total skeletal malformations	2 (2)	1 (1)	3 (3)	3 (3)	0	2 (2)	7 (5)	16 (9)*	0	0	2 (2)	2 (2)

TABLE 1—*Continued*

	2,4-D EHE[c]				2,4-D IPE[a]			
Dose (mg/kg/day)	0	15.1	45.2	135.7	0	12.3	36.9	123
2,4-D acid equivalent (mg/kg/day)	0	10	30	90	0	10	30	100
Maternal parameters								
No. bred	25	25	25	25	25	25	25	25
No. deaths	0	0	0	0	0	0	0	1
Maternal body weight-gain (GD 6–16)	76.2 ± 9.4	77.3 ± 12.0	71.3 ± 10.8	66.8 ± 13.1**	53 ± 8.0	49 ± 8.7	43 ± 7.2*	33 ± 22.8**
Reproductive parameters								
No. litters	22	22	24	24	25	24	25	21
% Implantations resorbed	8.9 (34/381)	3.9 (15/381)	8.0 (33/411)	5.0 (21/421)	6.9 (25/364)	6.3 (22/351)	9.1 (32/351)	10.2 (31/303)
% Litters with resorptions	72.7 (16/22)	40.9 (9/22)	75.0 (18/24)	58.3 (14/24)	60.0 (15/25)	45.8 (11/24)	72.0 (18/25)	33.3 (7/21)
Fetal body weight (g)	3.49 ± 0.2	3.61 ± 0.24	3.63 ± 0.42	3.29 ± 0.31*	3.6 ± 0.2	3.6 ± 0.2	3.4 ± 0.3	3.2 ± 0.3**
Fetal parameters								
External examination	351 (22)	366 (22)	378 (24)	400 (24)	339 (25)	329 (24)	319 (25)	272 (20)
Visceral examination	169 (22)	176 (22)	183 (24)	193 (24)	339 (25)	329 (24)	319 (25)	272 (20)
Skeletal examination	182 (22)	190 (22)	195 (24)	207 (24)	339 (25)	329 (24)	319 (25)	272 (20)
Skeletal								
Ribs								
Bent	0	0	0	0	2 (2)	3 (2)	9 (8)	5 (5)
Wavy†	0	6 (4)**	2 (1)	8 (3)**	0	0	0	0
Extra (cervical)†	0	0	1 (1)	0	3 (3)	9 (6)	5 (3)	15 (8)*
14th rudimentary rib(s)	0	0	0	0	3 (2)	4 (3)	6 (5)	26 (13)*
Total external malformations	0	0	0	0	2 (2)	1 (1)	2 (2)	0
Total visceral malformations	0	0	0	0	0	1 (1)	2 (2)	0
Total skeletal malformations	0	6 (4)	3 (2)	8 (3)	1 (1)	2 (2)	1 (1)	1 (1)

[a] WIL Research.
[b] Springborn Laboratories.
[c] Argus Laboratories.
[d] No. of fetuses (no. of litters).
[e] Bio/dynamic, Inc.
* Statistically different from concurrent controls ($p = 0.05$).
** Statistically different from concurrent controls ($p = 0.01$).
† Considered a malformation.

TABLE 2
2,4-D Salts and Esters Teratology: Maternal, Reproductive, and Fetal Parameters in Rabbits

	2,4-D Acid[a]				2,4-D-DEA[b]				2,4-D-DMA[a]			
Dose (mg/kg/day)	0	10	30	90	0	15	30	60	0	12	36.1	108.44
2,4-D acid equivalent (mg/kg/day)	0	10	30	90	0	10.2	20.3	40.6	0	10	30	90
Maternal parameters												
No. bred	20	20	20	20	20	20	20	20	20	20	20	20
No. of deaths	2	0	0	0	0	0	01	0	0	0	4	0
sacrificed	0	0	0	0	0	0	0	0	0	0	0	0
Aborted	0	0	0	2	0	3	0	1	0	1	1	0
Maternal weight gain (GD 6–19 (kg))	0.22	0.22	0.24	0.16	0.31	0.30	0.18**	0.15**	0.10	0.10	0.12	0.14
Reproductive parameters												
No. litters	16	18	16	16	18	17	16	17	20	16	18	14
No. implantations	117	124	116	129	130	155	134	150	177	133	163	112
% Implantations resorbed	11.1	3.2	13.8	10.1	2.3	5.2	5.2	14.7	4.0	5.3	4.9	11.6
% Litters with resorptions	56.3	22.2	37.5	25.0	16.7	29.4	33.3	52.9	30.0	43.8	33.3	57.1
Fetal body weight (g)	45.1 ± 5.0	46.1 ± 9.1	45.0 ± 7.1	44.4 ± 6.2	46.7 ± 5.4	41.5 ± 6.0*	42.2 ± 6.4	42.4 ± 5.1	43.6 ± 5.6	42.9 ± 7.1	40.9 ± 5.9	44.5 ± 7.0
Fetal parameters												
Fetuses examined	119 (16)	120 (18)	109 (16)	116 (16)	127 (18)	147 (17)	127 (16)	128 (17)	170 (20)	126 (16)	155 (18)	99 (14)
Visceral alterations												
Lung lobe, agenesis†	2 (2)	7 (5)	3 (2)	5 (2)	0	0	0	0	3 (3)	2 (1)	1 (1)	0
Skeletal alterations												
Ribs–7th cervical	0	0	0	0	0	5 (2)	1 (1)	7 (4)*	0	0	0	0
Total external malformations	0	1 (1)	0	3 (1)	0	1 (1)	0	0	0	0	1 (1)	1 (1)
Total visceral malformations	2 (2)	8 (6)	3 (2)	8 (3)	0	2 (1)	0	1 (1)	3 (3)	2 (1)	1 (1)	1 (1)
Total skeletal malformations	1 (1)	4 (3)	3 (3)	5 (2)	1 (1)	1 (1)	0	0	2 (2)	2 (1)	2 (2)	5 (5)

TABLE 2—*Continued*

	2,4-D IPA[c]				2,4-D TIPA[c]				2,4-D-BEE[c]				2,4-D EHE[a]			
Dose (mg/kg/day)	0	13	38	95	0	19	56	140	0	15	45	110	0	15.1	45.2	113.1
2,4-D acid equivalent (mg/kg/day)	0	10	30	75	0	10	30	75	0	10	30	75	0	10	30	75
Maternal parameters																
No. bred	20	20	20	20	18	18	18	18	24	24	24	24	20	20	20	20
No. of deaths	0	0	2	3	0	0	1	0	1	0	1	4	0	0	1	0
Moribund/sacrificed	0	0	0	4	0	0	0	3	0	0	1	4	0	0	0	2
Aborted	0	0	1	0	0	0	0	0	0	1	3	0	0	0	0	1
Maternal weight gain (GD 6–19 (kg))	0.20	0.13	0.11	–0.09*	0.24	0.20	0.13	0.10*	0.13	0.07	0.01	0.11	0.21	0.18	0.20	0.17
Reproductive parameters																
No. litters	20	16	16	13	13	16	13	15	21	20	15	14	18	16	14	17
No. implantations	144	113	129	104	85	135	96	107	162	145	79	102	131	133	118	112
% Implantations resorbed	8.3	6.2	5.4	2.9	2.4	9.6	5.2	5.6	3.7	3.4	16.5*	2.0	9.9	1.5	3.4	1.8
% Litters with resorptions	30	38	38	23	15	31	33	20	24	25	48*	14	17	12.5	27	6
Fetal body weight (g)	38.2 ± 3.1	40.4 ± 4.7	37.2 ± 7.2	38.0 ± 4.8	40.3 ± 7.1	38.2 ± 4.1	37.6 ± 5.3	37.9 ± 6.6	38.2 ± 5.0	37.4 ± 4.3	42.3 ± 5.7*	39.3 ± 3.6	46.6 ± 6.5	43.1 ± 3.8	44.5 ± 3.8	46.6 ± 5.1
Fetal parameters																
Fetuses examined	119 (16)	120 (18)	109 (16)	116 (16)	127 (18)	147 (17)	127 (16)	128 (17)	170 (20)	126 (16)	155 (18)	99 (14)	128	131	114	110
Visceral alterations																
Lung lobe, agenesis†	2 (2)	2 (2)	6 (5)	4 (2)	2 (2)	10 (5)	4 (3)	1 (1)	2 (2)	9 (3)	1 (1)	2 (1)	0	5 (2)**	3 (2)*	0
Skeletal alterations																
Ribs–7th cervical	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total external malformations	1 (1)	0	0	0	0	0	0	0	0	0	0	0	0	1 (1)	0	1 (1)
Total visceral malformations	7 (4)	2 (2)	7 (6)	4 (2)	5 (5)	10 (5)	5 (4)	2 (1)	4 (4)	9 (3)	1 (1)	2 (1)	0	5 (2)	3 (2)	4 (1)
Total skeletal malformations	2 (2)	0	2 (2)	2 (2)	2 (2)	0	1 (1)	1 (1)	1 (1)	3 (3)	3 (2)	1 (1)	1 (1)	0	2 (2)	1 (1)

Note. For 2,4-D IPA, 2,4-D-TIPA, and 2,4-D-BEE the maternal weight gain is GD 7–20 (kg). For all others maternal weight is GD 6–19 (kg).

[a] Argus Laboratories.

[b] Springborn Laboratories.

[c] Dow Laboratories.

* Statistically different from concurrent controls ($p = 0.05$).

** Statistically different from concurrent controls ($p = 0.01$).

† Considered a malformation.

associated with clinical signs of toxicity and mortality in the maternal animals.

A similar picture was seen in the incidence and severity of rat fetal alterations. With the exception of 2,4-D EHE, statistically significant treatment-related increases in fetal variations were observed with 2,4-D DEA, 2,4-D DMA, 2,4-D TIPA, 2,4-D BEE, and 2,4-D IPE only at dose levels of 90 mg/kg/day acid equivalent or above. These effects consisted of various measures of slightly delayed skeletal ossification (reduced ossification of the skull, incomplete or delayed ossification of the sternebrae, delayed ossification of the metatarsals and metacarpals) and the presence of extra ribs, either cervical or lumbar. With 2,4-D EHE, a statistically significant increase in the incidence of incomplete or unossified sternebrae was the only effect noted in fetuses at 30 mg/kg/day acid equivalent and above. These dose levels were also associated with slightly reduced maternal weight gain as noted above.

The most severe fetal effects were seen in rats at the high-dose level of 2,4-D TIPA (175 mg/kg/day acid equivalent). A statistically significant increase in the incidence of external, visceral, and skeletal malformations was seen in this dose group, consisting of malformations of the eyes (anophthalmia, microphthalmia, folded retina) and ribs (wavy, fused). However, it must be remembered that at this dose level, clinical signs of intoxication, 13% maternal mortality, and a 42% decrease in maternal-weight gain were observed, and this level exceeded an MTD. These types of malformations have been identified in the published literature to be frequently associated with maternal toxicity at Day 9 of gestation in the rat (Khera, 1985). Thus, these effects are likely secondary to the severe maternal toxicity elicited in these animals, rather than a direct effect of the compound.

The effects reported in the rat are consistent with previously published data. Schwetz *et al*. (1971) reported treatment-related embryotoxicity and fetotoxicity, but no teratogenic effects in Sprague-Dawley rats at dose levels of 50 mg/kg/day 2,4-D and above, with a developmental NOEL of 25 mg/kg/day. Unger *et al*. (1981) reported an increase in extra (14th) lumbar ribs, a minor variation, as the only statistically increased fetal anomaly in rats given 2,4-D propylene glycol butyl ether (PGBE) and isooctyl (IO) esters at acid equivalent doses of 87.5 mg/kg/day. Chernoff *et al* (1990) reported an increase in supernumerary (extra) ribs in Sprague-Dawley rats following gestation exposure to a dose level of 115 mg/kg/day. Likewise, Khera and McKinley (1972) reported an increased incidence of skeletal anomalies in Wistar rats at dose levels of 100 to 150 mg/kg/day 2,4-D. In all cases, these effects were seen in dose levels that produced maternal toxicity and/or were above the level at which renal clearance of 2,4-D is saturated.

In rabbits, embryonal and fetal development was essentially unaffected at maternally toxic doses. There were no effects on maternal reproductive measures such as litter size, resorption rates, etc., or on fetal body weights, and we observed no evidence of teratogenicity with 2,4-D acid, or its salts or esters. Low incidences of malformations were seen scattered throughout the various dose groups, including controls. The most common malformation observed in all studies across most groups, including controls, was a change in the lobation pattern of the lung, which was diagnosed as the absence of one of the lobes of the lung. The remaining malformations were seen at very low incidences, typically 1–3 fetuses, confined to 1–2 litters within a dose group. The incidences of the individual malformations observed were consistent with historical control data from the various laboratories in which these studies were conducted, as well as with published control data (Clemens *et al*., 1994; HRC, 1996; Palmer, 1980; Stadler *et al*., 1983). The only statistically significantly increased incidence of fetal alterations was the presence of 7th cervical ribs, a minor skeletal variation, in the group exposed to 2,4-D DEA at 40.6 mg/kg/day, which also produced clinical signs of toxicity and decreased maternal body weight gain.

The manifestation of toxic effects in pregnant animals at dose levels of 30–50 mg/kg/day ae and above is consistent with what is known about the metabolism and excretion of 2,4-D and its salts and esters, which undergo rapid acid and/or enzymatic hydrolysis to form 2,4-D acid. Erne (1966) reported only trace amounts of 2,4-D butyl ester (2,4-D BE) in blood, following oral exposure in pigs and rats, and Schulze *et al*. (1985) found no parent 2,4-D BE in the urine of rats. Likewise, only 2,4-D was found in the blood or urine of rats given 130 mg 2,4-D EHE/kg (Frantz and Kropscott, 1993). Parent 2,4-D is excreted by the kidneys via the organic acid active transport process (Berndt and Koschier, 1973; Gehring and Betso, 1978). Urinary excretion of 2,4-D in rats has been shown to be saturated at dose levels of 50 mg/kg and above (Gorzinski *et al*., 1987; Khanna and Fang, 1866; Smith *et al*., 1980). Sandberg *et al*. (1996) studied the distribution of 2,4-D in maternal and fetal rabbits following intravenous administration. At doses of 1 or 10 mg/kg, the ratio of a 2-h area under the curve (AUCs) for maternal kidney vs. plasma were ≥ 1; however, at 40 mg/kg, this ratio was approximately 0.67, suggesting saturation of renal organic acid active transport in rabbits. These authors also reported fetal plasma AUCs at doses of 1 and 10 mg/kg that were $<9\%$ of maternal values, but which increased to $>15\%$ at a dose of 40 mg 2,4-D/kg. Thus, the maternal toxicity observed in the present studies following repeated gavage doses of ≥ 30 mg/kg/day ae likely occurred under conditions of saturated renal clearance of 2,4-D.

The correlation of maternal toxicity to acid equivalence of 2,4-D, with little or no contribution of the different salt or ester moieties, is also consistent with data reported by Domaradzki *et al* (1993). These authors found that the fate of radiolabeled 2,4-D was unaffected by the simultaneous administration of 2,4-D TIPA, 2,4-D EHE, or 2,4-D BEE. Likewise, they found no effect of 2,4-D on the fate of the salt or ester moiety.

In conclusion, these studies confirm the pharmacokinetic and metabolism research in which the derivatives were rapidly and completely converted to 2,4-D acid after systemic absorp-

tion. Most importantly, the results of the teratology investigations using various salt and ester derivatives of 2,4-D are in excellent agreement with the findings from the 2,4-D acid studies, and are consistent with the conclusion that the derivatives are toxicologically equivalent to 2,4-D acid. The developing rat and rabbit fetuses were not uniquely sensitive to 2,4-D in any of its salt or ester forms. Adverse effects on the developing rat fetus exposed *in utero* to 2,4-D were observed only at dose levels that produced maternal toxicity. The severity of the maternal and fetal effects were correlated with the 2,4-D acid equivalent dose, with increasing dose levels which exceeded renal clearance, causing increasingly more severe maternal effects, with concomitant effects on the developing fetus. The overall maternal NOEL for all rat studies ranged from 8 to 17 mg/kg/day ae, and the overall developmental NOEL for all rat studies was approximately 30 mg/kg/day. Maternal toxicity in the rabbit studies, in the form of bodyweight gains and clinical signs of toxicity, was noted at dose levels of 30 mg 2,4-D acid equivalents/kg/day and above. At maternally toxic doses, embryonal and fetal development was essentially unaffected, and there was no indication of any teratogenic effect. Thus, the appropriate endpoint and NOEL for use in short-term occupational- or residential-exposure risk assessment is the developmental NOEL of 30 mg/kg/day.

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