Excretion and Metabolism of [(2,4-Dichlorophenoxy)acetyl]aspartic Acid and [(2,4-Dichlorophenoxy)acetyl]valine in the Rat

Robert Leon Buly and Ralph O. Mumma*

Adult rats were dosed orally with the aspartic acid or valine conjugates of [^{14}C]-(2,4-dichlorophenoxy)acetic acid (2,4-D) at 10 and 100 mg/kg, and the excretion profile and composition of the metabolites were determined. Urine and feces were collected at 6-h intervals up to 48 h and at 72 h. Each excreta sample was analyzed for radioactivity while selected samples were examined for metabolites. Neither conjugate exhibited appreciable body retention; over 90% of the ^{14}C label was excreted within the first 24 h. Urinary excretion peaked within the first 6 h and fecal excretion peaked during the second 6 h. Total excretion of the ^{14}C label was faster at the low dosage. With 2,4-D-Asp the urine contained a larger percentage of the low dosage compared to the high dosage (81% vs. 61%) while the feces of the larger dosage contained more of the ^{14}C label (33% vs. 16%). This trend did not occur with 2,4-D-Val. Total ^{14}C excretion is prolonged with rats intubated with 2,4-D-Val vs. rats treated with 2,4-D-Asp. Ingestion of 2,4-D-Asp gave rise to only two components in the excreta: 2,4-D-asp and primarily 2,4-D. Ingestion of 2,4-D-Val gave rise to three metabolites in the urine with 2,4-D predominating (ca. 86%). The feces contained at least eight metabolites of which 2,4-D and 2,4-D-Val predominated.

Conjugation of acids to various amino acids is now a well-recognized metabolic pathway of plants. Aspartic acid conjugates of indolacetic acid (Andrae and Good, 1957) and (2,4-dichlorophenoxy)acetic acid (2,4-D) have been detected in plants (Luckwill and Lloyd-Jones, 1960; Slife et al., 1962). Feung et al. (1971a) detected [(2,4-dichlorophenoxy)acetyl]aspartic acid (2,4-D-Asp) and [(2,4-dichlorophenoxy)acetyl]glutamic acid (2,4-K-Glu) as major metabolites of soybean callus tissue. At least five additional amino acid conjugates were subsequently identified: alanine, leucine, phenylalanine, tryptophan, and valine (Feung et al., 1971b, 1973). Five additional tissue cultures (carrot, jack bean, sunflower, tobacco, and corn), also yielded 2,4-D–amino conjugates (Feung et al., 1975). Recently Zama and Mumma (1983) reported that 132 ppm of 2,4-D-Asp and 2,4-D-Glu were found in soybean plants 4 days after treatment with 0.45 kg/0.40 ha of the propylene glycol butyl ether esters of 2,4-D. The 2,4-D–amino acid conjugates take on added significance with the discovery of their herbicidal properties in tissue culture and plants (Feung et al., 1974, 1977).

Concern over the extent of 2,4-D use has led to numerous studies investigating the metabolism of 2,4-D in animals and humans. Levey and Lewis (1947) revealed that 70–90% of oral doses of phenoxyacetic acid, (2-chlorophenoxy)acetic acid, and (4-chlorophenoxy)acetic acid were excreted unchanged in the urine of rabbits within 24 h of administration. Clark et al. (1964) noted that sheep given [^{14}C]-2,4-D orally excreted 96.0% of the dose in the urine and 1.4% of the dose in the feces, in an unaltered form in both cases. Khanna and Fang (1967) dosed rats orally with [1-^{14}C]-2,4-D and [2-^{14}C]-2,4-D in varying amounts. With dosages ranging from 1 to 10 mg/animal, 94–99% of the dose was recovered from the urine and feces within 48 h. With dosages ranging from 20 to 100 mg/animal, total recovery percentages diminished in proportion to dosages ranging from 91% down to 75%. They found 2,4-D residues in various tissues, the amount present being both time and dosage dependent. The vast majority of the radioactive metabolites were in the form of unaltered 2,4-D; however, small amounts of an unknown metabolite (ranging from 6.1% of the total radioactivity found in the liver at 80 mg/animal to an average of 0.25% in the composite urine samples spanning several dosage levels) were found in the urine and selected tissues. No radioactivity was found in the expired CO_2 trapped over a period of 3 days. Zielinski and Fishbein (1967) revealed that mice injected subcutaneously with 2,4-D excreted over 90% of the dose within 24 h. Erne (1966a) noted that pigs, rats, chickens, and calves all excrete the majority of an oral 2,4-D dose via the kidneys. Erne (1966b) also found an acid-hydrolyzable conjugate of 2,4-D in the urine of pigs.

Table I. Acute Oral LD_{50} Values for 2,4-D–Amino Acid Conjugates in Rats[a]

compound	mg/kg
2,4-D	750–800
2,4-D-Val	750
2,4-D-Ala	800–850
2,4-D-Ile	<900
2,4-D-Glu	900–950
2,4-D-Leu	>900
2,4-D-Asp	1000–1050

[a] Source: Mumma (1983).

Metabolism of 2,4-D has been studied with human subjects. Kohli et al. (1974) reported that 75% of an oral dose (5 mg/kg) was excreted unchanged in the urine after 96 h. Sauerhoff et al. (1976) found that an oral dose (5 mg/kg) of 2,4-D was excreted unchanged in the urine (82.3%) and as a 2,4-D conjugate (12.8%). The plasma half-life in this study was 11.7 h, and the body half-life based on urine excretion was 17.7 h. Body elimination and plasma clearance did not involve nonlinear kinetics. Feldmann and Maibach (1974) found 100% recovery of [^{14}C]-2,4-D in the urine following intravenous administration and 5.8% recovery in the urine following topical application. The intravenous half-life was calculated to be 13 h based on frequent urine determinations.

We must remain aware of the ultimate fate of any applied pesticide or subsequent metabolite, be it in plants, soils, animals, or man. Since 2,4-D and its derivatives are used to control unwanted plant species, we must be concerned with any implications of ingestion. The fate of the amino acid conjugated 2,4-D now becomes as important as the fate of ingested 2,4-D. The conjugation may sig-

Pesticide Research Laboratory and Graduate Study Center, Department of Entomology and Physiology Program, The Pennsylvania State University, University Park, Pennsylvania 16802.

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nificantly alter the absorption, transport, and subsequent elimination of 2,4-D from the body. Until now knowledge has been lacking as to the fate of ingested 2,4-D–amino acid conjugates in animals. The LD_{50} data on selected 2,4-D–amino acid conjugates are shown in Table I.

The objective of this study was to dose rats orally with two ^{14}C-amino acid conjugates of 2,4-D (aspartic acid and valine) at two different dosages and determine the excretion profile with time as well as composition of the metabolites. To accomplish this, methods were developed to synthesize pure ^{14}C-labeled conjugates and to develop analytical protocols by which metabolites could be characterized qualitatively and quantitatively.

MATERIALS AND METHODS

Reagents. The [1-^{14}C]-2,4-D used had a specific activity of 10.1 mCi/mmol (ICN, Chemical and Radioisotope Division, Irvine, CA). Unlabeled 2,4-D (Aldrich Chemical Co., Milwaukee, WI) was recrystallized twice from benzene. L-Valine and L-aspartic acid were purchased from Nutritional Biochemicals Corp., Cleveland, OH. All solvents used were either reagent or high-pressure liquid chromatography grade solvents. Nonradiolabeled 2,4-D–amino acid conjugate standards (2,4-D-Asp, 2,4-D-Val, 2,4-D-Gly) were previously synthesized by Dr. C.-S. Feung, Pesticide Research Laboratory, The Pennsylvania State University, University Park, PA. Carbodiimides used included *N*,*N*′-dicyclohexylcarbodiimide (99%, Aldrich Chemical Co.) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (Sigma Chemical Co., St. Louis, MO). Aqueous counting scintillant (Amersham Corp., Arlington Heights, IL) and Scinti Verse (Fisher Scientific Co., Fairlawn, NJ) were used for the preparation of liquid scintillation cocktails.

Materials and Instruments. Precoated thin-layer plates consisting of silica gel and a fluorescent indicator on plastic sheets (Eastman Kodak Co., Rochester, NY, and E. Merck, Darmstadt, West Germany, or MC/B Manufacturing Chemists, Cincinnati, OH) were used for thin-layer chromatography (TLC) procedures. X-ray exposure film (Eastman Kodak) was used for autoradiography of TLC plates. Stainless steel metabolism cages (Laboratory Animal Research Center, The Pennsylvania State University, University Park, PA) that permitted separation of urine and feces were used to house the experimental animals.

HPLC analyses were performed on a Model ALC/GPC 244 high-pressure liquid chromatograph with Model M-6000A pumps, a Model U6K injector, a Model 440 absorbance detector with a 280-nm filter, a 4 mm i.d. × 30 cm μBondapak C_{18} column, and a data module for determination of retention times and peak areas (Waters Associates, Milford, MA). Quantification of radioactive samples was determined on an LS 8000 liquid scintillation counter (Beckman, Fullerton, CA) complete with a data reduction program for dpm (disintegrations per minute) and corrections for quenching. A VirTis "45" tissue homogenizer (VirTis, Gardiner, NY) was used for feces and tissue extractions of metabolites. Solution concentration was performed with a Buchi Rotavapor-R water aspirator (Laboratoriums-Technik AG, Switzerland; Brinkmann Instruments, Westbury, NY) or with a stream of nitrogen. A Tri-Carb sample oxidizer, Model 305 (Packard, Downers Grove, IL), was used to combust dry samples for determination of radioactivity content.

Synthesis of Radiolabeled 2,4-D–Amino Acid Conjugates. Synthesis of nonradiolabeled 2,4-D–amino acid conjugates was performed by Dr. C.-S. Feung, employing the Schotten-Baumann reaction in a method described by Wood and Fontaine (1952). However, the radiolabeled 2,4-D–amino acid conjugates were prepared by using carbodiimides to form amide bonds as first described by Sheehan and Hess (1955) and Khorana (1955).

Synthesis of [^{14}C]-2,4-D-Val occurred in a reaction vial (5 mL) by mixing 400 mg (3.40×10^{-2} mmol) of L-valine dissolved in 2.0 mL of water and 22.5 mg (7.5 mg of radiolabeled and 15.0 mg of nonradiolabeled, for a total of 1.02×10^{-1} mmol) of 2,4-D dissolved in 1.0 mL of dimethylformamide (DMF). Total radioactivity was 250 μCi. Fifty-five milligrams (2.67×10^{-1} mmol) of DCC (*N*,*N*′-dicyclohexylcarbodiimide) was dissolved in a few drops of DMF (ca. 200 μL) and was added to the 2,4-D–valine solution while it was stirred vigorously. Stirring continued for 3 h, after which time the reaction mixture was plated onto eight precoated plastic-backed silica gel plates (20 cm × 20 cm × 0.2 mm). The thin-layer plates were developed with a solvent system composed of ethyl ether–petroleum ether (38–40 °C)–formic acid in a ratio of 70:30:2. Plates were allowed to dry under a hood for several hours to remove excess formic acid, after which time they were covered with polyethylene film and exposed to X-ray film overnight. TLC bands corresponding to 2,4-D-Val were cut from the plates, eluted with ethanol, and pooled. The unreacted, radiolabeled 2,4-D could be eluted and used again. The radiolabeled 2,4-D-Val was tested for purity by employing HPLC using a μBondapak C_{18} column and a mobile phase of 40% methanol–60% water at a flow rate of 1.5 mL/min. Fractions of the column effluent were collected every 10 s and measured for radioactivity. The retention times were compared to that of the 2,4-D-Val standard.

The unreacted 2,4-D was eluted from the TLC plates and the solvent taken to dryness. Ten milliliters of 0.1 N HCl was added, followed by four extractions with ethyl ether (10 mL). The ethyl ether was removed under a stream of nitrogen. A total of 3.25 mg of this radiolabeled 2,4-D (1.70×10^{-2} mmol), 0.76 mg of L-aspartic acid (5.65×10^{-3} mmol), and 11.65 mg of DCC (5.65×10^{-2} mmol) were reacted with the same volumes of water and DMF and in the same manner described for the synthesis of radiolabeled 2,4-D-Val. This reaction mixture was plated onto two precoated TLC plates, and the radiolabeled 2,4-D-Asp product was identified by using TLC, autoradiography, and HPLC. With 2,4-D-Asp, a mobile phase consisting of 25% methanol–75% buffer (0.005 M K_2HPO_4 and 0.005 M NH_4Cl) was used for HPLC analysis. In an alternative method for synthesizing radiolabeled 2,4-D-Asp, 3.25 mg of radiolabeled 2,4-D (1.70×10^{-2} mmol) and 0.76 mg of L-aspartic acid (5.65×10^{-3} mmol) were combined in 0.5 mL of H_2O. While this mixture was vigorously stirred, 10.83 mg (5.65×10^{-2} mmol) of EDC [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride] dissolved in 300 μL of water was added. Stirring continued for 3 h, after which the reaction mixture was plated onto two TLC plates, developed, exposed to X-ray film, eluted, and analyzed by HPLC.

Oral Administration of Radiolabeled 2,4-D–Amino Acid Conjugates. For each conjugate, 12 adult Wistar albino male rats were used; 5 rats received a radioactive dose of 100 mg/kg, 5 rats received a radioactive dose of 10 mg/kg, 1 received 100 mg/kg nonradiolabeled conjugate only, and 1 received only the buffer solution used to dissolve the conjugate in water.

The radioactive 2,4-D-Val solutions were prepared as to give each rat (228–250 g) approximately 1.0 mL containing 2.2×10^6 dpm. For the 100 mg/kg dose level, each milliliter contained 2,4-D-Val totaling 25.0 mg (15.0 μCi/

mmol), K_2HPO_4 (1.59×10^{-1} mmol), and tiny amounts of 1 N NaOH to adjust the pH to ~7.0. For the 10 mg/kg dose level, each milliliter contained 2,4-D-Val totaling 2.5 mg (156 μCi/mmol) in addition to the buffer. The rat that received nonradiolabeled 2,4-D-Val only at a dosage of 100 mg/kg was administered a solution containing 25.0 mg/mL 2,4-D-Val in the same buffer. The 12th rat received only the buffer solution.

The 2,4-D-Asp solutions were prepared in a similar manner. Each of the 12 rats (300–335 g) received a maximum of 1.0 mL. For the 100 mg/kg dose level, each milliliter contained 2,4-D-Asp totaling 33.5 mg (10.9 μCi/mmol), K_2HPO_4 (1.88×10^{-1} mmol), and a few drops of 1 N NaOH to make the pH ~7.0. Each milliliter used for the 10 mg/kg dose contained 2,4-D-Asp totaling 3.4 mg (102 μCi/mmol) and buffer. An 11th rat received nonradioactive 2,4-D-Asp for a dose level of 100 mg/kg. A 12th rat received only buffer.

The rats were fasted overnight. The following morning, each rat was administered his dose orally with a 1.0-mL tuberculin syringe and intubation needle and placed in separate metabolism cages. After dosing, the rats were given food and water ad libitum. A 12-h light/dark cycle was provided. The urine and feces for each animal were collected separately at 6, 12, 18, 24, 30, 36, 42, 48, and 72 h after the dosing. The sidewalls of the urine collector were washed down with a 50:50 mixture of ethanol and water and pooled with the urine at each collection to maximize recovery. The fecal pellets were placed in vials. After the 72-h collection, each rat was sacrificed with carbon dioxide. The metabolism cage floor and urine collector were then given a final thorough rinse with the ethanol–water solution. The rats, urine samples, and feces were all stored at −25 °C until analyzed.

Quantitation and Identification of Metabolites. The volume of each urine sample was measured. A 1.0-mL aliquot was removed from each and mixed with 10.0 mL of scintillation fluid and counted. Each feces sample was placed in a homogenization flask, followed by 50.0 mL of methanol. The sample sat covered at room temperature for 1 h followed by a 5-min homogenization. The methanol extract was then filtered (Whatman No. 1) with a water aspirator. An additional 10–40 mL of methanol was used to rinse the homogenization flask and blades and poured over the feces residue. The volume of the filtrate was measured. A 1.0-mL aliquot was removed, mixed with 10.0 mL of scintillation fluid, and counted. The feces residue for each sample was weighed. An aliquot was removed, weighed, and combusted in the oxidizer, to determine the total dpm left in the feces residue.

The urine and feces extract for each rat that contained the highest concentration of radioactivity was used to identify metabolites. Enough of each sample was used to obtain roughly 20 000 dpm. The samples were dried in a small pear-shaped flask (rotary evaporator, and the residues were taken up in 100–500 μL of methanol and plated onto precoated silica gel TLC plates (20 cm × 10 cm × 0.1 mm). Radioactive 2,4-D standard and radioactive 2,4-D-Asp or 2,4-D-Val standard were spotted onto each plate. The plates were developed with an ether–petroleum ether–formic acid (70:20:2) solvent system. The plates were then left to dry under the hood overnight to remove excess formic acid. The thin-layer plates were then covered with polyethylene film and X-ray film, placed in light-proof boxes, and exposed for 3 weeks. After development, R_f values were obtained and the radioactive bands were eluted from the plates. The elutant of each band was mixed with scintillation fluid and counted for radioactivity after a known volume was removed for HPLC analysis. For HPLC analysis of each conjugate's metabolites, varying amounts of buffered water (5 mM NH_4Cl and 5 mM K_2HPO_4) and methanol were used as the mobile phase. Aliquots of the elutant were then collected at 1-min intervals to provide a dpm profile for comparison to the ultraviolet absorption profile.

Statistical Analysis. To determine whether two averages differ significantly from each other, Cochran's modified t test was used (Ott, 1977).

RESULTS AND DISCUSSION

Excretion of ^{14}C Label from Rats Administered [^{14}C]-2,4-D-aspartic Acid. There appears to be no substantial retention of [^{14}C]-2,4-D-Asp in the animal bodies at either dosage. Rats administered [^{14}C]-2,4-D-Asp at 100 mg/kg excreted an average of 94.26% of the radioactivity via the urine and feces with 24 h. Rats dosed at 10 mg/kg excreted an average of 97.8% of the dose in a similar fashion within 24 h (Figure 1).

During the 72 h of the experiment, there were significantly different proportions of urinary and fecal excretion in the 100 vs. the 10 mg/kg dosage. At the 10 mg/kg dosage, 81.5% of the total radioactivity appeared in the urine, compared to 61% for the 100 mg/kg dosage (α = 0.01). Fecal excretion of the 10 mg/kg dosage level, which consisted of 16.3% of the radioactivity, was significantly lower than the 100 mg/kg dosage level, which consisted of 33.4% (α = 0.05). This is explained by the fact that the 100 mg/kg animals are presented with 10 times the amount of conjugate, thus overwhelming the absorbtive capacity of the gastrointestinal system. The excess conjugate passes out with the feces.

As expected, urinary clearance of [^{14}C]-2,4-D-Asp occurs faster than the fecal elimination at both dosage levels. Urinary excretion of [^{14}C]-2,4-D-Asp metabolites was greatest during the first 6 h, comprising 38.1% of the total dose at 100 mg/kg and 59.3% at 10 mg/kg. Fecal excretion was greatest between the 6th and 12th hours (hour 12), averaging 32.4% of the dose at 100 mg/kg and 12.1% at 10 mg/kg. This difference between the two types of excreta was due to the time needed for the luminal contents to traverse the gastrointestinal system.

The combined urine and feces elimination of the radiolabel at each collection time is represented graphically in Figure 2 and shows that total excretion occurs more rapidly in the 10 mg/kg dosage group ($t_{1/2}$ = 4.1 h). The 100 mg/kg dosage group, having to clear 10 times the amount of conjugate, may be slower due to saturation of absorptive sites in the small intestine and a greater dependence on the fecal route for elimination ($t_{1/2}$ = 6.6 h). At the end of 6 h, the low-dosage animals cleared 63.1% of the radioactivity, while the high-dosage animals cleared only 38.6% (α = 0.01). Total excretion for both dosages falls precipitously after the 12th hour.

Excretion of ^{14}C Label from Rats Administered [^{14}C]-2,4-D-valine. There appears to be no substantial retention of [^{14}C]-2,4-D-Val in the animal bodies. Rats administered [^{14}C]-2,4-D-Val at 100 mg/kg excreted an average of 91.1% of the radioactivity via the urine and feces within 24 h. However, rats dosed at 10 mg/kg excreted only 80% of the radioactivity within 24 h and a total of 81.8% by the end of 72 h (Figure 3).

With [^{14}C]-2,4-D-Asp, considerably more of the radioactivity appeared in the urine over 72 h with the low dosage compared to the high dosage. This is not the case with [^{14}C]-2,4-D-Val, in which rats excreted 75.9% at 100 mg/kg and only 69.3% at 10 mg/kg. This may be due to an increased adsorption rate for valine conjugates. Fecal

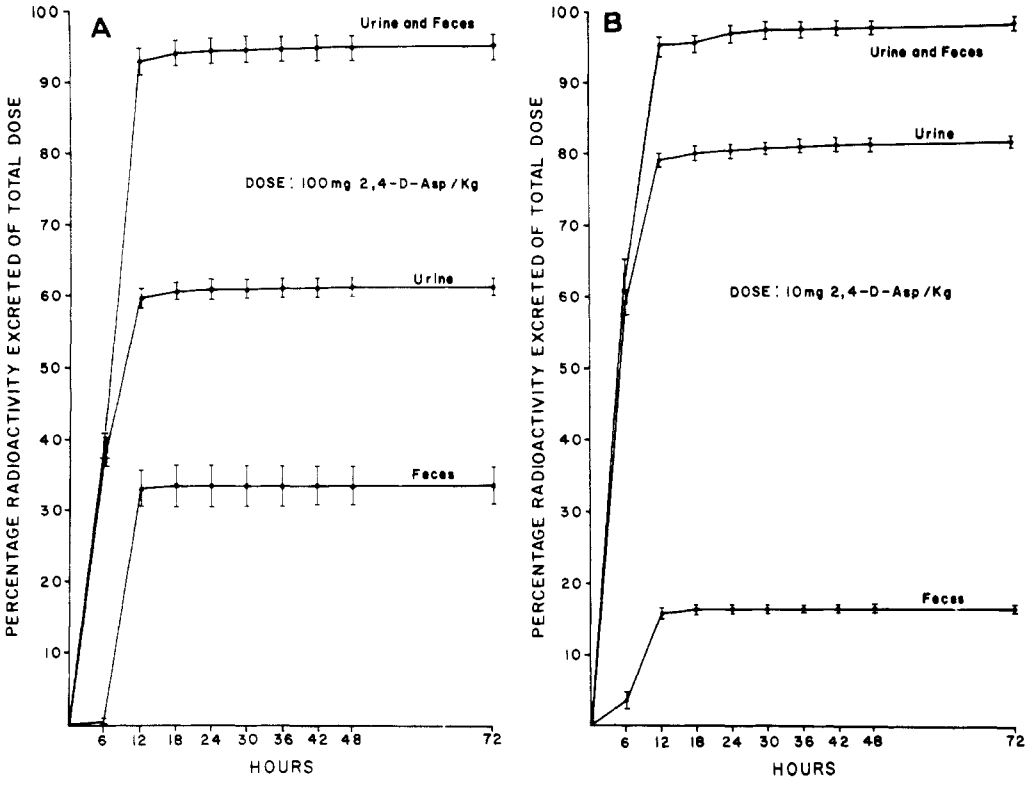

Figure 1. Cumulative excretion of the ^{14}C label in the urine and feces of four rats dosed with 100 mg/kg (A) and 5 rats dosed with 10 mg/kg (B) [^{14}C]-2,4-D-Asp vs. time. Bars represent the standard error of the mean.

excretion of the 100 mg/kg group comprised 17.3% of the radioactivity while the 10 mg/kg group excreted 12.5% of the dose in this manner.

Urinary clearance of [^{14}C]-2,4-D-Val proceeded at a faster rate than fecal elimination. Urinary excretion of the 10 mg/kg group peaked in the first 6 h with 50.4% of the radioactivity. The 100 mg/kg group was somewhat slower with 31.7% in the first 6 h and 37.5% in the second 6 h. Fecal excretion peaked in the second 6 h (hour 12) with 9.17% of the radioactivity for the high-dosage group and 7.51% for the low-dosage group.

Figure 4 shows the values for combined urine and feces elimination of radioactivity at each collection time. As was the case for [^{14}C]-2,4-D-Asp, total excretion of radioactivity takes longer at the higher dosage of [^{14}C]-2,4-D-Val ($t_{1/2}$ = 7.4 h) compared to the lower dosage ($t_{1/2}$ = 5.7 h). At the end of 6 h, the lower dosage group cleared significantly (α = 0.05) more radioactivity (51.9%) than the higher dosage group (32.4%).

Although the excretion profiles of [^{14}C]-2,4-D-Val do fall precipitously after the 12th hour, the drop is not as dramatic as the profiles of [^{14}C]-2,4-D-Asp. Excretion of [^{14}C]-2,4-D-Val is prolonged. From the 12th to the 18th hours (hour 18), total [^{14}C]-2,4-D-Val excretion in the 100 mg/kg group equals 10.1% compared to 1.25% for the comparable [^{14}C]-2,4-D-Asp group (α = 0.05). In this same time period for the 10 mg/kg group, [^{14}C]-2,4-D-Asp excretion totaled 4.45% while that for [^{14}C]-2,4-D-Asp was 1.23% (α = 0.01). The prolonged excretion time for the 2,4-D-Val is probably due to its lower polarity compared to the 2,4-D-Asp. This difference will be discussed later in regard to the types of metabolites formed with these two conjugates.

Metabolism of [^{14}C]-2,4-D-aspartic Acid. The urine and extracts of feces were analyzed by TLC and by HPLC as described under Materials and Methods. The metabolism of [^{14}C]-2,4-D-Asp was straightforward. Only two major components were excreted in the urine or feces, unaltered [^{14}C]-2,4-D-Asp and free [^{14}C]-2,4-D. No attempt was made to trap and analyze expired CO_2 for radioactivity. Khanna and Fang (1967) found no trace of radioactivity in expired CO_2 of rats dosed with various levels of [^{14}C]-2,4-D.

Although the exact cause of hydrolysis of 2,4-D-Asp is unknown, there are several possibilities. Hydrolysis may occur in the stomach due to acidic secretions that attack the peptide linkage of the conjugates. Intestinal peptidases may also hydrolyze the peptide bond. Bacteria in the small intestine, cecum, and colon may also be responsible for cleaving the [^{14}C]-2,4-D from its amino acid in an attempt to use either component as a metabolic substrate. Scheline (1973) in his review article mentions that mammals may harbor over 60 species of bacteria in the gut. These bacteria perform a whole host of metabolic transformations on foreign compounds, including amide bond hydrolysis.

Table II presents the mean percentages of [^{14}C]-2,4-D and [^{14}C]-2,4-D-Asp found in urine and feces extracts of all 10 animals at the 6- or 12-h collection period. For each animal, the most radioactive urine or feces sample was used. Free [^{14}C]-2,4-D was consistently present in larger amounts, ranging from an average of 83.0% in the urine of animals receiving the low dosage to 97.7% in the feces extracts of that same group. The urine and feces extracts of the higher dosage group contained an average [^{14}C]-2,4-D percentage of 88.3% and 86.2%, respectively.

In comparison of the low-dosage animals with the high-dosage animals, the former had a significantly higher (α = 0.05) percentage of [^{14}C]-2,4-D-Asp in their urine (17.0%) compared to the latter (11.7%). This probably reflects the faster absorption of the low dosage before hydrolysis can occur. This effect was even more pronounced in the feces. At the low dosage, whatever conjugate remained was thoroughly hydrolyzed, with only 2.3% of the radioactivity present as [^{14}C]-2,4-D-Asp. At the high dose, however, 13.8% of the fecal radioactivity was present in the form of [^{14}C]-2,4-D-Asp. This significant

Table II. Relative Percentage of [^{14}C]-2,4-D-aspartic Acid or [^{14}C]-2,4-D Found in Rat Urine or Feces following TLC of Extracts

urine[a]			feces[a]		
hour of collection	[^{14}C]-2,4-D-Asp	[^{14}C]-2,4-D	hour of collection	[^{14}C]-2,4-D-Asp	[^{14}C]-2,4-D
		Dosage: 100 mg of 2,4-D-Asp/kg			
6	11.7 ± 2.2[b]	88.3 ± 2.2	12	13.8 ± 8.7	86.2 ± 8.7
		Dosage: 10 mg of 2,4-D-Asp/kg			
6	17.0 ± 3.8	83.0 ± 3.8	12	2.3 ± 1.0	97.7 ± 1.0

[a] Pooled samples from four or five rats. [b] Standard error of the mean.

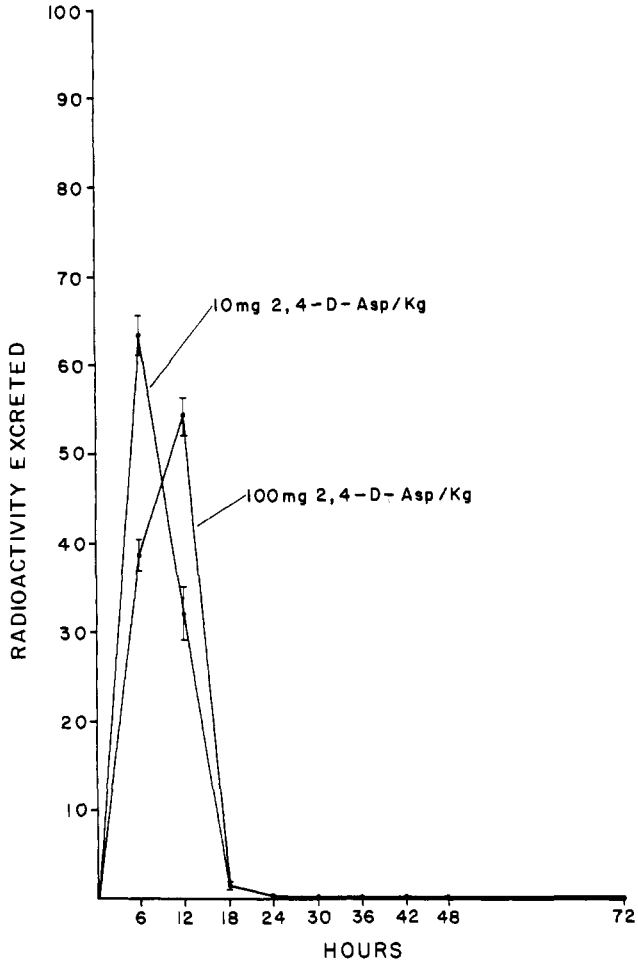

Figure 2. Hourly excretion profile of the ^{14}C label in the combined urine and feces of rats dosed with [^{14}C]-2,4-D-Asp. Five rats received 10 mg/kg and four rats received 100 mg/kg. Bars represent the standard error of the mean.

difference (α = 0.025) was due to the fact that the high-dosage animals were presented with 10 times the amount of conjugate to whatever forces were responsible for hydrolysis.

Metabolism of [^{14}C]-2,4-D-Valine. The metabolism of [^{14}C]-2,4-D-Val was much more complex than that of [^{14}C]-2,4-D-Asp. Figure 5 is an autoradiograph of a TLC plate of the urine extract for animal no. 2 taken between hours 6 and 12. This pattern was representative of the urine extracts for all 10 animals. Band 1 represents an unknown metabolite of [^{14}C]-2,4-D-Val that remained on the origin (R_f = 0.0). This unknown metabolite represented an average of 2.32% of the radioactivity in the high-dosage animals and 2.30% in the low-dosage animals. Band 2 represents [^{14}C]-2,4-D-Val. Band 3 is [^{14}C]-2,4-D. The identity of these components was confirmed by HPLC.

Table III. Relative Percentage of [^{14}C]-2,4-D-valine, [^{14}C]-2,4-D, and Other ^{14}C-Containing Metabolites Found in Rat Urine following TLC of Extracts[a]

hour of collection	band at origin	[^{14}C]-2,4-D-Val	[^{14}C]-2,4-D
	Dosage: 100 mg of 2,4-D-Val/kg		
6 or 12	2.32 ± 1.4[b]	11.4 ± 7.1	86.3 ± 6.7
	Dosage: 10 mg of 2,4-D-Val/kg		
6	2.30 ± 0.3	10.8 ± 2.8	86.9 ± 2.9

[a] Pooled samples from five rats. [b] Standard error of the mean.

Table IV. Relative Percentage of Radioactivity Found in Various TLC Bands Representing Different Metabolites of [^{14}C]-2,4-D-valine in Rat Feces

TLC band	animal no. 2, hour 18, %	animal no. 3, hour 18, %
	Dosage: 100 mg of 2,4-D-Val/kg	
1	13.65	29.63
2	5.80	7.52
3	12.86	7.63
4	3.27	2.25
5	8.26	8.56
6	28.20 (2,4-D-Val)	22.64 (2,4-D-Val)
7	6.97	7.18
8	20.98 (2,4-D)	14.59 (2,4-D)
	Dosage: 10 mg of 2,4-D-Val/kg	
TLC band	animal no. 6, hour 12, %	animal no. 7, hour 12, %
1	25.16	19.32
2	5.29	5.57
3	16.09	10.75
4	2.57	1.98
5	4.98	5.57
6	19.03 (2,4-D-Val)	22.93 (2,4-D-Val)
7	2.24	2.99
8	24.64 (2,4-D)	30.72 (2,4-D)

As was the case for [^{14}C]-2,4-D-Asp, the major urinary metabolite of [^{14}C]-2,4-D-Val was [^{14}C]-2,4-D (Table III). At the 100 mg/kg dose, 86.3% of the radioactivity was present as [^{14}C]-2,4-D compared with 86.9% for the 10 mg/kg dosage. In the high-dosage group, [^{14}C]-2,4-D-Val comprised 11.4% of the radioactivity compared with 10.8% in the low-dosage group. The remainder of the radioactivity consisted of the unknown metabolite(s) that remained at the origin. There were no significant differences in the average percentage of bands 1, 2, or 3 when comparing the high vs. the low dosages.

The fecal metabolites of [^{14}C]-2,4-D-Val presented a very complex pattern as shown in Figure 6. Band 8 is [^{14}C]-2,4-D and band 6 represents [^{14}C]-2,4-D-Val. However, there were numerous other metabolites present in the feces. All of these unknown metabolites were more polar

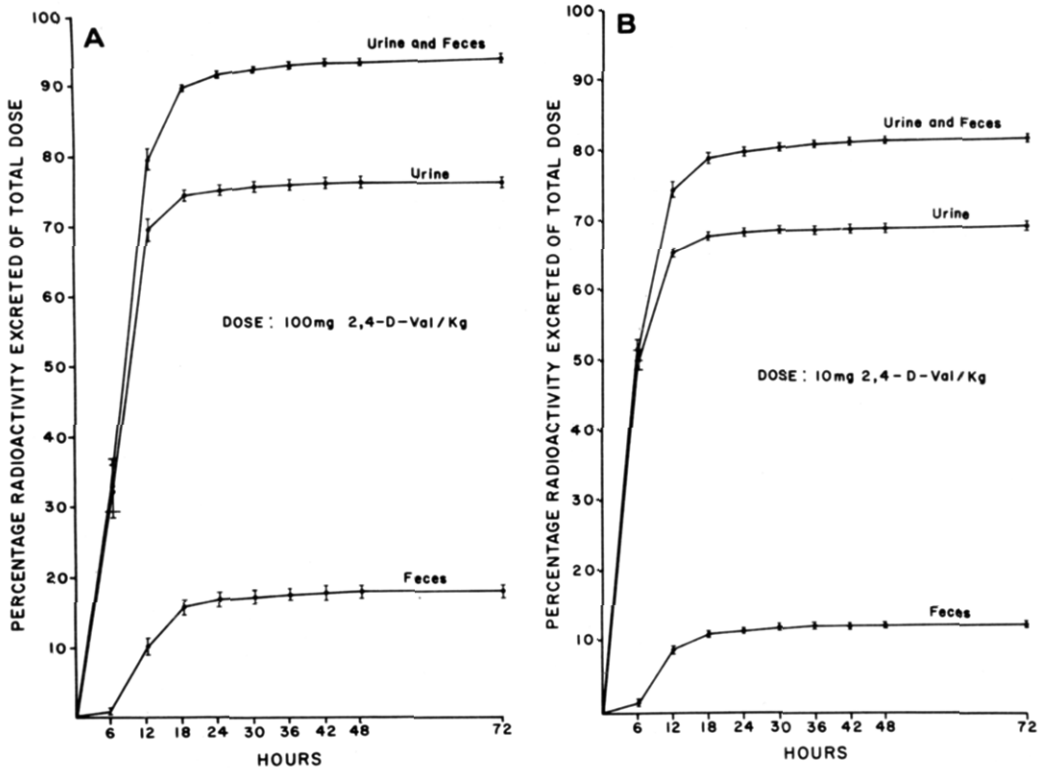

Figure 3. Cumulative excretion of the ^{14}C label in the urine and feces of five rats dosed with 100 (A) and 10 mg/kg (B) [^{14}C]-2,4-D-Val vs. time. Bars represent the standard error of the mean.

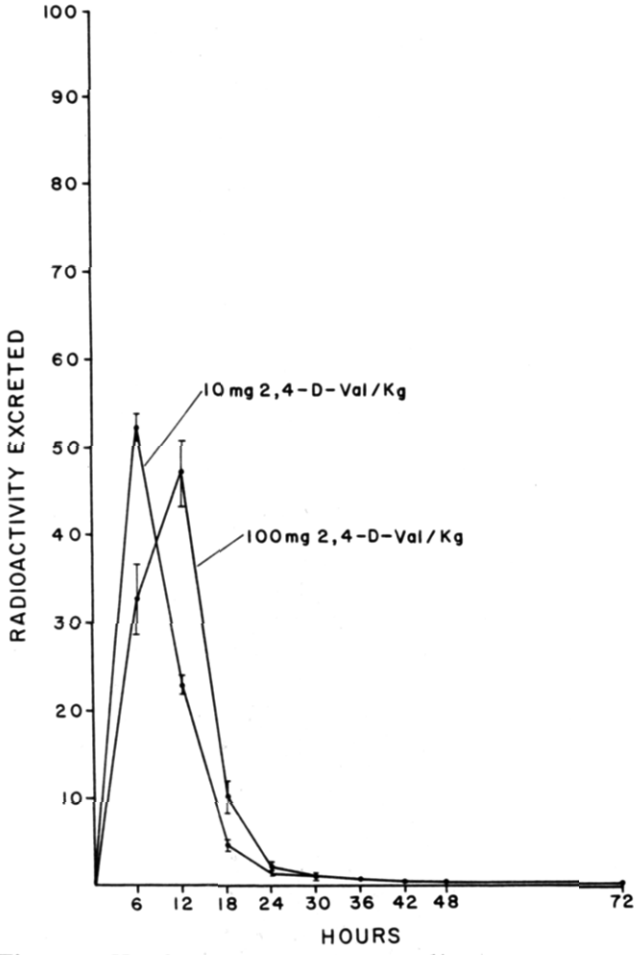

Figure 4. Hourly excretion profile of the ^{14}C label in the combined urine and feces of rats dosed with [^{14}C]-2,4-D-Val. Five rats received 10 mg/kg and five rats received 100 mg/kg. Bars represent the standard error of the mean.

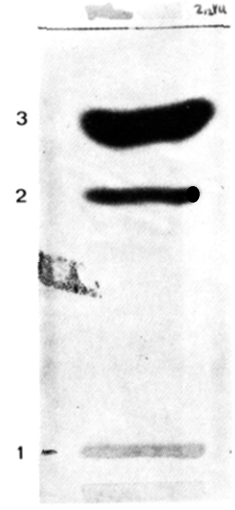

Figure 5. TLC autoradiograph of the urine extract from animal no. 2, 12 h after dosing with [^{14}C]-2,4-D-Val. Band 2 represents [^{14}C]-2,4-D-Val and band 3 represents [^{14}C]-2,4-D. The mobile phase is ether–petroleum ether–formic acid, 70:30:2 v/v/v.

than 2,4-D, and all but two were more polar than 2,4-D-Val. Table IV gives the percentages of fecal radioactivity found in the TLC bands from pooled feces of four animals. With the exception of animal no. 2, each sampling period represents the feces sample containing the highest concentration of radioactivity. Bands 1 (unknown), 6 ([^{14}C]-2,4-D-Val), and 8 ([^{14}C]-2,4-D) contain the greatest amounts of radioactivity in each example. Obviously some bands represent more than one metabolite.

There are many possibilities as to what these unknown metabolites may be. Gibaldi (1977) notes that rats tend to excrete drugs with a molecular weight under 300 via the urine and drugs with a molecule weight over 300 via the bile and feces. The molecular weight of 2,4-D-Val is 337 and that of 2,4-D-Asp is 353. With these molecular weights

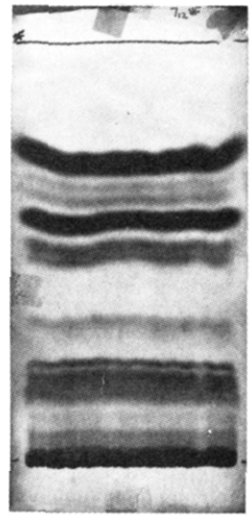

Figure 6. TLC autoradiograph of the feces extract from animal no. 7, 12 h after dosing with [^{14}C]-2,4-D-Val. Band 6 represents [^{14}C]-2,4-D-Val and band 8 represents [^{14}C]-2,4-D. The mobile phase is ether–petroleum ether–formic acid, 70:30:2 v/v/v.

so close to the transitional zone between the two possibilities, both renal clearance and bile secretion are to be expected. If biliary clearance did occur with 2,4-D-Asp, it was not evidently based upon the metabolites formed. The metabolites of 2,4-D-Val presented a different story. In addition to 2,4-D-Val and 2,4-D, at least six unknown metabolites were formed, most being more polar than 2,4-D-Val and probably represent additional oxidation and perhaps conjugation. These might be in part of microflora origin. These unknown metabolites represent no more than 5% of the applied dose and in most cases 1–2% of the applied dose. Future studies should be directed toward the identification of these metabolites.

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