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DRUG METABOLISM & DISPOSITION

SEX-DEPENDENT DIFFERENCES IN THE DISPOSITION OF 2,4-DICHLOROPHENOXYACETIC ACID IN SPRAGUE-DAWLEY RATS, B6C3F1 MICE, AND SYRIAN HAMSTERS

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ABSTRACT:

2,4-Dichlorophenoxyacetic acid (2,4-D), a widely used broadleaf herbicide, is under investigation in a study of peroxisome proliferators. To supplement that study, male and female rats, mice, and hamsters were dosed with ^{14}C-2,4-D orally at 5 and 200 mg/kg and tissue distributions were determined. Blood, liver, kidney, muscle, skin, fat, brain, testes, and ovaries were examined. At early time points tissues from female rats consistently contained higher amounts of radioactivity than did corresponding tissues from males (up to 9 times). By 72 hr, tissue levels were equivalent and males and females had excreted equal amounts of radioactivity. This sex difference was absent in mice. In hamsters, males had higher tissue levels than females. Taurine, glycine, and glucuronide conjugates of 2,4-D were excreted along with parent. Metabolite profiles differed between species qualitatively and quantitatively; however, differences between sexes were minimal. Plasma elimination curves were generated in male and female rats after iv and oral administration. Kinetic analysis revealed significant differences in elimination and exposure parameters consistent with a greater ability to clear 2,4-D by male rats relative to females. This suggests that at equivalent doses, female rats are exposed to higher concentrations of 2,4-D for a longer time than males and may be more susceptible to 2,4-D-induced toxicity. These sex-dependent variations in the clearance of 2,4-D in rats and hamsters may indicate a need for sex-specific models to accurately assess human health risks.

2,4-Dichlorophenoxyacetic acid (2,4-D) is a chlorophenoxy herbicide used worldwide (1–3). Human exposure to this chemical through agricultural use, food products, or through lawn and garden use has been demonstrated in several studies (2–6). The butyl ester of 2,4-D was a component of Agent Orange, a defoliant used extensively in the Vietnam war resulting in the potential for significant exposure to veterans of the war and to Vietnamese citizens (7).

The risk to human health, if any, that 2,4-D presents has not been completely assessed. 2,4-D has been shown to be cytotoxic to isolated rat hepatocytes, possibly *via* a depletion of reduced glutathione (8, 9). Single doses of 2,4-D at 60 mg/kg/day resulted in renal toxicity and decreased serum tetraiodothyronine levels in rats (10). Reversible neurobehavioral toxicity has also been reported in rats (11–13) after repeat dosing with 2,4-D at 150 mg/kg or more. While an association between malignant lymphoma in dogs exposed to 2,4-D after its use in their owners' yards has been reported (14), a recent subchronic study in the dog detected no significant toxicity in that species (15). A weak association between 2,4-D use and human birth defect rates has been demonstrated (16). The finding that 2,4-D is a weak peroxisomal proliferator in rats suggested that the chemical might be a rodent liver carcinogen upon chronic exposure (17–19). However, results from a large subchronic study in male and female rats and a chronic study in male and female rats and mice indicated no significant toxicities associated with 2,4-D (20, 21).

The National Toxicology Program is currently conducting a multispecies study into the mechanisms of peroxisomal proliferation. 2,4-D was selected as a model noncarcinogenic, weak peroxisomal proliferator. To interpret and extrapolate the results of this study, data on the metabolism, distribution, and pharmacokinetics of 2,4-D was required in the three test species, Sprague-Dawley rats, B6C3F1 mice, and Syrian Hamsters. These endpoints have previously been assessed in male, but not in female rats (1, 10, 22–26), as well as human males voluntarily or occupationally exposed (2, 3, 6, 27). The present study was conducted to examine the disposition of 2,4-D in male and female rats, mice, and hamsters. This was done to address dose-dependent effects and sex-dependent alterations in the disposition of 2,4-D and to provide fundamental data for the mouse and hamster to support a better extrapolation of the peroxisome proliferation results in rodents to humans of both sexes.

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Materials and Methods

Chemicals. 2,4-Dichlorophenoxyacetic acid (98%) was purchased from Aldrich (Milwaukee, WI). 2,4-dichlorophenoxyacetic acid [Ring-^{14}C] was obtained from New England Nuclear (Boston, MA). Glycine and taurine conjugates of 2,4-D were synthesized as previously described (32).

^{1}H NMR Spectra. ^{1}H NMR spectra were determined on a Varian Unity Plus (Palo Alto, CA) 500 MHz NMR spectrometer. The chemical shifts are reported relative to tetramethylsilane as the external standard.

Mass Spectrometry. FAB mass spectra were obtained on a VG ZAB-4F (VG Analytical, Manchester, UK) with glycerol as the matrix. Electrospray mass spectra were obtained on a VG 12–250 mass spectrometer/data system using a Vestec model 611B electrospray source (Vestec Corp., Houston, TX). Electron impact mass spectra (70 eV) were obtained on a Kratos Concept 1SQ Hybrid Mass Spectrometer (Manchester, UK) by direct probe inlet.

HPLC. The HPLC system employed two Waters Associates (Milford, MA) pumps and an automated gradient controller. A Beckman model 163 Variable Wavelength detector was used for UV detection and a Radiomatic (Tampa, FL) Flo-One beta radiochemical detector was used for quantitation of peaks.

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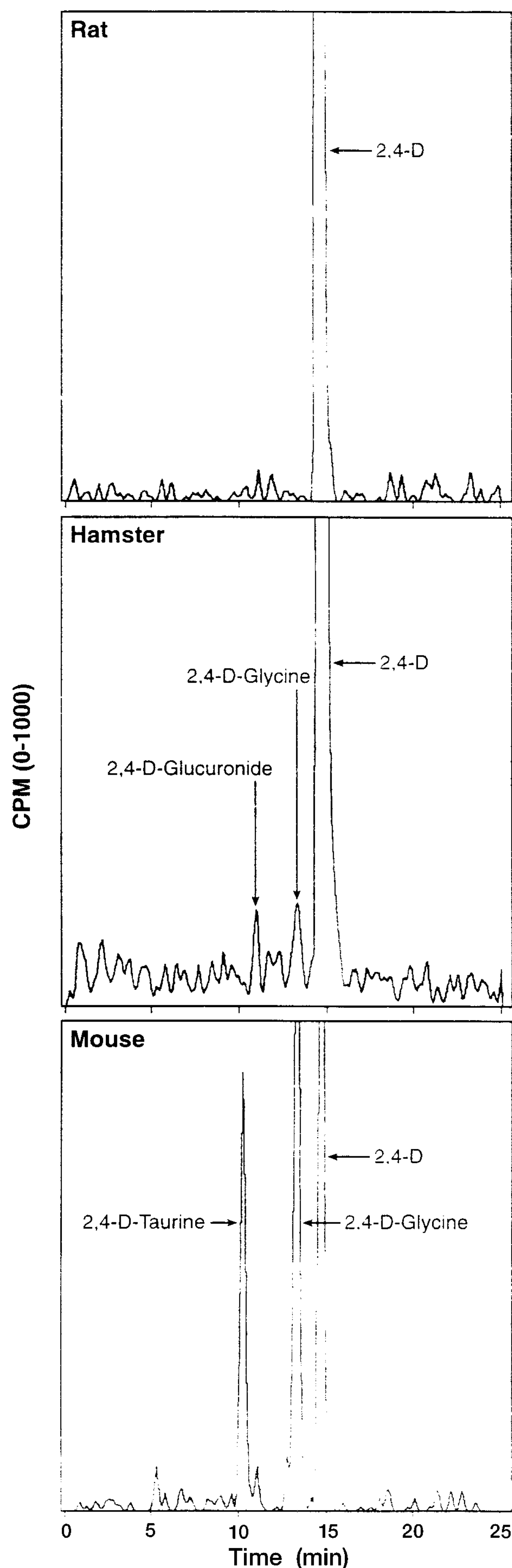

FIG. 1. *Radiochromatograms showing the separation by reversed phase HPLC of 2,4-D from its urinary metabolites in rats, mice, and hamsters.*

Separation was achieved on a C18, 5.0 mm, Rainin 4.6 X 250 mm Microsorb column (Rainin Instrument Co., Woburn, MA). The assay consisted of a linear gradient from 90% Solvent B (trifluoroacetic acid 0.1%)/10% Solvent A (acetonitrile containing 3% tetrahydrofuran) to 100% solvent A from 3 to 17 min. The flow rate was set at 1.5 ml/min.

Treatments. All animal procedures were approved by the institutional animal care and use committee. Male and female Sprague-Dawley rats (Charles River, Raleigh, NC), B6C3F1 mice (Taconic, Germantown, NY) and Syrian hamsters (Charles River, Canada) were allowed to acclimatize for at least 5 days after arrival. Animals (aged 6–8 weeks) were dosed orally (4 ml/kg for rats and hamsters and 10 ml/kg for mice) with ^{14}C-2,4-D (20.0 μcuries/rat and 5.0 μcuries/mouse or hamster) in 100mM sodium phosphate buffer at a final pH of 8 and at doses of 5 and 200 mg/kg.

Collection of Samples. Immediately after dosing, animals were placed in Jencons Metabowl glass metabolism cages (Hemel Hempstead, Hertfordshire, UK) for collection of urine, feces, and expired carbon dioxide. Carbon dioxide was trapped in 3:7 ethanolamine/ethylene glycol monomethylether. The animals were housed one per cage for 2, 8, 24, or 72 hr at which time the animals were sacrificed and tissues removed for the determination of 2,4-D disposition.

Sample Preparation. Urine was centrifuged at 1000 rpm for 10 min and injected directly on the HPLC. Feces were homogenized in sodium acetate buffer (pH 6.8, 100 mM) using a Kinematica Gmbh polytron (Brinkman Instruments, Westbury, NY). One ml of fecal homogenate was then precipitated with 3 ml of acetonitrile, vigorously vortexed, refrigerated overnight, and centrifuged at 1000 rpm for 10 min. The supernatant was passed through a C18 Sep-pak. Total radioactivity in urine samples was determined by directly counting aliquots of urine. Feces were dried and then oxidized in a Packard (Downers Grove, IL.) Model 306 Oxidizer for determination of total fecal radioactivity. Weighed aliquots of blood, liver, kidney, muscle, skin, fat, testes, ovaries, and brain were oxidized in triplicate to determine tissue disposition.

Metabolite Identification. Metabolites were isolated using the HPLC separation already described. Isolated peaks were identified by cochromatography with synthetic standards and by ^{1}H-NMR and mass spectrometry. Hamster urine was incubated with or without Type B1 β-glucuronidase for 24 hr at 37°C to determine the presence of 2,4-D-glucuronide.

Kinetic Analysis. Data were analyzed using PCNONLIN (SCI Software, Lexington, KY). The iv data best fit a two-compartment model for bolus iv administration. The data were weighted by the inverse square of the predicted values. All analyses were conducted on individual animal data.

Plasma Protein Binding. Plasma protein binding of 2,4-D was determined using the method described by Dix *et al.* (29). Briefly, blood was obtained from three male and three female SD rats by cardiac puncture. Centrifugation at 2000 rpm for 10 min yielded plasma samples. ^{14}C-2,4-D was added to the samples to yield concentrations of 6, 24, and 48 μg/ml. Aliquots of the spiked samples (450 μl) were placed in Centricon 10 (10,000 MW cutoff) and centrifuged at 4400 g for 5 min. Ten microliter aliquots of the filtrates and original spiked samples were counted. The free fraction was determined as the ratio of the activity in the filtrate divided by the activity in the original spiked sample.

Statistics. Data are presented as the mean ± the SE of the mean. Statistical comparisons were made using an unpaired Student's *t* test or a Mann-Whitney test ($p \leq 0.05$).

Results

Metabolite Identification. HPLC radiochromatograms of urine from rats, mice, and hamsters treated with ^{14}C-2,4-D are shown in fig. 1. 2,4-D, and its glycine and taurine conjugates were positively identified by cochromatography with authentic standards and by comparison of ^{1}H-NMR analysis of isolated peaks with the authentic standards. In hamster urine, an additional radioactive peak was present. This peak degraded to 2,4-D during attempts at isolation. It was not detected in aliquots of urine incubated with β-glucuronidase for 24 hr at 37°C. Aliquots of urine, blank-incubated for 24 hr at 37°C, still had readily detectable amounts of this peak. As a result this peak was tentatively identified as the acyl glucuronide of 2,4-D (fig. 2).

Recovery of Radioactivity. The cumulative recoveries of admin-

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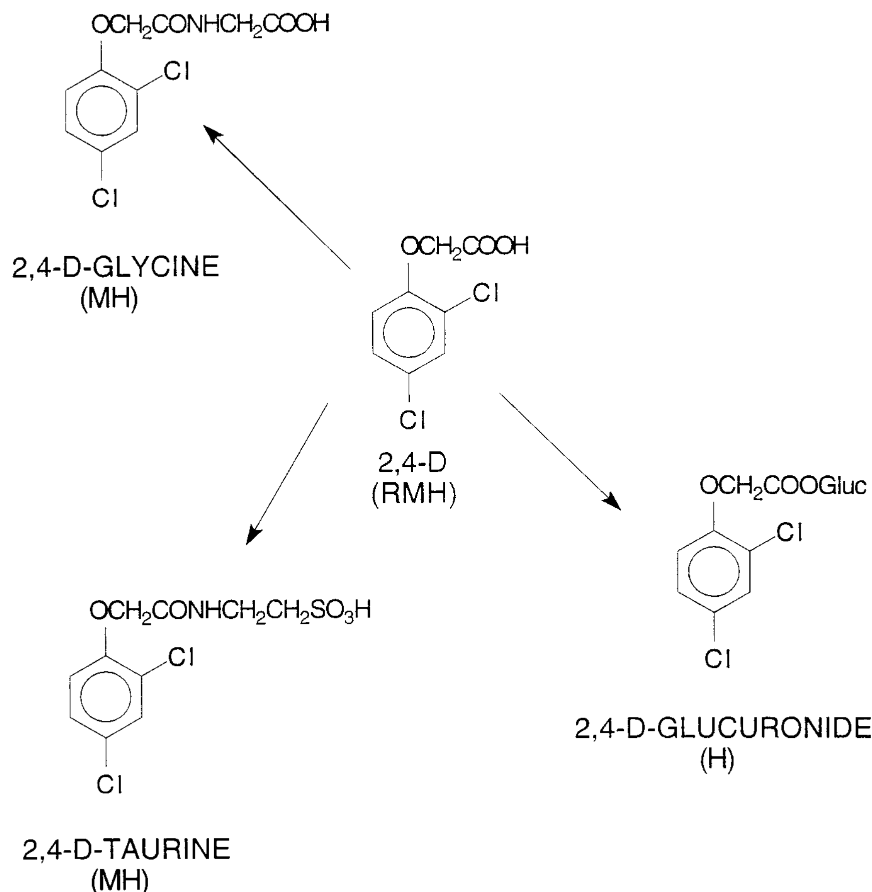

FIG. 2. *Urinary and fecal metabolites of 2,4-D in rats, mice and hamsters. R, rat metabolite; M, mouse metabolite; H, hamster metabolite.*

TABLE 1

Cumulative elimination of 2,4-D derived radioactivity in urine (% of administered dose)

Species	Sex	Dose	8 Hr	24 Hr	32 Hr	48 Hr	56 Hr	72 Hr
F344 rat	M	5 mg/kg	78.0 ± 1.9 ***	92.9 ± 1.7 ***	93.2 ± 2.8	94.3 ± 2.6	94.5 ± 2.6	97.0 ± 2.0
F344 rat	F	5 mg/kg	63.2 ± 6.2	85.1 ± 2.8	87.0 ± 2.9	88.3 ± 3.2	88.9 ± 3.2	92.2 ± 3.0
F344 rat	M	200 mg/kg	31.2 ± 4.5 ***	72.1 ± 6.5	82.5 ± 7.2	85.8 ± 7.2	86.2 ± 7.2	87.0 ± 7.2
F344 rat	F	200 mg/kg	16.5 ± 2.9	60.4 ± 3.9	75.6 ± 6.8	79.2 ± 8.0	79.9 ± 8.2	81.0 ± 8.4
B6C3F1 mice	M	5 mg/kg	17.6 ± 2.3	49.1 ± 7.1	56.4 ± 10.0	59.6 ± 12.0	60.0 ± 12.2	60.6 ± 12.4
B6C3F1 mice	F	5 mg/kg	13.7 ± 7.0	38.9 ± 11.0	43.0 ± 10.4	53.0 ± 9.7	53.5 ± 9.8	54.2 ± 9.9
B6C3F1 mice	M	200 mg/kg	12.7 ± 4.2 ***	31.0 ± 6.1 ***	40.3 ± 3.9 ***	67.4 ± 3.3 ***	71.1 ± 3.5 ***	73.0 ± 3.6 ***
B6C3F1 mice	F	200 mg/kg	26.8 ± 1.7	68.2 ± 8.4	74.0 ± 8.0	88.8 ± 6.2	91.2 ± 6.7	93.7 ± 7.2
Syr. hamster	M	200 mg/kg	4.9 ± 4.7	38.0 ± 12.2	44.4 ± 13.6	68.6 ± 2.4	79.1 ± 6.2	81.2 ± 7.03
Syr. hamster	F	200 mg/kg	33.9 ± 12.1	64.7 ± 9.6	68.1 ± 9.6	69.4 ± 9.6	70.2 ± 9.5	70.9 ± 9.6

*** Significant difference between sexes at same dose and time point by a Student's *t*-test ($p \leq 0.05$).
$N = 3$ in all cases except male hamsters ($N = 2$).

istered radioactivity in urine and feces are given in tables 1 and 2. 2,4-D was primarily excreted in the urine by all three species. It was more rapidly eliminated into urine by male rats than by female rats. The reverse was true for mice at the high dose. Less than 1% of the administered radioactivity was recovered in expired air.

Metabolite Profiles. In all three species and both sexes, 2,4-D was the major urinary metabolite (table 3). The glycine conjugate was detected in urine from mice and hamsters, the glucuronide only in hamster urine, and the taurine conjugate was detected in urine from mice and male hamsters. Rats excreted no detectable metabolites of 2,4-D in urine or feces. At the high dose, male mice metabolized 2,4-D to the glycine conjugate to a greater extent than females.

The only product present in feces from rats and hamsters was 2,4-D. In male mouse feces the taurine conjugate was 13.3 ± 2.4%, and 2,4-D was 86.7 ± 2.4% of the total fecal activity. In female mouse feces the taurine conjugate was 12.3 ± 2.3%, and 2,4-D was 87.7 ± 2.3% of the total fecal activity. HPLC analysis of rat blood and liver extracts also detected only the parent compound.

Disposition. The time-dependent tissue distribution of 2,4-D derived radioactivity in rats, mice, and hamsters is presented in tables

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TABLE 2

Cumulative elimination of 2,4-D derived radioactivity in feces (% of administered dose)

Species	Sex	Dose	24 Hr	48 Hr	72 Hr
F344 rat	M	5 mg/kg	1.8 ± 0.6	2.2 ± 0.7	2.3 ± 0.6
F344 rat	F	5 mg/kg	1.6 ± 0.2	2.0 ± 0.1	2.2 ± 0.1
F344 rat	M	200 mg/kg	2.7 ± 0.7	3.4 ± 1.0	3.7 ± 1.1
F344 rat	F	200 mg/kg	1.1 ± 0.1	1.5 ± 0.1	1.9 ± 0.2
B6C3F1 mice	M	5 mg/kg	18.1 ± 6.7	23.7 ± 7.0	24.4 ± 6.8
B6C3F1 mice	F	5 mg/kg	9.2 ± 4.6	11.6 ± 4.3	12.2 ± 4.2
B6C3F1 mice	M	200 mg/kg	2.8 ± 0.1	11.8 ± 2.1	13.2 ± 2.3
B6C3F1 mice	F	200 mg/kg	6.7 ± 1.8	8.7 ± 2.4	10.0 ± 2.2
Syr. hamster	M	200 mg/kg	2.5 ± 0.6	5.1 ± 0.6	6.1 ± 1.2
Syr. hamster	F	200 mg/kg	14.5 ± 6.2	16.0 ± 6.7	16.4 ± 6.8

$N = 3$ in all cases except male hamsters ($N = 2$).

4-8. In rats, tissue levels are consistently higher in females than in males at both doses and at all time points. The differences are the greatest at 24 hr at the high dose (almost 9 times) and at 2 hr at the low dose (up to 5 times). The sex-dependent differences were not always statistically significant, but of 64 comparisons, tissue levels of radioactivity were higher in males only seven times. In mice, there were a few cases where male tissue levels were higher with a statistically significant difference, but overall the differences were not consistent. In only about half of the comparisons was radioactivity in tissues higher in females than in males. In a smaller sample of hamsters the results suggest that tissue levels in male hamsters are higher than in females at the same doses and time points.

Kinetics. Male rats clear 2,4-D from plasma much more quickly than females. Pharmacokinetic analysis of the plasma data (table 9) indicate that the volume of distribution is significantly smaller in females which is consistent with the greater maximal concentration in females. Elimination parameters such as clearance and α and β half lives are clearly indicative of slower elimination in females. Consequently, measures of exposure such as area under the curve and mean residence time were significantly greater in females. Pharmacokinetic analysis of plasma concentrations of 2,4-D after oral administration (table 10) indicate that the rate of absorption was identical in male and female rats; however, area under the curve, elimination rate, Tmax, and Cmax were all significantly greater in females than in males.

Plasma Binding. Binding of 2,4-D to rat plasma proteins in male and female rats was determined at 6, 24, and 48 μg/ml. 2,4-D was approximately 97% bound in both sexes at all three concentrations.

TABLE 4

Tissue distribution of 2,4-D derived radioactivity in rats (200 mg/kg) nmoles/gram of tissue

Males	2 Hr	8 Hr	24 Hr	72 Hr
Blood	1026 ± 306	1501 ± 172	97 ± 42*	12 ± 7
Liver	608 ± 190	891 ± 101	48 ± 34*	7 ± 3
Kidney	659 ± 108	870 ± 89	247 ± 150	21 ± 2
Muscle	171 ± 69	226 ± 20	14 ± 7*	15 ± 4
Skin	293 ± 78	526 ± 89	57 ± 28*	39 ± 23
Fat	126 ± 40	174 ± 21	78 ± 19	73 ± 16
Testes	205 ± 66	293 ± 28	19 ± 12	4 ± 2
Brain	74 ± 34	99 ± 13	5 ± 4*	3 ± 2
Females				
Blood	1371 ± 154	1248 ± 143	861 ± 118	3 ± 1
Liver	885 ± 127	839 ± 132	323 ± 27	3 ± 1
Kidney	951 ± 95	941 ± 101	584 ± 49	22 ± 4
Muscle	314 ± 46	319 ± 49	129 ± 12	6 ± 4
Skin	463 ± 50	462 ± 60	270 ± 21	43 ± 10
Fat	204 ± 35	229 ± 29	113 ± 7	64 ± 16
Ovaries	554 ± 85	594 ± 99	357 ± 52	22 ± 7
Brain	169 ± 36	151 ± 31	40 ± 2	<1

* Significantly different from female results at that time point by a Student's *t*-test or a Mann-Whitney test ($p \leq 0.05$).
$N = 4$ at 2 and 8 hr and $N = 3$ at 24 and 72 hr.

Discussion

2,4-Dichlorophenoxyacetic acid has been the subject of toxicological investigation for many years. In the majority of these studies only males were used. Most of the disposition studies have been on the rat. These investigations have shown that 2,4-D is well absorbed after oral administration, does not concentrate in any tissue or organ, and is primarily excreted in the urine as parent compound along with traces of the taurine and glycine conjugates (1, 22–25, 27, 30). The pharmacokinetics in male rats has also been recently reported (26). A number of studies in humans also suggest that 2,4-D is rapidly absorbed after oral exposure, has a plasma half life of about 18 hr, and is excreted primarily in the urine (6, 28). A human metabolite has been detected but not identified (28). Disposition has been briefly examined in the mouse but not at all in the hamster (31). Biliary metabolism/elimination (32) and plasma pharmacokinetics (26) have been examined in rats, hamsters, and mice.

The most striking finding in the present study was the clear sex-dependence of 2,4-D elimination in rats. At both doses female rat tissues typically had higher concentrations of radioactivity than male rat tissues. This was clearly not because of a sex difference in

TABLE 3

Urinary metabolites of 2,4-D in male and female rats, mice and hamsters (% of total urinary radioactivity)

Species	Sex	Dose	Taurine	Glucuronide	Glycine	2,4-D
F344 rat	M	5 mg/kg	ND	ND	ND	100
F344 rat	F	5 mg/kg	ND	ND	ND	100
F344 rat	M	200 mg/kg	ND	ND	ND	100
F344 rat	F	200 mg/kg	ND	ND	ND	100
B6C3F1 mice	M	5 mg/kg	1.3 ± 0.6	ND	17.7 ± 2.4	80.1 ± 2.1
B6C3F1 mice	F	5 mg/kg	2.1 1.2	ND	10.9 ± 3.3	85.2 ± 4.0
B6C3F1 mice	M	200 mg/kg	14.6 ± 4.7	ND	34.9 ± 0.5 ***	50.6 ± 5.1 ***
B6C3F1 mice	F	200 mg/kg	7.0 ± 1.8	ND	13.1 ± 3.2	79.9 ± 1.6
Syr. hamster	M	200 mg/kg	2.0 ± 1.2	6.0 ± 2.6	4.8 ± 1.4	87.1 ± 4.5
Syr. hamster	F	200 mg/kg	ND	1.7 ± 0.1	0.9 ± 0.4	97.5 ± 0.4

*** Significant difference between sexes at same dose and time point by a Student's *t*-test ($p \leq 0.05$).
$N = 3$ in all cases except male hamsters ($N = 2$). ND, Not detected.

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TABLE 5

Tissue distribution of 2,4-D derived radioactivity in rats (5 mg/kg) nmoles/gram of tissue

Males	2 Hr	8 Hr	24 Hr	72 Hr
Blood	4.292 ± 0.798*	0.953 ± 0.310	0.170 ± 0.027*	0.021 ± 0.010
Liver	2.710 ± 0.485	0.599 ± 0.261	0.135 ± 0.036	0.032 ± 0.004*
Kidney	21.091 ± 6.387	3.878 ± 1.580	0.796 ± 0.184	0.111 ± 0.022
Muscle	1.355 ± 0.435	0.211 ± 0.065	0.090 ± 0.022	0.008 ± 0.005
Skin	1.352 ± 0.425*	0.261 ± 0.090	0.087 ± 0.028	0.020 ± 0.007*
Fat	1.052 ± 0.467	0.163 ± 0.027	0.092 ± 0.020	0.025 ± 0.008*
Testes	1.831 ± 0.282	0.226 ± 0.075	0.076 ± 0.015	0.001 ± 0.000
Brain	0.358 ± 0.114	0.048 ± 0.022	0.021 ± 0.005*	0.006 ± 0.004
Females				
Blood	24.586 ± 11.874	1.325 ± 0.429	0.543 ± 0.261	0.051 ± 0.001
Liver	13.356 ± 6.678	0.777 ± 0.291	0.343 ± 0.136	0.050 ± 0.003
Kidney	52.781 ± 16.276	11.899 ± 7.072	1.410 ± 0.538	0.199 ± 0.032
Muscle	2.603 ± 1.086	0.304 ± 0.118	0.200 ± 0.061	0.022 ± 0.005
Skin	5.549 ± 2.145	0.486 ± 0.196	0.156 ± 0.051	0.062 ± 0.017
Fat	1.583 ± 0.553	0.196 ± 0.086	0.172 ± 0.083	0.061 ± 0.013
Ovaries	10.676 ± 4.909	0.805 ± 0.291	0.271 ± 0.121	0.035 ± 0.010
Brain	1.213 ± 0.593	0.085 ± 0.031	0.051 ± 0.009	0.019 ± 0.004

* Significantly different from female results at that time point by a Student's *t*-test or a Mann-Whitney test ($p \leq 0.05$).
$N = 4$ at 2 and 8 hr and $N = 3$ at 24 and 72 h.

TABLE 6

Tissue distribution of 2,4-D-derived radioactivity in mice (200 mg/kg) nmoles/gram of tissue

Males	2 Hr	8 Hr	24 Hr	72 Hr
Blood	321.1 ± 75.4	175.8 ± 59.1	126.5 ± 18.6	1.6 ± 0.5
Liver	150.9 ± 31.5	174.1 ± 53.9	62.1 ± 15.6	2.3 ± 0.2*
Kidney	422 ± 42.1*	250.8 ± 45.5	248.3 ± 60.5	2.1 ± 0.2
Muscle	81.9 ± 15.3	78.9 ± 34.8	28.6 ± 7.7	0.7 ± 0.1
Skin	131.3 ± 30.2	108.1 ± 40.7	64.3 ± 15.2	2.9 ± 0.3
Fat	41.5 ± 8.0	48.2 ± 8.8	28.2 ± 6.0	2.0 ± 0.8
Testes	57.2 ± 13.1	39.2 ± 19.2	18.6 ± 5.3	1.4 ± 0.8
Brain	33.3 ± 9.4	37.8 ± 14.5	10.5 ± 3.5	0.3 ± 0.1*
Females				
Blood	316.9 ± 66.3	275.5 ± 87.6	131.6 ± 106.9	0.8 ± 0.1
Liver	141.7 ± 25.0	167.4 ± 59.9	59.1 ± 46.5	1.2 ± 0.3
Kidney	230.5 ± 30.0	215.2 ± 65.5	99.5 ± 67.2	1.7 ± 0.3
Muscle	130.1 ± 42.7	85.1 ± 34.6	32.4 ± 26.0	0.9 ± 0.4
Skin	137.8 ± 27.3	118.3 ± 43.5	57.4 ± 42.9	4.4 ± 1.4
Fat	64.3 ± 14.4	58.1 ± 9.2	12.5 ± 11.2	12.1 ± 4.9
Ovaries	201.5 ± 54.8	114.9 ± 34.0	66.1 ± 50.7	5.7 ± 5.4
Brain	42.6 ± 17.1	43.4 ± 26.2	9.2 ± 8.0	<0.1

* Significantly different from female results at that time point by a Student's *t*-test or a Mann-Whitney test ($p \leq 0.05$).
$N = 4$ at 2 and 8 hr and $N = 3$ at 24 and 72 hr.

TABLE 7

Tissue distribution of 2,4-D-derived radioactivity in mice (5 mg/kg) nmoles/gram of tissue

Males	2 Hr	8 Hr	24 Hr	72 Hr
Blood	1.98 ± 1.32	8.21 ± 4.11	0.47 ± 0.20	0.04 ± 0.01
Liver	1.25 ± 0.81	2.70 ± 0.99	0.23 ± 0.08	0.05 ± 0.01
Kidney	9.74 ± 6.36	23.33 ± 7.99	2.32 ± 0.80	0.13 ± 0.03
Muscle	0.54 ± 0.37	1.12 ± 0.47	0.12 ± 0.04	0.03 ± 0.02
Skin	1.26 ± 0.89	2.34 ± 0.83	0.27 ± 0.06	0.45 ± 0.38
Fat	0.49 ± 0.32	0.68 ± 0.26	0.16 ± 0.07	0.23 ± 0.22
Testes	0.87 ± 0.54	0.75 ± 0.26	0.10 ± 0.01	0.07 ± 0.01
Brain	0.19 ± 0.16	0.28 ± 0.11	0.03 ± 0.01	<0.01
Females				
Blood	0.28 ± 0.09	1.08 ± 0.23	0.98 ± 0.46	0.02 ± 0.01
Liver	0.36 ± 0.17	0.96 ± 0.13	0.49 ± 0.23	0.06 ± 0.03
Kidney	0.85 ± 0.24	5.21 ± 2.15	4.53 ± 2.19	0.12 ± 0.03
Muscle	0.09 ± 0.03	0.35 ± 0.08	0.28 ± 0.13	0.02 ± 0.00
Skin	0.24 ± 0.04	0.65 ± 0.18	0.43 ± 0.21	0.03 ± 0.00
Fat	0.12 ± 0.01	0.34 ± 0.10	0.28 ± 0.15	0.03 ± 0.01
Ovaries	0.17 ± 0.03	0.59 ± 0.12	0.88 ± 0.66	0.07 ± 0.01
Brain	0.29 ± 0.09	0.07 ± 0.01	0.04 ± 0.02	<0.01

* Significantly different from female results at that time point by a Student's *t*-test or a Mann-Whitney test ($p \leq 0.05$).
$N = 4$ at 2 and 8 hr and $N = 3$ at 24 and 72 hr.

metabolism because rats did not excrete any metabolites in urine or feces. The difference in elimination was neither owing to differences in plasma protein binding of 2,4-D nor to differences in absorption. A previous study in this laboratory demonstrated that there were no sex-dependent differences in biliary elimination or metabolites of 2,4-D in rats that could explain this response (32). The present work demonstrated there were no sex-dependent differences in route of elimination either. There were some indications that renal elimination in males is faster than in females and that after both iv and oral administration all elimination parameters were greater in males than in females. The mechanistic basis for this is not clear.

In mice there were no obvious sex-dependent differences in tissue distribution. Unlike rats, mice excreted significant amounts of two metabolites, the taurine conjugate in urine and feces and the glycine conjugate in urine. Males excreted more glycine conjugate than females in urine, but 2,4-D was always the major product excreted.

A more limited experimental design was used for hamsters. Disposition was examined at 8 and 72 hr only. Nevertheless a clear sex dependent difference in tissue distribution was detected. This suggests that male hamsters were less able to clear 2,4-D than females. This is the opposite of the effect seen in rats but was consistent with the prolonged secondary rise in plasma 2,4-D seen in male hamsters reported by Grizzle *et al.* (26). This is also consistent with the delayed urinary elimination of 2,4-D in male hamsters relative to females. This difference in urinary elimination was not statistically significant because the male group was $N = 2$ as a result of a loss of a sample for one animal at an early time point. There were some apparent sex-dependent differences in metabolism in hamsters. The taurine, gly-

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TABLE 8

Tissue distribution of 2,4-D-derived radioactivity in hamsters (200 mg/kg) nmoles/gram of tissue

	Sex	8 Hr	72 Hr
Blood	Male	1479 ± 77*	6.1 ± 2.4
Blood	Female	619 ± 71	2.5 ± 0.4
Liver	Male	1216 ± 86*	6.6 ± 2.2
Liver	Female	357 ± 62	3.3 ± 0.1
Kidney	Male	1166 ± 67*	5.0 ± 1.8
Kidney	Female	633 ± 26	4.3 ± 0.4
Muscle	Male	444 ± 34*	2.5 ± 1.3
Muscle	Female	196 ± 20	1.1 ± 0.6
Skin	Male	621 ± 45*	19.3 ± 7.7
Skin	Female	330 ± 24	7.2 ± 2.1
Fat	Male	162 ± 49	3.5 ± 0.5
Fat	Female	135 ± 20	9.5 ± 2.7
Gonads	Male	212 ± 25	0.6 ± 0.3*
Gonads	Female	315 ± 41	4.7 ± 1.2
Brain	Male	179 ± 22*	0.9 ± 0.4
Brain	Female	54 ± 9	1.6 ± 0.5

* Significant difference between sexes at same dose and time point by a Student's *t*-test ($p \leq 0.05$).

$N = 4$ at 8 hr and $N = 3$ at 72 hr.

TABLE 9

Toxicokinetic parameters in male and female Sprague Dawley rats treated with 2,4-D (5 mg/kg, iv)

Parameter	Males	Females
Volume of distribution (ml)	80.6 ± 4.5	50.2 ± 1.8 ***
Area under the curve (μg·min/ml)	492 ± 28	3706 ± 467 ***
$\alpha t_{1/2}$ (min)	17.0 ± 1.7	28.2 ± 3.8 ***
$\beta t_{1/2}$ (min)	138 ± 82	382 ± 102 ***
Maximal concentration (μg/ml)	16.5 ± 0.9	22.1 ± 0.8 ***
Clearance (ml/min)	2.70 ± 0.15	0.30 ± 0.04 ***
Mean residence time (min)	60 ± 23	458 ± 121 ***

*** Indicates a statistically significant difference between males and females by a Student's *t*-test ($p \leq 0.05$).

$N = 3$ for males and $N = 4$ for females.

TABLE 10

Toxicokinetic parameters in male and female Sprague Dawley rats treated with 2,4-D (5 mg/kg, oral)

Parameter	Males	Females
Rate of absorption (min^{-1})	0.063 ± 0.028	0.065 ± 0.008
Rate of elimination (min^{-1})	0.020 ± 0.005	0.005 ± 0.000 ***
Area under the curve (μg·min/ml)	639.0 ± 71.6	4026.8 ± 139.0 ***
Absorption half-life (min)	11.1 ± 4.9	10.7 ± 1.4
Elimination half-life (min)	34.6 ± 9.2	139.4 ± 7.8 ***
Time of maximal concentration (min)	26.8 ± 4.8	42.9 ± 3.5 ***
Maximal concentration (μg/ml)	7.5 ± 0.8	16.2 ± 0.6 ***

*** Indicates a statistically significant difference between males and females by a Student's *t*-test ($p \leq 0.05$).

$N = 5$ for males and $N = 4$ for females.

cine, and glucuronide metabolites were excreted by hamsters, but 2,4-D was metabolized to a greater extent in males and the taurine conjugate was not excreted by females. This suggests that enterohepatic recirculation of the metabolites might explain the longer retention of material in males. However, a difference in biliary elimination was not seen in hamsters in our earlier study (32).

The differences in clearance between sexes should be reflected in the toxicity of 2,4-D. There is little information on toxicity in mice and hamsters, but there is quite a bit on toxicity in rats. Where data exist for males and females, it appears that toxicity is indeed greater in females. For example, toxic effects on the central nervous system have been reported in male rats (11, 12) which have been linked to increased brain concentrations of 2,4-D (25). In the current study, female brain concentrations of 2,4-D are clearly higher than males. This suggests that females should be more susceptible to 2,4-D induced neurotoxicity, and this speculation is supported by a recent study (13). A recent bioassay in the dog detected no significant toxicities in that species although in females weight losses were higher than in males (15). Results from large subchronic and chronic studies in male and female rodents also indicated no significant toxicities in rats (20, 21). However, some minor toxic effects were reported, such as decreased weight gains, altered organ weights, retinal degeneration, and cataracts, which were often more evident, or appeared at lower doses, in females. The conclusion of that study was that the maximal tolerated dose in males was 150 mg/kg/day while in females it was 75 mg/kg/day (21). This is consistent with the sex-dependent kinetics difference described in this paper. In addition, there were no consistent sex-dependent differences in toxicity in mice in the chronic study. This is also consistent with the lack of a sex-dependent difference in tissue distribution in mice in the present study.

The relatively low toxicity of 2,4-D and similar herbicides has long been associated with the rapid elimination of these chemicals by renal tubular transport (33). There seem to be no studies comparing active anion transport of 2,4-D in females and males of any species. Studies on sex and species differences of the effect 2,4-D has on renal transport may offer an explanation for the toxicological and pharmacological differences observed. In fact, a study using renal slices was prompted by our observations. Greater transport of 2,4-D was observed in renal slices from male Sprague-Dawley and F344 rats compared with that in slices from females of the same strain (J. Pritchard and E. Lebetkin, unpublished results, 1997).

In the three species examined, the sex-dependent effects on 2,4-D elimination were different. There was no effect in mice, male rats cleared 2,4-D faster than females, and male hamsters cleared 2,4-D slower than females. Based on these results, there is a possibility that a sex-dependent difference in clearance of 2,4-D is present in humans. Human pharmacokinetic data on 2,4-D currently is available from males only. No human toxicity has been conclusively linked to 2,4-D. However, the current permissible exposure levels are presumably based on human male pharmacokinetic data and on the limited animal toxicity results. A difference in clearance in humans equivalent to that in rats might necessitate a decrease in permissible exposure limits.

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References

1. D. Knopp and F. Schiller: Oral and dermal application of 2,4-dichlorophenoxyacetic acid sodium and dimethylamine salts to male rats: investigations on absorption and excretion as well as induction of hepatic mixed function oxidase activities. *Arch. Toxicol.* **66,** 170–174 (1992).
2. P. K. Taskar, Y. T. Das, J. R. Trout, S. K. Chattopadhyay, and H. D. Brown: Measurement of 2,4-dichlorophenoxyacetic acid (2,4-D) after occupational exposure. *Bull. Environ. Contam. Toxicol.* **29,** 586–591 (1982).
3. R. Grover, C. A. Franklin, N. I. Muir, A. J. Cessna, and D. Riedel: Dermal exposure and urinary metabolite excretion in farmers repeatedly exposed to 2,4-D amine. *Toxicol. Lett.* **33,** 73–83 (1986).
4. P. Reynolds, J. S. Reif, and H. S. Ramsdell: Canine exposure to herbicide-treated lawns and urinary excretion of 2,4-dichlorophenoxyacetic acid. *Toxicologist* **13,** 1432 (1993).
5. S. A. Harris and K. R. Solomon: Exposure of homeowners, professional applicators and bystanders to 2,4-dichlorophenoxyacetic acid (2,4-D). *Toxicologist* **11,** 191 (1991).
6. J. D. Kohli, R. N. Khanna, B. N. Gupta, M. M. Dhar, J. S. Tandon, and K. P. Sircar: Absorption and excretion of 2,4-dichlorophenoxyacetic acid in man. *Xenobiotica* **4,** 97–100 (1974).
7. G. H. Oliveira and J. Palermo-Neto: Toxicology of 2,4-dichlorophenoxyacetate (2,4-D) and its determination in serum and brain tissue using gas chromatography-electron capture detection. *J. Anal. Toxicol.* **19,** 251–255 (1995).
8. C. M. Palmeira, A. J. Moreno, and V. M. C. Madeira: Thiols metabolism is altered by the herbicides paraquat, dinoseb and 2,4-D: a study in isolated hepatocytes. *Toxicol. Lett.* **81,** 115–123 (1995).
9. C. M. Palmeira, A. J. Moreno, and V. M. C. Madeira: Metabolic alterations in hepatocytes promoted by herbicides paraquat, dinoseb and 2,4-D. *Arch. Toxicol.* **68,** 24–31 (1994).
10. S. J. Gorzinski, R. J. Kociba, R. A. Campbell, F. A. Smith, R. J. Nolan, and D. L. Eisenbrandt: Acute, pharmacokinetic and subchronic toxicological studies of 2,4-dichlorophenoxyacetic acid. *Fundam. Appl. Toxicol.* **9,** 423–435 (1987).
11. I. Desi, J. Sos, J. Olasz, J. Sule, and V. Makkus: Nervous system effects of a chemical herbicide. *Arch. Environ. Health* **4,** 95–102 (1962).
12. G. E. Schulze and J. A. Dougherty: Neurobehavioral toxicity and tolerance to the herbicide 2,4-dichlorophenoxyacetic acid-*N*-butyl ester (2,4-D-ester). *Fundam. Appl. Toxicol.* **10,** 413–424 (1988).
13. A. M. Evangelista de Dufard, A. Bortolozzi, and R. O. Duffard: Altered behavioral responses in 2,4-dichlorophenoxyacetic acid treated and amphetamine challenged rats. *Neurotoxicology* **16,** 479–488 (1995).
14. H. M. Hayes, R. E. Tarone, and K. P. Cantor: On the association between canine malignant lymphoma and opportunity for exposure to 2,4-dichlorophenoxyacetic acid. *Environ. Res.* **70,** 119–125 (1995).
15. J. M. Charles, D. W. Dalgard, H. C. Cunny, R. D. Wilson, and J. S. Bus: Comparative subchronic and dietary toxicity studies on 2,4-dichlorophenoxyacetic acid, amine and ester in the dog. *Fundam. Appl. Toxicol.* **29,** 78–85 (1996).
16. V. F. Garry, D. Schreinemachers, M. E. Harkins, and J. Griffith: Pesticide appliers, biocides and birth defects in rural Minnesota. *Environ. Health Perspect.* **104,** 394–399 (1996).
17. M. A. Bacher and G. G. Gibson: Chlorophenoxyacid herbicides induce microsomal cytochrome P450 IVA1 (P452) in rat liver. *Chem.-Biol. Interactions* **65,** 145–156 (1988).
18. R. Mustonen, E. Elovaara, A. Zitting, K. Linnainmaa, and H. Vainio: Effect of commercial chlorphenolate, 2,3,7,8-TCDD and pure phenoxyacetic acids on hepatic peroxisome proliferation, xenobiotic metabolism and sister chromatid exchange in the rat. *Arch. Toxicol.* **63,** 203–208 (1989).
19. Y. Kawashima, H. Katoh, S. Nakajima, H: Kozuka and and M. Uchiyama: Effects of 2,4-dichlorophenoxyacetic and 2,4,5-trichlorophenoxyacetic acid on peroxisomal enzymes in rat liver. *Biochem. Pharmacol.* **33,** 241–245 (1984).
20. J. M. Charles, D. M. Bond, T. K. Jefferies, B. L. Yano, W. T. Stott, K. A. Johnson, H. C. Cunny, R. D. Wilson, and J. S. Bus: Chronic dietary toxicity/oncogenicity of 2,4-dichlorophenoxyacetic acid in rodents. *Fundam. Appl. Toxicol.* **33,** 166–172 (1996).
21. J. M. Charles, H. C. Cunny, R. D. Wilson, and J. S. Bus: Comparative subchronic studies on 2,4-dichlorophenoxyacetic acid, amine and ester in rats. *Fundam. Appl. Toxicol.* **33,** 161–165 (1996).
22. O. Pelletier, L. Ritter, J. Caron, and D. Somers: Disposition of 2,4-dichlorophenoxyacetic acid dimethylamine salt by Fischer 344 rats dosed orally and dermally. *J. Toxicol. Environ. Health* **28,** 221–234 (1989).
23. K. Erne: Distribution and elimination of chlorinated phenoxyacetic acids in animals. *Acta Vat. Scand.* **7,** 240–256 (1966).
24. W. Grunow and C. Bohme: Uber den Stoffwechsel von 2.4.5-T and 2.4-D bei ratten und mausen. *Arch. Toxicol.* **32,** 217–225 (1974).
25. H. A. Elo and P. Ylitalo: Distribution of 2-methyl-4-chlorphenoxyacetic acid and 2,4-dichlorophenoxyacetic acid in male rats: Evidence for the involvement of the central nervous system in their toxicity. *Toxicol. Appl. Pharmacol.* **51,** 439–446 (1979).
26. T. B. Grizzle, L. Buckley-Kedderis, B. J. Collins, T. J. Goehl, R. W. Handy, and D. S. Marsman: Toxicokinetics of 2,4-dichlorophenoxyacetic acid (2,4-D) in rats, mice and hamsters. *Internat. Congr. Toxicol.* **7,** 47-P-9 (1995).
27. S. Khanna and S. C. Fang: Metabolism of C14-labelled 2,4-dichlorophenoxyacetic acid in rats. *J. Agri. Food Chem.* **14,** 500–503 (1966).
28. M. W. Sauerhoff, W. H. Braun, G. E. Blau, and P. J. Gehring: The fate of 2,4-dichlorophenoxyacetic acid (2,4-D) following oral administration to man. *Toxicology* **8,** 3–11 (1977).
29. K. Dix, L. J. Deterding, L. T. Burka, and K. B. Tomer: Tris(2-chloroethyl) phosphate pharmacokinetics in the Fischer 344 rat: a comparison of conventional methods and in vivo microdialysis coupled with tandem mass spectrometry. *J. Pharm. Sci.* **83,** 1622–1629 (1994).
30. M. Kelley and D. A. Vessey: The effect of pretreatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic metabolism of 2,4,5-trichlorophenoxyacetate (2,4,5-T) and 2,4-dichlorophenoxyacetate (2,4-D). *Toxicol. Appl. Pharmacol.* **91,** 295–298 (1987).
31. N. G. Lindquist and S. Ullberg: Distribution of the herbicides 2,4,5-T and 2,4-D in pregnant mice: accumulation in the yolk sac epithelium. *Experientia* **27,** 1439–1441 (1971).
32. R. J. Griffin, J. Salemme, J. Clark, P. Myers, and L. T. Burka: Biliary elimination of oral 2,4-dichlorophenoxyacetic acid and its metabolites in male and female Sprague-Dawley rats, B6C3F1 mice and Syrian hamsters. *Toxicol. Environ. Health*, In Press (1997).
33. W. O. Berndt and F. Koschier: In vitro uptake of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) by renal cortical tissue of rabbits and rats. *Toxicol. Appl. Pharmacol.* **26,** 559–570 (1973).

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