cottonseed when cross-fertilized. No other effects of treatment were noted, either on the treated plants or in their hybrid progeny. The application of either pantothenic acid or D-ribose partially reversed the effect of the chlorinated acids.

Susceptibility to dalapon may be under partial genetic control. Scott reported rather widely differing results in his gametocide experiments with several varieties of cotton. Funderburk and Davis (*14*) reported that hybrid varieties of corn differed in their susceptibility to dalapon, and Buchholz at Wisconsin as well as Behrens (*4*) studied a number of inbred lines of corn which differ widely in tolerance to dalapon.

Although higher plant systems seem to have little effect on the various chlorinated aliphatic acids, these acids have varied and profound effects on higher plants. Obviously a number of plant processes are affected, and it is likely that more than a single pathway is inhibited. The evidence points to multiple pathways and to more than one site of action.

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Received for review August 1, 1963. Accepted November 22, 1963. Division of Agricultural and Food Chemistry, 144th Meeting, ACS, Los Angeles, Calif., April 1963.

End of Symposium

ANIMAL METABOLISM OF HERBICIDES

The Fate of 2,4-Dichlorophenoxyacetic Acid in Sheep

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THE EFFECTIVENESS of 2,4-dichlorophenoxyacetic acid (2,4-D) and related compounds as plant growth regulators has been recognized for a number of years (*1*, *4*). The herbicidal activity of 2,4-D has been attributed to its hormonelike activity rather than to direct dehydration or necrosis of plant tissues (*3*). Although previous studies have shown that phenoxyacetic acid is excreted unchanged by man and dogs (*6*, *8*) and almost quantitatively in urine by rats and rabbits within 24 hours (*2*), no work has been done on the metabolism of 2,4-D or related compounds in ruminants. Previous observations have shown that sheep can tolerate rather large quantities of 2,4-D salts and esters for extended periods of time (*7*). However, whether 2,4-D is metabolized, stored, or excreted unchanged by the sheep has not been established.

Apparatus

The instrument used for carbon-14 quantitation was a thin-window (mica) Geiger-Müller tube enclosed in a Tracerlab SC-59S Shielded Manual Sample Changer and attached to a Tracerlab "Versamatic II" Scaler.

Reagents

Chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid, disodium salt, dihydrate): 0.05% in concentrated sulfuric acid.

2-Phenoxyethanol–silver nitrate reagent: 1.7 grams of silver nitrate in 5 ml. of water. Add 20 ml. of 2-phenoxyethanol and dilute to 200 ml. with acetone. If the solution darkens, 1 to 5 drops of 30% hydrogen peroxide may be added.

Electrophoresis buffer: dissolve 5.4 grams of KH_2PO_4 and 0.93 gram of NaH_2PO_4 in 1 liter of water and adjust pH to 6.0 with Na_3PO_4.

Procedure

Administration of 2,4-D and Sampling Procedure. A gelatin capsule containing 539.6 μc. in 106.3 mg. of 2,4-dichlorophenoxyacetic acid-2-C^{14} (Tracerlab, Inc.) in 95% ethanol was administered orally to a yearling ewe (weight 26.6 kg.). The dose of 4.0

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Approximately 96% of an orally administered dose of 2,4-D-C^{14} to a sheep was excreted unchanged in the urine within 72 hours. Slightly less than 1.4% of the administered radioactivity was excreted in the feces over the same period. The nature of the urinary C^{14} was established by paper chromatography and electrophoresis. In no case was there a test for chloride, carbon-14, or phenoxyacetic acid groups at any R_f or migration other than that of standard 2,4-D. From the results, it was concluded that 2,4-D is excreted unchanged by the sheep. Very little residual radioactivity was found in edible tissue.

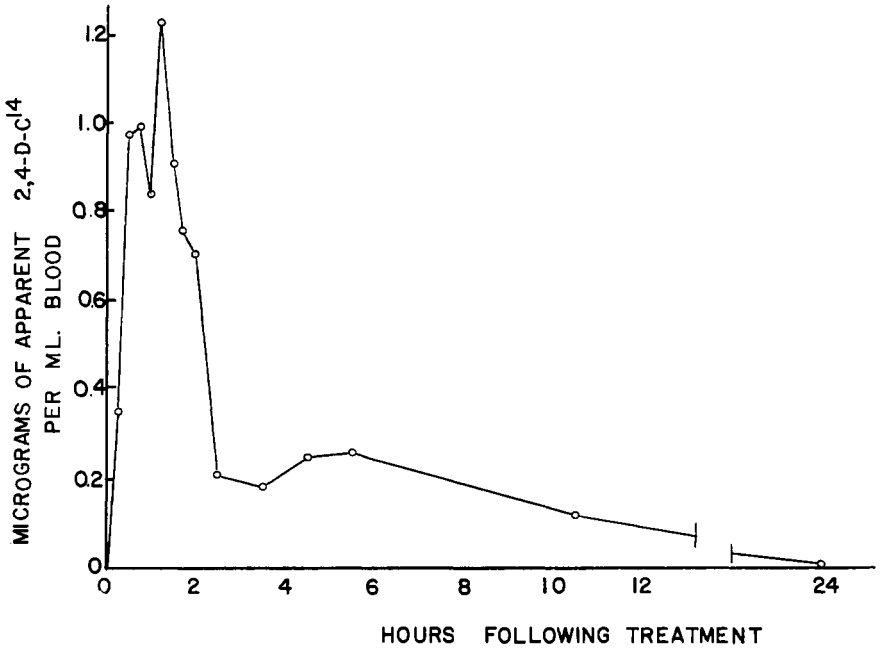

Figure 1. Appearance of C^{14} in the blood of a sheep after oral administration of 2,4-D-C^{14}

mg. of 2,4-D per kg. of sheep weight was calculated as the approximate minimum daily dose a sheep would ingest from grazing on a pasture treated with the herbicide. Continuous samples of urine were taken by means of an in-dwelling catheter which fed into a collection bottle attached to the side of the sheep. Fecal samples were obtained by a plastic collecting bag taped to the animal. Blood samples were withdrawn from the jugular vein at timed intervals following administration of the dose.

On the fourth day post-treatment, the animal was anesthetized and exsanguinated. At necropsy, samples of various tissues and organs were removed, weighed, and frozen for assay of carbon-14.

Sample Preparation. FECES. To 20 grams of fresh feces was added 120 ml. of solvent which consisted of a 50:50 mixture of 95% ethanol and diethyl ether (v./v.) plus 1% by volume of concentrated HCl. The mixture was stirred and allowed to stand 30 minutes at room temperature. The slurry was then power-filtered through Whatman No. 42 filter paper and the residue washed twice with 50 ml. of solvent and once with 50 ml. of chloroform. The filtrate was made slightly alkaline, concentrated, and assayed. Recovery of a sample spiked with 2,4-D-C^{14} and treated in this manner was 99.3%.

URINE. Samples of whole urine were diluted 1:100 with 95% ethanol. One-milliliter aliquots were evaporated to dryness and assayed for C^{14} without further treatment since it was determined that little loss due to self-absorption occurred.

BLOOD. High recoveries (above 90%) were obtained from blood by extraction with hot 70% ethanol (adjusted to pH 1 with HCl) followed by chilling and filtration.

TISSUE. The extraction method used was unsatisfactory for carbon-14 assay where infinitely thin plates are required. However, allowing for 50% absorption of carbon-14 by the residues in the planchets, a confidence level of 0.05 p.p.m. was established.

Results

Appearance of 2,4-D-C^{14} in Blood Following Oral Administration. Samples of blood were withdrawn from the sheep at 15-minute intervals for 2 hours following administration of 2,4-D-C^{14}. Thereafter, samples were taken at various intervals over 24 hours' duration.

Levels of carbon-14 in ethanol extracts of these blood samples are given in Figure 1. Activity rose rapidly during the first half-hour, reached a peak at $1^1/_4$ hours, and diminished rapidly thereafter. By 24 hours post-treatment, the blood radioactivity had diminished to essentially background levels.

Excretion of 2,4-D-C^{14}. About 15% of the original dose of 2,4-D-C^{14} was found in the urine collected during the first $1^3/_4$ hours post-treatment. At $8^1/_2$ hours post-treatment, 50% had been recovered in the urine, and by the end of 28 hours, over 90% of the dose had been excreted. By 60 hours, the total recovery exceeded 95%; thereafter, urine radioactivity was negligible. The total urinary excretion of 2,4-D-C^{14} by 70 hours was 95.8%. Extraction of total feces collected over the 70-hour period following administration of 2,4-D-C^{14} yielded approximately 1.4% of the dose.

Identification of Excreted 2,4-D-C^{14} by Paper Chromatography and Paper Electrophoresis. Urine from the sheep treated with 2,4-D-C^{14} was compared chromatographically and electrophoretically with pure 2,4-D-C^{14}. The urine sample was a pool representing the total 70-hour collection following administration of the herbicide. Comparisons of R_f values and electrophoretic migrations from pure 2,4-D-C^{14} samples were made with pooled urine samples and with pooled urine to which pure 2,4-D-C^{14} was added. The data obtained from the various chromatographic systems and electrophoresis are given in Tables I and II.

The papers used for ascending chromatography included Whatman No. 1, washed; Whatman No. 1, washed and impregnated with 2% sodium carbonate; and glass fiber strips (Hurlburt Paper Company No. 934-A4). For electrophoresis, Beckman Electrophoresis Paper No. 300-028 and glass fiber strips were used.

C^{14} was detected by radioautography. The chromatograms were covered with a thin sheet of Saran Wrap (Dow Chemical Co.) and then placed in contact with nonscreen x-ray film for approximately 1 week. For identification of organic chloro compounds, the method of Mitchell (5) was employed.

Table I. Paper Chromatography of 2,4-Dichlorophenoxyacetic Acid

Stationary Phase	R_f[a] Mobile Phase: Acetone : urea : water (60 : 0.5 : 40) v./w./v.	Ethanol, 95%	Acetic acid, 2%	Benzene : acetic acid : water (1 : 1 : 2)
	2,4,-D-C^{14}			
Whatman No. 1	0.86, 0.85	0.31, 0.30	0.63, 0.63	0.84, 0.83
Whatman No. 1 and 2% Na_2CO_3	0.65, 0.64			
Glass fiber	0.81, 0.81	0.83, 0.83	0.76, 0.78	0.90, 0.88
	Urine			
Whatman No. 1	0.79, 0.80	0.24, 0.25	0.62, 0.60	0.82, 0.83
Whatman No. 1 and 2% Na_2CO_3	0.67, 0.64			
Glass fiber	0.83, 0.83	0.80, 0.80	0.90, [b]	0.91, [b]
	Urine and 2,4-D-C^{14}			
Whatman No. 1	0.82, 0.82	0.24, 0.25	0.62, 0.58	0.84, 0.83
Whatman No. 1 and 2% Na_2CO_3	0.68, 0.64			
Glass fiber	0.83, 0.83	0.75, 0.75	0.90, [b]	0.88, [b]

[a] The first R_f value with the papers is by radioautography for C^{14}, and the second for organic chloride with 2-phenoxyethanol–silver nitrate–acetone. With the glass fiber, the first value is by radioautography and the second by chromotropic–sulfuric acid for the phenoxyacetic acid group.
[b] Spot near R_f 1.0 masked by impurities.

Table II. Paper Electrophoresis of 2,4-Dichlorophenoxyacetic Acid

Sample	Strip	Migration, Cm.[a]
2,4-D-C^{14}	Paper	−3.8, −4.0
	Glass	−2.0 to +1.5
Urine	Paper	−4.1, −4.1
	Glass	−1.0 to +2.0
Urine and 2,4-D-C^{14}	Paper	−3.6, −3.5
	Glass	−2.0 to +2.0

[a] For the paper strips, the first value was obtained by radioautography and the second by test for organic chloride with 2-phenoxyethanol–silver nitrate–acetone. For the glass strips, although the pattern is a smear, in all cases the same pattern was found by both the radioautography and by chromotropic–sulfuric acid detection methods.

Chromatographic identification of phenoxyacetic acid was accomplished by a modification of the chromotropic acid–sulfuric acid reagent. The use of glass fiber filter strips instead of paper is essential when using this technique because of the corrosive nature of sulfuric acid. The glass strip chromatogram is dipped momentarily into the chromotropic acid solution at 140° to 150° C. and observed immediately for violet spots or bands indicative of 2,4-D.

Electrophoresis of samples containing 2,4-D was carried out on a Spinco Paper Electrophoresis apparatus with Durham cells. The papers used included glass fiber filter strips as well as the usual electrophoresis paper strips. The buffer was a solution of KH_2PO_4/NaH_2PO_4, pH 6.0. The time of migration varied from 5 to 6 hours at a current time of 9.0 ma.

The identity of 2,4-dichlorophenoxyacetic acid in the urine was judged by the following criteria: a single band or spot on each chromatogram as evaluated by radioautography; coincidence of a positive test for organic chloride with the band or spot obtained by radioautography; coincidence of a positive test for phenoxyacetic acid with the band or spot obtained by radioautography; a single band or spot in electrophoretic patterns as evaluated by radioautography, organic chloride, and phenoxyacetic acid determinations; R_f values and electrophoretic migrations of the urine C^{14} coincident with those obtained from 2,4-D-C^{14} alone and/or urine C^{14} to which pure 2,4-D-C^{14} had been added; and in no instance was there more than one spot identified by either chromatography or electrophoresis. By these criteria, the radioactive material excreted in the urine by the sheep was identified as 2,4-D-C^{14}.

Tissue Residues. Although the radiometric method for tissue carbon-14 left much to be desired, all the edible tissues assayed contained less than 0.05 p.p.m., and in most cases was far below this level. Exceptions to this were the thyroid and the urinary bladder, which indicated 0.56 and 0.50 p.p.m., respectively. No attempt was made to identify the nature of the residual radioactivity.

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Received for review July 26, 1962. Accepted August 14, 1963.