Gas Chromatographic Measurement of Disappearance Rates of 2,4-D and 2,4,5-T Acids and 2,4-D Esters in Mice

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The rates of disappearance of 2,4-D and 2,4,5-T acids and 2,4-D butyl and isooctyl esters were followed by electron-capture gas chromatographic analysis of extracts from whole mice. The data revealed the disappearance of esters was more rapid than the free acids, with the butyl ester rate greater than the octyl ester. A relatively prolonged body residence time was observed for 2,4,5-T. No 2,4-dichlorophenol was detected in the analyses of animals which had been injected with 2,4-D acid or its butyl or isooctyl esters. Pretreatment with the same herbicide revealed an enhanced disappearance rate only for the 2,4-D butyl ester.

Ester derivatives, and the free acid forms of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Hildebrand, 1946), are well known for their herbicidal activity. Gas chromatography was used for the measurement of 2,4-D in citrus fruit (Erickson and Hield, 1962; Meagher, 1966), in forage plants (Hagin and Linscott, 1965), and in fish (Coakley *et al.*, 1964); 2,4,5-T in apricots (Crane *et al.*, 1965); and 2,4,5-trichlorobenzoic acid in soil and plants (Kirkland and Pease, 1964). Clark and his coworkers (1967) recently measured 2,4-dichlorophenol following hydrolysis of 2,4-D in extracts from sheep body tissue by the same technique.

Owing to interest in toxicity evaluations for chlorinated pesticides as environmental poisons, investigations have been conducted on 2,4-D and 2,4,5-T in dogs (Drill and Hiratzka, 1953); 2,4-D in monkeys (Hill and Carlisle, 1947), man (Seabury, 1963; Nielsen *et al.*, 1965), and on its influence on rat thyroxine (Florsheim *et al.*, 1963); and on the 2,4-D butyl and isooctyl esters applied dermally, in rabbits (Kay *et al.*, 1965).

The authors' interests stem from an extensive pesticides-carcinogenesis study in mice which has been in existence in this laboratory for the past three years. The investigation reported herein was undertaken in order to ascertain the rate of disappearance of various pesticides from the body of mice, as measured by electron-capture gas chromatography on extracted whole animals.

EXPERIMENTAL

Standards. The 2,4-D and 2,4,5-T acids, and the 2,4-D butyl and isooctyl esters were obtained from the Dow Chemical Co., Midland, Mich. The standards were used as received following a purity check by gas chromatography and infrared spectra.

Treatment and Extraction of Animals. Female C 57 BL/6 mice, weighing 22 to 24 grams each, received a single 100-mg. per kg. subcutaneous injection of herbicide in dimethylsulfoxide, were sacrificed by cervical dislocation at specified times, and immediately homogenized in toto in a Waring Blendor. For the 2,4-D and 2,4,5-T acids, 75 ml. of 13 to 1 chloroform–concentrated hydrochloric acid were added to the blender with the animal. The sample was blended at high speed for 5 minutes and the supernatant poured through a glass wool mat into a collection flask. The mascerated animal residue was re-extracted with two 35-ml. portions of chloroform. The combined extract was passed through a 0.5-inch Celite blanket over 1.5 inches of anhydrous sodium sulfate in a sintered glass funnel on a suction flask. Light vacuum or simple gravity drip was used. The filtrate was quantitatively transferred to a 250-ml. 24/40 ⅁ round-bottomed flask with chloroform. The flask was connected to a Rinco flask evaporator and the solvent removed under a stream of hot tap water. The filtrate transfer was made in several steps in order to avoid using a large flask in this concentration step. The solvent-free oily liquid residue was transferred with a minimal amount of ethyl ether, 15 ml. of 2,2-dimethoxypropane, 15 ml. of anhydrous methanol, and 2 drops of concentrated hydrochloric acid to a 125-ml. Erlenmeyer flask and allowed to methylate overnight (Lorette and Brown, 1959). The following morning, 40 ml. of water were added, and the herbicide methyl esters were extracted twice with ethyl ether, washed thoroughly with water, and filtered through anhydrous sodium sulfate on a glass wool mat into a 10-ml. volumetric flask for gas chromatographic analysis. Care was taken to keep the ether extract below 10 ml. prior to filtration. If greater amounts of ether were obtained in the extraction, the filtrate was concentrated to less than 10 ml. under a nitrogen stream prior to transfer to the volumetric flask. The efficiency of this methylation procedure was essentially 100%.

Animals injected with 2,4-D butyl or isooctyl esters were blended initially with 70 ml. of 1 to 1 methylene chloride–hexane followed by two 35-ml. portions of the same solvent mixture. The extraction procedure was identical to that described above, with the exception that full vacuum was used on the sintered glass funnel, and the stripped oily liquid residue from the Rinco flask was transferred with 1 to 1 methylene chloride–hexane directly to a 10-ml. volumetric flask for gas chromatographic analysis.

Gas Chromatography. A ⅛-inch O.D. × 3-foot coiled borosilicate glass column, packed with 4% QF-1 fluorosilicone on 80- to 100-mesh acid-washed, DMCS-pretreated Chromosorb G and housed in an Aerograph HyFI Model

Bionetics Research Laboratories, Inc., Falls Church, Va.

600-B gas chromatograph containing a 250-mc. titanium tritide concentric foil electronic-capture detector, was employed for the analyses. Column temperature was controlled by a Barber-Colman Model 293C Capacitrol and was set at 120° C. for 2,4-D and 2,4,5-T methyl esters, 140° C. for 2,4-D butyl ester, and 175° C. for 2,4-D isooctyl ester. Nitrogen carrier gage pressure was 30 p.s.i. for all compounds except the 2,4-D isooctyl ester, which was maintained at 40 p.s.i.g. Under these conditions, elution in minutes was 2.0 for 2,4-D methyl ester, 4.1 for 2,4,5-T methyl ester, and 3.9 for the 2,4-D butyl and isooctyl esters.

To evaluate the possible influence of repeated herbicidal injections on compound disappearance rates from the animal (possibly via influences on the microsomal enzyme systems), an alternative group of mice were pretreated by five successive daily injections of the same dose of the same herbicide, the last pretreatment dose being administered 24 hours prior to the test injection. The analytical values of herbicide obtained by gas chromatographic analysis were then compared for the two groups of animals.

For quantitative measurements, the appropriate standard herbicide ester was injected after each analysis of two samples containing the same herbicide. The sample herbicide chromatogram peak area (square millimeters, by triangulation) was mathematically compared with the peak area per nanogram injected for the standard to obtain the level of herbicide present in the sample. The injection volume was 1.0 μl. (containing 50, 100, 25, and 10 nanograms, respectively, for standard injections of 2,4-D butyl and isooctyl esters, and 2,4-D and 2,4,5-T acids). Representative sensitivities obtained for the various esters were: 13 for methyl 2,4-D, 72 for methyl 2,4,5-T, 15 for butyl, 2,4-D, and 38 sq. mm. per nanogram for isooctyl 2,4-D.

RESULTS AND DISCUSSION

Recoveries of the herbicide standards from samples of solvent and animal tissue, which had been fortified prior to sample blending and subsequently taken through the appropriate extraction procedure, are given in Table I. The recoveries were slightly lower from spiked tissue samples than from solvent as one might expect; the values were, however, in excess of 80%, illustrating the efficacy of the extraction procedures chosen for these studies. Values of 82 to 84% recovery of the 2,4-D methyl and isopropyl esters from fortified potato samples have been reported by Bevenue *et al.* (1963).

2,4-D Butyl Ester. Animals which had been injected with this ester were serially sacrificed at 0, 0.5, 1, 2, 4, and 6 hours following injection. Six animals were used for each point. The same protocol was employed for animals receiving pretreatment with the 2,4-D butyl ester. The disappearance of the ester is indicated by the mean values from extraction of the entire animal (Table II). The pretreated animals appear to eliminate this ester somewhat more rapidly than nonpretreated animals. Control animals for all experiments showed no detectable herbicide or interfering peaks on the chromatogram.

2,4-D Isooctyl Ester. Animals were sacrificed at 0, 1, 2, and 4 hours following injection of the ester. Six animals were used for each point, and the disappearance rate was compared with animals receiving pretreatment by the isooctyl ester as illustrated in Table II.

2,4-D Acid. The rate of disappearance of this compound from the body of mice was much slower than for that of the esters. Sacrifice times were extended up to 24 hours following injection (0, 1, 2, 4, 6, 16, and 24 hours). Six animals were used per point. Pretreatment 24 hours prior to injection was not used with this compound. Its long residence time is shown in Table II.

2,4,5-T Acid. The disappearance of 2,4,5-T was even slower than that of 2,4-D. The elimination of this herbicide appeared to be sufficiently slow effectively to produce higher recoveries in the animals receiving pretreatment with 2,4,5-T (Table II).

No interfering chromatogram peaks were observed in any of the samples analyzed. Extraneous peaks were noted for several extracts containing 2,4-D acid; none, however, overlapped that of the 2,4-D methyl ester.

Kinetics. One may see, by inspection of the disappearance rates (slopes) in Table III, that the linearity of disappearance is a function of both time and compound,

Table I. Recovery Data from Extraction Procedures

Compound	Fortifying Medium	No. of Analyses	Recovery, %	Standard Deviation
2,4-D	Solvent	4	93.0	16.4
2,4-D	Tissue	5	85.9	6.9
2,4,5-T	Solvent	6	96.0	3.6
2,4,5-T	Fetuses	1	97.4	...
2,4,5-T	Amniotic fluid	1	101.0	...
Butyl 2,4-D	Solvent	4	100.5	7.5
Butyl 2,4-D	Tissue	11	83.0	2.8
Isooctyl 2,4-D	Solvent	3	96.9	5.9

Table II. Disappearance of Esters and Acids from Mice

Sacrifice Time, Hr.	Recovery, % Nonpretreated	Pretreated
	2,4-D Butyl Ester	
0	81.3 ± 3.3	84.9 ± 3.9
0.5	64.1 ± 11.5	56.4 ± 6.9
1	50.3 ± 11.5	33.4 ± 13.8
2	23.2 ± 7.8	16.4 ± 6.3
4	6.6 ± 6.1	
6	4.9 ± 3.8	
	2,4-D Isooctyl Ester	
0	81.8 ± 3.0	71.4 ± 7.0
1	64.0 ± 2.1	55.3 ± 7.3
2	56.8 ± 2.4	55.6 ± 6.8
4	36.5 ± 1.8	29.8 ± 11.5
	2,4-D Acid	
0	77.5 ± 1.8	
1	54.0 ± 4.6	
2	58.1 ± 2.6	
4	58.2 ± 6.3	
6	61.3 ± 4.8	
16	5.3 ± 7.8	
24	9.4 ± 6.7	
	2,4,5-T Acid	
0	77.1 ± 5.0	111.0 ± 6.5
16	56.9 ± 4.2	83.3 ± 5.7
24	23.7 ± 3.6	65.8 ± 4.8

Table III. Slopes[a] of the Disappearance Curves

Compound	t, Hr. 0–0.5	0.5–1	0–1	1–2	2–4	4–6	6–16	0–16	16–24
2,4-D butyl ester	34.2	27.6	31.0	27.1	8.3	0.85			
2,4-D butyl ester[b]	57.0	46.0	51.0	17.0					
2,4-D isooctyl ester			17.8	7.2	10.2				
2,4-D isooctyl ester[b]			61.1	Zero[c]	12.9				
2,4-D			23.5	Zero[c]	Zero[c]	Zero[c]	5.6	4.5	Zero[c]
2,4,5-T								1.3	4.1
2,4,5-T[b]								1.7	2.2

[a] Values are expressed in per cent disappearance per hour.
[b] Animals receiving pretreatment.
[c] Negative slopes were designated zero.

and with the butyl ester in particular, of pretreatment as well. A test of significance on the slopes for the two sets of animals receiving the butyl ester was highly significant ($t_{0.01}$ = 3.6 for the 0- to 2-hour period). Figure 1 was obtained from the data in Table III assuming approximate linearity during the time periods of: 0 to 2 hours for the butyl ester; 0 to 1 hour for the butyl ester, pretreated; 0 to 4 hours for both the isooctyl ester and the isooctyl ester, pretreated; 0 to 16 hours and 0 to 24 hours for both 2,4,5-T and 2,4,5-T, pretreated. The slopes were drawn from a relative 100% recovery, converting the ordinate designation to relative per cent disappearance. The half-turnover rates were 1.11 and 0.85 hours for the butyl ester (nonpretreated and pretreated, respectively) for the 0- to 2-hour period; 3.80 and 5.56 hours (0- to 2-hour period) and 3.45 and 3.18 hours (0- to 4-hour period) for the isooctyl ester (nonpretreated and pretreated, respectively). That $t_{1/2}$ (0- to 16-hour) value for 2,4-D was 4.12 hours and that for 2,4,5-T (nonpretreated and pretreated, respectively) was 14.1 and 31.8 (0- to 24-hour period).

A study of the disappearance rates of 2,4-D and 2,4,5-T, and of the butyl and isooctyl esters of 2,4-D by gas chromatography of extracted whole mice showed the following: butylester > isooctyl ester > 2,4-D acid ≫ 2,4,5-T acid. Crosby and Tutass (1966) have reported that 2,4-D in the presence of ultraviolet light and moisture degrades via hydrolysis of the phenoxide and by C-hydroxylation (by replacement of the 2-chloro, then the 4-chloro atoms). Beta-oxidation of 4-(2,4-dichlorophenoxy)butyric acid to 2,4-D has also been reported in plants (Gutenmann and Lisk, 1963; Linscott, 1964). Radioisotope studies, however, of 2,4-D-C^{14} in sheep (Clark *et al.*, 1964) and in rats (Khanna and Fang, 1966) have indicated that, while a small level of unidentified metabolite may be formed, 2,4-D is essentially unaltered in the excreta. Metabolic cages for collection of excreta were not employed in the present study. Phenoxyacetic acid has also been reported as being quantitatively excreted from man and dogs (Nencki and Giacosa, 1880; Thierfelder and Schempp, 1917) and from rats and rabbits (Levey and Lewis, 1947). The rapid elimination of the esters measured in the present study suggests a similar route for these herbicides; however, pretreatment may alter the elimination rate, as was indicated for the butyl ester. Detection of 2,4-D upon methylation of ester extracts was not observed in these experiments. Comparable recoveries of the herbicides from mice pretreated

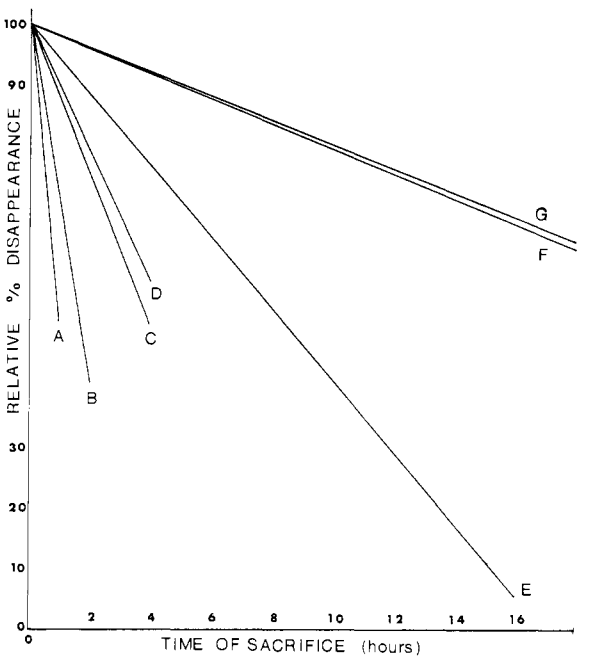

Figure 1. Relative per cent disappearance *vs.* time of sacrifice

A. 2,4-D butyl ester, pretreatment
B. 2,4-D butyl ester
C. 2,4-D isooctyl ester
D. 2,4-D isooctyl ester, pretreatment
E. 2,4-D
F. 2,4,5-T pretreatment
G. 2,4,5-T

with dimethylsulfoxide indicated that this vehicle did not appear to influence the disappearance rates. Chlordan had a stimulatory effect on hepatic microsomal drug metabolism in the rat (Hart *et al.*, 1963). Pretreatment did not appear to influence the disappearance rate of the isooctyl ester, or 2,4,5-T. Cleavage of 2,4-D or 2,4-dichlorophenol was not detected in bean plants by Crosby (1964). Similarly, 2,4-dichlorophenol was undetected in this study during chromatography of extracts from mice receiving 2,4-D or its butyl or isooctyl esters.

ACKNOWLEDGMENT

The authors acknowledge the technical assistance of Melba Bryan, Margaret Cavanaugh, Susan Sieber, and Thomas Welsko.

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Received for review March 15, 1967. Accepted May 18, 1967. Study supported by Research Contract PH 43-64-57, National Cancer Institute, National Institutes of Health, Public Health Service, and represents paper No. 42 of this contract.