at 100° C. and, after cooling, was added cautiously to 25 grams of ice. The product was isolated by ether extraction and removal of the solvent to give 830 mg. of crude 2,6-dinitro-4-trifluoromethylchlorobenzene. This was purified by preparative thin-layer chromatography (system A) and was recovered by ether elution in a small column. The oil crystallizes on standing.

N-Alkyl- and N,N-Dialkyl-2,6-dinitro-4-trifluoromethylanilines. METHOD I. Equimolar amounts of 2,6-dinitro-4-trifluoromethylchlorobenzene and the amine hydrochloride in dry triethylamine (2 ml. for 0.5-mmole run) were heated overnight at 80° C. After cooling, ether was added, and the mixture was washed with 1N HCl, water, 1N NaOH, and water. Purification was accomplished by thin-layer chromatography.

METHOD II. To 1 mmole of 2,6-dinitro-4-trifluoromethylchlorobenzene in 25 ml. of ether was added 4.25 mmoles of the N,N-dialkylamine, and the mixture was allowed to stand overnight. Six milliliters of 1N HCl was added, and then, the ethereal solution was washed with water. The ether was removed under reduced pressure, and the product was purified by thin-layer chromatography.

Acknowledgment

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2,4-D METABOLISM

Metabolism of C^{14}-Labeled 2,4-Dichlorophenoxyacetic Acid in Rats

SUCHITRA KHANNA and S. C. FANG
Department of Agricultural Chemistry, Oregon State University, Corvallis, Ore.

C^{14}-labeled 2,4-dichlorophenoxyacetic acid (2,4-D-1-C^{14} or -2-C^{14}) was fed to adult rats at a dose of 1 to 100 mg. per rat, and the expired air, urine, feces, internal organs, and tissues were analyzed for radioactivity. No C^{14} was found in the expired air during a three-day period following dosing. The rate of 2,4-D elimination was dosage dependent. Radioactivity was found in all organs and tissues examined. The maximum radioactivity in all tissues was generally reached at 6 to 8 hours after dosing, and started to decrease immediately for 1-mg. 2,4-D dosage. At 100-mg. dosage, the peak concentration persisted until about 17 hours. The urine and the extracts of several tissues contained mainly unchanged 2,4-D residue. A study of the intracellular distribution of 2,4-D in six organs has shown that the soluble fraction of the cells contained the major portion of radioactivity, followed by the nuclear fraction, and the mitochondrial and microsomal fractions.

FOR A NUMBER of years, 2,4-dichlorophenoxyacetic acid (2,4-D) has been widely used as a selective herbicide. In 1947, Levey *et al.* (*4*) reported a study of the metabolism of phenoxyacetic acid and monochloro- derivatives in rabbits. They found that when phenoxyacetic acid was given orally, 96% of the administered dose was excreted in the urine in 24 hours. In the case of *o*- or *p*-monochlorophenoxyacetic acid, the recovery was 70 to 90% in 24 hours. There was a difference in the rate of excretion between these two isomers. Clark *et al.* (*2*) reported on the metabolism of 2,4-D-1-C^{14} in sheep. They found that approximately 96% of a dose of 2,4-D was excreted unchanged in the urine and 1.4% in the feces in 72 hours. Very little residual radioactivity was found in sheep tissues after 72 hours. The purpose of the present investigation was to study the metabolic fate, tissue accumulation, cellular incorporation, and excretory pattern in adult rats receiving varying amounts of C^{14}-labeled 2,4-D.

Materials and Methods

Adult rats of the Wistar strain, approximately 4 to 6 months old and weighing 350 to 400 grams for males, and 225 to 275 grams for females, were used in this study. Aqueous solution of 2,4-D-1-C^{14}, 3.03 mc. per mmole, or 2,4-D-2-C^{14}, 1 mc. per mmole, at a concentration ranging from 1 to 50 mg. per ml. was prepared by dissolving it in water containing equal molar amounts of tribasic potassium phosphate. Neither labeled 2,4-D contains any isotopic impurity as revealed by paper chromatography. The same amount of 1 mg. of labeled 2,4-D was used at all dose levels with the balance being made up of nonlabeled 2,4-D acid in the case where doses were greater than 1 mg. The herbicide was administered to the rat by stomach tube at different dose levels, ranging from 1 to 100 mg. per animal—approximately 3 to 300 mg. per kg. of body weight. The oral LD_{50} for 2,4-D in rats has been found to be 300 to 1000 mg. per kg. of body weight (*3*). After dosing, the animals were placed individually in metabolism cages, and the urine and feces were collected periodically. The expired CO_2 was trapped in a dilute sodium hydroxide solution, which was changed at hourly intervals and checked for radioactivity for three days following 2,4-D administration. During this period, no radioactivity was found in the expired air from rats fed 2,4-D labeled either at the 1-C or 2-C position. The radioactivity in all urine samples was measured in a Packard Tricarb liquid scintillation spectrometer Model 314S. Aliquots of urine

sample, 0.5 ml., were pipetted into low potassium counting vials containing 10 ml. of methylcellosolve–naphthalene–toluene phosphor solution which contained 4 grams of 2,5-diphenyloxazol (PPO), 50 mg. of 1,4-bis-2-(5-phenyloxazoly-benzene (POPOP) and 25 grams of naphthalene per liter. The methylcellosolve–toluene ratio was 1 to 1. The counting efficiency of this medium was 48%. Each sample was corrected for color and chemical quenching by using benzoic acid–C^{14} as an internal standard. The radioactivity of feces was measured also by liquid scintillation procedure after extracting the feces sample with 50% ethanol and determining the radioactivity in the ethanol extract. The alcohol-insoluble residue of feces was checked by G-M detector and no radioactivity was found, indicating that the radioactivity in the feces was completely soluble in alcohol.

Tissue Accumulation. Two series of experiments were conducted in this study. In the first series, six rats were given a dose of 1 mg. of 2,4-D per rat, and in the second series, seven rats were given a dose of 80 mg. of 2,4-D. After dosing, the rats were sacrificed at predetermined intervals of time ranging from 1 to 41 hours. Various organs and representative tissues were removed and freeze-dried. These dry tissues were finely ground, and 0.1-gram aliquots of each were evenly placed on planchets, and their radioactivity was determined in a windowless, gas-flow G-M counter. The values were averaged and converted either into $BaCO_3$ counting or into absolute radioactivity by pre-established factors. These factors were obtained by determining the radioactivity of the dried tissue both directly and, after conversion to $BaCO_3$, by a wet combustion technique (*1*). The radioactivity of $BaCO_3$ was also measured by a liquid-scintillation procedure to establish its absolute radioactivity.

Cellular Incorporation. For the determination of the incorporation of 2,4-D into the cellular constituents, rats were given an oral dose of 1, 2, or 80 mg. of 2,4-D-1-C^{14}, and were sacrificed after 3 or 6 hours. The organs were weighed and immediately homogenized with 5 volumes of 0.25*M* sucrose solution in a Potter-Elvehjem tissue grinder (*5*). They were then fractionated by differential centrifugation (nuclear, 700 × G, 10 minutes, twice washed; mitochondrial, 10,000 × G, 10 minutes, once washed; microsomal, 35,000 × G, 90 minutes). Each fraction was brought up to a known volume. Aliquots of each fraction were plated on preweighed planchets and dried. The planchet was weighed again to determine the mass of cellular constituents, and its radioactivity was determined with a windowless gas-flow counter. Corrections were made for self-absorption and background. The resulting values were averaged.

Paper Chromatography and Radioautography. Urine samples or tissue extracts were spotted on Whatman No. 1 filter paper and chromatographed one dimensionally using 2-propanol–NH_4OH–H_2O (8:1:1), or 1-butanol–acetic acid–H_2O (8:2:3) as a developing solvent. Known 2,4-D-1-C^{14} was also chromatographed on a separate strip with each run. The chromatograms were scanned directly with a Ferro gas-flow paper chromatogram scanner for radioactivity spots, and in some cases radioautograms were prepared from these paper chromatograms in order to confirm the presence of minor radioactive spots which are detected by direct scanning.

Countercurrent Extraction. The urine samples or alcoholic extracts of tissues were acidified and extracted with benzene continuously for 48 hours in a liquid-liquid extractor to remove quantitatively the radioactivity in the sample. In case of alcoholic extract of tissues, the alcohol was partially evaporated, and the residue was redissolved in sufficient amounts of water before being subjected to liquid-liquid extraction. The benzene extracts were concentrated to the proper volume and submitted directly to fractionation by a countercurrent distribution method with a 100-tube all-glass Craig countercurrent apparatus. One-tenth molar phosphate buffer, pH 4.5, was the lower phase, and benzene was the upper phase. In preliminary experiments, phosphate buffer at pH 7.0 or 8.5 was also used. After completion of 50 transfers the upper layer of each tube was assayed for radioactivity.

Results and Discussion

Excretion Pattern of 2,4-D. A study of 21 rats showed that no C^{14} was present in the exhaled CO_2 at any dose level either with 2,4-D-1-C^{14} or with 2,4-D-2-C^{14}. The excretion of the radioactivity in the urine and feces varied with the dose. In rats receiving from 1 to 10 mg. of 2,4-D there was almost complete excretion of the herbicide in the urine and feces in 48 hours, the range being from 94 to 99% (Table I). Of this, 93 to 96% was excreted in the first 24 hours and was almost entirely in the urine. Only 1 to 3% was excreted in the next 24 hours. The recovery pattern at these lower levels is in agreement with that of Clark *et al.* (*2*) who found that when 2,4-D in 4-mg.-per-kg. dosage was administered to sheep, the recovery in the urine and feces was 97% in 72 hours. In rats fed higher doses of 2,4-D—i.e., from 20 to 100 mg.—there was a linear decrease in the percentage recovered in the urine and feces with an increase of dosage (Table I). There was also a change in the pattern of excretion with less being excreted in the first 24 hours and more in the second 24 hours, as compared with the percentages excreted in rats fed lower doses of 2,4-D (Table I). This linear relationship of dose to recovery was observed both in males and females, and in the case of both 2,4-D-1-C^{14} and 2,4-D-2-C^{14}. This changed pattern of excretion indicates slower absorption and, hence, slower excretion of the herbicide at the higher dose levels. Because of the lower recovery of radioactivity at the higher dose levels, an attempt was made to determine whether or not 2,4-D might be converted to some volatile radioactive metabolites and be given out through the expired air. This was done by passing the expired air through a series of cold traps cooled with dry ice–acetone mixture, or through a trap containing ethanol. However, no radioactivity was found in either case.

Accumulation and the Rate of Elimination of 2,4-D in Tissues. Since 48 hours were required for the almost complete excretion of 2,4-D at the lower dose levels, and since elimination of 2,4-D from the body was not complete even at the end of 144 hours in animals receiving larger doses, a study was made to determine the sites of accumulation and the rate of elimination for both 1- and 80-mg. doses. In all, 12 tissues were examined (Table II), and all showed the presence of some radioactivity. Some accumulation was observed as early as 1 hour after dosing, indicating how rapidly the herbicide was absorbed from the stomach and distributed throughout the body. A difference in the rates of accumulation

Table I. Average Per Cent Recovery of Radioactivity in Urine and Feces of Rats Fed Varying Amounts of 2,4-D-C^{14}

	2,4-D Dosage, Mg. per Rat							
Time, Hr.	1 (2)[a]	5 (2)	10 (2)	20 (2)	40 (4)	60 (1)	80 (7)	100 (1)
0–24	92.5	91.9	95.8	88.8	63.2	49.2	59.3	42.3
24–38	1.4	1.0	3.0	2.1	23.2	36.0	16.5	31.0
48–72	0.3	0.1	0.5	...	0.2	0.2	1.1	2.1
72–144	...	...	...	0.4[b]	0.5	0.2	1.0	0.1
Total recovery	94.2	93.0	99.3	91.3	87.1	87.4	77.9	75.5

[a] Number of rats used.
[b] 48-hour to 144-hour urine samples.

Table II. 2,4-D Content in Organs of Rats Fed Two Levels of Labeled 2,4-D

2,4-D content, µg./0.1 gram dry tissue

Tissues	*80-Mg. Dosage, Time after Dosing, Hr.*							*1-Mg. Dosage, Time after Dosing, Hr.*							
	4	*8*	*17*	*20*	*24*	*30*	*41*	*1*	*2*	*4*	*6*	*8*	*12*	*16*
Blood	41.4	80.5	66.6	23.5	26.8	1.1	0.7	0.53	0.29	0.54	1.50	1.00	0.01	0.01
Liver	19.2	59.5	44.5	19.2	14.1	2.3	1.5	0.20	0.08	0.17	0.43	0.44	0.01	0.01
Kidney	32.2	64.2	70.2	37.6	41.9	1.3	1.8	1.73	0.71	0.81	2.70	5.20	0.03	0.02
Heart	17.7	43.1	35.1	14.2	13.8	0.7	0.7	0.15	0.12	0.25	0.80	0.46	0.01	0.01
Lungs	16.3	53.4	49.3	17.4	12.7	0.3	0.7	0.18	0.20	0.31	0.39	0.25	0.09	0.00
Spleen	11.0	25.2	25.5	8.2	6.1	0.1	0.1	0.04	0.01	0.06	0.05	0.24	0.01	0.00
Muscle	6.8	8.1	16.6	3.8	5.0	0.1	0.5	0.02	0.01	0.03	0.15	0.09	0.03	0.01
Brain	2.0	6.6	10.2	3.1	1.6	0.1	0.1	[a]	[a]	[a]	0.07	0.06	0.00	0.00
Testicles	14.4	29.4	45.6	19.5	13.2	0.2	0.5	0.11	0.06	0.14	0.36	0.56	0.01	0.01
Urogenital organ	16.2	15.6	12.8	12.0	7.8	0.3	0.5	0.12	0.04	0.10	0.28	0.49	0.06	0.01
Stomach	690.0	895.0	502.5	511.5	1690.0	4.5	0.3	8.92	10.70	11.30	13.20	8.72	0.21	0.31
Intestine	17.8	46.5	28.2	33.5	7.6	0.2	0.0	0.06	0.03	0.05	0.38	0.31	0.01	0.02

[a] Not determined.

and elimination of 2,4-D was observed between rats fed 1 mg. and those fed 80 mg. of 2,4-D. In the rats receiving 1 mg. of the labeled herbicide, the peak concentration in the tissues was reached at 6 to 8 hours after dosing. After this, there was a very rapid decrease in the concentration, and the tissues showed no detectable radioactivity at 24 hours. The rate of elimination was thus very rapid as indicated by the biological half-life of the isotope, which averages 0.58 hour in the first six tissues listed in Table II (range 0.5 to 0.8 hour) with the exception of lungs (2 hours). In rats fed 80 mg. of 2,4-D, radioactivity was observed in the tissues even after 41 hours. The peak concentration of radioactivity in the organs was reached at 8 hours, and there was essentially no change in this level until about 17 hours. At 20 hours after dosing, a distinct lowering in the concentration was observed (Figure 1). The averaged biological half-life of 2,4-D in the first six organs listed in Table II was 3.1 hours (range 3 to 3.5 hours). There was also some indication that there may be two rates of elimination. One rate was from 0 to 30 hours after dosing and the other from 30 hours onward. The plateau observed at the peak concentration could be attributed to the continuing absorption at slower rates of 2,4-D from the stomach. This is borne out by the fact that almost 7% of the radioactivity fed to the rat was still in the stomach at the end of 24 hours when an 80-mg. dose was given, but only 0.4% of the radioactivity was present in the stomach 12 hours after administration of 1 mg. of 2,4-D.

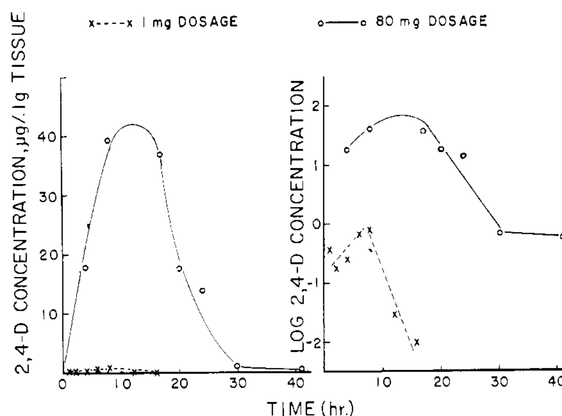

Figure 1. Average 2,4-D content in five vital organs (liver, kidney, heart, lung, spleen) of rats following a single oral dose of 2,4-D

Nature of the Radioactivity in Urine and Tissue Extracts. Paper chromatography was used initially to determine the nature of the radioactivity in the urine and tissue extracts; aliquots of these were chromatographed one dimensionally in two solvent systems. In both cases, only one radioactive spot, which coincided with 2,4-D, was observed. Clark *et al.* (2) in their work on sheep urine, using paper chromatography and electrophoresis, concluded that 2,4-D was excreted unchanged in the urine. In their studies in rabbits with phenoxyacetic acid, *o*- and *p*-chlorophenoxyacetic acid, Levey and Lewis (4) found that the rabbits excrete these compounds unchanged. When urine and tissue extracts were subjected to countercurrent separation, both contained a metabolite(s) of 2,4-D (Figure 2). The amount of this metabolite was very small and would escape detection by paper chromatography. The average amount of this metabolite in nine urine samples was 0.25% of the radioactivity found in the urine. The tissue extracts from animals sacrificed 1 or 2 hours after dosing showed a range of 0.7% of this metabolite in the lung to 6.1% in the liver (Table III). If a comparison were made between the radioactive metabolite present in rat liver from animals killed 2 or 8 hours after dosing, or between 1- and 80-mg. doses, it appears that the formation of the metabolite might be dependent on both the time after dosing and the dosage used. The liver could be the site of metabolite formation and storage, because it contains the largest amount of the metabolite among the organs analyzed. Slow elimination of the

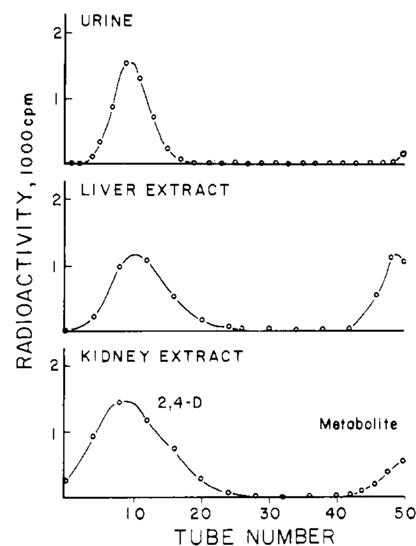

Figure 2. Countercurrent distribution patterns of radioactivity for the urine or tissue extracts from rats fed 2,4-D-1-C^{14} after 50 transfers using solvent system benzene over buffered phosphate solution (pH 4.5)

metabolite in the urine undoubtedly would cause an increase in its accumulation in the liver after a longer length of time or higher doses of the herbicide.

Table III. Percentage of 2,4-D Metabolite Present in Urine and Organs of Rats Fed 2,4-D, by Countercurrent Extraction Method

Sample	Metabolite, %
Urine (9)[a]	0.25 ± 0.10
Liver extract, 1-mg. dose (2)	3.63
Liver extract, 80-mg. dose (1)	6.10
Blood extract (1)	1.13
Kidney extract (1)	1.33
Lung extract (1)	0.71

[a] Number of samples analyzed.

Table IV. Percentage Distribution of C^{14} in Cellular Fractions of Rat Organs after Administration of 2,4-D-1-C^{14}

Organ	Nuclear			Mitochondrial			Microsomal			Soluble		
	A	B	C	A	B	C	A	B	C	A	B	C
Kidney	29	24	21	6	3	2	7	5	12	59	70	65
Liver	27	38	34	11	3	3	4	2	2	58	56	60
Spleen	15	18	16	3	2	1	2	2	2	79	78	80
Brain	30	25	26	5	2	1	2	2	2	63	69	70
Heart	8	9	12	4	2	1	3	2	1	84	86	86
Lungs	10	12	10	2	2	1	1	2	2	88	84	88

A. Average of 3 rats; 1 male fed 1 mg. and 2 females fed 2 mg. each and sacrificed after 3 hours.
B. Average of 2 rats; females fed 80 mg. each and sacrificed after 3 hours.
C. One female fed 80 mg. and sacrificed after 6 hours.

Paper chromatography of the metabolite (pooled sample from countercurrent separation) revealed that it has the same R_f value as 2,4-D in BAW solvent (0.88–0.90), but a slightly higher R_f than 2,4-D in 2-propanol-NH_4OH-H_2O solvent system. (R_f for 2,4-D 0.55 to 0.59, R_f for metabolite 0.67 to 0.69.)

Cellular Incorporation. Since so much difference in the rate of elimination at high (80-mg.) and low (1-mg.) doses was observed, a study was made on the cellular incorporation of this herbicide at both dose levels. All four fractions—i.e., nuclear, mitochondrial, microsomal, and soluble—contained significant radioactivity. The majority of the radioactivity—i.e., 57% in liver to 86% in lungs (Table IV)—was found in the soluble fraction. An average of 9% in the heart to 32% in the liver was found in the nuclear fraction while the mitochondrial and microsomal fractions had an average ranging from 1.4 to 6.7%. Although the range of per cent radioactivity in various fractions varied greatly among different tissues, the results were quite consistent for each tissue. There was no significant difference in the percentage in the various fractions when different dose levels were administered, or when the animals were sacrificed 3 and 6 hours after dosing. Hence, incorporation of 2,4-D in the cellular components cannot be used to explain the differences in the elimination rates at the two-dose levels. The radioactivity in the soluble fraction from all tissues could be easily extracted by ether, and the radioactivity in the ether extract was shown to be unchanged 2,4-D by paper chromatography. No attempt was made to determine whether or not the ether extract may contain 2,4-D metabolite by countercurrent extraction method. This observation suggests that the 2,4-D molecule in the soluble fraction is not protein- or peptide-bound as found in the plant tissues.

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INSECTICIDE DETERMINATION

Colorimetric Determination of O,O-dimethyl(1-hydroxy-2,2,2-trichloroethyl)-phosphonate and Its Higher Homologs

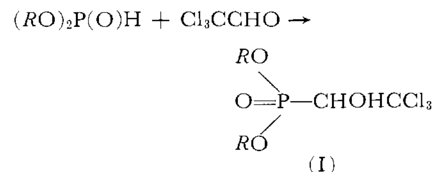
AHMED MUSTAFA, MAHMOUD M. SIDKY,[1] ZAKARIA I. EL-DARAWY, and HUSSEIN KAMEL

Department of Chemistry, Cairo University and the National Center for Social and Criminological Research, Cairo, Egypt

A rapid colorimetric method for the estimation of Dipterex and its higher homologs involves the cleavage of the phosphorus–carbon bond in the α-hydroxyphosphonate derivative, and the determination of the formed dialkyl phosphite by interaction with 3,5-dinitrobenzoic acid in the presence of alkali, whereby a stable violet-blue color develops. As little as 20 p.p.m. of Dipterex can be determined easily. The method allows differentiation between Dipterex and its dehydrochlorinated compound (DDVP).

The condensation of chloral with dialkyl phosphites leads to the formation of a series of *O,O*-dialkyl-(1-hydroxy-2,2,2-trichloroethyl)phosphonates (I) (*3, 10*) the lower members of which

$(RO)_2P(O)H + Cl_3CCHO \rightarrow$



(Ia), $R = CH_3$
(Ib), $R = C_2H_5$
(Ic), $R = C_3H_7$-*i*

possess remarkable insecticidal activity. Thus, for example, compound Ia, the dimethyl ester of (2,2,2,-trichloro-1-hydroxyethyl)phosphonic acid (Bayer L 13/59) marketed under the trade name Dipterex, recently attracted much interest as an insecticide effective against DDT-resistant houseflies (*6, 7*). It is widely used in United Arab Republic as

[1] Present address, Wayne State University, Detroit, Mich.