

Life-Stage-, Sex-, and Dose-Dependent Dietary Toxicokinetics and Relationship to Toxicity of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Rats: Implications for Toxicity Test Dose Selection, Design, and Interpretation

Shakil A. Saghir,* Mary S. Marty,^{†,1} Carol L. Zabloutny,[†] Julie K. Passage,[†] Adam W. Perala,[†] Barbara H. Neal,[‡] Larry Hammond,[§] and James S. Bus[‡]

*Intrinsic Environmental Sciences Inc., Mississauga, Ontario, Canada L5T 0A3, and Aga Khan University, Karachi, Pakistan 74800; [†]The Dow Chemical Company, Midland, Michigan 49684; [‡]Exponent, Alexandria, Virginia 22314; and [§]Industry Task Force II on 2,4-D Research Data, Washington, District of Columbia 20006

¹To whom correspondence should be addressed. Fax: 989-638-9863. E-mail: mmarty@dow.com.

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Life-stage-dependent toxicity and dose-dependent toxicokinetics (TK) were evaluated in Sprague Dawley rats following dietary exposure to 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D renal clearance is impacted by dose-dependent saturation of the renal organic anion transporter; thus, this study focused on identifying inflection points of onset of dietary nonlinear TK to inform dose selection decisions for toxicity studies. Male and female rats were fed 2,4-D-fortified diets at doses to 1600 ppm for 4-weeks pre-mating, <2 weeks during mating, and to test day (TD) 71 to parental (P1) males and to P1 females through gestation/lactation to TD 96. F1 offspring were exposed via milk with continuing diet exposure until postnatal day (PND) 35. As assessed by plasma area under the curve for the time-course plasma concentration, nonlinear TK was observed ≥ 1200 ppm (63 mg/kg/day) for P1 males and between 200 and 400 ppm (14–27 mg/kg/day) for P1 females. Dam milk and pup plasma levels were higher on lactation day (LD) 14 than LD 4. Relative to P1 adults, 2,4-D levels were higher in dams during late gestation/lactation and postweaning pups (PND 21–35) and coincided with elevated intake of diet/kg body weight. Using conventional maximum tolerated dose (MTD) criteria based on body weight changes for dose selection would have resulted in excessive top doses approximately 2-fold higher than those identified incorporating critical TK data. These data indicate that demonstration of nonlinear TK, if present at dose levels substantially above real-world human exposures, is a key dose selection consideration for improving the human relevance of toxicity studies compared with studies employing conventional MTD dose selection strategies.

Key Words: 2,4-dichlorophenoxyacetic acid; 2,4-D; toxicokinetics; TK; pharmacokinetics; PK; development; perinatal; toxicity; rats.

2,4-Dichlorophenoxyacetic acid (2,4-D) was first introduced into commerce as a herbicide in the mid-1940s and

continues to be one of the world's most widely used herbicides (Munro *et al.*, 1992). In part because of its lengthy history and widespread use, the toxicity and potential human health effects of 2,4-D have been extensively studied and reviewed (Bus and Hammond, 2007; Garabrant and Philbert, 2002; Munro *et al.*, 1992). Subchronic and chronic rat toxicity studies have identified the kidney as a primary target organ (Charles *et al.*, 1996a,b; Gorzinski *et al.*, 1987), consistent with the observation that 2,4-D is accumulated in renal proximal tubules through the action of a saturable, metabolically active renal organic anion transporter, OAT1 (Berndt and Koschier, 1973; Hasegawa *et al.*, 2003; Hook *et al.*, 1974). The OAT1 transporter plays a critical role in the dose-dependent systemic renal clearance of 2,4-D in rats and is saturated at oral gavage and dietary doses of approximately 50 mg/kg, resulting in distinct nonlinear toxicokinetic (TK) behavior (Gorzinski *et al.*, 1987; Saghir *et al.*, 2006; Timchalk, 2004; van Ravenzwaay *et al.*, 2003). Importantly, OAT1 is the primary transporter responsible for renal clearance of 2,4-D in humans (Nozaki *et al.*, 2007), and thus, 2,4-D would be expected to exhibit analogous dose-dependent nonlinear TK in humans.

The Society of Toxicology Task Force to Improve the Scientific Basis of Risk Assessment has commented that findings of animal toxicity studies observed at doses well in excess of real-world human exposures may have limited, if any, value to actual human risk due to the onset of high-dose restricted modes of action (Conolly *et al.*, 1999). Such dose-dependent modes of action include saturation of metabolic and/or other systemic clearance mechanisms including renal anion transporters (Counts and Goodman, 1995; Slikker *et al.*, 2004a,b). In an extension of these reports, the International Life Sciences Institute Health and Environmental Sciences Institute, Agricultural Chemical Safety Assessment Technical

Committee (ACSA) recommended that TK data should be an integral consideration in the design and interpretation of animal toxicological studies (Barton *et al.*, 2006; Carmichael *et al.*, 2006; Cooper *et al.*, 2006; Doe *et al.*, 2006). The ACSA committee emphasized that *a priori* evidence of nonlinear TK in test animals should be a determinant in dose selection decisions for toxicological studies, and that limiting the highest test dose to the inflection point of the onset of nonlinear behavior was justified if real-world human exposures were well below the TK-identified saturation point.

Human biomonitoring studies of applicators and bystanders have reported mean daily doses of <5 µg/kg/day of 2,4-D (Alexander *et al.*, 2007; Harris and Solomon, 1992; Morgan *et al.*, 2008; Thomas *et al.*, 2010; Yeary, 1986), and a mean dose of 11 µg/kg/day has been reported in a worst-case exposure scenario of forestry workers applying 2,4-D with backpack sprayers (Zhang *et al.*, 2011). All of these exposures are substantially below both the dose associated with the onset of nonlinear TK in rats, approximately 50 mg/kg, and the overall no observed adverse effect level (NOAEL) of 5 mg/kg/day derived from rodent chronic bioassays (Charles *et al.*, 1996a,b). Based on the 5 mg/kg/day of NOAEL, regulatory agencies have established acceptable population-level daily human exposures of 50 µg/kg/day for 2,4-D (United States Environmental Protection Agency [USEPA], 2011).

As part of a regulatory reregistration/re-evaluation of 2,4-D in North America designed to provide new data to evaluate potential human risks, both the USEPA (2005) and Health Canada Pest Management Regulatory Agency (PMRA, 2008) issued a data call-in requiring that 2,4-D be further evaluated for reproductive, endocrine, developmental neurotoxicity, and immunotoxicity in rats using state-of-the-art test protocols (USEPA, 2005). The objective was to assess these endpoints using technical refinements in these toxicity testing methods compared with those previously used for 2,4-D toxicity assessments, as well as to further evaluate potential for endocrine effects and developmental neurotoxic or immunotoxic effects of 2,4-D. In consultation with USEPA and Health Canada PMRA, the 2,4-D Task Force (the Industry Task Force II on 2,4-D Research Data is a consortium of companies responsible for the manufacture and sale of 2,4-D in North America and is composed of Dow AgroSciences, LLC, Nufarm Americas Inc, and AGRO-GOR) agreed to implement the regulatory testing requirement with a newly developed ACSA extended 1-generation test protocol, which was modified to address all of the endpoints of interest in a single F1-extended 1-generation study (Cooper *et al.*, 2006; Marty *et al.*, 2013). To improve the human relevance of such toxicological studies as recommended by ACSA, the purpose of this study was to assess both the toxicity and the dose-dependent dietary TK of 2,4-D across life stages to provide a rational basis for dose selection for the planned F1-extended 1-generation reproductive toxicity study (Marty *et al.*, 2013). Given the wide margin of exposure between real-world human exposures to 2,4-D and the dose levels signaling onset of nonlinear TK in

rats, a particular intent was to provide information such that the top dose selected for the F1-extended 1-generation reproductive toxicity study would be at or slightly above the threshold for renal saturation as recommended by ACSA (Cooper *et al.*, 2006).

In addition to the value of TK data for guiding dose selection decisions for a complex state-of-the-art rat toxicity test, the data presented in this study provide novel information about the life-stage-, sex-, and dose-dependent TK of a chemical known to be subject to saturation of systemic clearance, and, importantly, following administration by the human-relevant dietary mode of exposure. Assessment of 2,4-D TK during the perinatal period is particularly important and informative in that expression of OAT1 does not reach full maturity in rats until approximately postnatal day (PND) 30 (Buist *et al.*, 2002). Understanding systemic dosimetry during this critical period of development has improved both the design and interpretation of the extended 1-generation reproductive toxicity study (Marty *et al.*, 2013). Finally, this TK data set provides useful insights not only for informing past and future toxicity test design and interpretation considerations for 2,4-D but also more broadly informs risk assessment implications for a spectrum of other low-molecular-weight environmental contaminants similarly subject to nonlinear TK behaviors.

MATERIALS AND METHODS

Test material and test diet preparation. 2,4-Dichlorophenoxyacetic acid (2,4-D; 99% pure) was provided by Nufarm Americas Inc. For the targeted premix, 2,4-D was air milled (particle size approximately 149 µm; Howard Industries, Columbus, Ohio), mixed with finely ground rodent chow and then mixed with ground rodent chow. 2,4-D-fortified diets were prepared by further diluting the premix with ground feed. Control diets were prepared using a similar procedure without 2,4-D. The concentration and homogeneity of diet mixes were confirmed at multiple time points during the study; test diets ranged from 87% to 106% of the targeted concentrations (data not shown). The stability of 2,4-D in rodent chow was confirmed to be at least 27 days at the concentrations used in this study (data not shown); test diets were prepared and used within this stability period.

Animals and animal husbandry. A total of 96 male and 120 female Crl:CD(SD)IGS BR rats were purchased from Charles River Laboratories Inc (Portage, Michigan) and acclimated to the laboratory conditions for at least 7 days. Following health examinations and weighing, 9- to 10-week-old rats were randomly assigned by body weight to treatment groups.

Adult (parental, P1) animals were individually housed in wire-mesh cages suspended above catch pans. Pregnant and lactating females were kept in plastic litter boxes with ground corncob bedding from gestation day (GD) 19 until weaning of their litters on lactation day (LD) 21. Animals were housed in rooms designed to maintain adequate conditions (22°C ± 3°C, 40%–70% relative humidity, 12-h light/dark cycle, 12–15 times/h air exchange). Rats were *ad libitum* fed on control or 2,4-D-fortified ground Certified Rodent LabDiet 5002 (PMI Nutrition International, St Louis, Missouri). A pressure-activated lixit-valve watering system allowed *ad libitum* access to municipal water. The animal facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The institutional animal care and use committee of The Dow Chemical Company approved the experimental protocols, and the study followed good laboratory practice guidelines.

Dosing. Groups of 10 male and 10 female rats were initially fed diets supplying 0, 100, 400, 1000, 1600, or 2000 ppm of 2,4-D. Due to excessive toxicity (marked decreases in body weight and feed consumption), the 2000-ppm dose was reduced to 1200 ppm on test day (TD) 20. To optimize dose spacing, the 1000-ppm dose was simultaneously lowered to 800 ppm. The approximate mg/kg/day doses of 2,4-D provided to adult prebreeding animals were 0, 6, 25, 50, 75, or 100 (for actual mg/kg/day doses at different life stages after dose adjustment, see Table 1). Throughout this study, the lower adjusted dietary concentrations are used to identify these dose groups. Exposure of P1 males continued for approximately 7 weeks after the initiation of the mating with final sacrifice on TD 71. P1 females continued on the test diets through gestation, lactation, and postweaning until termination on TD 96. To avoid potentially confounding stress due to blood sampling in pregnant animals designated to deliver pups, a terminal satellite group of P1 females (6 animals/dose) was included for the assessment of 2,4-D TK on GD 17. Details of the study design including TK sampling times are outlined in Figure 1.

To further refine characterization of the point of TK nonlinearity in female rats, a follow-up study was conducted in which adult female rats (4/dose group)

were exposed to 0, 100, 200, 400, 600, or 800 ppm of 2,4-D-fortified diets for 4 weeks. The additional TK study followed the design outlined for the pre-mating females in Figure 1.

Mating. In the main study, females were transferred to the cages of randomly selected males from the same exposure group after 4 weeks of dosing. Satellite females were transferred to the cages of nonexposed males. All females were examined daily for the evidence of mating (copulatory plug or sperm-positive vaginal smear). The animals were separated on the day of confirmed mating (designated GD 0). Females with no evidence of mating after 14 days were separated from the males without further opportunity for mating and not monitored thereafter.

Parturition and litter data. All females were allowed to naturally deliver their litters. The day of parturition was designated as LD 0 for dams and PND 0 for pups. The number of live and dead pups, sex, body weights, and thorough clinical examinations (hand-held detailed evaluation of skin, fur, mucous membranes, respiration, palpable masses, and central nervous system functions including tremors and convulsions, diarrhea, animal behavior) were conducted on LD 0, 1, 4, 7, 14, and 21.

TABLE 1
Daily Dietary Ingestion and Corresponding Systemic Dose of 2,4-D to P1 and F1 Male and Female Rats

Diet (ppm)	Dose		AUC _{24h} (µg/h/ml)		Dose		AUC _{24h} (µg/h/ml)	
	(mkd) ^a	Fold Difference ^b	Plasma	Fold Difference ^b	(mkd) ^a	Fold Difference ^b	Plasma	Fold Difference ^b
	P1 male (test day 28)				P1 male (test day 71)			
100	5	1×	14	1×	5	1×	18	1×
400	21	4×	60	4×	19	4×	84	5×
800	41	8×	147	11×	40	8×	160	9×
1200	69	13×	179	13×	63	13×	579	33×
1600	79	15×	579	41×	81	16×	672	38×
	P1 female (test day 29)				P1 female (test day 95)			
100	6/7	1×	26/21	1×	7	1×	25	1×
200 ^c	14	2×	68	3×	ND	ND	ND	ND
400	25/27	4×	213/233	8/11×	25	4×	201	8×
600 ^c	41	6×	651	31×	49	8×	1628	65×
800	52/56	8/8×	1002/1285	39×	ND	ND	ND	ND
1200	75	12×	4139	160×	73	11×	3618	144×
1600	99	16×	3113	120×	— ^d	—	—	—
	P1 female (gestational day 17)				P1 female (lactational day 14)			
100	7	1×	34	1×	15	1×	87	1×
400	29	4×	311	9×	58	4×	887	10×
800	61	9×	1631	48×	118	8×	2470	29×
1200	90	12×	2893	85×	140	9×	5759	66×
1600	114	16×	4253	126×	173	11×	6449	74×
	P1 female (lactational day 4)				P1 female (lactational day 14)			
100	10	1×	72	1×	15	1×	87	1×
400	37	4×	347	5×	58	4×	887	10×
800	71	7×	4703	65×	118	8×	2470	29×
1200	92	9×	5835	81×	140	9×	5759	66×
1600	124	13×	6992	97×	173	11×	6449	74×
	F1 male pup (postnatal day 35)				F1 female pup (postnatal day 35)			
100	14	1×	38	1×	14	1×	35	1×
400	57	4×	204	5×	58	4×	305	9×
800	123	9×	1655	43×	121	9×	2439	71×

Notes. Data are mean of 4 animals. Gray-shaded area indicates doses at which nonlinear TK was observed.

Abbreviations: AUC_{24h}, area under the time-course plasma concentration curve; 2,4-D, 2,4-dichlorophenoxyacetic acid; mkd, mg/kg/day; ND, not determined; TK, toxicokinetics.

^aCells with 2 values show data from the original TK (first) and TK refinement (second) studies.

^bFold differences in actual intake and daily systemic dose (AUC_{24h}) of 2,4-D with reference to the lowest dose.

^c200- and 600-ppm doses were added to the study to further refine the point of departure from linearity.

^dData are not available as these dams were removed from study prior to lactation day 21 due to excessive toxicity.

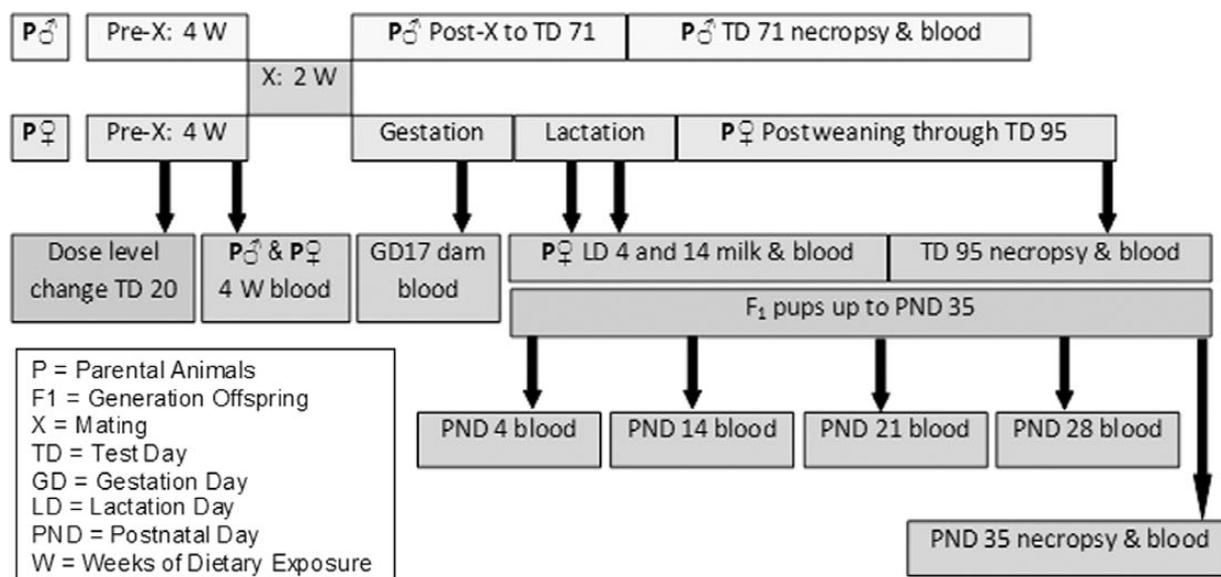


FIG. 1. Study design/TK sampling from parents and offspring after exposure of 2,4-D through diet. Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; TK, toxicokinetics.

Culling and weaning. To minimize variation in pup growth due to differences in litter size, litters were culled on LD 4 to 10 pups (5 males and 5 females, whenever possible). Culled pups were euthanized by exsanguination and grossly examined. All litters were weaned on PND 21. Weanlings were group housed with same sex littermates after weaning from PND 21–28 and individually housed from PND 28–35.

Observations, body weights, and body weight gains. Cage-side examinations for apparent signs of toxicity or injury were conducted at least twice a day on all rats. All adult rats were weighed immediately pre-exposure and weekly during the 4-week pre-mating periods. Males were weighed weekly until termination. Following confirmed pregnancy, females were weighed on GD 0, 7, 14, and 20 (GD 0, 7, 14, and 17 for satellite rats) and on LD 0, 1, 4, 7, 14, 21, and 22 (termination); weaned offspring were weighed on PND 28 and 35. Body weight gains of pregnant females were calculated for GD 0–7, 7–14, 14–20 (14–17 satellite rats), 0–20 (0–17), and LD 1–4, 4–7, 7–14, 14–21, and 1–21 intervals.

Feed consumption and test material intake. Feed consumption was determined pre-exposure and during the same intervals over which body weights were collected by weighing feed containers at the start and end of a measurement cycle. Feed consumption was measured in males and prebreed females at weekly intervals, for pregnant females on GD 0, 7, 14 (17 satellite rats), and 20, and for lactating females on LD 1, 4, 7, 11, 14, 17, 19, and 21. Feed consumption was measured for weaned offspring on PND 28–35. Test material intake (TMI) was calculated from feed consumption data, feed concentrations, and body weights; TMI in dams was not determined during the last week of lactation due to competing feed consumption from the pups.

Parturition and litter data. The day of parturition, number of live and dead pups on LD 0, 1, 4, 7, 14, and 21, sex, and body weight of each pup on LD 1, 4 (before and after culling), 7, 14, and 21 were recorded. Clinical observations on pups were conducted on days 0, 1, 4, 7, 14, and 21 postpartum. Prior to weaning on PND 21, pups that died or were euthanized due to excessive toxicity were discarded without additional data collection.

Necropsy and pathology. On the morning of termination, parental (P1) males (TD 71), female adult rats (TD 96), fasted for approximately 16 h, were weighed, anesthetized with isoflurane; blood was collected from the orbital sinus; the rats were euthanized by decapitation; and a complete necropsy was conducted. Based on previous 2,4-D organ toxicity data (Charles *et al.*,

1996a,b), kidney weights were recorded for organ-to-body weight ratios and preserved in neutral phosphate-buffered 10% formalin for histopathological examination. Kidneys were processed for sectioning (approximately 6- μ m thick), stained with hematoxylin and eosin. Histopathological examinations of kidneys were initially conducted in high-dose adult rats and preceded in descending order until histopathological changes were not observed for an entire lower dose group. F1 offspring were euthanized at scheduled necropsy (PND 35) and examined grossly.

Blood collection. Blood samples (approximately 200 μ l from the jugular vein of nonanesthetized rats) were collected at 6 AM, 9 AM, and 5 PM on the designated days from randomly selected adult animals (4 rats/sex/dose) at the end of the prebreeding exposure (TD 28 or 29) and prior to euthanasia of the TD 71 males and TD 96 females (Fig. 1). This sampling protocol was previously demonstrated to effectively quantitate plasma C_{max} , C_{min} , and diurnal area under the time-course plasma concentration curve (AUC_{24h}) for 2,4-D administered by diet to rats (Saghir *et al.*, 2006). To determine potential changes in the diurnal systemic dose of 2,4-D during lactation, 3 blood samples were collected from lactating dams (4 females/dose group) on LD 4, 14, and 21 as described previously.

Three blood samples were collected from the satellite P1 females on GD 17 (4 rats/dose group) as described previously. After the last blood collection (5 PM), satellite females were anesthetized with isoflurane, examined internally to confirm pregnancy, and humanely euthanized. Fetuses were also euthanized by an IP injection of Socumb euthanasia solution (Veterinary Laboratories Inc, Lenexa, Kansas).

Systemic exposure of 2,4-D to nursing pups was determined by collecting terminal blood samples at different stages of postnatal development (3 blood samples were not collected from pups \leq PND 28 due to insufficient blood volume). Blood samples were collected from 1 randomly selected culled pup/sex/litter (4 litters/dose) on PND 4 and 1 randomly selected pup/sex/litter (4 litters/dose) on PND 14, 21, and 28. At all ages, pups were anesthetized using isoflurane, and the blood was collected in heparinized tubes after nicking the left ventricle of the heart. Following blood collection, pups were grossly examined and humanely euthanized. In some cases, PND 4 samples were pooled from additional culled littermates of the same sex to yield greater blood volumes.

To determine changes in the kinetic parameters of 2,4-D in pups after weaning, 3 blood samples as described previously were collected on PND 35 from at

least 1 pup/sex/litter (4 litters/dose). After the final blood collection, pups were anesthetized, grossly examined, and humanely euthanized.

Milk collection. Milk (approximately 300 μ l) was collected from 4 dams/dose on LD 4 and 14. Approximately, 4 h before milk collection (ie, 10–11 AM), dams were separated from the pups. Shortly before milk collection, dams were administered SC 2 United States Pharmacopeia (USP) units oxytocin in 2 split doses, 5–10 min apart. Dams were anesthetized by isoflurane inhalation, and milk was collected into nonheparinized hematocrit tubes by gently squeezing the gland and the nipple area. Pups were returned to dams immediately after recovery from anesthesia. To limit stress to the lactating dams, different dams were used for milk and blood collection on LD 4 and 14.

Analysis. Immediately after collection, blood was transferred into heparinized hematocrit tubes and centrifuged to obtain plasma. Both plasma and milk were stored in the -80°C freezer until analyzed. The stability of 2,4-D in plasma and milk (stored at -80°C) was confirmed at 2 and 5 weeks after sample collection (data not shown). Internal standard (uniformly ring-labeled ^{13}C -2,4-D) was added to the weighed plasma/milk samples followed by the precipitation of proteins by adding acetonitrile and vortexing. The precipitated proteins were removed by filtration, and the supernatant was analyzed for 2,4-D by liquid chromatography-mass spectrometer with electrospray ionization as described by Saghir *et al.* (2006).

Data analysis. The systemic toxicity data were analyzed for the equality of variances by Bartlett's test ($\alpha = 0.01$), and ANOVA was conducted for the comparison of controls and treated groups, followed by Dunnett's test ($\alpha = 0.05$; Winer, 1971). Data from the neonates/litters were analyzed by Wilcoxon test (Haseman and Hoel, 1974) with Bonferroni's correction.

The TK data were used to estimate plasma C_{max} , C_{min} , and daily systemic dose ($\text{AUC}_{24\text{h}}$) of 2,4-D. The $\text{AUC}_{24\text{h}}$ was calculated from the 3 plasma samples extrapolated to 24 h assuming steady-state plasma concentrations and similar daily fluctuations of the systemic dose due to the feeding pattern of the rats (McCoy *et al.*, 2012; Saghir *et al.*, 2006, 2012). Dose-dependent TK behavior was determined graphically and by comparing ratios of plasma 2,4-D $\text{AUC}_{24\text{h}}$ at any selected dose to the lowest tested dose (100 ppm) and assuming linear TK behavior at the 100-ppm dose. The comparison was made both for the nominal (ie, ppm in diet) and the actual ingested (mg/kg/day) doses. Microsoft Excel was used to calculate TK parameters.

RESULTS

Body Weight/Body Weight Gain and Feed Consumption

No statistically significant differences in body weights/body weight gains and feed consumption of P1 male rats were identified at any dose during the course of the study (Supplementary Tables 1–3 and 5). At pre mating, TD 31, females were given 800-, 1200-, and 1600-ppm doses weighed 3%, 8%, and 10% less than controls; body weight gains were 19%, 50%, and 64% less than controls, respectively. No body weight effects were observed in the 100- and 400-ppm dose groups during the study. Over the entire gestational period (GD 0–20), no significant effects on body weights/body weight gains were observed. Feed consumption during lactation was significantly decreased (7%–19%, 14%–39%, and 20%–36% below control) in females exposed to 800-, 1200-, and 1600-ppm 2,4-D, respectively. Due to a marked decrease in feed consumption in the 1600-ppm dams and associated pup effects (see Toxicity to Pups section), litters of the 1600-ppm group were removed from the study on LD 14. For similar reasons, litters of the 1200-ppm group were terminated around the time of weaning (LD 21). Animals in

all other treatment groups survived until scheduled termination. Dietary concentrations of 1200- and 1600-ppm 2,4-D clearly exceeded the maximum tolerated dose (MTD) for both P1 females and F1 animals and thus were determined to be unacceptably high for the subsequent extended 1-generation study.

Daily TMI

Daily doses of 2,4-D expressed as mg/kg/day, when averaged over the periods of pre mating (males and females), post mating (males), gestation, lactation and post weaning (females), and PND 35 (pups), were consistent with dietary intake (Table 1). The inversion in pre breeding (TD 29) $\text{AUC}_{24\text{h}}$ at 1200- and 1600-ppm doses in females was due to the intentional decrease in dose levels from 2000 to 1200 ppm on TD 20 in response to excessive decreases in body weight gain. Saturation of elimination from kidney at 2000 ppm led to much slower elimination of the systemic dose. Due to fixed dietary concentrations of 2,4-D, lactating females received higher daily doses of 2,4-D associated with increased feed consumption during this period. TMI was not calculated for the LD 14–21 period because maternal feed consumption values were confounded by pup feed intake. The increased daily TMI in PND 28–35 pups relative to adults corresponded to increased feed consumption per kilogram body weight during this period of rapid growth.

Toxicity to P1 Animals

No treatment-related effects were observed at any exposure level on conception, time to mating, gestation length, or pup-sex ratio (Table 2; Supplementary Table 4). The male and female fertility (female fertility index = [number of females with evidence of pregnancy/number paired] \times 100; male fertility index = [number of males siring a litter/number paired] \times 100) and mating (female mating index = [number of females with evidence of mating/number paired] \times 100; male mating index = [number of males with evidence of mating/number paired] \times 100) indices were 70% and 80%, respectively, at 1600 ppm compared with 100% in control rats. However, because the toxicity assessments in this study were designed only as a dose range-finding exercise supporting the conduct of an extended 1-generation reproductive toxicity study, the small sample sizes, omission of uterine staining for implantation sites, and lack of gross or microscopic examination precluded the determination of whether these findings were treatment-related effects. There were no effects on the mean number of live or dead pups born per litter, which makes an effect on fertility less likely. There were no findings on any reproductive parameters at lower exposure concentrations (up to 1200 ppm) that might be attributable to treatment.

No significant differences were observed in the terminal body weights of either male or female animals. Relative kidney weights were significantly increased in males only at 1600-ppm 2,4-D; in females, both absolute and relative kidney weights were significantly increased in the 800- and 1200-ppm dose groups. Males given 400, 800, 1200, or 1600 ppm and females

TABLE 2
Terminal Body and Kidney Weights and Histopathological Results of P1 Male and Female Rats

Endpoint	Dietary Concentration (ppm)					
	0	100	400	800	1200	1600
P1 males						
Terminal body weights (g) ^a	531	538	526	534	511	509
Absolute kidney weights (g)	3.64	3.87	3.73	3.92	3.85	4.08
Relative kidney weights (g/100 g bw)	0.68	0.72	0.71	0.74	0.75	0.81*
Kidneys (examined) ^b	10	10	10	10	10	10
Very slight	1	1	6	8	4	7
Slight	0	0	0	0	4	1
P1 females						
Terminal body weights (g) ^a	288	300	301	283	282	—
Absolute kidney weights (g)	1.97	2.10	2.15	2.24*	2.33*	—
Relative kidney weights (g/100 g bw)	0.68	0.70	0.72	0.79*	0.83*	—
Kidneys (examined) ^b	10	10	10	10	10	—
Very slight	0	0	0	5	3	—
Slight	0	0	0	0	2	—

Note. Bold italicized values were interpreted to be treatment related.

Abbreviation: bw, body weight.

^aData are mean of 5–10 animals.

^bKidneys were examined for degeneration, multifocal, proximal convoluted tubule, outer strip, outer zone and classified as very slight and slight.

*Statistically different from control mean by Dunnett's test, alpha = 0.05.

given 800 or 1200 ppm of 2,4-D had treatment-related very slight or slight microscopic degenerative multifocal lesions in the kidneys involving the proximal convoluted tubules in the outer stripe of the outer zone of the medulla.

Toxicity to Pups

Decreased pup survival during lactation was observed among litters of the dams exposed to 1200 and 1600 ppm of 2,4-D (Supplementary Tables 2, 4, and 5). Decreased pup body weight was observed at doses ≥ 800 ppm (Supplementary Table 2). Decreased pup growth coincided with marked decreases in dam feed consumption during lactation (Supplementary Table 3). F1 offspring in the 1200- and 1600-ppm groups had a greater incidence of "stomach void of milk," "cold to touch," and "decreased activity" (data not shown). Most of the pup mortality occurred during the last third of lactation (\geq LD 17; Supplementary Table 5) coinciding with the period of largest feed consumption in lactating dams and self-feeding in the offspring (Table 1). Hanley and Watanabe (1985) have shown that nursing rat pups begin to eat significant quantities of parental diet after PND 15.

Systemic Dose to P1 Males on TD 28 and 71

The daily systemic dose after 28 days of dietary exposure to 2,4-D-fortified diet, as measured by AUC_{24h} was dose-proportional up to the second highest dose (69 mg/kg/day; 1200 ppm; Table 1; Supplementary Table 6). The TK became distinctly nonlinear at the highest (1600 ppm) dose; mean AUC_{24h} values for rats fed 1600 ppm was 41-fold higher than the 100-ppm dose group, despite only a 15-fold difference in ingested 2,4-D (5.5 vs 79.2 mg/kg/day; gray highlighted cells in Table 1), which is consistent with earlier findings of Saghir et al. (2006)

in male rats. Exposure for 10 weeks resulted in saturation of renal elimination of 2,4-D in males at the 1200-ppm dietary concentration (gray highlighted cells in Table 1).

Systemic Dose to P1 Females on TD 29 and 95

Female rats had lower capacity for 2,4-D clearance than did male rats; females had higher 2,4-D AUC_{24h} values across all dose groups, and this correlated with increased systemic toxicity as measured by decreased body weights (Table 1; Supplementary Table 7). After 4 weeks of exposure to 2,4-D-fortified diet (TD 29), nonlinear TK was apparent at ≥ 400 ppm (gray highlighted cells in Table 1). A 4-, 8-, 12-, or 16-fold increase in the dose from 100 ppm resulted in an increase in the diurnal systemic dose (AUC_{24h}) by >8 -, 39-, 160-, or 120-fold, respectively. The plasma AUC_{24h} at 1600 ppm on TD 29 was slightly lower than 1200 ppm, which was likely due to residual effects from the reassignment of animals at 2000- to 1200-ppm dietary concentration on TD 20. The interval between the reassignment of the dose and blood collection (9 days) likely was not long enough for female rats to clear the steady-state body burden of 2,4-D from the previously assigned 2000-ppm dietary exposure, resulting in a higher plasma concentration than the animals fed diets containing 1600 ppm. The pattern of TK behavior in females after completing the life stages of gestation and lactation (95 days of exposure to 2,4-D-fortified diet, prior to necropsy on TD 96) was similar to that after 29 days of exposure, with nonlinearity occurring ≥ 400 ppm (gray highlighted cells in Table 1).

The initial TK assessment indicated that females fed 400 ppm of 2,4-D were substantially above the point of TK nonlinearity (8-fold increase in AUC_{24h} vs 4-fold increase in TMI), thus, a supplementary 28-day TK study was conducted to refine the

potential point for the onset of nonlinear TK. Treatment with 200 ppm of 2,4-D yielded a 3-fold increase in plasma 2,4-D levels, which was associated with only a 1.9-fold increase in dose as measured by TMI. These data suggest that for female rats, 200 ppm is at, or slightly above, the point of nonlinear dietary TK. The addition of the 600-ppm dose group to the supplemental study further confirmed that 600 ppm was substantially above the threshold for nonlinear TK (31-fold increase in AUC_{24h} vs 6-fold increase in TMI). The close overlap of TK data (AUC_{24h}) between the original and subsequent dose refinement study in female rats also indicated the reproducibility of the TK measurement methodology (Table 1; Supplementary Table 6).

Systemic Dose to P1 Females on GD 17

As expected, pregnancy slightly increased dietary intake, which resulted in an average of 17% higher TMI on GD 17 when compared with the intake for the same rats prior to pregnancy (TD 29; Table 1; Supplementary Table 8). The higher TMI translated into 31%–46% higher AUC_{24h} of 2,4-D on GD 17 than TD 29, with nonlinear TK apparent at doses ≥ 400 ppm (Table 1; Supplementary Tables 7 and 8).

Systemic Dose to P1 Females on LD 4 and 14

During LD 1–4, feed consumption increased 57% in control animals (data not shown) and 23%–57% in 2,4-D-treated animals relative to feed consumption by preparturient females (TD 29; Table 1; Supplementary Table 9). A higher TMI was apparent not only from increased TMIs but also from the 2.8-, 1.6-, 4.7-, 1.4-, and 2.2-fold higher AUC_{24h} at 100-, 400-, 800-, 1200-, and 1600-ppm dietary concentrations, respectively, when compared with nonlactating rats (Table 1; Supplementary Table 9). When

measured against the baseline of 100-ppm AUC_{24h} value, nonlinear TK behavior in LD 4 rats was highly apparent at doses ≥ 800 ppm (gray highlighted cells in Table 1). It is important to note, however, that the baseline 100-ppm AUC_{24h} (72 $\mu\text{g}/\text{h}/\text{ml}$) in LD 4 rats was slightly above the AUC_{24h} of the 200-ppm TD 29 nonpregnant rats (67.6 $\mu\text{g}/\text{h}/\text{ml}$), a dose established as either at or slightly above nonlinear performance (Table 1). Thus, the inability to conclusively identify the 400-ppm LD 4 dose group as being above the point of nonlinear TK may have been attributable to the high AUC_{24h} baseline associated with the 100-ppm LD 4 treatment group. This conclusion is further evidenced by the observation that the 400-ppm LD 4 AUC_{24h} was approximately 13-fold higher than nonpregnant TD 29 100-ppm AUC_{24h} and 10-fold higher than GD 17 100 ppm. However, additional systemic clearance of 2,4-D into milk may have in part contributed to the failure to observe nonlinear TK in the 400-ppm treatment group (Table 3).

With increasing size of the pups and higher demand for milk, the dams further increased dietary intake during mid-lactation. By LD 14, control dams increased feed consumption by 2.3-fold compared with preparturient feed consumption (data not shown). Increased dietary intake among 2,4-D-treated dams on LD 14 was 1.8- to 2.5-fold higher when compared with TD 29. The increased diet intake in control and exposed rats on LD 14 translated into 2- to 4-fold higher systemic doses when compared with nonlactating rats on TD 29 (Table 1). On LD 14, the systemic dose became nonlinear ≥ 400 ppm (gray highlighted cells in Table 1).

Elimination of 2,4-D in Milk by P1 Females

2,4-D was excreted in milk on both LD 4 and 14 (Table 3; Supplementary Table 10). On LD 4, the concentration of 2,4-D

TABLE 3
Daily Ingested 2,4-D and Its Relationship With Levels in Dam Blood, Milk, and Nursing Rat Pups

Diet (ppm)	Systemic Dose		Dam Plasma		Milk		Pup Plasma ^a		Ratio ^b (%)
	(mkd)	Difference ^c	($\mu\text{g}/\text{ml}$)	Difference ^c	($\mu\text{g}/\text{g}$)	Difference ^c	($\mu\text{g}/\text{ml}$)	Difference ^c	
Lactational/postnatal day 4									
100	9.9	1 \times	2.9 \pm 1.5	1 \times	1.1 \pm 0.5	1 \times	0.8 \pm 0.7	1 \times	28
400	37.4	3.8 \times	15.5 \pm 11.5	5 \times	9.3 \pm 4.3	9 \times	4.7 \pm 3.0	6 \times	30
800	71.3	7.2 \times	194.5 \pm 38.5	67 \times	30.8 \pm 9.2	29 \times	94.2 \pm 33.7	118 \times	48
1200	92.5	9.4 \times	243.0 \pm 51.2	84 \times	74.8 \pm 19.4	69 \times	173.6 \pm 41.7	217 \times	71
1600	123.6	12.5 \times	292.5 \pm 65.4	101 \times	106.9 \pm 59.9	99 \times	195.4 \pm 32.3	244 \times	67
Lactational/postnatal day 14									
100	15.5	1 \times	3.7 \pm 1.7	1 \times	2.4 \pm 1.1	1 \times	2.8 \pm 4.2	1 \times	76
400	57.5	3.7 \times	36.9 \pm 16.3	10 \times	14.7 \pm 4.0	6 \times	46.1 \pm 19.0	16 \times	125
800	117.5	7.6 \times	102.1 \pm 19.3	28 \times	68.9 \pm 20.1	29 \times	173.3 \pm 33.3	62 \times	170
1200	139.6	9.0 \times	237.8 \pm 51.3	64 \times	104.7 \pm 20.0	45 \times	305.4 \pm 68.4	109 \times	128
1600	173.0	11.2 \times	266.4 \pm 25.3	72 \times	127.6 \pm 76.2	54 \times	360.5 \pm 32.4	129 \times	135

Notes. Data are mean of 4 animals. Gray-shaded area indicates doses at which nonlinear TK was observed.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; mkd, mg/kg/day; TK, toxicokinetics.

^aAverage of male and female pup plasma concentration of 2,4-D.

^bRatio of the concentration of 2,4-D in dam versus pup blood ($\text{blood}_{\text{pup}} \div \text{blood}_{\text{dam}}$).

^cFold differences in actual intake and concentration of 2,4-D with reference to the lowest dose.

in milk was 1.7- to 6.3-fold lower than the concentration in the circulating dam plasma; the ratio was reduced to 1.5- to 2.5-fold on LD 14 (Table 3; Supplementary Table 10). The approximate doubling of the concentration of 2,4-D in milk on LD 14 was due to the approximate doubling of TMI on LD 14 relative to LD 4 (Tables 1 and 3; Supplementary Table 10). Paralleling dam plasma concentrations, milk concentrations increased in a dose-dependent nonlinear manner at doses ≥ 400 ppm relative to the 100-ppm treatment group (gray highlighted cells in Table 3).

Systemic Exposure of F1 Pups on PNDs 4 and 14

As expected from the concentrations of 2,4-D in the milk, nursing-pup plasma on PND 4 had quantifiable levels of 2,4-D (Table 3; Supplementary Table 10). Unlike the differential plasma concentrations observed in male and female adults treated with the same dietary concentration of 2,4-D, concentrations of 2,4-D in the plasma of male and female pups treated with equivalent doses were approximately equal on PND 4 and averaged 2.2 ± 0.8 - and 2.6 ± 1.3 -fold lower than that of the dams, respectively. Nonlinear TK was exhibited in both male and female pups at doses ≥ 800 ppm on PND 4 as plasma concentration of 2,4-D markedly exceeded the proportional increase in dietary concentrations administered to lactating dams (gray highlighted cells in Table 3). A 118-fold increase in pup plasma 2,4-D was observed between the 100- and 800-ppm doses; however, there was only a 7-fold increase in dam TMI for these same dose intervals (Table 3; Supplementary Table 10).

With the increase in the dietary intake by dams on LD 14 and corresponding higher concentration of 2,4-D in milk, 2- to 10-fold higher plasma concentrations of 2,4-D were observed in the PND 14 when compared with PND 4 pups (Table 3; Supplementary Table 10). Again, concentrations of 2,4-D in the plasma of male and female pups were approximately equal on PND 14. On LD 14, plasma concentrations of 2,4-D in

100-ppm pups were 1.3-fold lower than in the dams; however, at higher doses (400–1600 ppm), plasma concentrations were higher (1.3- to 1.7-fold) in the pups than in the dams. Nonlinear TK was observed in PND 14 pups at maternal doses ≥ 400 ppm, with plasma concentrations in pups markedly exceeding the proportional increase in dietary concentrations administered to lactating dams (gray highlighted cells in Table 3). However, as observed with the P1 LD 14 dams, the 2.8- $\mu\text{g}/\text{ml}$ pup plasma concentration seen at the 100-ppm comparator dose may have been at or slightly exceeding renal saturation in that this pup plasma concentration was similar to mean plasma concentrations of 2.30–3.67 $\mu\text{g}/\text{ml}$ observed in TD 29 adult females treated with 200 ppm of 2,4-D (Supplementary Table 7).

Systemic Exposure of F1 Pups on PND 21

Pups were weaned on day 21; thus, pups sampled on PND 21 received test material through both milk and feed consumption (Table 4; Supplementary Table 10). Pup feed consumption also increases markedly late in lactation (Hanley and Watanabe, 1985). Consequently, the average of male and female plasma 2,4-D levels in PND 21 pups were 3.2-, 2.5-, and 1.7-fold higher at dietary concentrations of 100, 400, and 800 ppm, respectively, when compared with the plasma concentration of 2,4-D in PND 14 pups (Tables 3 and 4; Supplementary Table 10). At 1200 ppm, the average of male and female pup plasma concentrations of 2,4-D was slightly lower on PND 21 than PND 14. This decrease may have been due to the onset of systemic toxicity in either or both the dams and pups as reflected by prolonged and severe decreases in feed consumption in this dose group during lactation and in postweaning pups (Supplementary Tables 1 and 2). Plasma concentrations of 2,4-D in male and female pups on PND 21 were 19- to 51-fold and 6- to 12-fold higher than male and female adults on TD 28 and 29, respectively (data not shown). Similarly, continuous exposure to test material through both diet and milk resulted in a higher plasma concentration of 2,4-D in PND 21 pups when compared with

TABLE 4
Plasma Concentrations of 2,4-D in F1 Male and Female Rat Pups on PND 21 and 28

Diet (ppm)	Male		Female	
	Difference ^a	($\mu\text{g}/\text{g}$)	Difference ^a	($\mu\text{g}/\text{g}$)
F1 pup plasma 2,4-D on PND 21				
100	1 \times	11.2 ± 8.0	1 \times	6.41 ± 4.0
400	4 \times	126.2 ± 45.7	11 \times	106.6 ± 48.8
800	8 \times	282.2 ± 44.8	25 \times	291.7 ± 62.1
1200	12 \times	262.9 ± 59.1	23 \times	311.6 ± 18.1
F1 pup plasma 2,4-D on PND 28				
100	1 \times	1.9 ± 1.2	1 \times	1.6 ± 0.4
400	4 \times	78.2 ± 49.1	42 \times	108.1 ± 35.7
800	8 \times	223.0 ± 77.6	120 \times	182.0 ± 109.2

Note. Data are mean \pm SD of 4 animals.

Abbreviation: PND, postnatal day.

^aFold differences in dietary and plasma concentration of 2,4-D with reference to the lowest dose.

PND 28 and 35 pups (Tables 1 and 4; Supplementary Tables 10 and 11). Nonlinear TK of 2,4-D were observed in both male and female PND 21 pups at ≥ 400 ppm, as evidenced by plasma concentrations far exceeding the proportional increase in dietary treatments to dams (gray highlighted cells in Table 4). The mean pup plasma concentration of 11.2 $\mu\text{g}/\text{ml}$ in the 100-ppm PND 21 pups was similar to that of 7.16–11.57 $\mu\text{g}/\text{ml}$ seen in adult 400-ppm TD 29 and 95 females, a dose identified as saturating renal clearance (Table 1; Supplementary Table 7), and thus, the 100-ppm dose may be above renal saturation in PND 21 pups.

Systemic Exposure of F1 Pups on PND 28

Plasma concentrations of 2,4-D in the PND 28 pups ranged from approximately 5-fold lower at 100 ppm to ≤ 1.6 -fold lower at 400 and 800 ppm than plasma concentrations in PND 21 pups (Table 4; Supplementary Table 10). The mean pup plasma concentration at 100 ppm was slightly higher than those seen in adult animals (Supplementary Tables 6 and 7), indicating this dose level was below renal saturation. These data are consistent with switching to test material fortified diet alone after weaning and indicative of a significant contribution of milk to total 2,4-D dose in late preweaning rats. However, plasma concentration of 2,4-D in PND 28 pups remained 1.4- to 12-fold higher in females and 3- to 35-fold higher in males compared with the preweaning (male, TD 28; female, TD 29) adults (Table 4 and Supplementary Tables 6 and 7). These results are due to increased feed intake/kg body weight during this period of early postweaning development when compared with young adults. Nonlinear TK of 2,4-D was clearly evident in both male and female PND 28 pups at ≥ 400 ppm (gray highlighted cells in Table 4).

Systemic Exposure of F1 Pups on PND 35

Corresponding to the rapid growth of pups, ingestion of diet relative to the body weight (ie, g/kg) was substantially higher in all groups of PND 35 rats when compared with preweaning adults (Table 1; Supplementary Tables 3 and 11). Consequently, male and female PND 35 offspring ingested $175\% \pm 19\%$ and $130\% \pm 4\%$ higher test material, respectively, than the 4-week exposed adults prior to mating (Table 1; Supplementary Table 3). In male pups, the increase in the systemic dose appeared linear up to 400 ppm, evidenced by the almost dose-proportional increase in the $\text{AUC}_{24\text{h}}$, but was distinctly nonlinear in the 800-ppm dose group (gray highlighted cells in Table 1). The striking nonlinearity in male PND 35 pups at ≥ 800 ppm contrasted to TK performance in adult males in which nonlinearity was observed at 1600 ppm on TD 28 or ≥ 1200 ppm on TD 71 (gray highlighted cells in Table 1) and was attributed to higher TMI in these young animals and possibly incomplete maturation of OAT1 transporter expression. In female pups, nonlinear TK was apparent at 400 ppm (gray highlighted cells in Table 1).

Life-Stage-Dependent Plasma Concentrations From 100- and 400-ppm 2,4-D Treatment

The change in plasma concentration of 2,4-D at different life stages (adult females at TD 29, GD 17, LD 4, LD 14, TD 95; pups at PND 4, PND 14, PND 21, PND 28, and PND 35) of rats exposed to 100 and 400 ppm of 2,4-D in diet is graphically summarized in Figure 2. Relative to plasma concentrations in TD 28–29 adult animals, these data illustrate that 2,4-D plasma levels increase as dams enter lactation and are accompanied by corresponding surges in pup plasma concentrations that are further augmented as late preweaning pups ingest maternal diet. As postweaning pups mature, however, plasma concentrations return to levels approaching those in adult animals.

DISCUSSION

The results of this range-finding study, which identified the point of TK nonlinearity associated with dietary exposure of 2,4-D at various life stages in Sprague Dawley rats, affirmed the value and use of TK data for dose selection decisions for substances like 2,4-D for which there is clear evidence of onset of nonlinear TK at dose levels substantially separated from human exposures.

Both human adults and children have substantially lower systemic doses to 2,4-D than the animals exposed in this TK study. Alexander *et al.* (2007) indicated that children living on farms on which 2,4-D was being actively applied had biomonitored systemic exposures (geometric mean) of 0.12 $\mu\text{g}/\text{kg}$ (children >12 years old) to 0.32 $\mu\text{g}/\text{kg}$ (children 4–11) based on 3-day-recovery values in urine after application. These dose levels were 51, 875- to 171, 667-fold below the NOAEL of 16.6–20.6 mg/kg/day in a recently conducted extended 1-generation reproduction toxicity study intended to evaluate the perinatal toxicity potential of 2,4-D, and in which no toxicity was observed in either parental or offspring animals at this dose level (Marty *et al.*, 2013). The margins of exposure (MOE) for children would be even greater if measured against the dose identified as the inflection point for onset of nonlinear TK in female rats (approximately 25 mg/kg/day; Table 1) and which was targeted for the approximate top dose level selection in female rats in the extended 1-generation reproduction toxicity study. Large MOEs similarly exist for adults biomonitored for 2,4-D exposure in Alexander *et al.* (2007); geometric mean doses of 2.46 and 0.8 $\mu\text{g}/\text{kg}$ were found for applicators and spouses, respectively. Importantly, low adult and children 2,4-D exposures (<5 $\mu\text{g}/\text{kg}$) have been confirmed in a variety of other biomonitoring studies (Harris and Solomon, 1992; Morgan *et al.*, 2008; Thomas *et al.*, 2010; Yeary, 1986).

As recommended by ACSA, the life-stage- and dose-dependent TK assessment demonstrates the value of this type of information for rational dose selection decisions for rodent toxicity studies. Consideration of TK data in concert with MOE information between humans and potential animal toxicity test doses

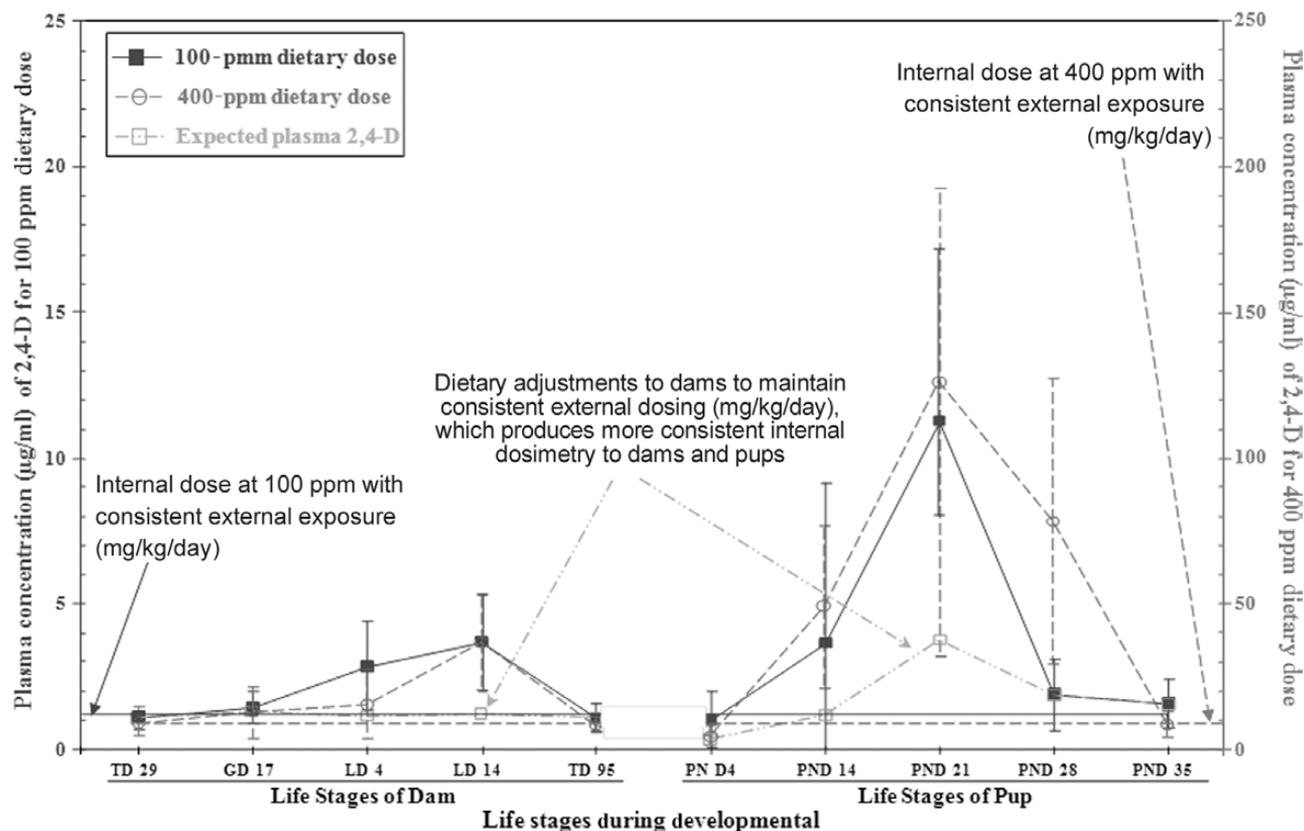


FIG. 2. Plasma concentrations of 2,4-D in dams and pups in various life stages following exposure to 100- or 400-ppm 2,4-D-fortified diet. Without adjusting dietary concentrations of 2,4-D, dose levels (mg/kg/day) were markedly greater than nominal during the lactation period in dams, which impacted internal dosimetry in both dams and pups during this period. Furthermore, offspring dose levels (mg/kg/day) were higher than nominal from PND 21 through PND 35, which also increased internal doses in the F1 offspring. Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; LD, lactation day; PND, postnatal day; TD, test day.

avoids selection of high test dose(s) in experimental animals that are irrelevant to real-world exposures due to high-dose-specific saturation of metabolic processes (Carmichael *et al.*, 2006; Conolly *et al.*, 1999; OECD, 2011; Saghir *et al.*, 2012; Slikker *et al.*, 2004a,b). Such inappropriately high dose levels are not informative of either hazard identification or potential human health risks, and ultimately detract from the quality of the risk assessment by artificially limiting the number of doses available for consideration of dose-response evaluation that are in the range of nonsaturated biological function. Conventionally, high dose levels in toxicity studies are selected based on systemic toxicity using the MTD approach, which is based largely on consideration of body weight decreases and/or other generally marked evidence of systemic toxicity. However, the ACSA project and others have strongly recommended that TK information should be factored into selection of the high dose used in toxicity studies, and that with evidence of TK saturation, the high dose should be set at or slightly above the point of TK nonlinearity (Carmichael *et al.*, 2006; Creton *et al.*, 2012; OECD, 2011; Saghir *et al.*, 2006, 2012), provided that the dose selected provides adequate margins of safety based on human exposures. Thus, selection of high dose levels based on evidence of TK nonlinearity will produce toxicity data that are more meaningful for extrapolation of human risk.

Recently, consideration of TK in the design of animal toxicity tests including life-stage-specific studies has been officially adapted in the OECD 443 toxicity test guideline for conduct of extended 1-generation reproduction studies (OECD, 2011). The guideline states that “TK data from previously conducted dose range-finding or other studies are extremely useful in the planning of the study design, selection of dose levels and interpretation of results. Of particular utility are data which: (1) verify exposure of developing fetuses and pups to the test compound (or relevant metabolites), (2) provide an estimate of internal dosimetry, and (3) evaluate for potential dose-dependent saturation of kinetic processes.” The design of this 2,4-D study, which fundamentally followed the OECD guidance, provides a practical demonstration of the implications of both the ACSA and OECD guidance for facilitating dose selection in life-stage-specific toxicity studies (Marty *et al.*, 2013, Table 5). If the toxicity data alone from this pilot study had been employed in the conventional MTD or toxicity-derived maximum dose approach for dose selection, the top dose levels for parental males and females for a life-stage toxicity test would have been set at 2- to 4-fold higher than top doses identified incorporating TK information identified in this study (Table 5). This differential in dose selection strategy was also apparent on examination of

TABLE 5
Comparison of Conventional MTD and K-MD of 2,4-D in Different Life Stages of Rats

Group and Life Stage	Dietary Dose (ppm)		
	LOEL ^a	MTD ^b	KMD ^c
Adult male			
Adult	400	>1600	Between 800 and 1200
Adult female			
Premating		800	Between 200 and 400
Gestation to termination	800	Between 400 and 800	400
F1 offspring			
Pups	800	800	400
Postweaning	800	800	400
PND 35 (termination)	800	800	400 (females) and 800 (males)

Note. Data are mean of 5–10 animals.

Abbreviations: LOEL, lowest observed effect level; KMD, kinetically derived maximum dose; MTD, maximum tolerated dose; PND, postnatal day.

^aLOEL values were based on kidney histopathology in adult males, increased kidney weights and histopathology in adult females, and decreased body weights in pups.

^bDose level where significant systemic toxicity is seen. MTD values were based on evidence of overt toxicity (decreased body weights/gains; [Supplementary Tables 1–3](#)) because there were no clinical signs of toxicity and kidney effects were not severe.

^cDose level where kinetics become nonlinear.

pup toxicity and TK. The concept of using TK as a key determinant for dose selection relative to a conventional MTD has been termed “kinetically derived maximum dose” or KMD ([Saghir et al., 2012, Table 5](#)). Thus, top doses of 1600 ppm for parental males and 800 ppm for females ([Table 5](#)) would have been selected had a conventional MTD dose selection strategy been applied in a recently conducted 2,4-D extended 1-generation reproduction study using only the toxicity data from this pilot study ([Marty et al., 2013](#)). Such doses clearly would have been toxicologically irrelevant for human risk assessment given the very large MOEs between actual human exposures and onset of nonlinear TK in test animals, and would have had the practical consequence of providing only 2 dose levels for evaluation of a key element of risk assessment, dose response.

The life-stage-specific examination of TK in this study also provided clear evidence for the need to adjust 2,4-D dose levels to both lactating dams and early postweaning pups in rat reproduction studies to avoid overdosing that would occur during these life stages if test material is offered at a constant ppm level in diet administered *ad libitum* ([Fig. 2](#)). A 2- to 4-fold increase in feed consumption usually occurs in dams during lactation to support the adequate production of milk for the offspring ([Widdowson, 1976](#)), and postweaning pup food consumption per kg of body weight is likewise higher to meet energy demands during this period of rapid growth. The data in [Figure 2](#) illustrate that in the absence of dose adjustments during these periods of differential food consumption relative to body weight, nonlinear TK behavior is further accentuated for compounds such as 2,4-D in which TK is saturated. Thus, the data in this study indicate that consideration of TK data during altered food intake periods in life-stage studies support dietary concentration adjustments during lactation for more consistent dosimetry (mg 2,4-D/kg body weight/day) over a given dose

level and life stage to avoid significant nonlinear TK excursions due to increased feed intake by lactating dams and postweaning pups. As emphasized previously, the magnitude of the MOEs between demonstrated human exposures and dose levels used in animal toxicity studies indicates that such extreme TK excursions further limit the value of any toxicity data collected under such high dose conditions, and fully justifies the use of dietary adjustments to keep the lactating dams and early postweaning pups as near as possible to the threshold for nonlinear kinetics with chemicals like 2,4-D ([Fig. 2; Marty et al., 2013](#)).

2,4-Dichlorophenoxyacetic acid is rapidly excreted unchanged in the urine by OATs in both animals and humans ([Arnold and Beasley, 1989; Gehring and Betso, 1978; Hasegawa et al., 2003; Nozaki et al., 2007; Timchalk, 2004](#)). With the exception of dogs, 2,4-D kinetics appear to be similar in other mammalian species examined, including humans who also share renal OATs. Because of extensive plasma protein binding, approximately 93%–97% over a broad range of concentrations ([Griffin et al., 1997; van Ravenzwaay et al., 2003](#)), these compounds are not readily filtered at the glomerulus but rather are actively secreted into urine via a saturable OAT1 carrier protein located on the basolateral membrane of renal proximal tubules. The results of this study and many others are consistent with a dose-dependent saturation of 2,4-D clearance associated with the OAT1 transporter ([Gehring and Betso, 1978; Gorzinski et al., 1987; Kim et al., 1994; Smith et al., 1980; Timchalk, 2004; van Ravenzwaay et al., 2003](#)). Interestingly, 2,4-D also is eliminated from brain by the same OAT1 mechanism located at the plasma membrane of rat brain capillaries ([Kusuhara and Sugiyama, 2001a,b](#)). Disproportionately high plasma levels of 2,4-D resulting from saturation of renal clearance could thus result in increased distribution to brain and the associated possibility of onset of secondary nonlinear TK in that organ

attributable to saturation of brain OAT1 clearance. However, the potential for high-dose-specific neurotoxicity associated with elevated brain concentrations of 2,4-D is dependent on such a sequential coupling of both kidney and brain OAT1 saturation that it would not be relevant to human risk assessment given the wide separation between doses required for saturation and actual human exposures.

The gender differences observed in dietary TK in this study are entirely consistent with sex-dependent differences in 2,4-D clearance mediated by OAT1 (Gorzinski *et al.*, 1987; Timchalk, 2004). Reyes *et al.* (1998) demonstrated that male rats have higher renal excretion rates for organic acids than females. This difference was hypothesized to be due to a testosterone-induced increase in the number of functional transporters available in the kidney for organic acid secretion, a finding consistent with the observation that adult male rats express higher levels of OAT1 than adult females (Buist *et al.*, 2002). These gender differences in OAT1 and organic acid transport are consistent with the current TK data, wherein female rats exhibited nonlinear TK at lower doses than males. The slower elimination of 2,4-D in females also was reported by van Ravenzwaay *et al.* (2003) after PO gavage dosing.

The 2,4-D life-stage-specific TK findings of this study also are consistent with the developmental ontogeny of OAT1. The dose-dependent clearance of 2,4-D in male and female pups was similar on PND 4, 21, and 28, but by PND 35, saturation of renal clearance was apparent in females at lower doses relative to males (Tables 1 and 4). Functionally, accumulation of the organic acid *p*-aminohippuric acid in rat kidney slices increased by approximately 2-fold between GD 20 fetuses and PND 6 pups, and approximately 3-fold between GD 20 fetuses and adults (Nakajima *et al.*, 2000). These results are consistent with the developmental expression of OAT1, where expression increased 4-fold between PND 5 and 35 in both male and female rats (Buist *et al.*, 2002). This developmental delay in achieving adult levels of OAT1 transport capacity, when coupled with increased dose (g/kg body weight) in pre- and early postweaning rats, likely contributed to the increased blood levels of 2,4-D at these early postnatal life stages. By PND 35, males express more OAT1 messenger RNA than females, and this difference in OAT1 expression becomes significant by PND 40 (Buist *et al.*, 2002). The timing of the gender-related difference in OAT1 expression is consistent with the appearance of gender-related differences in 2,4-D AUC_{S_{24h}} on PND 35.

The results of this extensive dietary and life-stage-dependent determination of 2,4-D TK also demonstrate that toxicity resulting from PO gavage dosing regimens potentially overestimates both hazard and risk potential relative to studies using equivalent daily dietary doses of 2,4-D. This potential is most clearly evidenced by comparing dietary male and female plasma C_{max} concentrations observed in this study (Supplementary Tables 6 and 7) to those reported in rats treated by PO gavage (van Ravenzwaay *et al.*, 2003). Plasma C_{max} concentrations were 0.73 and 1.29 µg/g, respectively, for male and female rats

treated with approximately 5 mg/kg/day (100 ppm TD 28 and 29; Table 1) in the diet, whereas plasma C_{max} were elevated to 9.84 and 14.26 µg/g, respectively, for rats dosed at 5 mg/kg/day by PO gavage. The differences in plasma C_{max} were magnified to a greater extent as the daily doses approached or exceeded renal saturation. A daily dietary dose of 41 mg/kg/day (800 ppm, TD 28; Table 1) resulted in a plasma C_{max} of 10.14 µg/g in male rats compared with 189.8 µg/g in rats treated at the approximate equivalent dose of 50 mg/kg/day by PO gavage. A daily dietary dose of 52 mg/kg/day (800 ppm, TD 29; Table 1) in female rats produced a C_{max} of 48.58 µg/g versus 266.6 µg/g in females treated at the equivalent 50 mg/kg/day by PO gavage. 2,4-Dichlorophenoxyacetic acid is rapidly absorbed and exhibits nearly complete bioavailability by both PO gavage and dietary routes (Timchalk, 2004). Thus, the higher C_{max} values via PO gavage are related to rapid absorption of a bolus dose of 2,4-D, which more quickly saturates renal clearance. The slower dose rate achieved with the spreading of dietary exposures over the course of a day both prolongs the time to renal saturation for a given dose of 2,4-D as well as speeds up the time for recovery from saturation during noneating periods, resulting in lower C_{max} values via the dietary route. Because dietary exposure more closely represents the mode of exposure concern for the general human population, ie, pesticide residues in foods (Cooper *et al.*, 2006), these disparate plasma 2,4-D concentrations suggest that toxicity studies conducted by PO gavage likely overestimate toxicity potential in humans.

Although many of the toxicity studies used to support the registration of 2,4-D as a pesticide are indeed conducted by dietary administration, 2,4-D developmental toxicity studies in particular have routinely been performed using PO gavage dosing (Charles *et al.*, 2001). Finally, it is important to note that the dietary TK observed in parental rats treated up to 71 (males) and 95 (female) days in this study indicate that all of the reported systemic toxicity responses in adult rats, which have been used to establish regulatory health standards for 2,4-D (thyroid, adrenals, eye, ovaries/testes, and nervous system), with the exception of kidney, are associated with saturation of renal clearance (USEPA, 2005). In this study, only very slight kidney toxicity was seen in male rats at a subsaturating dose of 400 ppm (Table 2), whereas female kidney toxicity was only observed at the highly renal clearance saturating dose of 800 ppm. Although the mode of action of the sex-specific sensitivity of male rats to kidney toxicity is uncertain, it may be attributable to the higher level of expression of OAT1 and associated higher threshold for renal saturation in male relative to female rats, allowing for a higher delivered dose of 2,4-D to proximal tubule cells.

In summary, the example of 2,4-D illustrates how variations in TK over both dose and life stage can be used to define a scientifically sound and robust approach for dose selection in the design of an extended 1-generation reproduction study, including revealing systemic dose implications associated with significant changes in food intake occurring during periods

of rapid postnatal development in rats (Marty *et al.*, 2013). Importantly, the findings presented in this study have several broader implications for both improvements to future toxicity test design and for understanding the human risk relevance of previously reported high-dose-specific toxicity findings. These data are perhaps the first comprehensive examination of dietary dose- and life-stage-specific TK performance for a compound exhibiting dose-dependent saturation of metabolic clearance, and as such, illustrate how the KMD approach, which when coupled to human exposure information, significantly improves confidence in human risk extrapolation relative to conventional MTD-based study designs. Thus, for 2,4-D, application of the KMD approach confirmed that the highest toxicity test dose should be 2- to 4-fold lower than that suggested by an MTD approach, ultimately allowing for selection and/or interpretation of experimental doses more informative of real-world human exposures. This study findings are also yet another example demonstrating that mode of dose delivery is a potentially important factor impacting expression of toxicity, for example, **PO** gavage studies may overestimate toxicity potential for compounds subject to saturation of metabolic processes controlling toxicity and whose primary human **PO** exposures are through diet or drinking water. Of course, it is recognized that 2,4-D represents a relatively simple example of how saturation of a metabolic process(es) influences KMD evaluations in that 2,4-D does not undergo significant and potentially complicating toxification/detoxification metabolism, and its TK is largely influenced just by dose-dependent saturation of the OAT1 renal transporter in both animals and humans. Finally, this study has also shown that sex-dependent physiologic and/or metabolic factors also can be additional variables in KMD-based evaluations.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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