

DAVIS v. DOW

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Exhibits 51 - 100

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rottened 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 treated 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 (12) reported that hybrid varieties of corn differed in their susceptibility to dalapon, and Buchholz at Wisconsin as well as Behrens (1) 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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End of Symposium

ANIMAL METABOLISM OF HERBICIDES

The Fate of 2,4-Dichlorophenoxyacetic Acid in Sheep

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, 2). 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 (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.

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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_2PO_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.

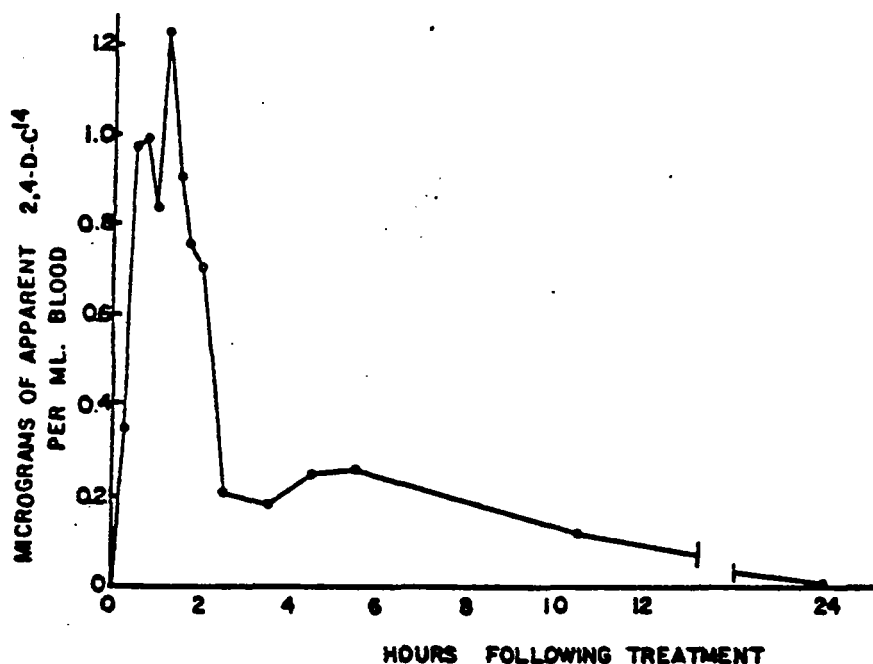


Figure 1. Appearance of C^{14} in the blood of a sheep after oral administration of 2,4-D- C^{14} .

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 1½ 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 1½ hours post-treatment. At 8½ 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.

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

Stationary Phase	Mobile Phase			
	Acetone: water (60:0.5:40) v./v./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 ₂ CO 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 ₂ CO ₃	0.67, 0.64			
Glass fiber	0.83, 0.83	0.80, 0.80	0.90, *	0.91, *
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 ₂ CO ₃	0.68, 0.64			
Glass fiber	0.83, 0.83	0.75, 0.75	0.90, *	0.88, *

* The first R_f value with the papers is by radioautography for C¹⁴, 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 chromatotropic-sulfuric acid for the phenoxyacetic acid group.

* Spot near R_f 1.0 masked by impurities.

Chromatographic identification of phenoxyacetic acid was accomplished by a modification of the chromatotropic 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 chromatotropic 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 KH₂PO₄/NaH₂PO₄, 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 C¹⁴ coincident with those obtained from 2,4-D-C¹⁴ alone and/or urine C¹⁴ to which pure 2,4-D-C¹⁴ 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-C¹⁴.

Tissue Residues. Although the radiometric method for tissue carbon-14

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

Sample	Strip	Migration, Cm.*
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

* 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 chromatotropic-sulfuric acid detection methods.

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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REPORT TO

THE NATIONAL AGRICULTURAL CHEMICALS ASSOCIATION

REPEATED DERMAL TOXICITY STUDIES
ON THREE FORMULATIONS OF
2,4 DICHLOROPHENOXYACETIC ACID

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REPORT TO
THE NATIONAL AGRICULTURAL CHEMICALS ASSOCIATION
REPEATED DERMAL TOXICITY STUDIES ON
THREE FORMULATIONS OF 2,4 DICHLOROPHENOXYACETIC ACID

I. Introduction and Outline of Investigation

At the request of The National Agricultural Chemicals Association (NACA), a repeated dermal toxicity study, using albino rabbits as test animals, was conducted on the following three commercially available formulations of 2,4 dichlorophenoxyacetic acid (2,4-D):

2,4-D Dimethylamine Salt*
2,4-D Isooctyl Ester**
2,4-D Butyl Ester***

The procedure employed in the study was that supplied by NACA.

- * Weed-Rhap Herbicide A-4; Hercules Powder Company, Inc., Wilmington, Delaware
- ** Monsanto 2,4-D Low Volatile Ester Weed Killer; Monsanto Chemical Company, St. Louis, Missouri
- *** Estron 76 BE Weed Killer; The Dow Chemical Company, Midland, Michigan

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Skin application of the test materials, prepared as dilutions in water and/or oil, were made seven hours per day, five days per week for three weeks according to the outline presented in Table I. Fifteen milliliter volumes of the appropriately prepared test dilutions were used for the skin applications.

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TABLE I

Repeated Dermal Toxicity Study - Albino Rabbits

Outline of Experiment

Group Number	Number of Rabbits		Condition of Skin	Test Material and Concentration Employed (%, w/v)	Per Cent 2, 4-D Acid Equivalent
	Male	Female			
C-I	4	4	Intact	water control	-
	4	4	Abraded	water control	-
C-II	4	4	Intact	*oil control	-
	4	4	Abraded	oil control	-
T-I	2	2	Intact	1.53 dimethylamine salt in water	0.626
	2	2	Abraded	1.53 dimethylamine salt in water	0.626
T-II	2	2	Intact	7.65 dimethylamine salt in water	3.13
	2	2	Abraded	7.65 dimethylamine salt in water	3.13
T-III	2	2	Intact	1.36 isooctyl ester in water	0.626
	2	2	Abraded	1.36 isooctyl ester in water	0.626
T-IV	2	2	Intact	6.8 isooctyl ester in water	3.13
	2	2	Abraded	6.8 isooctyl ester in water	3.13
T-V	2	2	Intact	1.0 butyl ester in water	0.626
	2	2	Abraded	1.0 butyl ester in water	0.626

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TABLE I Continued
Repeated Dermal Toxicity Study - Albino Rabbits
Outline of Experiment

Group Number	Number of Rabbits Male Female		Condition of Skin	Test Material and Concentration Employed (%, w/v)	Per Cent 2, 4-D Acid Equivalent
T-VI	2	2	Intact	5.0 butyl ester in water	3.13
	2	2	Abraded	5.0 butyl ester in water	3.13
T-VII	2	2	Intact	1.36 isooctyl ester in oil	0.626
	2	2	Abraded	1.36 isooctyl ester in oil	0.626
T-VIII	2	2	Intact	6.8 isooctyl ester in oil	3.13
	2	2	Abraded	6.8 isooctyl ester in oil	3.13
T-IX	2	2	Intact	1.0 butyl ester in oil	0.626
	2	2	Abraded	1.0 butyl ester in oil	0.626
T-X	2	2	Intact	5.0 butyl ester in oil	3.13
	2	2	Abraded	5.0 butyl ester in oil	3.13

* LP-429, 433 Furnace Oil #2; Texaco, Inc., Lockport, Illinois

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The rabbits in all groups were housed individually over the period of investigation and observations were made with respect to body weight effects, incidence of mortality and behavioral and local skin reactions. In addition, hematologic studies and clinical blood chemistry determinations were conducted.

At the end of the test period, final mortality tabulations were made and all surviving rabbits from each test and control group were sacrificed for pathologic studies. Gross autopsies were performed and a complete set of representative tissues and organs was taken from each rabbit and preserved in formalin. Microscopic examinations of selected tissues and organs were made to ascertain the presence or absence of histopathologic change as a result of dermal applications of the test material. Also, at the time of gross autopsy, heart, liver, kidneys, spleen and testes were weighed and organ-to-body weight ratios calculated.

II. Investigational Procedure

A. Experimental Animals

The animals employed in the repeated dermal toxicity study were adult, New Zealand strain albino rabbits. The body weights of the test animals were in the two-to-three kilogram range. A total of 112 adult rabbits were selected from a larger population after examination of each animal for general physical well-being. Each animal selected for the experiment was housed individually in a wire-bottomed stainless steel rabbit cage for the duration of the test. All rabbits were fed the standard laboratory rabbit ration* plus water ad libitum.

B. Method of Application

In preparation for the test, the back of each rabbit was shaved using electric clippers. The exposure site thus prepared on each animal constituted approximately ten per cent of the total body surface area. The animals were then returned to their stock cages and 24 hours were allowed to elapse before the first dermal applications were made. This period permitted the skin to recover from the slight disturbance of the stratum corneum caused by the clipping procedure and also permitted the healing of any microscopic abrasions possibly produced during the shaving procedure.

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* Rockland Rabbit Ration, Teklad, Inc., Monmouth, Illinois

At the end of the 24-hour recovery period, just prior to the first application of the test material, the shaved skin of two males and two females in each test group (four males and four females in each control group) were abraded by making a series of parallel epidermal incisions, every two or three centimeters longitudinally, over the area of exposure. These incisions were made sufficiently deep to penetrate the stratum corneum, but not to disturb the derma. The skin of the remaining animals in each group was left intact.

Dosing was accomplished in the following manner: an 8-ply gauze patch (4" x 3") was affixed over the application site of each animal. Fifteen milliliters of the respective test dilution was placed on this gauze patch and immediately covered with a piece of plastic sheeting (6" x 4"). The plastic sheeting was held in place by thin strips of adhesive tape. Finally, the entire trunk of the animal was covered by a sleeve of gauze elastic stockinette which helped to hold both gauze patch and plastic sheeting in place. The test material was allowed to contact the skin for a period of seven hours. At the end of the contact period the coverings were removed and all residual test material was washed from the skin with soap and water.

The animals in the control groups were treated in the same manner as those in the test groups with the exception that either tap water or oil was applied to the skin.

The above schedule was followed 5 days per week for three weeks or a total of 15 applications.

C. Body Weight Effects

Each animal used in the study was weighed twice during the week prior to the inception of the experiment. Thereafter, the animals were weighed three times during each week i.e., Monday, Wednesday and Friday, the last weighing being immediately prior to sacrifice and autopsy.

D. Mortality and Reactions

Checks for mortality and abnormal behavioral reactions were made daily during the three-week test period. Special emphasis was placed on observations for local skin reactions in order to determine the degree of skin irritation produced by the test material.

E. Hematologic and Clinical Blood Chemistry Studies

Blood studies including determinations of hemoglobin concentration, hematocrit value, erythrocyte count and both total and differential leukocyte counts were made at the beginning of the study and at the end of the three-week test period. These determinations were conducted upon each individual animal.

Clinical blood chemistry studies including determinations of urea nitrogen concentration (BUN) and alkaline phosphatase activity (SAP) were conducted on each animal at the end of the three-week test period.

F. Gross and Microscopic Pathologic Studies

Arrangements were made to subject any animal which might die during the test to a gross autopsy. Also, in those instances where post-mortem changes were not advanced, sections of representative tissues and organs were scheduled to be taken for histopathologic study.

At the conclusion of the investigational period, all surviving rabbits in each group were sacrificed and subjected to gross pathologic examination. At this time, the heart, liver, kidneys, spleen and testes were removed, trimmed and weighed. The organ weights were then tabulated and expressed as the percentage of the total body weight.

Also at the time of gross pathologic examination, the following tissues and organs were fixed in formalin and prepared for histological examination: brain, spinal cord, peripheral nerves (sciatic and femoral), thyroid, adrenals, gonads, skeletal muscle (thigh) heart, liver, spleen, pancreas, kidney, urinary bladder and mediastinal and mesenteric lymph nodes. All sections were stained with Hematoxylin and Eosin. In addition, sections of the brain, spinal cord and peripheral nerves were prepared and stained with Luxol-Fast Blue to evaluate possible myelin sheath damage.*

* Margolis, George, M. D., Picket, John Phillip, H. T.; New Applications of the Luxol Fast Blue Myelin Stain; Lab. Invest., 5, pp. 459-474; 1956.

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III. Results

A. Mortality

The mortality data are presented separately for each test material in Tables II, III and IV. A summary of the mortality data for all materials is presented in Table V.

Table VI presents a summary of individual mortality data with respect to day of death and number of applications received.

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TABLE II

TEST MATERIAL: Dimethylamine Salt of 2, 4-D

Repeated Dermal Toxicity Study - Albino Rabbits

Mortality Data

Group	Mortality*			
	Intact Skin		Abraded Skin	
	Male	Female	Male	Female
C-I	0/4	0/4	1/4	1/4
T-I	0/2	0/2	0/2	0/2
T-II	0/2	0/2	0/2	1/2

* Number Dead/Number Tested

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TABLE III

TEST MATERIAL: Isooctyl Ester of 2,4-D
Repeated Dermal Toxicity Study - Albino Rabbits
Mortality Data

Group	Mortality*			
	Intact Skin		Abraded Skin	
	Male	Female	Male	Female
C-I	0/4	0/4	1/4	1/4
C-II	1/4	0/4	0/4	0/4
T-III	0/2	0/2	1/2	0/2
T-IV	0/2	0/2	0/2	0/2
T-VII	0/2	0/2	1/2	1/2
T-VIII	0/2	0/2	1/2	0/2

* Number Dead/Number Tested

TABLE IV
TEST MATERIAL: Butyl Ester of 2,4-D
Repeated Dermal Toxicity Study - Albino Rabbits

Mortality Data

Group	Mortality*		Mortality*	
	Intact Skin Male	Intact Skin Female	Abraded Skin Male	Abraded Skin Female
C-I	0/4	0/4	1/4	1/4
C-II	1/4	0/4	0/4	0/4
T-V	0/2	0/2	0/2	0/2
T-VI	0/2	0/2	1/2	0/2
T-IX	1/2	0/2	0/2	1/2
T-X	0/2	1/2	0/2	0/2

* Number Dead/Number Tested

TABLE V
Repeated Dermal Toxicity Study - Albino Rabbits
Summary of Mortality Data

Group	Mortality*		Total
	Number Dead/Number Tested	Male	Female
C-I	1/8	1/8	2/16
C-II	1/8	0/8	1/16
T-I	0/4	0/4	0/8
T-II	0/4	1/4	1/8
T-III	1/4	0/4	1/8
T-IV	0/4	0/4	0/8
T-V	0/4	0/4	0/8
T-VI	1/4	0/4	1/8
T-VII	1/4	1/4	2/8
T-VIII	1/4	0/4	1/8
T-IX	1/4	1/4	2/8
T-X	0/4	1/4	1/8

* Figures represent total number of animals employed, i.e. intact and abraded skin combined.

TABLE VI
Repeated Dermal Toxicity Study - Albino Rabbits
Summary of Individual Mortality Data

Group	Animal Number and Sex	Day of Death	Number of Applications Received
C-I	7-Male	19	15
	11-Female	13	10
C-II	20-Male	22	15
T-II	46-Female	20	15
T-III	51-Male	18	14
T-VI	75-Male	8	6
T-VII	83-Male	10	8
	87-Female	12	10
T-VIII	91-Male	7	5
T-IX	97-Male	9	7
	103-Female	9	7
T-X	109-Female	21	15

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B. Reactions

1. Behavioral Reactions

No significant untoward behavioral reactions were noted among rabbits receiving dermal applications of the respective test materials in the form of aqueous solutions. Behavior of these animals was the same as that of their corresponding water controls.

Hyperirritability became evident after the third or fourth day of testing among all animals (test and control) receiving dermal applications of oil or oil dilutions of the respective test materials. These animals cried upon application of the test material after the fourth or fifth dosing. These reactions persisted throughout the remainder of the test period.

2. Local Skin Reactions

Local skin reactions were noted after the fifth or sixth application among all animals receiving the respective test materials as aqueous dilutions. However, these reactions were identical to those seen in the water control animals and were characterized by mild to moderate erythema, wrinkling and drying of the skin.

Rabbits receiving oil dilutions of the respective test materials as well as the oil control animals exhibited the same type of local skin reactions after approximately three applications. The reactions were characterized by severe erythema, moderate edema,

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drying, cracking and bleeding of the skin. Loss of hair at the application site and surrounding areas was also noted. As the test progressed, the skin at the application site became necrotic followed by the formation of eschar tissue.

C. Body Weight Effects

Salient individual body weight data for each control and test group are presented separately in Tables VII through XVIII.

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TABLE VII

GROUP: C-I

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
Male	1-I	2.35	2.30	2.21	2.27	2.26
	2-I	2.24	2.41	2.55	2.52	2.35
	3-I	2.36	2.41	2.43	2.43	2.44
	4-I	2.88	3.08	3.17	3.23	3.34
	5-A	2.27	2.31	2.46	2.41	2.66
	6-A	2.44	2.48	2.26	2.35	2.61
	7-A	2.48	2.46	2.55	2.72	(2.72)
	8-A	2.69	2.70	2.72	2.83	2.89
Female	9-I	2.82	3.09	3.26	3.37	3.48
	10-I	2.55	2.59	2.72	2.83	3.01
	11-I	2.80	2.91	2.86	(2.78)	-
	12-I	2.28	2.39	2.52	2.55	2.61
	13-A	2.45	2.57	2.60	2.66	2.81
	14-A	2.96	3.17	3.37	3.49	3.00
	15-A	2.65	2.70	2.89	2.95	3.34
	16-A	2.20	2.29	2.15	1.87	1.84

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TABLE VIII

GROUP: C-II

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				Final
		-5	1	8	15	
Male	17-I	2.38	2.43	2.75	2.81	2.41
	18-I	2.29	2.34	2.41	2.47	2.47
	19-I	2.60	2.65	2.63	2.83	2.83
	20-I	2.44	2.48	1.98	1.76	(1.47)
	21-A	2.39	2.41	2.46	2.58	2.58
	22-A	2.34	2.29	2.18	2.21	2.18
	23-A	3.35	3.33	3.34	3.49	3.52
Female	24-A	2.22	2.27	2.41	2.61	2.84
	25-I	2.25	2.20	1.87	1.90	1.89
	26-I	2.21	2.20	1.89	1.76	1.64
	27-I	2.33	2.35	2.26	2.27	2.43
	28-I	2.51	2.44	2.52	2.52	2.52
	29-A	2.33	2.34	2.46	2.41	2.26
	30-A	2.33	2.22	2.15	2.29	2.58
	31-A	2.54	2.38	2.49	2.58	2.61
	32-A	2.27	2.33	2.38	2.38	2.41

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TABLE IX

GROUP: T-I

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
Male	33-I	2.66	2.64	2.75	2.75	2.83
	34-I	2.58	2.59	2.80	2.92	2.98
	35-A	2.36	2.50	2.66	2.72	2.77
	36-A	2.29	2.40	2.49	2.58	2.63
Female	37-I	2.25	2.25	2.26	2.35	2.35
	38-I	2.66	2.74	2.55	2.52	2.01
	39-A	2.58	2.78	2.72	2.98	3.11
	40-A	2.38	2.64	2.63	2.69	2.81

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TABLE X

GROUP: T-II

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				Final
		-5	1	8	15	
Male	41-I	2.46	2.44	2.60	2.75	2.86
	42-I	2.41	2.60	2.29	2.21	2.38
	43-A	2.25	2.23	2.24	2.32	2.52
	44-A	2.38	2.59	2.63	2.52	1.81
Female	45-I	2.69	2.78	2.94	3.06	3.12
	46-I	2.26	2.26	2.26	2.29	(2.10)
	47-A	2.28	2.42	2.46	2.52	2.24
	48-A	2.43	2.58	2.75	2.89	2.92

Figure in parenthesis represents autopsy weight

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TABLE XI
GROUP: T-III

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				Final
		-5	1	8	15	
Male	49-I	2.53	2.68	3.00	3.06	3.20
	50-I	2.43	2.37	2.46	2.35	2.41
	51-A	2.27	2.31	2.38	2.35	(2.04)
	52-A	2.26	2.34	2.46	2.58	2.72
Female	53-I	2.78	2.77	2.94	3.00	2.72
	54-I	2.57	2.66	2.72	2.72	2.84
	55-A	2.38	2.41	2.69	2.75	2.75
	56-A	2.31	2.55	2.63	2.72	2.63

Figure in parenthesis represents autopsy weight

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TABLE XII

GROUP: T-IV

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
Male	57-I	2.25	2.04	2.04	2.24	2.41
	58-I	2.43	2.68	2.72	2.89	2.97
	59-A	2.46	2.63	2.75	2.78	2.86
	60-A	2.90	3.06	3.17	3.17	3.26
Female	61-I	2.53	2.58	2.60	2.72	2.78
	62-I	2.82	2.89	2.86	2.98	3.06
	63-A	2.32	2.28	2.43	2.49	2.58
	64-A	2.32	2.43	2.60	2.72	2.86

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TABLE XIII

GROUP: T-V

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				Final
		-5	1	8	15	
Male	65-I	2.57	2.73	2.69	2.78	2.86
	66-I	2.45	2.48	2.72	2.81	2.89
	67-A	2.67	2.45	2.26	2.47	2.66
	68-A	2.26	2.36	2.49	2.58	2.66
Female	69-I	2.46	2.61	2.75	2.83	2.86
	70-I	2.33	2.14	2.04	2.29	2.12
	71-A	2.32	2.41	2.52	2.63	2.75
	72-A	2.41	2.19	2.38	2.38	2.49

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TABLE XIV
GROUP: T-VI
Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
Male	73-I	2.40	2.58	2.72	2.83	2.77
	74-I	2.27	2.23	2.12	2.10	2.13
	75-A	2.27	2.16	(1.58)	-	-
	76-A	2.26	2.79	2.77	2.86	2.92
Female	77-I	2.28	2.23	2.32	2.35	2.44
	78-I	2.41	2.57	2.72	2.86	2.89
	79-A	2.52	2.48	2.41	2.41	2.38
	80-A	2.58	2.71	2.75	2.98	3.06

Figure in parenthesis represents autopsy weight

TABLE XV

GROUP: T-VII

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
Male	81-I	2.37	2.35	2.32	2.47	2.64
	82-I	2.48	2.53	2.43	2.61	2.69
	83-A	2.27	2.42	1.72	(1.72)	-
	84-A	2.66	2.82	2.86	2.98	3.00
Female	85-I	2.42	2.62	2.58	2.66	2.86
	86-I	2.67	2.62	2.60	2.69	2.86
	87-A	2.81	2.82	2.09	(1.87)	-
	88-A	2.46	2.46	2.38	2.38	2.44

Figures in parenthesis represent autopsy weights

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TABLE XVI

GROUP: T-VIII

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
Male	89-I	2.39	2.61	2.49	2.52	2.49
	90-I	2.25	2.14	1.98	2.04	2.18
	91-A	2.24	2.02	(1.50)	-	-
	92-A	2.37	2.27	2.29	2.52	2.55
Female	93-I	2.67	2.60	2.60	2.75	2.79
	94-I	2.25	2.42	2.32	2.44	2.32
	95-A	2.42	2.64	2.52	2.69	2.89
	96-A	2.48	2.53	2.24	2.41	2.12

Figure in parenthesis represents autopsy weight

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TABLE XVII

GROUP: T-IX

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				
		-5	1	8	15	Final
	97-I	2.61	2.67	2.12	(2.18)	-
Male	98-I	2.29	2.35	2.35	2.41	2.55
	99-A	2.48	2.53	2.46	2.55	2.52
	100-A	2.26	2.40	2.29	2.38	2.55
	101-I	2.47	2.52	2.46	2.35	2.52
Female	102-I	2.81	2.86	2.55	2.86	2.95
	103-A	2.30	2.00	1.45	(1.36)	-
	104-A	2.23	2.18	2.12	2.18	2.38

Figures in parenthesis represent autopsy weights

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TABLE XVIII

GROUP: T-X

Repeated Dermal Toxicity - Albino Rabbits

Body Weight Data

Sex	Animal Number and Skin Condition	Individual Body Weights (kilograms)				
		Test Day Number:				Final
		-5	1	8	15	
Male	105-A	2.41	2.24	2.12	2.35	2.49
	106-I	2.36	2.54	2.52	2.66	2.72
	107-A	2.66	2.73	2.66	2.75	2.24
	108-A	2.66	2.74	2.38	2.66	2.72
Female	109-I	2.51	2.65	2.63	2.72	(2.04)
	110-I	2.28	2.47	2.41	2.49	2.52
	111-A	2.36	2.38	2.26	2.30	2.47
	112-A	2.36	2.49	2.41	2.55	2.66

Figure in parenthesis represents autopsy weight

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D. Hematologic and Clinical Blood Chemistry Studies

The results of the blood studies performed at the beginning and again at the conclusion of the investigational period did not reveal any significant differences between any of the test groups and their respective oil or water control groups. All values were considered to be within the normal range.

Determinations of urea nitrogen concentration (BUN) and alkaline phosphatase activity (SAP) conducted at the conclusion of the study did not reveal any abnormal findings. Values for all test animals were comparable to those of their corresponding controls.

E. Gross Pathologic Findings

With the exception of the skin at the application site, no gross pathologic alterations were noted in the tissues and organs of any of the test or control group animals. Gross findings with respect to skin were the same as those described in the section on local skin reactions (Sect. III, B2).

F. Microscopic Pathologic Findings

Microscopic examination of tissues and organs taken from animals receiving dermal applications of the test materials as aqueous dilutions as well as the water control animals revealed histopathologic alterations in the skin at the application site. These findings were characterized by the presence of slight subepithelial fibrosis and mono-nuclear infiltrate. No significant differences could be observed between

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either the incidence or the severity of these findings among test animals as compared to controls. No other histopathologic alterations were noted in the tissues and organs examined from these animals.

Again, with the exception of the skin at the application site, no significant histopathologic alterations were noted in the tissues and organs of animals receiving dermal applications of oil dilutions of the test materials. The skin of these animals also revealed subepithelial fibrosis with mononuclear infiltrate. However, in this instance, the incidence and severity of the reactions found for test animals was somewhat increased over that noted among controls. The histopathologic findings for the skin of these test and control rabbits are shown in Table XIX.

TABLE XIX
Repeated Dermal Toxicity - Albino Rabbits
Histopathologic Changes - Skin

Group	Number of Animals Examined	Findings	Incidence	Average Grade
C-II	15	Subepithelial Fibrosis	1	+
		Mononuclear Infiltrate	1	+
T-VII	6	Subepithelial Fibrosis	4	++
		Mononuclear Infiltrate	4	+
T-VIII	7	Subepithelial Fibrosis	4	++
		Mononuclear Infiltrate	2	+
T-IX	6	Subepithelial Fibrosis	3	++
		Mononuclear Infiltrate	1	+
T-X	8	Subepithelial Fibrosis	4	+++

Grading System

+ = slight

++ = mild

+++ = moderate

G. Organ Weights and Organ/Body Weight Ratios

Individual organ weights, viz. liver, kidneys, spleen, heart, and testes, are presented separately for each test and control group in Tables XX through XXXI.

Individual organ/body weight ratios for each test and control group are presented separately in Tables XXXII through XLIII.

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TABLE XX

Group C-I

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
1-M	101.9	20.7	1.5	6.2	1.4
2-M	88.4	16.8	0.8	6.0	3.2
3-M	94.9	17.8	0.6	5.6	1.2
4-M	148.2	19.3	1.7	7.6	4.9
5-M	148.1	21.6	1.0	5.8	4.0
6-M	159.2	20.5	1.0	6.6	2.2
8-M	113.8	24.7	1.2	6.1	4.5
9-F	145.2	21.4	1.7	9.3	-
10-F	149.9	23.8	2.4	9.6	-
12-F	87.1	18.2	1.1	6.7	-
13-F	105.0	15.9	1.2	7.1	-
14-F	132.8	19.2	1.1	6.8	-
15-F	137.9	25.1	2.0	8.3	-
16-F	79.1	12.8	1.0	4.9	-

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TABLE XXI

Group C-II

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
17-M	138.7	20.5	1.4	6.5	2.6
18-M	103.4	22.9	1.6	6.3	3.4
19-M	130.6	19.5	1.1	7.3	5.3
21-M	137.0	19.1	1.2	6.6	4.9
22-M	84.8	18.3	1.0	6.1	2.7
23-M	151.8	27.2	1.6	8.7	6.1
24-M	179.8	20.6	1.3	6.6	4.5
25-F	104.2	18.5	0.9	5.6	-
26-F	68.3	17.0	0.8	5.6	-
27-F	141.4	17.9	1.6	5.9	-
28-F	96.6	15.3	1.0	7.0	-
29-F	117.9	22.5	0.9	7.5	-
30-F	150.3	18.6	1.2	7.4	-
31-F	99.7	18.3	1.2	7.1	-
32-F	144.2	19.5	1.2	6.4	-

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TABLE XXII

Group T-I

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
33-M	79.1	20.0	4.1	9.5	7.3
34-M	136.1	23.4	2.2	9.6	6.2
35-M	110.0	19.4	2.3	9.5	6.3
36-M	127.9	23.3	1.9	7.6	3.2
37-F	71.1	13.5	1.2	5.4	-
38-F	68.5	22.6	1.3	6.5	-
39-F	159.0	24.7	1.7	7.6	-
40-F	147.1	23.4	1.3	7.8	-

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TABLE XXIII

Group T-II

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Spleen	Heart	Testes
41-M	170.3	33.1	1.2	7.3	5.6
42-M	139.4	27.4	1.0	7.0	4.1
43-M	158.9	20.8	2.8	8.5	6.6
44-M	175.9	27.1	0.7	6.0	3.1
45-F	151.8	33.0	2.6	9.6	-
47-F	96.4	26.2	2.0	7.9	-
48-F	112.0	25.4	4.6	8.5	-

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TABLE XXIV

Group T-III

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Spleen	Heart	Testes
49-M	140.0	24.1	1.7	8.7	4.8
50-M	117.7	19.2	2.1	6.7	5.7
52-M	192.0	19.3	1.0	5.7	2.8
53-F	83.5	26.8	1.3	6.7	-
54-F	146.8	16.3	1.7	6.5	-
55-F	136.4	21.7	1.4	7.0	-
56-F	79.9	18.0	1.0	6.8	-

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TABLE XXV

Group T-IV

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
57-M	146.9	22.1	1.5	8.3	3.3
58-M	167.1	22.9	1.7	9.1	5.2
59-M	108.1	15.5	0.8	7.2	4.7
60-M	162.5	24.3	1.8	10.9	7.2
61-F	86.8	16.5	1.6	7.6	-
62-F	138.3	19.8	1.7	9.3	-
63-F	128.5	17.9	1.2	7.9	-
64-F	128.9	19.1	2.0	8.3	-

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TABLE XXVI

Group T-V

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
65-M	143.6	16.4	2.3	8.5	5.0
66-M	76.3	21.5	0.9	7.2	5.0
67-M	142.4	24.3	1.1	8.0	3.7
68-M	118.3	22.5	1.5	9.9	3.4
69-F	135.8	22.7	2.5	7.2	-
70-F	81.5	20.1	1.1	6.1	-
71-F	157.5	13.8	1.6	7.8	-
72-F	106.1	19.1	1.0	9.1	-

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TABLE XXVII

Group T-VI

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
73-M	103.0	18.7	1.2	8.5	4.2
74-M	78.7	23.1	1.0	5.8	3.7
76-M	120.6	22.8	1.3	8.4	6.0
77-F	105.8	16.6	1.0	7.1	-
78-F	130.3	22.4	2.1	8.2	-
79-F	115.3	19.2	1.4	5.9	-
80-F	159.0	19.8	0.9	7.7	-

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TABLE XXVIII

Group T-VII

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Spleen	Heart	Testes
81-M	125.6	20.8	0.9	5.8	5.2
82-M	109.9	18.3	0.9	5.2	4.8
84-M	128.5	20.1	1.2	6.1	4.7
85-F	126.1	20.5	1.8	8.4	-
86-F	135.5	19.5	0.6	7.2	-
88-F	143.7	19.0	0.9	7.1	-

DOW 500385

TABLE XXIX

Group T-VIII

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
89-M	117.6	21.4	1.0	7.1	1.8
90-M	127.2	22.1	0.8	6.6	1.4
92-M	137.4	21.5	1.3	6.2	3.5
93-F	136.5	19.9	2.2	8.3	-
94-F	136.7	20.4	1.1	7.6	-
95-F	178.3	24.7	1.6	6.7	-
96-F	100.7	19.0	0.9	6.0	-

DOW 500386

TABLE XXX

Group T-IX

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
98-M	158.0	21.3	1.4	7.9	5.6
99-M	97.3	18.2	1.9	7.4	3.9
100-M	167.4	16.7	1.0	6.1	2.8
101-F	134.2	18.6	1.0	6.8	-
102-F	156.8	19.7	1.2	8.1	-
104-F	130.9	17.4	0.8	6.9	-

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TABLE XXXI

Group T-X

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ Weights

Animal Number and Sex	Organ Weights (grams)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
105-M	128.8	19.3	0.8	6.9	4.5
106-M	140.7	21.8	1.6	7.7	4.9
107-M	95.1	17.3	0.9	8.6	1.3
108-M	125.0	17.0	1.3	6.9	6.0
110-F	114.1	20.6	1.9	7.7	-
111-F	116.2	19.8	1.0	6.6	-
112-F	137.1	17.4	1.3	7.6	-

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TABLE XXXII

Group C-I

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body weight Ratios (g/100 g)				
	Liver	Kidney	Spleen	Heart	Testes
1-M	4.49	0.912	0.065	0.274	0.062
2-M	3.76	0.715	0.034	0.255	0.142
3-M	3.89	0.730	0.025	0.230	0.049
4-M	4.44	0.578	0.051	0.228	0.146
5-M	5.57	0.813	0.038	0.218	0.150
6-M	6.10	0.785	0.038	0.253	0.084
8-M	3.94	0.854	0.042	0.211	0.156
9-F	4.17	0.615	0.049	0.267	-
10-F	4.98	0.794	0.080	0.320	-
12-F	3.34	0.669	0.042	0.257	-
13-F	3.74	0.567	0.043	0.260	-
14-F	4.43	0.640	0.060	0.227	-
15-F	4.13	0.755	0.038	0.249	-
16-F	4.30	0.695	0.054	0.267	-

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TABLE XXXIII

Group C-II

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Liver	Kidney	Spleen	Heart	Testes
17-M	5.75	0.851	0.058	0.271	0.108
18-M	4.20	0.927	0.065	0.256	0.138
19-M	4.62	0.690	0.040	0.259	0.188
21-M	5.31	0.740	0.046	0.256	0.190
22-M	3.90	0.841	0.046	0.281	0.124
23-M	4.32	0.774	0.046	0.248	0.174
24-M	6.33	0.725	0.046	0.232	0.159
25-F	5.50	0.980	0.048	0.296	-
26-F	4.17	1.037	0.049	0.342	-
27-F	5.83	0.738	0.066	0.243	-
28-F	3.84	0.608	0.040	0.278	-
29-F	5.21	1.000	0.040	0.332	-
30-F	5.83	0.721	0.046	0.287	-
31-F	3.83	0.703	0.046	0.272	-
32-F	6.00	0.810	0.050	0.266	-

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TABLE XXXIV

Group T-I

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ-Body Weight Ratios

Animal Number and Sex	Organ/Body weight Ratios (g/100 g)				
	Organ:				
	Liver	Kidney	Spleen	Heart	Testes
33-M	2.80	0.708	0.145	0.336	0.258
34-M	4.57	0.785	0.074	0.323	0.208
35-M	3.97	0.700	0.083	0.343	0.228
36-M	4.86	0.885	0.072	0.289	0.122
37-F	3.03	0.575	0.051	0.230	-
38-F	3.41	1.124	0.065	0.324	-
39-F	5.11	0.795	0.055	0.244	-
40-F	5.23	0.834	0.060	0.278	-

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TABLE XXXV

Group T-II

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body weight Ratios

Animal Number and Sex	Organ/Body weight Ratios (g/100 g)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
41-M	5.95	1.16	0.042	0.356	0.196
42-M	5.87	1.15	0.042	0.294	0.172
43-M	6.31	0.825	0.111	0.338	0.262
44-M	9.72	1.50	0.039	0.332	0.171
45-F	4.87	1.059	0.084	0.308	-
47-F	4.30	1.170	0.089	0.353	-
48-F	3.84	0.870	0.158	0.292	-

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TABLE XXXVI

Group T-III

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Organ:				
	Liver	Kidney	Spleen	Heart	Testes
49-M	4.39	0.753	0.053	0.272	0.150
50-M	4.89	0.798	0.067	0.278	0.237
52-M	7.06	0.710	0.037	0.210	0.103
53-F	3.07	0.985	0.048	0.246	-
54-F	5.16	0.575	0.060	0.229	-
55-F	4.96	0.790	0.051	0.255	-
56-F	3.05	0.685	0.038	0.259	-

TABLE XXXVII

Group T-IV

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Organ:				
	Liver	Kidney	Spleen	Heart	Testes
57-M	6.10	0.919	0.062	0.346	0.137
58-M	5.64	0.772	0.057	0.306	0.175
59-M	3.68	0.542	0.028	0.252	0.164
60-M	4.99	0.745	0.055	0.334	0.221
61-F	3.13	0.594	0.058	0.274	-
62-F	4.52	0.648	0.056	0.340	-
63-F	4.99	0.695	0.046	0.306	-
64-F	4.51	0.669	0.070	0.290	-

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TABLE XXXVIII

Group: T-V

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
65-M	5.03	0.574	0.080	0.297	0.175
66-M	2.64	0.745	0.031	0.249	0.173
67-M	5.37	0.810	0.041	0.301	0.139
68-M	4.45	0.846	0.056	0.372	0.128
69-F	4.75	0.794	0.088	0.252	-
70-F	3.85	0.950	0.052	0.288	-
71-F	5.73	0.502	0.058	0.284	-
72-F	4.26	0.767	0.040	0.366	-

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TABLE XXXIX

Group T-VI

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Organ:				
	Liver	Kidney	Spleen	Heart	Testes
73-M	3.72	0.675	0.043	0.307	0.152
74-M	3.69	1.082	0.047	0.272	0.174
76-M	4.14	0.781	0.044	0.288	0.206
77-F	4.34	0.681	0.041	0.292	-
78-F	4.51	0.774	0.073	0.284	-
79-F	4.85	0.806	0.059	0.248	-
80-F	5.20	0.648	0.029	0.252	-

DOW 500396

TABLE XL

Group T-VII

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Liver	Kidney	Organ: Spleen	Heart	Testes
81-M	4.76	0.788	0.034	0.220	0.197
82-M	4.08	0.680	0.033	0.193	0.178
84-M	4.29	0.671	0.040	0.204	0.157
85-F	4.41	0.683	0.063	0.294	-
86-F	4.75	0.718	0.021	0.252	-
88-F	5.89	0.780	0.037	0.291	-

TABLE XLI

Group T-VIII

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Organ:				
	Liver	Kidney	Spleen	Heart	Testes
89-M	4.72	0.859	0.040	0.286	0.072
90-M	5.84	1.015	0.037	0.303	0.064
92-M	5.40	0.845	0.051	0.244	0.141
93-F	6.67	0.713	0.079	0.298	-
94-F	5.89	0.880	0.048	0.328	-
95-F	6.17	0.855	0.056	0.232	-
96-F	4.75	0.896	0.042	0.283	-

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TABLE XLII

Group T-IX

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Liver	Kidney	Spleen	Heart	Testes
98-M	6.20	0.835	0.055	0.310	0.220
99-M	3.87	0.723	0.076	0.294	0.155
100-M	6.57	0.655	0.039	0.240	0.110
101-F	5.34	0.740	0.040	0.270	-
102-F	5.31	0.670	0.041	0.275	-
104-F	5.50	0.731	0.034	0.290	-

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TABLE XLIII

Group T-X

Repeated Dermal Toxicity - Albino Rabbits

Individual Organ/Body Weight Ratios

Animal Number and Sex	Organ/Body Weight Ratios (g/100 g)				
	Liver	Kidney	Spleen	Heart	Testes
105-M	5.17	0.775	0.032	0.278	0.181
106-M	5.17	0.803	0.060	0.284	0.180
107-M	4.24	0.773	0.040	0.268	0.058
108-M	4.60	0.625	0.048	0.254	0.221
110-F	4.54	0.819	0.068	0.306	-
111-F	4.71	0.803	0.040	0.268	-
112-F	5.16	0.655	0.049	0.286	-

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IV. Summary

The following summarizes the results of subacute dermal application of water and/or oil dilutions of the dimethylamine salt, the butyl ester and the isooctyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) to albino rabbits:

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A. Mortality

Deaths occurring during the test period were relatively few and were scattered among the various groups. No correlation was noted between the incidence of lethal responses and any given test material or dose of test material.

B. Reactions

Hyperirritability and pain upon dermal applications were observed among animals treated with the oil dilutions of the butyl and isooctyl esters of 2,4-D but were also noted among control rabbits receiving oil applications. These reactions were not seen among any animals of those groups where water was employed as the solvent.

Local skin reactions were observed among all animals including controls. Among groups where water was employed as the solvent, only relatively mild to moderate inflammatory reactions were seen and the reactions occurred to essentially the same incidence and degree among both test and control animals. Inflammatory reactions among the groups for which oil was the solvent were severe and progressed to necrosis and eschar formation. Again, however, no significant

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differences were observed in either incidence or degree between test and control animals.

C. Body Weight Effects

Body weight data tabulated for animals of the test groups did not differ materially from those recorded for their corresponding controls.

D. Hematologic and Clinical Blood Chemistry Studies

No remarkable deviations from normal ranges were found for the hematologic and clinical blood chemistry parameters studied in either test or control animals.

E. Pathologic Findings

The only tissue disclosing any significant pathologic alteration among test or control animals was the skin. Evidence of the local inflammatory reactions already described (See IV. B above) were seen both grossly and microscopically among both test and control animals. Grossly, no differences could be discerned between test animals and their corresponding controls, i. e., those receiving the same solvent employed as a diluent, either oil or water. This was also the case microscopically for the water-treated groups. However, among the oil treated groups the incidence and degree of microscopic change, characterized by subepithelial fibrosis and mononuclear infiltrate, appeared to be increased somewhat in test animals as compared to the corresponding controls.

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F. Organ Weights and Organ/Body Weight Ratios

Gross inspection of the organ weight and ratio data disclosed a number of intergroup differences. Except for the observed effect of the dimethylamine salt of 2,4-D significantly increasing kidney weights and ratios in both males and females, none of the deviations observed were believed to be outside the normal range of variation for the albino rabbit and were considered unassociated with dermal application of the test materials. Moreover, in view of the lack of gross or microscopic findings in the kidneys of the rabbits treated with the dimethylamine salt, even the increased kidney weights and ratios noted may have been coincidental.

Respectfully submitted,

INDUSTRIAL BIO-TEST LABORATORIES, INC.

Richard J. Palazzolo

Report prepared by: Richard J. Palazzolo, B.S.
Departmental Director
Acute Toxicity Department

John H. Kay

Report approved by: John H. Kay, Ph. D.
Associate Director

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Director

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March 13, 1964

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ADDENDUM REPORT TO
THE NATIONAL AGRICULTURAL
CHEMICALS ASSOCIATION
REPEATED DERMAL TOXICITY STUDIES ON
THREE FORMULATIONS OF
2,4 DICHLOROPHENOXYACETIC ACID

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ADDENDUM REPORT TO
THE NATIONAL AGRICULTURAL CHEMICALS ASSOCIATION
REPEATED DERMAL TOXICITY STUDIES ON
THREE FORMULATIONS OF
2,4 DICHLOROPHENOXYACETIC ACID

I. Introduction

This addendum report presents the detailed results of the hematologic and clinical blood chemistry studies conducted on each animal during a three-week repeated dermal toxicity study previously reported¹ on the three commercially available formulations of 2,4 dichlorophenoxyacetic acid (2,4-D) indicated below:

2,4-D Dimethylamine Salt²

2,4-D Isooctyl Ester³

2,4-D Butyl Ester⁴

An outline of the investigation as abstracted from the prior report is presented in Table I.

- 1 See report to The National Agricultural Chemicals Association "Repeated Dermal Toxicity Studies on Three Formulations of 2,4 Dichlorophenoxyacetic Acid," March 13, 1964.
- 2 Weed-Rhap Herbicide A-4; Hercules Powder Company, Inc., Wilmington, Delaware.
- 3 Monsanto 2,4-D Low Volatile Ester Weed Killer; Monsanto Chemical Company, St. Louis, Missouri.
- 4 Estron 76 BE Weed Killer; The Dow Chemical Company, Midland, Michigan.

III. Summary

No remarkable deviations from normal ranges were found for the hematologic and clinical blood chemistry parameters studied in either test or control animals.

Respectfully submitted,

INDUSTRIAL BIO-TEST LABORATORIES, INC.

Richard J. Palazzolo

Report prepared by: Richard J. Palazzolo, B.S.
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March 16, 1964

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Kirk-Othmer

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Chlorine
to
Colors for Foods,
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CHLOROPHENOLS

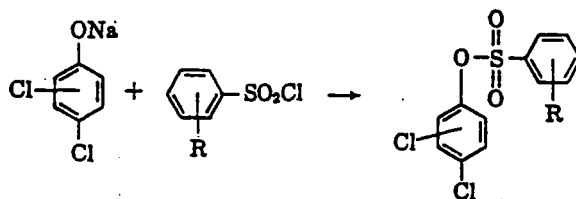
This article discusses in detail the methods of manufacture and the physical and chemical properties, toxicology, and uses of the major chlorophenols while presenting briefer information on those compounds that are of no commercial importance. In general, chlorophenols containing other substituents on the ring, for example, chloronitrophenols, chloroalkylphenols, chlorobromophenols, etc., are not treated. Such compounds, when of commercial importance, are discussed in connection with the specific chlorophenol from which they are derived. An exception, however, is made in the case of some of the commercially important chlorinated cresols and xylenols, which are discussed at the end of the article.

Properties and General Reactions

With the exception of *o*-chlorophenol, all of the chlorophenols are solids at room temperature, and all have a pungent, medicinal odor. They are generally insoluble in methanol and acetone.

The chlorophenols are stronger acids than phenol itself: Unlike the latter, they can be converted to the sodium salts from sodium carbonate solutions. This property affords a method of separating phenol from chlorophenols. In addition, other metallic salts, eg, salts of barium, potassium, calcium, zinc, etc., are also easily prepared. The chlorophenols and their salts show actual or potential utility in a wide range of applications. They exhibit outstanding germicidal and insecticidal properties and have demonstrated utility as flea repellents, fungicides, wood preservatives, mold inhibitors, etc. In general, effectiveness increases with the degree of chlorine substitution. References 1-4 typify the many studies which have been made on these materials (see Antiseptics and disinfectants; Fungicides).

Generally, the chlorophenols react very much the same as phenol itself. The methyl, ethyl, propyl, and butyl ethers of all of the chlorophenols are well known and are readily available by the reaction of the sodium chlorophenoxides with the corresponding alkyl halides. An extension of the etherification reaction involving the reaction of the sodium chlorophenoxides with α -halo-aliphatic acids has considerable commercial significance. Thus, the reaction of sodium 2,4-dichlorophenoxide with chloroacetic acid leads to 2,4-dichlorophenoxyacetic acid, $\text{Cl}_2\text{C}_6\text{H}_3\text{OOCCH}_3$, or 2,4-D; similarly, sodium 2,4,5-trichlorophenoxide leads to 2,4,5-T; these two compounds and other related compounds have gained a widespread reputation as weed killers (qv). Their evaluation and importance are discussed in several excellent review articles (5-7). Another important reaction is that of the sodium salts of chlorophenols with aromatic sulfonyl chlorides to form sulfonates:



All of the commercially important chlorophenols have been converted to the sulfonates and tested in various germicidal and insecticidal applications. The sul-

Table 1. Physical Properties of the Chlorophenols

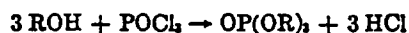
Material	Bp at 760 mm, °C	Fp, °C	Dissociation constant at 25°C, K_a
<i>o</i> -chlorophenol	175-176	8.7	3.2×10^{-9}
<i>p</i> -chlorophenol	219	40-41	6.6×10^{-10}
<i>m</i> -chlorophenol	215-217	32.8	1.4×10^{-9}
2,4-dichlorophenol	210-211	43-44	2.1×10^{-8}
2,6-dichlorophenol	219-220	67	1.6×10^{-7}
2,3-dichlorophenol	206	58	3.6×10^{-7}
2,5-dichlorophenol	212-213	58	4.5×10^{-7}
3,4-dichlorophenol	253	65	4.1×10^{-8}
3,5-dichlorophenol	233	68	1.2×10^{-7}
2,4,6-trichlorophenol	246	68	3.8×10^{-8}
2,4,5-trichlorophenol	245-246	68	3.7×10^{-8}
2,3,4-trichlorophenol		83.5	2.2×10^{-8}
2,3,5-trichlorophenol	255	62	4.3×10^{-8}
2,3,6-trichlorophenol	272	101	7.4×10^{-8}
3,4,5-trichlorophenol	275	101	1.8×10^{-8}
2,3,4,6-tetrachlorophenol	164/23 mm	69-70	4.2×10^{-8}
2,3,4,5-tetrachlorophenol		116-117	1.1×10^{-7}
2,3,5,6-tetrachlorophenol		115	3.3×10^{-8}
pentachlorophenol	309-310	190	1.2×10^{-8}

fonates of the lower substituted chlorophenols show insecticidal properties equivalent to those of phenylarsenous acid in apple sprays (8).

The chlorophenols undergo substitution reactions, eg, nitration, alkylation, acetylation, much the same as phenol itself except when positions occupied by the chlorines preclude further reaction.

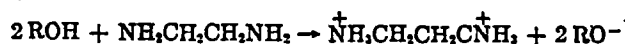
The lower substituted chlorophenols condense with formaldehyde to form phenolic resins which in turn serve as intermediates for the preparation of resoles and novolaks (see Phenoplasts). Generally, the resulting polymers have greater flame resistance than the usual resins prepared from phenol itself. The positions of the chlorine atoms on the phenol nucleus play a significant role in the utility of chlorophenols as starting materials for such resins. The resins, to be useful, must have a high degree of cross linkage which, in turn, is possible only if the starting phenol has open ortho or para positions. For this reason, *o*- and *p*-substituted chlorophenols have no real utility in this application. *m*-Chlorophenol and 3,5-dichlorophenol have shown promise, but high costs limit their utility to specialty applications. Small amounts of *p*- and *o*-chlorophenol are, however, used in phenolic resins where a greater degree of fire resistance is desired.

The mono-, di-, and triphosphate esters are formed by the reaction of chlorophenols with phosphorus oxychloride. The products have utility as flameproofing agents, fungicides, wood preservatives, etc (9,10). Esterification proceeds by the general equation



where R represents $\text{Cl}_n\text{C}_6\text{H}_{5-n}$.

Chlorophenols form salts with amines. The salts formed with diamines, such as ethylenediamine and propylenediamine, can be employed as bactericides, insecticides, fungicides, etc (11).



In many cases these salts are very stable. Salts similarly formed with certain alkanolamines, such as *N*-cyclohexylmonoethanolamine, have similar applications (12). More specific reactions of the chlorophenols are discussed in the sections describing individual members of the family that have commercial significance.

Toxicity

The toxicity of the chloro-substituted phenols varies from isomer to isomer within a group. However, we may make the following generalizations: The *monochlorophenols* are likely to be moderate in acute oral toxicity. Most of them are likely to be corrosive to the skin and eyes, and are readily absorbed through the skin in toxic amounts. Their vapors or dusts are very irritating and toxic. In handling these materials, precautions should be taken to prevent skin and eye contact and avoid inhalation of vapors or dusts.

The *dichlorophenols* may be somewhat less toxic by skin and eye contact than the monochlorophenols and are less likely to be absorbed through the skin. Their dusts, however, are very irritating. Precautions for handling should include prevention of skin and eye contact and avoidance of dust inhalation.

The *trichlorophenols* are usually considered to be the least toxic of the chlorophenols. Most of the isomers should cause only moderate skin irritation. Eye contact may result in marked irritation, possibly some corneal injury. The dusts are likely to be very irritating. Precautions should be taken to avoid skin and eye contact and to avoid inhalation of dusts.

While the *tetrachlorophenols* are perhaps somewhat less toxic than pentachlorophenol, all of these highly chlorinated phenols are considered to present essentially the same degree of hazard on handling. They may be moderately to highly toxic when ingested, and highly toxic when absorbed by the skin. Strong solutions are especially hazardous in this respect. Eye contact is likely to cause marked irritation, or even a burn. Continuous daily skin contact has been known to cause acneform dermatitis in humans. Dusts of these materials are very irritating to the respiratory tract and to the eyes. In handling, precautions should be directed toward avoiding eye or skin contact with the dusts. The inhalation of dusts should be avoided.

None of the chloro-substituted phenols has been reported to cause skin sensitization. The manufacturer should be contacted for specific toxicological information before any of these chlorophenols are handled in industrial quantities.

General Methods of Preparation

There are several general methods by which chlorophenols can be prepared. These include direct chlorination with various chlorinating agents, hydrolysis of chlorinated benzenes, conversion of the diazonium salts of various chlorinated anilines, and chlorination of phenolsulfonic acids and benzenesulfonic acids, followed by removal of the sulfonic acid group. The isomer desired determines the synthetic route.

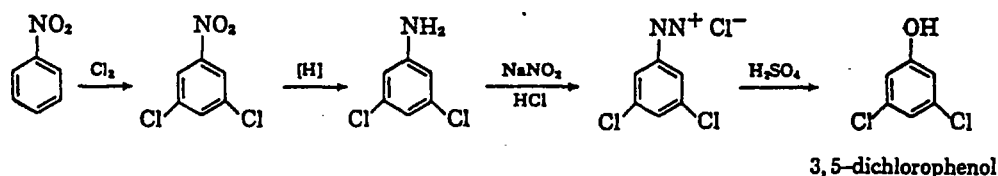
Direct Chlorination. One of the most widely used methods is the direct chlorination of phenols with molecular chlorine. The reaction proceeds stepwise and can lead to all possible products from monochlorophenol to pentachlorophenol.

Generally, chlorination proceeds readily at the lower levels of substitution by the direct addition of gaseous chlorine to molten phenol in the absence of catalysts. When greater degrees of chlorination are desired, catalysts, such as FeCl_3 , AlCl_3 , and

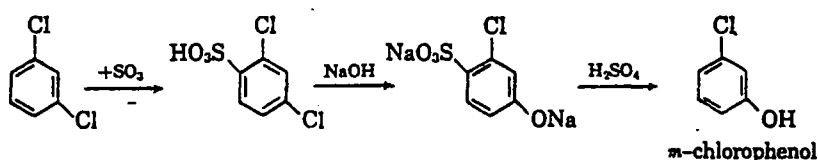
SbCl_3 , are employed in concentrations of 0.05–1.0%. Usually, the reactions are carried out in the absence of solvents, but carbon tetrachloride, acetic acid, and water have been used as diluents. Since the phenolic hydroxyl is ortho-para-directing, the product of monochlorination is a mixture of *o*- and *p*-chlorophenol, and the products of dichlorination are chiefly the 2,4- and the 2,6-isomer. The product of trichlorination is chiefly the 2,4,6-isomer and, of tetrachlorination, primarily the 2,3,4,6-isomer. Other chlorinating agents which have been used include hypochlorous acid, sodium hypochlorite solutions, and sulfuryl chloride. Substitution occurs in the same positions as with molecular chlorine; however, there is evidence for a greater degree of ortho substitution (13). Various mechanisms for the direct chlorination of phenols have been proposed (14–16), but none has as yet won general acceptance.

Hydrolysis of Chlorinated Benzenes. The hydrolysis of polychlorobenzenes is another widely used method of preparing chlorophenols. This can be an attractive commercial route, since chlorinated benzenes are produced in large volumes in the United States. It is used chiefly to obtain chlorophenol isomers not readily available by direct chlorination. Generally, hydrolysis is carried out in aqueous alkaline solutions at high temperatures and under pressure. Very often solvents such as methanol or ethanol are added to the reaction media to increase solubility and, thus, reaction rate; however, some methyl or ethyl ether of the chlorinated phenol is then obtained through side reactions. Reaction temperatures vary from 250 to 300°C, reaction times are from 15–90 min, pressures vary from atmospheric to 500 psi, and conversions are 85–98%. Some of the chlorinated phenols prepared by this method are 2,5-di-, 2,4,5-tri-, 2,3,5,6-tetra-, and pentachlorophenol.

Other Methods of Preparation. Since both the direct chlorination of phenols and the hydrolysis of polychlorobenzenes result chiefly in similar *o*- and *p*-substituted products, other routes must be used to prepare substituted chlorophenols such as *m*-chlorophenol and 3,5-dichlorophenol. Two methods are commonly utilized. The first of these involves preparation of the diazonium salt of a chlorinated aniline, and its subsequent conversion to the desired product. The preparation of 3,5-dichlorophenol can be used as an example.



The other method can be illustrated by the preparation of *m*-chlorophenol. It involves sulfonation, followed by removal of the sulfonic acid group, as illustrated below:



Either method can be used for the preparation of *m*-substituted products, depending on the starting materials. These processes are, of course, more expensive than direct

chlorination or the hydrolysis of polychlorobenzenes. For this reason, *m*-substituted chlorophenols have never reached large-scale commercial volume.

Analysis

Several of the classic analytical methods used for phenol can be applied to the chlorophenols. When the ortho or para, or both, positions are open, the bromination method (17) may be used. The acetylation or phthalation of the hydroxyl group is an applicable method.

The higher chlorophenols, ie, tri-, tetra-, and pentachlorophenol, have a pH sufficiently low to permit their being titrated potentiometrically with standardized sodium hydroxide solutions in the presence of appropriate indicators. For instance, 2,4,5-trichlorophenol is dissolved in an excess of aqueous standard NaOH solution and the excess NaOH is titrated with standard hydrochloric acid solution; thymolphthalein serves as the indicator. Tetra- and pentachlorophenol can be titrated directly by dissolving the sample in alcohol and adding water in the amount of 50% of the volume of alcohol used. Aqueous, standard NaOH solution is employed; *metacresol* purple is a suitable indicator. These methods, as well as other chemical analyses, are well covered in the literature (18).

Infrared spectroscopy is particularly important in that it can be used to identify and determine the individual chlorophenols. An excellent review on the use of these spectrographic techniques on phenols in general has been published by Bellamy (19). These materials may also be assayed by determining the quantity of chlorine by one of the standard chloride determinations after an ignition procedure. Depending on the degree of accuracy required, the ignition can be carried out in an oxygen bomb, in the Parr sodium peroxide bomb, or by calcium hydroxide ignition. This last type of ignition is particularly appropriate for the determination of chlorophenols in wood, paper, pulp, etc. In this application, it should be realized that chlorides, as a rule, are naturally present in these products, and a blank should be run on some of the same material that has not been treated with the chlorophenol.

Other methods include ultraviolet absorption techniques; these are also applicable to both the chlorophenols or the derivatives formed from the chlorophenols. Various colorimetric procedures are available, such as the Gibbs method, in which 2,6-dibromoquinone chloroimide is used. The chlorophenols can often be separated from other materials by distillation or by absorption on suitable ion exchange resins.

Individual Products

o-Chlorophenol is sparingly soluble in water at room temperature (<0.1 g/100 g); soluble in ethanol and ether (>200 g/100 g); and volatile with steam, even from aqueous sodium hydroxide solutions. Melting point is not a good criterion of purity, since 20% phenol present as an impurity lowers the melting point only 1.7°C.

o-Chlorophenol is made most economically by the direct chlorination of phenol with molecular chlorine or by the hydrolysis of *o*-dichlorobenzene. Direct chlorination is carried out by passing gaseous chlorine into molten phenol at temperatures of 50–150°C. This process has two disadvantages: (1) Chlorine ratios must be controlled to avoid the formation of di- and trisubstituted products; and (2) *p*-chlorophenol is also produced. Considerable work has been done to develop conditions favoring ortho substitution; however, no positive ways have been found. Chlorination temperature

or the presence of solvents has little effect. The chlorophenols can be separated from unreacted phenol by adding aqueous sodium carbonate to the reaction mixture, thus converting the chlorophenols to the sodium salts. The unreacted phenol is then removed by extraction. After acidification of the sodium chlorophenoxides, washing, and drying, *o*-chlorophenol is separated from *p*-chlorophenol by fractional distillation, since the difference in boiling points is greater than 45°C.

Various methods have been proposed for the partial hydrolysis of *o*-dichlorobenzene to *o*-chlorophenol. The hydrolysis can be carried out in the vapor phase with steam and finely divided silicates, bauxite, magnesite, etc, at 500–700°C, or in aqueous methanol or ethanol at high pressures and temperatures in the presence of alkali, alkaline earths, or alkaline carbonates. Both of these procedures depend on distillation to recover pure products. Both likewise require strict control to avoid complete hydrolysis to *o*-dihydroxybenzene (catechol); however, the liquid-phase hydrolysis results in the formation, as by-products, of some methyl or ethyl ethers of the chlorophenol.

More exotic synthetic routes for the preparation of *o*-chlorophenol have also been studied. These include chlorination of phenol with ethyl hypochlorite (20) or *N,N'*-dichlorourea (21), diazotization and subsequent decomposition of *o*-chloroaniline (22), reaction of 2-chlorophenol-4-sulfonic acid with water at 180–200°C under pressure (23), and reaction of chlorine with *p*-phenolsulfonic acid in nitrobenzene at 55°C (24).

o-Chlorophenol can be further chlorinated to 2,4- and 2,6-dichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol. It can be brominated, iodinated, and nitrated to the mono- and disubstituted derivatives. Condensation of *o*-chlorophenol with formaldehyde under the proper conditions produces phenolic resin intermediates, or the reaction can be controlled to form 3,3'-dichloro-4,4'-dihydroxydiphenylmethane. Treatment of *o*-chlorophenol with methyl chloride or methyl bromide in the Reimer-Tiemann reaction gives 3-chloro-2-hydroxybenzaldehyde and 3-chloro-4-hydroxybenzaldehyde, and reaction with phthalic anhydride yields dichlorophenolphthalein. The ring hydroxyl of chlorophenols behaves very much the same as that of phenol in etherification reactions, Claisen condensations, allylic rearrangements, esterifications with phosphorus oxychloride, etc. In the United States most of the *o*-chlorophenol that is used commercially is recovered as a by-product from the manufacture of *p*-chlorophenol by direct chlorination. Only small quantities are sold, and most of the production is used as an intermediate to higher chlorophenols.

p-Chlorophenol has a disagreeable, persistent odor. It is sparingly soluble in water (2.71 g/100 g water) at room temperature, but is readily soluble in ethanol, ether, benzene, chloroform, and carbon disulfide. Like *o*-chlorophenol, it can be volatilized with steam even from aqueous sodium hydroxide solutions.

p-Chlorophenol can be prepared by the same general routes described above for *o*-chlorophenol, except that proper positional isomers of halogenated benzenes are required when the latter serve as the starting materials. Commercial production is chiefly via the route of direct chlorination of phenol (see the procedure for *o*-chlorophenol).

p-Chlorophenol undergoes the same reactions as described for *o*-chlorophenol or, more generally, the same reactions as phenol itself. In the United States, the majority of *p*-chlorophenol produced is utilized as a raw material for the manufacture of other materials. Since further chlorination of *p*-chlorophenol results in a high conversion to the 2,4-isomer (chlorination of *o*-chlorophenol yields a mixture of 2,4-

and 2,6-isomers), large quantities of *p*-chlorophenol are used to manufacture 2,4-dichlorophenol. Reaction of *p*-chlorophenol with phthalic anhydride and concentrated sulfuric acid results in the formation of 1,4-dihydroxyanthraquinone (quinizarin). Reaction with 4-sulphthalic anhydride in the presence of sulfuric acid results in 1,4-dihydroxy-6-anthraquinonesulfonic acid (6-sulfoquinizarin). Likewise, *p*-chlorophenol reacts with α -alkylacetoacetates in the presence of phosphorus pentoxide to produce chromones. For example, *p*-chlorophenol and ethyl α -ethylacetoacetate give 6-chloro-3-ethyl-2-methylchromone. *p*-Chlorophenol couples with diazonium salts, such as benzenediazonium chloride, to form products which, upon acidification, give chloro-hydroxy-azo derivatives such as 4-chloro-2-(benzene-azo)phenol. It also reacts with indene hydrochloride, *o*-nitrobenzenesulfonic acids, etc., to produce indophenols. The above materials are useful in the manufacture of dyes and pigments.

The reaction of *p*-chlorophenol and benzyl chloride gives 4-chloro- α -phenol-*o*-cresol (Santophen I), a widely used germicide that is sold by the Monsanto Company. *p*-Chlorophenol can be reacted with ethyl chloride to produce *p*-chlorophenetole, which can then be treated with ammonia to produce phenetidine. Phenetidine can then be converted to the *N*-acetyl derivative, acetophenetidine (phenacetin), a commercial analgesic (see Vol. 2, p. 390). *p*-Chlorophenol can be reacted with *p*-chlorobenzenesulfonic chloride to produce *p*-chlorophenyl-*p*-chlorobenzene-sulfonate, a miticide sold under the trademark Ovex by The Dow Chemical Company.

In addition to its use as a chemical intermediate, *p*-chlorophenol is employed as a selective solvent in refining mineral oils and as a denaturant for ethanol. Certain salts, such as those of sodium, potassium or copper, are used as antigumming agents for gasoline, wash liquids for fuel gas purification, and germicides. *p*-Chlorophenol and its alkali metal salts are considered to be better germicides than *p*-cresol or phenol. United States production figures are not available for this material, but quoted prices approximate 38¢/lb in drum quantities.

m-Chlorophenol has an odor similar to phenol and is almost insoluble in water (260 mg/100 g), but is soluble in ethanol, ether, benzene, and carbon disulfide. Like *p*- and *o*-chlorophenol, *m*-chlorophenol is soluble in sodium carbonate solutions at room temperature and is reprecipitated by the addition of carbon dioxide.

m-Chlorophenol can be synthesized by the diazotization of *m*-chloroaniline (prepared by the chlorination of nitrobenzene and subsequent reduction of the nitro group) followed by treatment with water. Yields of 60–65% have been reported (25). Hodgeson (26) has described a commercial method for the manufacture of *m*-chlorophenol by the diazotization of *m*-chloroaniline sulfate, followed by treatment with boiling water and sulfuric acid. During the latter step, steam is introduced into the reaction mixture, and the chlorophenol is steam-distilled out as soon as it is formed. *m*-Chlorophenol may also be prepared by treating *m*-chlorobenzene with water in the presence of methanol and dimethyl ether at 180°C for 30 hr.

m-Chlorophenol on fusion with potassium hydroxide can be converted to *m*-dihydroxybenzene (resorcinol). *m*-Chlorophenol will react with formaldehyde to form phenol-formaldehyde resins. Upon curing, the resulting products have a higher degree of fire retardancy than similar resins prepared from phenol. The high price of *m*-chlorophenol has prohibited its use in resorcinol manufacture or the preparation of any phenol-formaldehyde resins except specialty items. *m*-Chlorophenol reacts much the same as *o*- or *p*-chlorophenol, and phenol itself. Most of the derivatives described for these materials have been prepared from *m*-chlorophenol also.

2,4-Dichlorophenol has an unpleasant, persistent odor suggesting iodoform and is sparingly soluble in water but easily soluble in ethanol, ether, benzene, and methyl chloride. Generally, 2,4-dichlorophenol behaves as a weak acid and is soluble in aqueous alkaline solutions. It is volatile with steam but, unlike the monochlorophenols, is not volatile from aqueous alkaline solutions. This difference affords a method for separating monochlorophenols from dichlorophenols.

2,4-Dichlorophenol may be prepared by the direct chlorination of phenol with two moles of chlorine. The resulting product usually contains some 2,6-dichlorophenol and 2,4,6-trichlorophenol as by-products. Foster and Bennett (27) have patented a manufacturing process whereby chlorine is passed into molten phenol at 80–100°C until a product with a melting point of 34–36°C is obtained. In this method, only very small amounts of the 2,6-isomer and trichlorophenol are formed; 2,4-dichlorophenol may be isolated in 80–90% yield. 2,4-Dichlorophenol of high purity may also be manufactured by the chlorination of *p*-chlorophenol; however, close control of the chlorine ratio is required to avoid the incidental preparation of trichlorophenols. The further chlorination of *o*-chlorophenol results in mixtures of the 2,4- and 2,6-isomers; thus, *o*-chlorophenol is not normally used as a raw material for the commercial manufacture of pure 2,4-dichlorophenol. 2,4-Dichlorophenol can also be prepared from phenol and *N,N'*-dichlorourea in HCl solution (21) or from phenol and SO₂Cl₂ (28).

2,4-Dichlorophenol may be brominated, iodated, and nitrated, with substitution occurring in the 6 position. It yields condensation products with Formalin (37% formaldehyde solution in water) alone, Formalin in hydrochloric acid, etc. The product 2,2'-dihydroxy-3,5,3',5'-tetrachlorodiphenylmethane may also be obtained by the reaction of 2,4-dichlorophenol with methylal, methylene diacetate, or methylene diiodide in the presence of sulfuric acid. It has found application as a mothproofing compound, antiseptic, and seed disinfectant. 2,4-Dichlorophenol may be reacted with benzenesulfonyl chloride to prepare 2,4-dichlorophenyl benzenesulfonate. This compound is sold by Allied Chemical Corporation under the trademark Genite-EM-923, as a miticide for fruit trees. The most important use for 2,4-dichlorophenol is in the manufacture of 2,4-dichlorophenoxyacetic acid (2,4-D) and its derivatives. Commercial synthesis of 2,4-D involves reacting the 2,4-dichlorophenol with chloroacetic acid in the presence of sodium hydroxide. The powerful plant-growth regulatory activity of 2,4-D was discovered at the Boyce Thompson Institute in the early 1940s and initial production occurred in 1946. In 1962, approximately 43,000,000 lb of 2,4-D was produced in the United States. While it is also possible to produce 2,4-D by the chlorination of phenoxyacetic acid, the yields are 70% or lower and it is difficult to purify the product. Reaction with alkali metal salts results in 2,4-dichlorophenates, which have found utility as germicides, antiseptics, etc. The germicidal activity of 2,4-dichlorophenol and its salts has been discussed in detail in the literature (3,29). Based on estimates from 2,4-D manufacture, United States production of 2,4-dichlorophenol amounted to 32,000,000 lb in 1962. Average quoted selling price was 32¢/lb in drum quantities.

2,6-Dichlorophenol has a penetrating odor resembling *o*-chlorophenol or, if dilute, iodoform. It can be crystallized from petroleum ether, is miscible with ethanol and diethyl ether, and is volatile with steam. 2,6-Dichlorophenol can be prepared by the chlorination of *p*-hydroxybenzoic acid in acetic acid, followed by the elimination of carbon dioxide by heating in quinoline, or by the sulfonation of phenol, followed by

chlorination in nitrobenzene and final removal of the sulfonic acid by hydrolysis. Mixtures containing 2,6-dichlorophenol can be obtained by the further chlorination of *o*-chlorophenol; however, 2,4-dichlorophenol and 2,4,6-trichlorophenol are also formed. Although 2,6-dichlorophenol undergoes the same reactions as 2,4-dichlorophenol, it has found no commercial application. Material produced in the United States coincidentally with other chlorinated phenols is used primarily as starting material for the manufacture of trichlorophenols, tetrachlorophenols, and pentachlorophenol.

2,5-Dichlorophenol has a strong, persistent phenolic odor, is sparingly soluble in water, and is easily soluble in ethanol, diethyl ether, and benzene. It is volatile with steam. 2,5-Dichlorophenol can be prepared in 90% yield by the diazotization of 2,5-dichloroaniline followed by decomposition of the diazonium salt with boiling water and sulfuric acid. Agfa Chemical Company has a patent (30) for the manufacture of 2,5-dichlorophenol by the hydrolysis of 1,2,4-trichlorobenzene in the presence of sodium methylate at 180°C. The material has found no commercial utility.

3,4-Dichlorophenol is soluble in benzene, ethanol, diethyl ether, and petroleum ether, and is volatile with steam. 3,4-Dichlorophenol can be prepared by the diazotization of 3,4-dichloroaniline followed by the hydrolysis of the diazonium salt, and by the hydrolysis of 4-fluoro-1,2-dichlorobenzene in the presence of sodium methylate (31). It reacts with phthalic anhydride in the presence of sulfuric acid and boric acid to produce 2-chloro-1,4-dihydroxyanthraquinone (2-chloroquinizarin), which can be used as a dye intermediate (32). Further chlorination results in 2,3,4-trichlorophenol. Because of its expensive preparative route, 3,4-dichlorophenol has not enjoyed any commercial activity.

3,5-Dichlorophenol is slightly soluble in cold water and quite soluble in hot water. It is also soluble in ethanol, diethyl ether, and benzene. 3,5-Dichlorophenol can be prepared by the diazotization of 3,5-dichloroaniline followed by hydrolysis of the diazonium salt. It can be readily nitrated and brominated to the trinitro and tribromo derivatives. 3,5-Dichlorophenol reacts with formaldehyde to produce a typical phenol-formaldehyde resin composition having a high degree of fire retardancy. Although this product looks extremely interesting, the high cost of the starting chlorophenol has precluded commercial development.

2,3-Dichlorophenol can be obtained as crystals from petroleum ether. It is volatile with steam and its odor resembles *o*-chlorophenol and iodoform. It can be prepared from 2,3-dichloroaniline via the diazo reaction and from 3-amino-2-chlorophenol via the Sandmeyer method. This material has not achieved commercial importance.

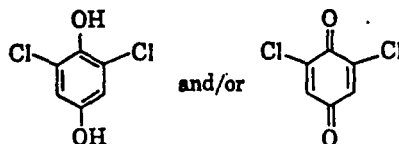
2,4,6-Trichlorophenol is insoluble in water, but readily soluble in methanol (525 g/100 g), acetone (500 g/100 g), toluene (100 g/100 g), and petroleum solvents (16 g/100 g). It is volatile with steam but, unlike *o*- and *p*-chlorophenol, it is not volatile from alkaline solutions. This offers a method of separating it from the latter materials. 2,4,6-Trichlorophenol behaves as an acid and can be titrated with 0.1*N* aqueous sodium hydroxide.

2,4,6-Trichlorophenol is sold in commercial quantities by a number of producers. It is available from The Dow Chemical Company under the trademark Dovicide 2S. Manufacture is readily accomplished by the direct chlorination of phenol. Mono- and dichlorophenols, obtained as coproducts, are easily removed by distillation because of the wide spread in boiling points. These mono- and dichlorinated compounds

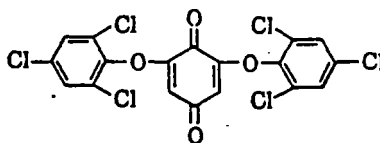
are subsequently converted to the desired product by recycling and further chlorination. In industry, manufacturers of *p*-chlorophenol and 2,4-dichlorophenol dispose of by-product *o*-chlorophenol and 2,6-dichlorophenol by chlorinating to 2,4,6-trichlorophenol. 2,4,6-Trichlorophenol has also been prepared by the chlorination of phenol with sodium or potassium hypochlorite solutions in the presence of hydrochloric acid, and by the diazotization of 2,4,6-trichloroaniline followed by hydrolysis of the diazonium salt.

Considerable work has been done on the use of 2,4,6-trichlorophenol as a bactericide and fungicide and in general antiseptic action. It has been found that 2,4,6-trichlorophenol is a more effective germicide than *o*- or *p*-chlorophenol or 2,4-dichlorophenol. The literature provides detailed descriptions of the utility of 2,4,6-trichlorophenol as a wood preservative (9), glue preservative (33), insecticide ingredient (34), bactericide (35), and antimildew treatment for textiles (36).

Oxidation of 2,4,6-trichlorophenol results in 2,6-dichlorohydroquinone and/or 2,6-dichloro-1,4-benzoquinone,



or 2,6-bis(2',4',6'-trichlorophenoxy)-1,4-benzoquinone,



depending on the reaction conditions, or the degree of oxidation achieved. Treatment of 2,4,6-trichlorophenol with chlorine and fuming sulfuric acid results in the preparation of 2,3,5,6-tetrachloro-1,4-benzoquinone (chloranil). Chloranil is used as a seed protectant for a large number of food crops and ornamentals. Further chlorination of 2,4,6-trichlorophenol results in 2,3,4,6-tetrachlorophenol and pentachlorophenol. It can also be brominated to the mono- and disubstituted derivatives. The direct nitration of 2,4,6-trichlorophenol has not been reported, presumably because nitration reagents readily oxidize the material to the benzoquinone derivative. 2,4,6-Trichlorophenol reacts with formaldehyde to form the bis(methylene) derivative, and with SCl_2 to give 3,3'-dihydroxy-2,4,6,2',4',6'-hexachlorodiphenyl sulfide, both soap germicides. The phenolic hydroxyl of 2,4,6-trichlorophenol reacts much the same as that of phenol; ethers, esters, amine adducts, etc. have been prepared. United States production quantities are not available.

2,4,5-Trichlorophenol can be recovered as colorless needles from ethanol and ligroin. It is volatile with steam and sublimes. 2,4,5-Trichlorophenol behaves as a weak monobasic acid, but can be titrated with 0.1*N* aqueous sodium hydroxide.

Like its 2,4,6-isomer, 2,4,5-trichlorophenol is available commercially under several trademark designations; Dow sells the product as Dovicide 2. 2,4,5-Trichlorophenol is manufactured by the hydrolysis of 1,2,4,5-tetrachlorobenzene. Hydrolysis is carried out continuously in a coil reactor at 160°C in the presence of methanol and sodium hydroxide. Contact time is approx 7 hr and yields of 80-85% are achieved. The

chief impurity is 2,4,5-trichloroanisole, which results from the reaction of 2,4,5-trichlorophenol with some of the methanol. After the hydrolysis is complete, the reaction mass is extracted with an organic solvent to remove the bulk of the anisole derivative, acidified with hydrochloric acid, and distilled to recover the trichlorophenol. 2,4,5-Trichlorophenol may also be prepared by the diazotization of 2,4,5-trichloroaniline followed by decomposition of the diazonium salt with water and sulfuric acid.

2,4,5-Trichlorophenol forms a sparingly soluble potassium salt, and in the presence of toluene and methanolic caustic forms the anhydrous sodium salt which can be crystallized from water as the pentahydrate. The lithium, calcium, and barium salts have also been prepared. These salts have found utility as fungicides (37) and wood preservatives (9). 2,4,5-Trichlorophenol can be brominated and nitrated to the mono- and disubstituted derivatives. It reacts with formaldehyde to form the bis-(methylene) derivative, and with sulfuryl chloride to yield the thiobis derivative. Both of these materials have found application as soap germicides. 2,4,5-Trichlorophenol reacts with thiophosphoryl chloride and then with sodium methylate in the presence of methanol to produce 2,4,6-trichlorophenol, *O,O*-dimethylphosphorochlorodithioate, which is used for the systemic control of grubs in cattle. The Dow name for this product is Ronnel.

The largest single use for 2,4,5-trichlorophenol is in the manufacture of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and derivatives. Commercial preparation involves reacting the 2,4,5-trichlorophenol with chloroacetic acid and caustic soda in a manner analogous to that used for 2,4-D. 2,4,5-T is used for brush control, being particularly effective against brambles, certain oak species, and woody vines. Total United States production of 2,4,5-T in 1962 was 8,400,000 lb. A closely related use for 2,4,5-trichlorophenol is in the manufacture of α -(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP). 2,4,5-TP was introduced in 1953 by The Dow Chemical Company to control brush and bushes resistant to 2,4,5-T, and has also found use in killing dandelions, plantains, and chickweed. Technical grades of 2,4,5-trichlorophenol and its sodium salt are sold in flake form for germicidal use. Total United States production in 1962 amounted to 12,000,000 lb.

2,3,4-Trichlorophenol is too weakly acid to be titrated, although it is soluble in alkaline solutions. 2,3,4-Trichlorophenol can be prepared by the direct chlorination of 3,4-dichlorophenol or by the nitration and reduction of 1,2,3-trichlorobenzene followed by diazotization and hydrolysis. It has achieved no commercial importance.

2,3,5-Trichlorophenol is volatile with steam, and can be titrated with 0.1N sodium hydroxide using phenolphthalein as an indicator. When 2,3,5-trichlorophenol is dissolved in hot solvents and cooled, gels result. 2,3,5-Trichlorophenol can be prepared from 2,3,5-trichloroaniline by diazotization and subsequent hydrolysis of the diazonium salt. This material has no commercial utility.

3,4,5-Trichlorophenol is too weakly acid to be titrated, although it is soluble in alkaline solutions. 3,4,5-Trichlorophenol can be prepared by the diazotization of 3,4,5-trichloroaniline, followed by decomposition of the resulting salt, and also by the cleavage of 3,4,5-trichloroanisole. It has not achieved any commercial importance.

2,3,4,6-Tetrachlorophenol is insoluble in cold water (100 ppm at 25°C), partially soluble in hot water (500 ppm at 70°C), and soluble in methanol (319 g/100 g), acetone (570 g/100 g), and toluene (175 g/100 g). 2,3,4,6-Tetrachlorophenol behaves as an acid, and can be titrated with 0.1N aqueous sodium hydroxide.

see
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2,3,4,6-Tetrachlorophenol is sold by The Dow Chemical Company under the designation Dowicide 6. It can be prepared by the direct chlorination of phenol; however, long reaction periods are required to achieve the desired product. Reaction time can be shortened by using catalysts such as iodine, antimony chloride, or iron chloride. Highest yields of relatively pure product are achieved through the chlorination of 2,4,6-trichlorophenol. In industry, the starting material for the production of 2,3,4,6-tetrachlorophenol can be a mixture containing *o*-chlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol. These are materials available if the company is a primary producer of *p*-chlorophenol, 2,4-dichlorophenol, and 2,4,6-trichlorophenol. 2,3,4,6-Tetrachlorophenol can also be prepared by the reduction of 1,2,4,4,5,6,6-heptachlorocyclohexen-1-one-3 with stannous chloride/hydrochloric acid/acetic acid or with potassium iodide/acetic acid. Alkali salts are easily prepared in mixtures of organic solvents. The chief applications of 2,3,4,6-tetrachlorophenol and its salts include use as bactericides for latex preservation (38), insecticides, wood preservatives (34), and leather preservatives (4). In 1960, approximately 9,000,000 lb of 2,3,4,6-tetrachlorophenol was produced and sold in the United States either as the phenol or as salts.

2,3,4,5-Tetrachlorophenol behaves as a weak acid with a neutral equivalent of 232. 2,3,4,5-Tetrachlorophenol can be prepared by the diazotization of 2,3,4,5-tetrachloroaniline, followed by hydrolysis of the diazonium salt. It can also be prepared by the hydrolysis of pentachlorobenzene in the presence of methanol and sodium methylate. It has found no commercial utility.

2,3,5,6-Tetrachlorophenol is a fairly strong acid and can be directly titrated with 0.1N aqueous sodium hydroxide having a neutral equivalent of 232. 2,3,5,6-Tetrachlorophenol can be prepared from 2,3,5,6-tetrachloroaniline via diazotization and subsequent hydrolysis of the diazonium salt. It is also produced by the hydrolysis of the pentachlorobenzene as a coproduct with 2,3,4,5-tetrachlorophenol. It has found no commercial utility.

Pentachlorophenol is insoluble in water, but is soluble in acetone (53 g/100 g), diethyl ether (158 g/100 g), ethanol (143 g/100 g), methanol (202 g/100 g), and toluene (16 g/100 g). Pentachlorophenol has a low odor level when cold, but a strong pungent odor when hot. Pentachlorophenol is acidic, and in alcohol solution titrates quantitatively (bromothymol blue indicator). It has a pK_a of 4.86. It is slightly volatile with steam, but not from alkaline solutions.

Pentachlorophenol is sold by The Dow Chemical Company under the designation Dowicide 7. It can be manufactured by the direct chlorination of phenol or polychlorophenols. One commercial process (39) uses a mixture of phenol, *o*-chlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol as the starting material. Chlorination is carried out in the absence of solvents and catalyst until tetrachlorination is achieved. At that point, a catalyst such as $FeCl_3$, $AlCl_3$, $SbCl_3$, etc, is added to take the reaction to pentachlorophenol. The end point of the reaction is determined by the freezing point. When the proper freezing point is achieved, the chlorination is stopped, and the material is flaked and packaged. Pentachlorophenol is also manufactured by the hydrolysis of hexachlorobenzene with 5-15% sodium hydroxide in methanol at 130-140°C (40). The hexachlorobenzene is easily prepared by the liquid-phase iron-catalyzed chlorination of benzene. Metallic salts are easily prepared by reacting pentachlorophenol with the appropriate base.

Pentachlorophenol is a broad-spectrum biotoxicant. It is too phytotoxic to use on crops. As a fungicide and insecticide, its chief use is in wood preservation, where it competes with creosote. Wood preservation with pentachlorophenol started in the late 1930s. An excellent detailed review of pentachlorophenol and its salts in wood treatment has been written by Sproule (41). Pentachlorophenol is quite dusty and exposure to the dust can lead to respiratory difficulties. To avoid this problem, several companies produce an oiled pentachlorophenol. United States production in 1962 was approximately 39,000,000 lb; the average selling price was 22¢/lb in drum quantities.

4-Chloro-2-methylphenol melts at 48–49°C. It can be manufactured by the direct chlorination of *o*-cresol at low temperatures without catalyst or solvents. The resulting product is a mixture of 60–65% 4-chloro-*o*-cresol and 35–40% 6-chloro-*o*-cresol. A purer product can be manufactured by using sulfuryl chloride as the chlorinating agent. If this process is used along with catalysts such as ferric or aluminum chloride, the 4-chloro-2-methylphenol content can be raised to as high as 90% in the crude chlorinated product (42). The use of sulfuryl chloride can be made still more economical by mixing the off-gas from the chlorination with chlorine and passing the mixture through charcoal where the sulfur dioxide is reconverted to sulfuryl chloride (43). The chief use for 4-chloro-2-methylphenol is in the manufacture of 2-methyl-4-chlorophenoxyacetic acid (MCPA) and its derivatives. MCPA was developed concurrently with 2,4-D as a plant-growth regulator. MCPA is produced by a process analogous to that used for 2,4-D, eg, 4-chloro-2-methylphenol is reacted with chloroacetic acid in the presence of sodium hydroxide. The MCPA formulation is usually sold as the potassium salt and occasionally as the sodium salt. These salt formulations have the advantage over 2,4-D amine salts that they are more water-soluble. MCPA and derivatives are widely used in Europe, but have not found wide acceptance in the United States. Agricultural methods and climatic conditions are probable reasons for this.

4-Chloro-3,5-dimethylphenol. This product melts at 115–116°C and boils at 246°C at 760 mm Hg. It is relatively insoluble in water, but is soluble in ethanol (86 g/100 g) and isopropyl alcohol (50 g/100 g) and slightly soluble in benzene (6 g/100 g) and glycerine (1.5 g/100 g). 4-Chloro-3,5-dimethylphenol can be prepared by the chlorination of 3,5-dimethylphenol with SO_2Cl_2 in chloroform solution or with chlorine in acetic acid solution. Yields of 65–70% have been achieved. It should be noted that 2-chloro-3,5-dimethylphenol is also formed as a coproduct in this reaction and must be removed to prepare the pure 4-chloro derivative. 4-Chloro-3,5-dimethylphenol has received considerable attention as an antiseptic, disinfectant, preservative, bactericide, etc. The material has found utility as a preservative in latex paints, adhesives, cosmetics, and pharmaceuticals. It has also found utility as an antiseptic in liniments, liquid soaps, waterless hand cleaners, etc (44). Its utility has been enhanced due to its relatively nontoxic nature. 4-Chloro-3,5-dimethylphenol is noncorrosive, nonirritating, and nonsensitizing; therefore, it can be used in many applications where other chlorinated phenols cannot be tolerated. Joseph (45) has carried out detailed toxicological studies on this material. 4-Chloro-3,5-dimethylphenol is manufactured and sold in the United States as well as in Europe. The product is commercially available and sold under the name Ottasept by Ottawa Chemical Company.

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T36.25-66681-4

DOW 749555

THE FORMATION OF FOLLICULITIS IN THE RABBIT EAR FOLLOWING A SINGLE
SUBCUTANEOUS INJECTION OF SYMMETRICAL TETRACHLORODIBENZODIOXIN IN
BENZENE

Problem

To produce folliculitis in the rabbit ear by subcutaneously in-
jecting various concentrations of symmetrical tetrachlorodibenzo-
dioxin.

Materials Used

0.02 Per cent, 0.01 per cent, 0.001 per cent, 0.0001 per cent,
0.00001 per cent and 0.000002 per cent symmetrical tetrachloro-
dibenzodioxin isolated from caustic insoluble oil. Vehicle -
benzene.

Experimental Procedure

0.01 Milliliter of each solution described above was injected
subcutaneously in rabbit ears. A control rabbit for the solvent
was incorporated into the study. One rabbit was used on each
concentration of symmetrical tetrachlorodibenzodioxin.

A total of seven animals was used in the evaluation.

Results and Conclusions

Results of the study indicated that a subcutaneous injection of 0.01 ml of 0.02 per cent symmetrical tetrachlorodibenzodioxin produced a discernible folliculitis after eleven days exposure and that a severe folliculitis developed after 21 days.

Also, that an injection of 0.01 ml of as low a concentration as 0.001 per cent produced a slight folliculitis after 26 days exposure. Concentrations less than 0.001 per cent produced no discernible folliculitis under the conditions of the experiment.

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Table of Results

First Exposed 2/11/65

Animal Number	Rack And Cage	Solution Strength (%)	Date of First Folliculitis	Appearance At Autopsy	Autopsy		
					Lung	Liver	Kidney
752	134-1	0.02	2/22/65	Severe folliculitis	OK	+	OK
756	-2	0.01	2/25/65	Moderate folliculitis	OK	OK	OK
758	-3	0.001	3/9/65	Slight folliculitis	OK	OK	OK
760	-4	0.0001	None	None	OK	OK	OK
762	-5	0.00001	None	None	OK	OK	OK
764	-6	0.000002	None	None	OK	+	OK
765	-7	Benzene	None	None	OK	OK	OK

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POW 749557

First exposed 2/11/65

Animal Number	Rack And Cage	Solution Strength (%)	Date Of First Folliculitis	Appearange At Autopsy	Autopsy		
					Lung	Liver	Kidney
752	134-1	0.02	2/22/65	Severe folliculitis	OK	+	OK
756	-2	0.01	2/25/65	Moderate folliculitis	OK	OK	OK
758	-3	0.001	3/9/65	Slight folliculitis	OK	OK	OK
760	-4	0.0001	None	None	OK	OK	OK
762	-5	0.00001	None	None	OK	OK	OK
764	-6	0.000002	None	None	OK	+	OK
765	-7	Benzene	None	None	OK	OK	OK

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T36.25-66681-6

DOW 749568

THE INEFFECTIVENESS OF WASHING AS A PREVENTIVE MEASURE IN THE
FORMATION OF FOLLICULITIS IN THE RABBIT EAR FOLLOWING REPEATED
APPLICATIONS OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN IN CORN
OIL

Problem

To examine the effectiveness of washing with soap and water as a prophylactic measure in the control of folliculitis produced in the rabbit ear by repeated applications of various concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin in corn oil. A previous report (T36.25-66681-2) summarized the effect of washing the material from the ear when applied in benzene as a solvent. The material is relatively soluble in benzene. It was of interest to employ similar techniques by applying the dioxin in oil in which it is essentially insoluble.

Materials Used

- 0.01 Per cent and 0.001 per cent 2,3,7,8-tetrachlorodibenzo-p-dioxin isolated from caustic insoluble oil.

Solvent -- Corn oil

Soap -- Dial

Tap water

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March 26, 1965

Washing Procedure

Ears were thoroughly washed with rich soap lather for 30 seconds and subsequently rinsed in copious amounts of freely-flowing tap water, after which they were blotted dry with paper towels.

Exposure Times Selected For Study

- (1) Five seconds (time taken to uniformly spread solutions described above on rabbit ear).
- (2) Two hours.
- (3) Four hours.

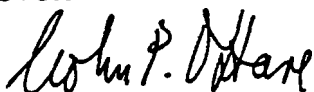
Experimental Procedure

The effectiveness of washing with soap and water as a prophylactic measure was studied in the lab, by repeatedly applying the solutions described above to rabbit ears -- 0.1 cc/ear/day -- and allowing the material to remain for controlled periods of time before washing. Two animals were used to demonstrate each point represented by a chemical concentration and a particular exposure time. Controls for each chemical concentration and for the solvent were incorporated into the study. A total of 18 animals were used in the evaluation.

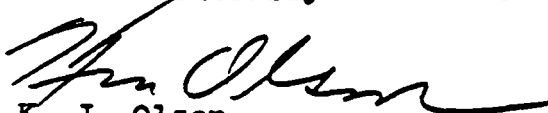
Results And Conclusions

Results of this study indicate that concentrations as low as 0.001 per cent 2,3,7,8-tetrachlorodibenzo-p-dioxin in corn oil produced a discernable folliculitis as a result of sixteen applications in 24 days. When compared to a previous similar experiment in which benzene was incorporated as the solvent, results indicate that the use of benzene hastens the development of folliculitis.

Under the conditions of the experiment it appears that washing with soap and water is quite ineffective as a preventive measure even when the dioxin is applied in an essentially insoluble form.



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Summary of Results

Solution Strength	Number Of Applications	Days Exposed	Washed	Ear Appearance	First Appearance of Folliculitis	
					Applications	Days Exposed
0.01%	16	24	No	Moderate-severe folliculitis	12	18
0.01%	14	24	No	Severe folliculitis	12	18
0.001%	16	24	No	Slight folliculitis	16	24
0.001%	16	24	No	Slight folliculitis	16	24
0.01%	11	17	At once	Severe folliculitis	9	13
0.01%	14	20	At once	Severe folliculitis	12	18
0.001%	16	24	At once	Slight folliculitis	16	24
0.001%	16	24	At once	Very slight folliculitis	16	24
0.01%	7	13	2 hours	Severe folliculitis	6	10
0.01%	6	11	2 hours	Slight folliculitis (Animal died)	6	11
0.001%	16	24	2 hours	Slight folliculitis	16	24
0.001%	14	20	2 hours	Severe folliculitis	11	17
0.1%	16	24	4 hours	Moderate folliculitis	15	21
0.01%	16	24	4 hours	Moderate folliculitis	15	21
0.001%	16	24	4 hours	Slight folliculitis	16	24
0.001%	16	24	4 hours	Slight folliculitis	16	24
Corn oil Control	16	24	No	O.K.		
Corn oil Control	16	24	2 hours	O.K.		

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THE DOW CHEMICAL COMPANY

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DOW 119709

ANALYTICAL METHOD

June 22, 1965

MLW.65.11

THE DETERMINATION OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN IN 2,4,5-TRICHLOROPHENOXYACETIC ACID BY GAS-LIQUID CHROMATOGRAPHY

1. Scope

This method is applicable to the determination of 2,3,7,8-tetrachlorodibenzo-p-dioxin in 2,4,5-trichlorophenoxyacetic acid. The dioxin can be detected at the one ppm level with a lower limit of 0.5 ppm possible at optimum operation conditions.

2. Principle

The 2,3,7,8-tetrachlorodibenzo-p-dioxin is separated from the 2,4,5-trichlorophenoxyacetic acid by means of an extraction with chloroform. The chloroform extract is concentrated and then chromatographed. The 2,3,7,8-tetrachlorodibenzo-p-dioxin in the sample is measured and compared to a known standard.

3. Safety Precautions

2,3,7,8-Tetrachlorodibenzo-p-dioxin is capable of causing a severe delayed skin response (chloracne) upon minimal contact. Samples suspected of containing any of this compound should be handled so as to prevent all skin contact and inhalation. Wear impervious gloves (rubber, polyvinyl chloride, etc.) at all times when contact is a possibility. Clean all equipment with acetone followed by a chloroform wash. Dispose in such a manner as to prevent all skin contact, any potentially contaminated equipment or materials which are not readily cleaned with chloroform, i.e., towels, gloves, etc.

4. Apparatus

- (a) Gas chromatograph, Aerograph A-600-D with flame ionization detector, Wilkins Instrument and Research, Inc., Walnut Creek, California, or equivalent.
- (b) Recorder, -0.05 to +1.05 millivolt, full span, one-second full response time.
- (c) Syringe, Hamilton microliter, No. 701N, or equivalent.
- (d) Syringe, Multifit 5 cc, Becton, Dickinson and Company, or equivalent.
- (e) Syringe, Yale 1/4 cc, Becton, Dickinson and Company, or equivalent.

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June 22, 1965

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- (f) Centrifuge
- (g) Injector insert, Pyrex glass for A-600-D. Available from Wilkins Instrument and Research, Inc., Walnut Creek, California (Note 11a).
- (h) Column, 1/8-inch O.D., 0.081-inch I.D., stainless steel tubing, five feet in length packed with reagent 5(c).

5. Reagents

- (a) Solid support, Chromosorb W, 60/80 mesh, Johns-Manville.
- (b) Partitioning agent, SE-30, Silicone gum rubber-methyl (Note 11b).
- (c) Column packing, five percent by weight of SE-30 on 60/80 mesh Chromosorb W. Available from Wilkins Instrument and Research, Inc., Walnut Creek, California.
- (d) Carrier gas, nitrogen, commercial grade.
- (e) Chloroform, ACS grade.
- (f) 2,3,7,8-Tetrachlorodibenzo-p-dioxin, available from The Dow Chemical Company, Midland, Michigan.
- (g) Sodium hydroxide, 1 N solution. Dissolve 40 grams of reagent grade sodium hydroxide in one liter of water.

6. Chromatographic Conditions

- (a) Oven temperature, 225°C.
- (b) Inlet temperature, 260°C.
- (c) Carrier gas flow rate, 75 ml. per minute as determined by the moving soap bubble technique.
- (d) Attenuation, such that a response of at least 50% of scale is obtained from a 1.0 microliter sample of a standard containing 100 micrograms of 2,3,7,8-tetrachlorodibenzo-p-dioxin in one milliliter of chloroform.

7. Preparation of Standard

- (a) Again read Section 3.
 - (b) Weigh, using a micro-balance, one milligram of 2,3,7,8-tetrachlorodibenzo-p-dioxin into a ten ml. volumetric flask.
 - (c) Dilute to the mark with chloroform.
 - (d) Inject a 1.0 microliter sample into the chromatograph.
- See Figure I for a typical chromatogram.

8. Procedure

- (a) Weigh 10.0 grams of the sample into a four-ounce bottle.
- (b) Add 20.0 milliliters of chloroform and shake for one hour.
- (c) Place the solution in a centrifuge tube and, with proper balancing, centrifuge for five minutes.
- (d) Using an eye-dropper, draw off as much of the clear chloroform layer as possible into a two-ounce bottle.

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June 22, 1965

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- (e) Add 25 ml. of 1 N sodium hydroxide to the chloroform extract and shake for 15 minutes (Note 11c).
- (f) Centrifuge for five minutes.
- (g) Using a five-milliliter syringe, draw off as much of the bottom chloroform layer as is possible into a small vial. Note this volume.
- (h) Evaporate to dryness in a hood.
- (i) Take up with chloroform to 5.0% of the volume noted in step (g). This final solution represents ten grams of sample per ml. of chloroform.
- (j) Inject 1.0 microliter into the chromatograph and measure the response of the 2,3,7,8-tetrachlorodibenzo-p-dioxin. Figure 11 shows a representative chromatogram.

9. Calculations

Let:

- A = The area of the 2,3,7,8-tetrachlorodibenzo-p-dioxin in the sample.
- B = The attenuation of the chromatograph for the sample.
- C = The micrograms per milliliter of the 2,3,7,8-tetrachlorodibenzo-p-dioxin in the standard.
- D = The area of the response from the 2,3,7,8-tetrachlorodibenzo-p-dioxin in the standard.
- E = The attenuation of the chromatograph for the standard.

Then.

$$\text{ppm of 2,3,7,8-tetrachlorodibenzo-p-dioxin} = \frac{A \times B \times C}{D \times E \times 10}$$

10. Accuracy

The accuracy of this method is $\pm 5\%$, or less, relative.

11. Notes

- (a) Glass inlet liners have been found to be necessary to provide reproducible results.

June 22, 1965

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(b) Silicone FS-1265 (fluro) has been found to work well as a stationary phase. It also is available from Wilkins Instrument and Research, Inc., Walnut Creek, California.

(c) Any 2,4,5-trichlorophenoxyacetic acid which has dissolved in the chloroform extract must be removed as it will interfere with the chromatographic analysis of the 2,3,7,8-tetrachlorodibenzo-p-dioxin.

* * * * *

The analytical procedures given herein have been adapted from literature sources or developed upon the basis of experimental data which are believed to be reliable. In the hands of a qualified analyst they are expected to yield results of sufficient accuracy for their intended purposes. However, The Dow Chemical Company makes no representation or warranty whatsoever concerning the procedures or results to be obtained and assumes no liability in connection with their use. Users are cautioned to confirm the suitability of the methods by appropriate tests. Anyone wishing to reproduce or publish the material in whole or in part should request written permission from The Dow Chemical Company.

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June 22, 1965

FIGURE I

MLW.65.11

Attenuation 1 x 1

2,3,7,8-Tetrachlorodibenzo-p-dioxin
114.4 micrograms/ml.

DOW 119713

Recorder Response (m.v.)

0001000

5481

0
Start

2

4

6

8

Retention Time (minutes)

June 22, 1965
Attenuation 32 x 10

FIGURE II

MLW. 65.11

DOW 119714

Attenuation 1 x 1

2,3,7,8-Tetrachlorodibenzo-p-dioxin
2.5 ppm

Recorder Response (m.v.)

Attenuation
32 x 1

2931000

5482

Start

Retention Time (minutes)

58

58

5483

35.10 - 61346
file m. wolf
35.12 - 38323
35.12 - 44793

**ACUTE FISH TOXICITY OF DANTRON[®], TORSEM[®], AND DURSBAH[®]
TO THREE SPECIES OF FISH**

By
Howard C. Alexander
Terry L. Batchelder

Waste Control
The Dow Chemical Company
Midland, Michigan

**For presentation on April 14, 1966 at the annual
meeting of the Midwest Benthological Society at
Central Michigan University.**

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INTRODUCTION

The past few years much attention has been focused on the effects of pesticides, and in particular insecticides, upon natural habitats. Some insecticides have been observed to be toxic in very small amounts not only to insects, its primary victims, but also to other living animals. The ecology of the aquatic community may likewise be upset by pesticides.

Suggested toxicity test procedures for birds, fish, and marine mollusks have been outlined by the U. S. Fish and Wildlife Service in their "Procedures for Evaluation of Acute Toxicity of Pesticides to Fish and Wildlife", revised December 14, 1964. The purpose is to aid industry in the determination of pesticide toxicity on fish and wildlife for data to support requests for label registration. The techniques for fish are similar to those used by the research stations of the Bureau of Commercial Fisheries and Sport Fisheries and Wildlife, and are considered to give a reasonable basis for judging acute fish toxicity.

One of the main objectives is to protect fish and wildlife resources from hazardous compounds, including those which cause acute and chronic toxicities. Consideration of the total hazard must include the stability of the compound in nature and the proposed use pattern. Those utilized repeatedly, or that persist, may cause chronic toxicity and thus have a greater significance in total potential toxicity

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even though the compound exhibits no acute toxicity at low concentrations. All compounds that may contaminate our water resources should at least be tested for acute fish toxicity.

Three Dow pesticides, known under the trademarks Daxtron[®], Tordon[®], and Dursban[®], were tested for acute toxicity to three species of fresh water fish. Daxtron, a general herbicide, is still in the experimental stage; Tordon herbicide is used for killing a wide variety of deep-rooted perennial herbaceous weeds and woody plants; Dursban insecticide is in the experimental stage. Rainbow trout (Salmo gairdneri Richardson) were used as cold water representatives; bluegills (Lepomis macrochirus Rafinesque) and channel catfish (Ictalurus punctatus Rafinesque) as warm water fish. The bluegill data is acceptable for evaluation of possible effects on marine fish. The common goldfish (Carassius auratus Linnaeus) may also be used as a warm water fish.

METHODS AND MATERIAL

Water used for the test was aerated distilled water with the following additives: 30 mg/l CaSO₄, 30 mg/l MgSO₄, 48 mg/l Na₂CO₃, and 3 mg/l KCl. This distilled water solution was made up in a 55 gallon plastic coated drum and aerated. Water was added to the test vessels and aeration was continued until the addition of the test compound. There was no aeration during the test period.

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Test fish were acclimated for at least 13 days in constant flow storage drums. Dry trout pellets were fed for 10 days; three days prior to testing, no food was given so as to empty the digestive tract. Dead or diseased fish were removed immediately. After acclimation, five fish were placed in each five gallon wide mouth glass jug or 10 fish in each 10 gallon aquarium.

The fish were introduced into the test vessels 24 hours prior to the addition of the test compound. The compound was added in the following manner: 15.5 liters of water were placed in each five gallon jug, five fish added, then at the end of 24 hours the chemical was added with two more liters of water making a total of 17.5 liters. Each ten gallon aquarium was filled with 31.5 liters of prepared water and ten fish were added. After 24 hours, the compound was introduced with four more liters of water. The vessels were then placed in constant temperature water troughs: 60°F for the cold water fish, and 80°F for the warm water fish. Plastic mesh was laid across the tops of the containers to prevent fish loss.

Ten fish were exposed to each concentration of the compound. The fish were observed and the data recorded daily at the same time for four days (96 hours).

A reference DDT toxicity was made for each test lot on each species of fish.

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After each test, the fish vessels were thoroughly scrubbed and rinsed twice, using a commercial "chlorine" cleanser. Care must be taken to insure that only vessels of non-porous materials be used. Several slate-bottomed aquaria had to be discarded because of contamination that could not be removed by this method.

All test solutions were prepared on a weight-volume basis immediately prior to administering the test pesticide.

Suitable test fish are not as readily available as stated in the Fish and Wildlife procedure. Rainbow trout in the proper size range may be obtained most months of the year from several sources within Michigan. However, bluegills and channel catfish in this size range are available for only a couple of months during the year and a source for these fish could not be found within the state. Trout were obtained from the Michigan Department of Conservation, Harrietta Hatchery and Baldwin Rearing Ponds; the bluegills and channel catfish from the U. S. Fish and Wildlife Service, National Fish Hatcheries at Hebron, Ohio, and Senecaville, Ohio.

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RESULTS

The test solution concentrations and determination of TL_m values were in accordance with the bioassay methods stated in "Standard Methods for the Examination of Water and Wastewater", 11th Edition, 1960, pages 457-471. The 24, 48, 72, and 96-hour TL_m for each species of fish was determined for each compound. The TL_m is the median tolerance limit or the concentration of the material being tested at which 50 percent of the test animals are able to survive for a specific time interval. The TL_m corresponds to the 50 percent lethal or LC_{50} used in the fish section of the Fish and Wildlife test procedure.

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DAXTRON

Candidate pesticide: Manufacturer - The Dow Chemical Company

A. Trade Mark - Daxtron

Active Ingredient - 2,3,5-trichloro-4-pyridinol potassium salt

B. Reference - 3-1350-11 91% potassium salt

DOW 765067

Solvents used:

A. Identity and quantity used for reference pesticide:

0.1 mg/ml in acetone

B. Identity and quantity used for candidate pesticide:

100 mg/ml in water

Fish:

A. Rainbow trout

Source - Baldwin, Michigan

Date: 7-23-65

Average weight (g.): 1.8

Range (g.): 1.5 - 2.1

B. Bluegill

Source: Hebron, Ohio

Date: 9-14-65

Average weight (g.): 1.2

Range (g.): 1.0 - 1.3

C. Channel catfish

Source: Senecaville, Ohio

Date: 9-14-65

Average weight (g.): 1.9

Range (g.): 1.8 - 2.0

Bioassay vessels:

A. Vessel type and size: glass - 5 gallon jar

glass - 10 gallon aquarium

B. Loading (grams of fish/liter of test medium)

less than 0.5 gm fish/liter of water

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C. Number of fish/vessel: 5 gallon jar - 5 fish
10 gallon aquarium - 10 fish

Water: Distilled water with the following compounds added:

30 mg/l calcium sulfate
30 mg/l magnesium sulfate
48 mg/l sodium carbonate
3 mg/l potassium chloride

DOW 765068

Results with pesticides expressed as TL_m on each species

<u>Species</u>	<u>Daxtron TL_m mg/l</u>			
	<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	142	100	100	82
Bluegill at 80°F	615	425	320	320
Channel catfish at 80°F	138	134	130	130

Reference pesticide (p,p' - DDT)

<u>Species</u>	<u>TL_m mg/l</u>			
	<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	.0160	.0143	.0125	.0115
Bluegill at 80°F	.0078	.0066	.0066	.0046
Channel catfish at 80°F	.0145	.0134	.0128	.0128

Results with controls expressed as survival over 96 hours

<u>Species</u>	<u>Total</u> <u>Fish</u>	<u>Number of fish surviving at</u>			
		<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	10	10	10	10	10
Bluegill at 80°F	20	19	19	18	18
Channel catfish at 80°F	20	20	19	19	19

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TORDON

Candidate pesticide: Manufacturer - The Dow Chemical Company

A. Trade Mark: Tordon

Active Ingredient: 4-Amino-3,5,6-trichloropicolinic acid as the
Lot #962 potassium salt

Solvents used:

A. Identity and quantity used for reference pesticide:

0.1 mg/ml in acetone

B. Identity and quantity used for candidate pesticide:

12.5 mg/ml as formulated in water

Fish:

A. Rainbow trout

Source: Harrietta, Michigan

Date: 6-22-65

Average weight (g.): 1.8

Range (g.): 1.7 - 2.1

B. Bluegill

Source: Hebron, Ohio

Date: 9-14-65

Average weight (g.): 1.2

Range (g.): 1.0 - 1.3

C. Channel catfish

Source: Senecaville, Ohio

Date: 9-14-65

Average weight (g.): 1.9

Range (g.): 1.8 - 2.0

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Bioassay vessels:

- A. Vessel type and size: 5 gallon glass jar
10 gallon glass aquarium
- B. Loading (grams of fish/liter of test medium)
less than 0.5 gm fish/liter
- C. Number of fish/vessel: 5 gallon jar - 5 fish
10 gallon aquarium - 10 fish

Water: Distilled water with the following compounds added:

30 mg/l calcium sulfate
30 mg/l magnesium sulfate
48 mg/l sodium carbonate
3 mg/l potassium chloride

Results with pesticides expressed as TL_m on each species

<u>Species</u>	Tordon TL_m mg/l			
	<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	27	13	13	13
Bluegill at 80°F	69	69	45	24
Channel catfish at 80°F	41	24	16	14

Reference pesticide (p,p' - DDT)

<u>Species</u>	TL_m mg/l			
	<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	.0091	.0074	.0068	.0063
Bluegill at 80°F	.0078	.0066	.0066	.0046
Channel catfish at 80°F	.0145	.0134	.0128	.0128

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Results with controls expressed as survival over 96 hours

<u>Species</u>	<u>Total Fish</u>	Number of fish surviving at			
		<u>24 hours</u>	<u>48 hours</u>	<u>72 hours</u>	<u>96 hours</u>
Rainbow trout at 60°F	10	10	10	10	10
Bluegill at 80°F	20	19	19	18	18
Channel catfish at 80°F	20	20	19	19	19

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DURSBAN

Candidate pesticide: Manufacturer - The Dow Chemical Company

A. Trade Mark - Dursban

Active Ingredient - O,O-diethyl-O,3,5,6-trichloro-2-pyridyl
phosphorothioate

Lots 2 and 3 99% pure

Solvents used:

A. Identity and quantity used for reference pesticide:

0.1 mg/ml in acetone

B. Identity and quantity used for candidate pesticide:

0.1 mg/ml in acetone

Fish:

A. Rainbow trout

Source: Baldwin, Michigan

Date: 7-23-65

Average weight (g.): 1.8

Range (g.): 1.5 - 2.1

B. Bluegill

Source: Hebron, Ohio

Date: 8-12-65

Average weight (g.): 1.8

Range (g.): 1.6 - 2.0

C. Channel catfish

Source: Senecaaville, Ohio

Date: 9-14-65

Average weight (g.): 1.9

Range (g.): 1.8 - 2.0

Bioassay vessels:

A. Vessel type and size: 5 gallon glass jar

10 gallon glass aquarium

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B. Loading (grams of fish/liter of test medium)
less than 0.5 gm fish/liter of water

C. Number of fish/vessel: 5 gallon jar - 5 fish
10 gallon aquarium - 10 fish

Water: Distilled water with the following compounds added:

30 mg/l calcium sulfate
30 mg/l magnesium sulfate
48 mg/l sodium carbonate
3 mg/l potassium chloride

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Results with pesticides expressed as TL_m on each species

<u>Species</u>	<u>Dursban TL_m mg/l</u>			
	<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	.0075	.0047	.0033	.0030
Bluegill at 80°F	.0040	.0036	.0033	.0033
Channel catfish at 80°F	.0198	.0142	.0134	.0134

Reference pesticide (p,p' - DDT)

<u>Species</u>	<u>TL_m mg/l</u>			
	<u>24</u> <u>hours</u>	<u>48</u> <u>hours</u>	<u>72</u> <u>hours</u>	<u>96</u> <u>hours</u>
Rainbow trout at 60°F	.0160	.0143	.0125	.0115
Bluegill at 80°F	.0128	.0056	.0042	.0042
Channel catfish at 80°F	.0145	.0134	.0128	.0128

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Results with controls expressed as survival over 96 hours

<u>Species</u>	<u>Total Fish</u>	Number of fish surviving at			
		<u>24 hours</u>	<u>48 hours</u>	<u>72 hours</u>	<u>96 hours</u>
Rainbow trout at 60°F	10	10	10	10	10
Bluegill at 80°F	10	10	10	10	10
Channel catfish at 80°F	10	9	9	9	9

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DISCUSSION AND CONCLUSIONS

The results show that Daxtron and Tordon herbicides are not particularly toxic to the fish. Dursban insecticide is toxic to the fish studied in low concentrations, parts per billion. However, it should be remembered that laboratory tests using clear water and standard conditions serve only as a preliminary indication of the possible problems that need to be evaluated under practical use conditions.

Environmental factors such as the effect of temperature, sunlight, sorption, and biological stability of the compound can only be evaluated by an actual field test. Formulation controlling release of the toxicant; pH, organic sediment, and foliage density may all modify the selectivity of the chemical to various aquatic faunal species. The hazard of a chemical under specified uses in specified locations may be greatly different than the toxicity shown in laboratory conditions. Such factors are being determined for Dursban insecticide at the present time.

Pesticides used improperly can cause serious damage. Always read the label and follow the instructions given on the label. Use recommended procedures and equipment for application. Avoid contamination of any other areas. Never allow spills or water for cleanup to contaminate sewers, ditches or the ground. Properly dispose of unused application mixtures, contaminated clothing and containers.

To be sure, Read the Label!

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C. R. Youngson

C. R. Youngson 12/17/66

A BIOASSAY STUDY ON THE DETOXIFICATION OF AQUEOUS SOLUTIONS OF TORDON BY
SUNSHINE IN DEEP CONTAINERS UNDER STILL AND CIRCULATING CONDITIONS

Evaluation of the effect of sunlight on photodetoxification of Tordon in
water in 12-foot deep containers has shown:

1. Circulating Water

a. Rate of loss calculated for 94 days' exposure during May to
October was at least 0.35 lb. per acre per day.

b. Rate of loss calculated for 56 days' exposure during September and
October was 0.35 lb. per acre per day. Percent detoxification was
roughly proportional to exposure period during the course of the
experiment. (Continued)

Work by: C. A. I. Goring and C. R. Youngson

Record books: GS-1450, 1457, 1472, 1475, 1480, 1485, 1501, 1508

GS-781

Report No.

165076

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*Seattle, Washington (JRF)
*Wayside Field Group (RCH)
*Washington (RGH)
*Dow Latin America (AWS)
*Dow International (ETC)
*Dow Canada (LJM, FFW)
*Dow Europe (LLC)

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SUMMARY (Continued)

2. Calm Water

Rate of loss was 0.19 lb. per acre per day calculated for 154 days' exposure. Loss was uniform throughout the depth of the container, even though sunlight rarely penetrated to the bottom.

A survey of data from 4 reports has shown that rate of photodetoxification of Tordon in water is markedly affected by such factors in the environment as brightness of sun, geographical location, time of year, cloudiness, dustiness, haziness, etc. Photodetoxification in water can occur in a variety of water environments, probably including the surface of leaves. However, when water evaporates leaving dry films of Tordon on surfaces, the loss rate is about 100 times less than that in water. Furthermore, when Tordon is present in muddy water or soil, sorption of UV radiation by the soil particles either drastically curtails or eliminates photodetoxification.

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In feeding studies of 2,4-D with dairy cows and steers (Lisk et al., 1963; Gutenmann et al., 1963a,b; Bache et al., 1964a,b), 2,4-D was found unchanged in the urine only. No evidence of beta-oxidation was found. Similar findings were obtained with sheep. Ninety-six percent of an orally administered dose of 2,4-D-C¹⁴ to a sheep was excreted unchanged in the urine in 72 hours and slightly less than 1.4% in the feces. Very little residual radioactivity was found in edible tissue (Clark et al., 1964). Cows fed 2,4,5-T and silvex excreted both as soluble salts in their urine. Kuron was hydrolyzed to silvex prior to elimination (St. John et al., 1964).

Treatment of lemons with C¹⁴ labeled 2,4-D isopropylester indicated that the ester was hydrolyzed and that part of the 2,4-D then reacted with some plant constituent to form an ester-like complex. Ester-like residues were also found after treatment with the sodium, diethanolamine, or triethanolamine salts (Erickson and Nield, 1962; Erickson et al., 1963). Hydrolysis (Crafts, 1960; Morre and Rogers, 1960) and decarboxylation (Edgerton and Hoffman, 1961; Basler, 1964) of 2,4-D by other plants has also been shown.

Plants are capable of hydroxylating phenoxyacetic acids (Wilcox et al., 1963; Thomas and Loughman, 1963). When bean plants were treated with 2,4-D, three compounds were found (Crosby, 1964). One corresponded roughly to that of 2,4-dichloroanisole; one was a water-soluble, ether-insoluble ester derivative; and the third, an ether-soluble compound with a basic

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structural change. The methyl derivative was less volatile than 2,4-D methyl ester, but more volatile than the 5-hydroxy-2,4-D methyl ester. It might be one of the other two hydroxy derivatives; however, 6-hydroxy-2,4-D was not detected (Holley, 1952; Jaworski and Butts, 1952; Jaworski et al., 1955; Evans and Smith, 1954; Bach, 1961).

The free acid has been recovered from bean and corn plants after treatment with 2,4-D butoxyethanol and propylene glycol butyl esters (Hagen et al., 1949; Fang et al., 1951; Fang and Butts, 1954; Hay and Thimann, 1956; Szabo, 1963). On cotton, cucumbers, beans, and grain sorghum, labeled 2,4-D gave rise to $C^{14}O_2$ (Holley et al., 1950; Weintraub et al., 1952a,b). Pea and tomato plants have also been studied (Fang, 1958). In young leaves and bolls of cotton, material chromatographically different from 2,4-D was formed. Sorghum converted 2,4-D to a complex different than that found in cotton (Morgan, 1963; Nencki and Giacosa, 1880; Weintraub et al., 1950, 1952a,b, 1953, 1954, 1956; Slife et al., 1962).

Amino acids have been implicated in the formation of some compounds, as in the case of 2,4-dichlorophenoxyacetylaspartic acid (Andreae and Good, 1957; Bach and Fellig, 1961). Evidence indicates that 2,4-D moves through plants as a protein complex, which can be recovered after aqueous extraction and NaOH hydrolysis, into the roots where most of the degradation occurs (Canny and Markus, 1960). Glucose esters have also been suggested (Klamt, 1961; Crosby, 1964). Recent studies have shown that glucoside complexes are formed. From stem tissues of oats (Avena sativa), 1-O-(2,4-dichlorophenoxyacetyl)- β -D-glucose was isolated (Thomas et al., 1964b), and from stems of the kidney bean (Phaseolus vulgaris), the 2,5- and 2,3-

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dichlorophenoxyacetic acid glucosides have been obtained (Thomas and Loughman, 1964a).

Degradation of compounds related to 2,4-D has also been studied (Wain, 1954). MCPB gave rise to MCPA when fed to dairy cows (Bache et al., 1964a, b, c). In laboratory tests, bluegill (Lepomis gibbosus) converted 4-(2,4-DB) to 2,4-D (Gutenmann and Lisk, 1965). When 4-(2,4-DB) was fed to dairy cows, however, 2,4-D was not found, although it does not decompose in the rumen. The major portion of 4-(2,4-DB), therefore, is presumably degraded by a mechanism other than β -oxidation (Lisk et al., 1963; Gutenmann and Lisk, 1963c). In silage (Linscott and Hagin, 1963; Linscott, 1964), timothy, birdsfoot trefoil, or pea plants (Fertig et al., 1964), decomposition of 4-(2,4-DB) has been shown to proceed by β -oxidation. It also has been shown that related compounds could be degraded by α - and β -oxidation in plants (Levey and Lewis, 1947; Fawcett et al., 1954, 1958; Moore and Rogers, 1960). γ -(2,4,5-Trichlorophenoxy)butyric acid was metabolized to its acetic acid derivative in wheat but not in pea stems (Balayannis et al., 1965b).

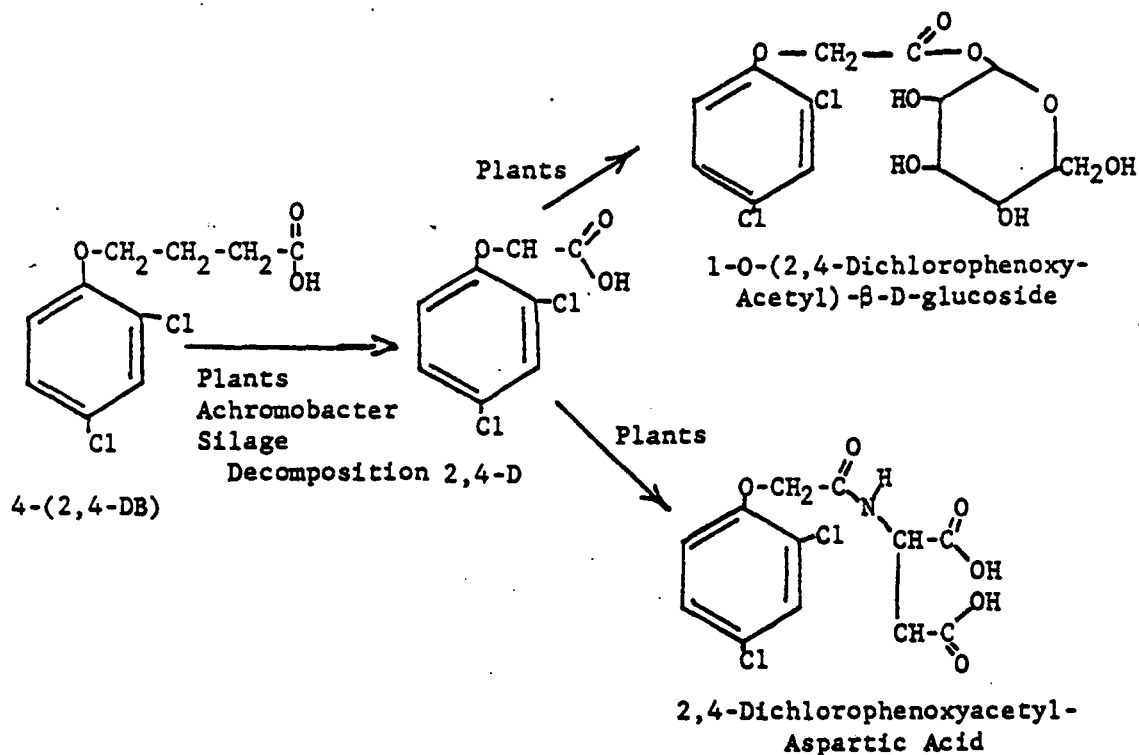
The biotransformation of 2,4-dichlorophenoxyalkanoic acids and related compounds by soil microflora has been extensively studied (Audus, 1949, 1950, 1951, 1952b; Audus and Symonds, 1955; Newman and Thomas, 1950; Steenson and Walker, 1956; Byrde et al., 1956, 1957, 1958; Jensen and Peterson, 1952; Evans and Smith, 1954, 1957; Fawcett et al., 1954; Webley et al., 1955, 1957, 1958; Bell, 1957; Henderson, 1957; Thomas and Loughman, 1957, 1963; Faulkner and Woodcock, 1961; Thiels, 1962; Clifford and Woodcock, 1964). Phenoxyalkanoic acids with an even number of carbons in the fatty

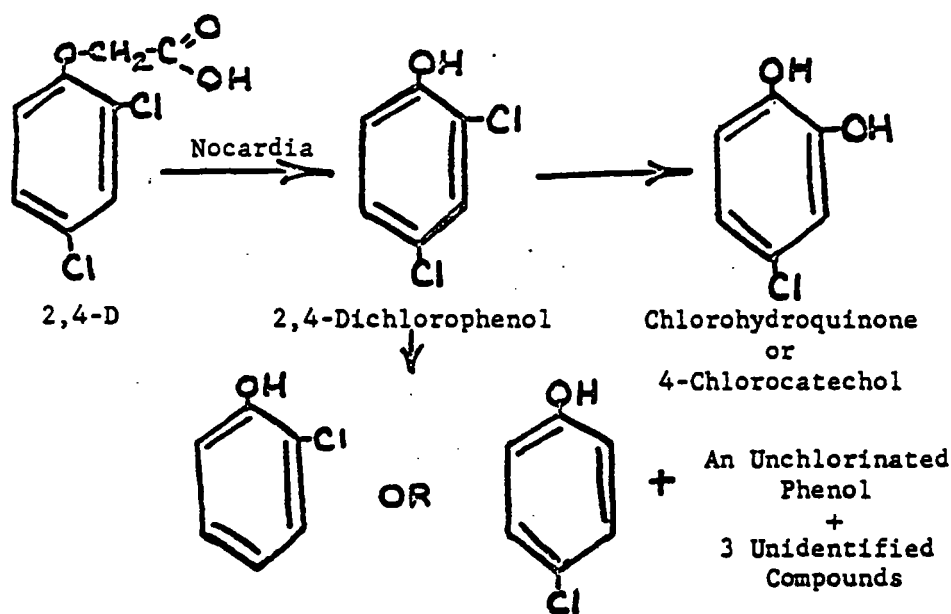
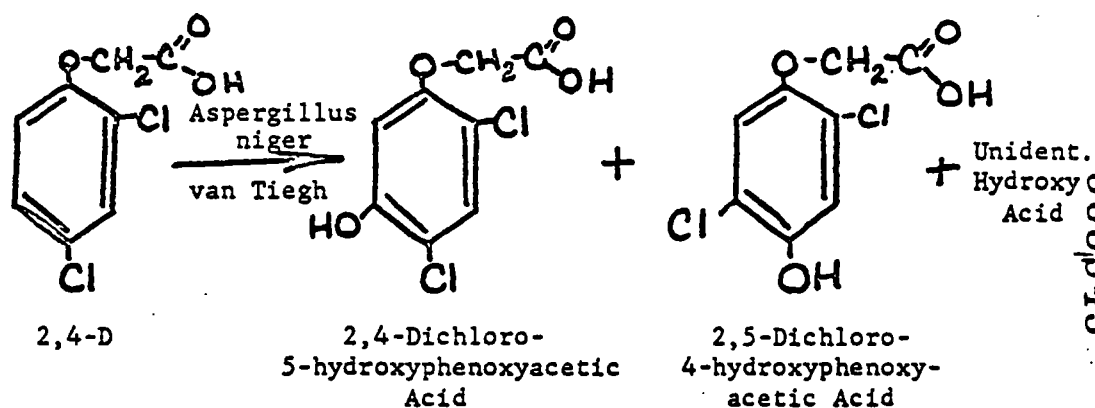
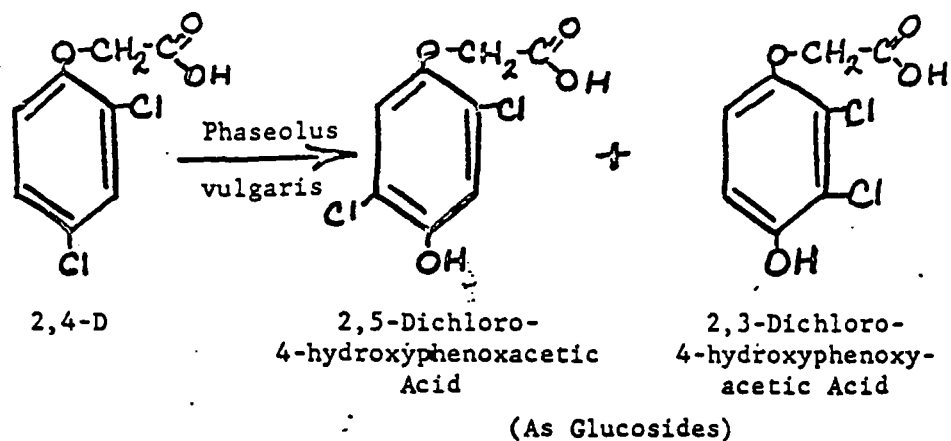
acid were converted by β -oxidation to products with an even number of carbons (Levey and Lewis, 1947; Webley et al., 1955, 1957, 1958; Gutenmann et al., 1964a, c). A second mechanism involved cleavage of the ether linkage (Canny and Markus, 1960; MacRae et al., 1963a b, 1964; Audus, 1964; Bocks et al., 1964).

Evidence has been obtained that 2,4-D is dissimilated by a variety of microorganisms (Reid, 1960; Alexander and Aleem, 1961) through 2,4-dichlorophenol and 4-chlorocatechol and that MCPA is dissimilated through 4-chloro-2-cresol (Audus, 1952b). MCPB was degraded by the bacteria Nocardia opaca via crotonic and β -hydroxy acid to 2,4-D (Webley et al., 1957, 1958). A product from the degradation of 2,4-D by bacteria of the genus Pseudomonas has been identified as β -chloromuconic acid. A second species of Pseudomonas gave rise to α -chloromuconic acid (Fernley and Evans, 1959). Pure cultures of a Nocardia species and an Achromobacter strain of bacteria rapidly degraded 2,4-D and the presence of 2,4-dichlorophenol, chlorohydroquinone, a monochlorophenol, an unchlorinated phenol, and three other unidentified compounds have been demonstrated (Newman and Thomas, 1950; Audus, 1951; Bell, 1957, 1960; Steenson and Walker, 1957, 1958; Faulkner and Woodcock, 1961; Taylor and Wain, 1962). The main product of 2,4-D metabolism by the mold Aspergillus niger van Tiegh was 2,4-dichloro-5-hydroxyphenoxyacetic acid. By means of infrared and mixed melting points, a second metabolite was identified as the 2,5-dichloro-4-hydroxyphenoxyacetic acid - the first time such a rearrangement was reported (Faulkner and Woodcock, 1964, 1965). Another unidentified acid, not the 3- or 6-hydroxyacid, was also found. Under similar conditions, MCPA gave rise to

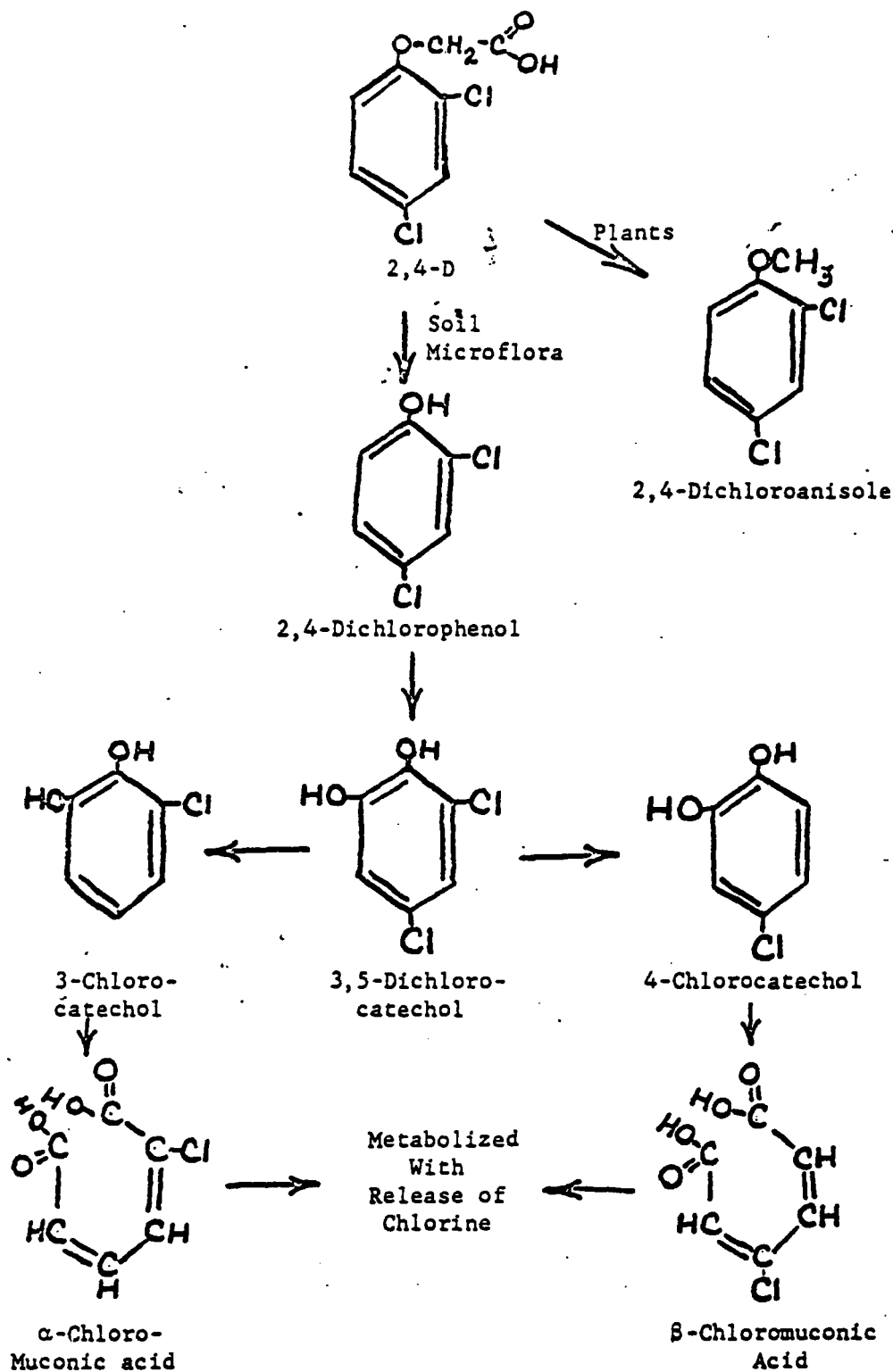
2-methyl-4-chloro-5-hydroxyphenoxyacetic acid (Faulkner and Woodcock, 1964, 1965). Studies with 4-(2,4-DB) showed that it is detoxified by Flavobacterium sp. (MacRae and Alexander, 1965), releasing chlorine and cleaving the ring (Burger et al., 1962).

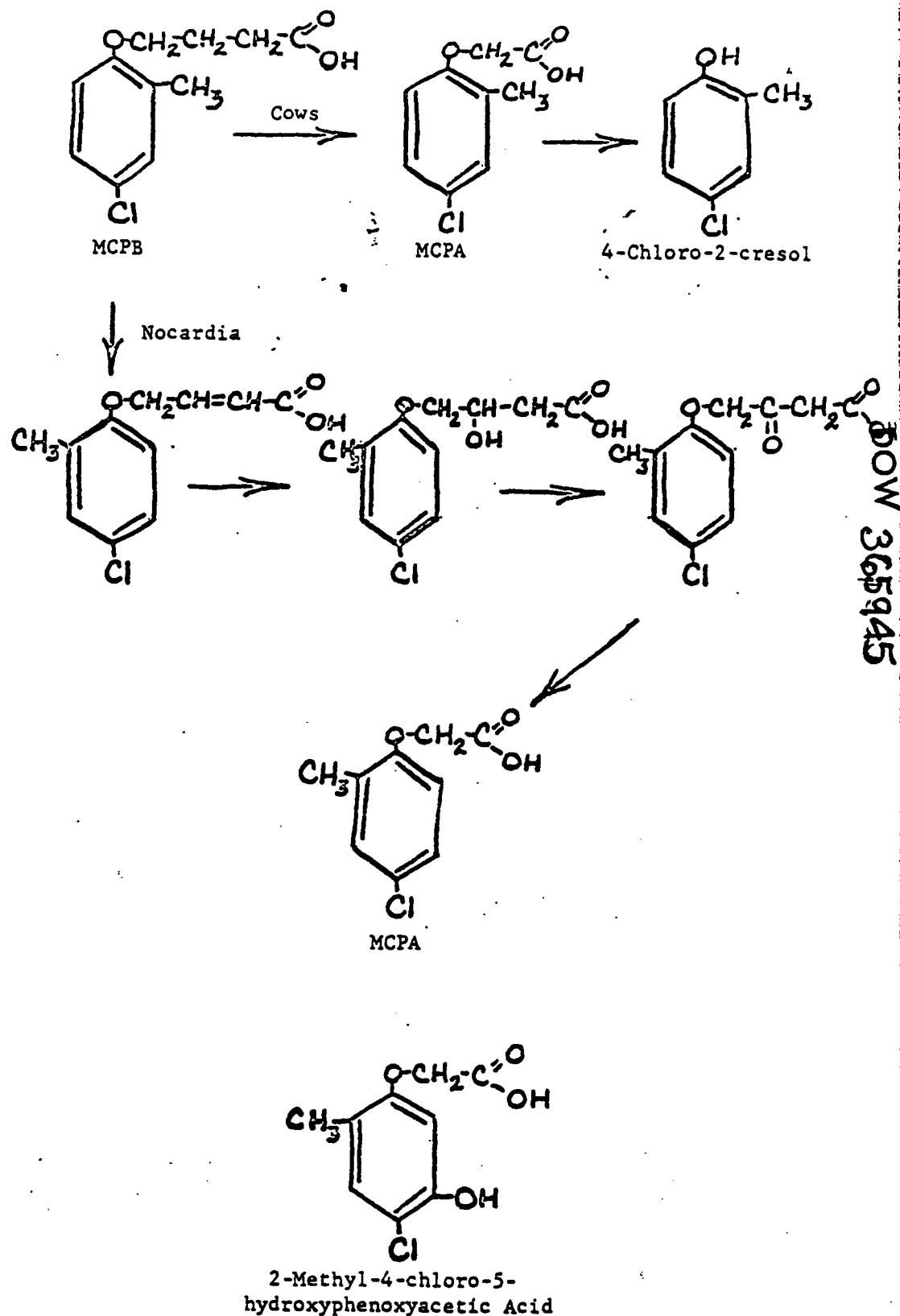
In natural surface waters, 2,4-D, its salt and i-propyl or butyl esters, were hydrolyzed by microorganisms to 2,4-D acid and the corresponding alcohol (Aly and Faust, 1964). Under the influence of ultra-violet, aqueous solutions of 2,4-D sodium salt gave rise to 2,4-dichlorophenol, and subsequently to 4-chlorocatechol. Complete dechlorination and polymerization followed (Hansen and Buchholtz, 1952; Tutass and Crosby, 1965). In the presence of riboflavin, compounds containing more than one aromatic nucleus were probably also formed in addition to 2,4-dichlorophenol. Products differed according to the original pH and concentration of the treated solution (Bell, 1956; Hansen and Buchholtz, 1952).



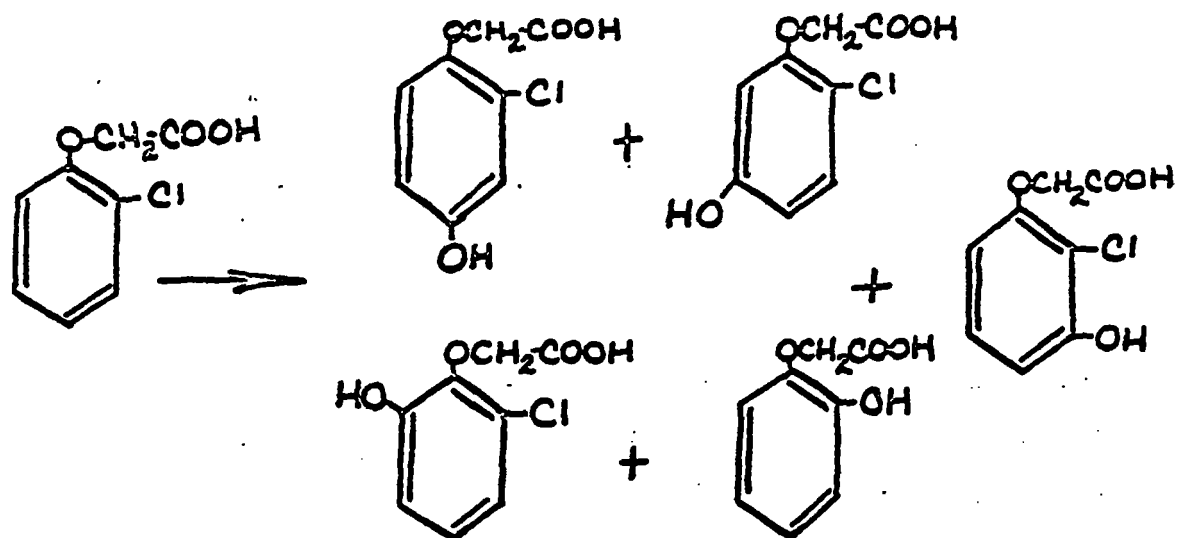
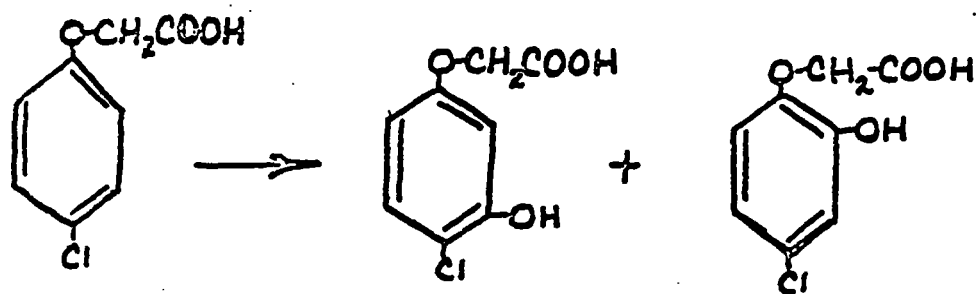
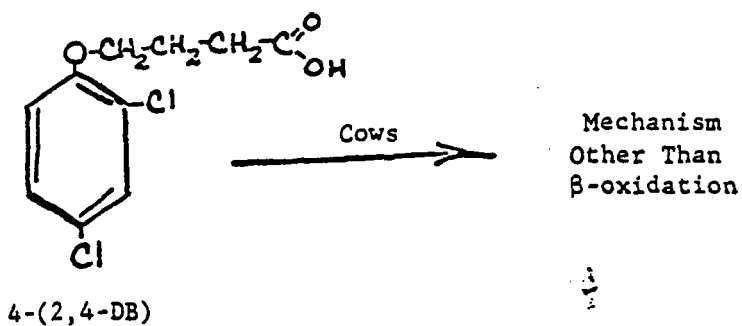


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FROM THE NATIONAL VETERINARY INSTITUTE,
STOCKHOLM, SWEDEN

STUDIES ON
THE ANALYTICAL CHEMISTRY AND TOXICOLOGY
OF PHENOXY HERBICIDES

BY

KURT ERNE

STOCKHOLM 1966

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Foreword

The present survey covers studies mainly accounted for in the following publications.

- I. *Erne, K.*: Detection and determination of chlorophenoxyacetic acid derivatives in water. *Acta chem. scand.* 1963, 17, 1663—1676.
- II. *Erne, K.*: Determination of phenoxyacetic herbicide residues in biological materials. *Acta vet. scand.* 1966, 7, 77—96.
- III. *Erne, K.*: Distribution and elimination of chlorinated phenoxyacetic acids in animals. *Acta vet. scand.* 1966, 7, 240—256.
- IV. *Erne, K.*: Studies on the animal metabolism of phenoxyacetic herbicides. *Acta vet. scand.* 1966, 7, 264—271.
- V. *Björklund, N.-E. & K. Erne*: Toxicological studies of phenoxyacetic herbicides in animals. *Acta vet. scand.* 1966, 7, 274—280.

In this survey these papers will be referred to by the roman numerals.

The investigations have been carried out at the National Veterinary Institute, Stockholm, during the years 1960—1966.

To professor Hans-Jörgen Hansen, Director of the Institute, I wish to express my sincere gratitude for his keen interest in my work and for his steady support and encouragement.

To the Dean of the Royal Veterinary College, Professor Carl Schmiterlöv, and to the Board of Professors of the college, I am indebted for permission to submit this thesis at the College.

My immediate chief, Professor Hans Wanntorp, Head of the Chemistry Department, has furthered my work by many helpful discussions and by taking an extra share of ponderous work during my preoccupation with this study, for which I am deeply grateful to him.

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Nils-Olof Lindgren, V.M.D., for constructive criticism of the manuscripts of this and the preceding publications, as to both subject matter and linguistic form,

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to Mrs. Gun Sätterström and Miss Helga Holz, whose skilful and devoted technical assistance has been of invaluable help to me,

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Mr. Ernst Nilsson and Mr. Knut Hultkvist aided in carefully looking after the experimental animals, and Mr. Inge Ericsson skilfully prepared the photographs. To all of them I extend my hearty thanks.

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Svenska AB Philips, Stockholm, and AB Ewos, Södertälje, generously supplied herbicide formulations and reference grade chemicals.

Stockholm, October, 1966.

Kurt Erne

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Introduction

In the battle against those living organisms ("pests") which may interfere with man's production or storage of food, feed, or other commodities, or with his health or comfort, chemical methods of control are being used to an increasing extent. Some 200 basic chemicals are in current world-wide use as pesticides (or "biocides"), commercially presented in thousands of different formulations and under numerous trade names. According to the intended target organism, these materials may be classified as insecticides, acaricides, fungicides, herbicides, rodenticides, etc.

On a quantitative basis, the herbicides constitutes the major group. Broadly speaking, herbicides may be defined as agents intended for the chemical control of weeds or other unwanted vegetation. Such agents are being used for a multitude of purposes in agriculture, forestry, horticulture, and many other areas of human activity. Typical applications include the control of weeds competing with food or forage crops, or with other useful crops, of low-value arboreal vegetation in forests, of plant vectors for insect, fungal, or viral pests, and of terrestrial or aquatic vegetation interfering with traffic, drainage, or irrigation. At lower dosage, herbicidal agents may be used for regulating physiological processes such as root induction, leaf abscission, fruit setting and maturation, and seed development. Herbicides were used in Sweden during 1965 to a value of more than 25 million Swedish crowns, the total quantity amounting to some 4000 ton of formulated products.

Among modern herbicides, chlorinated phenoxyaliphatic acids (phenoxy herbicides or "hormone weed killers") hold a dominating position, although a variety of other organic compounds also are being widely used, such as halogenated aliphatic and aromatic acids, halo- and nitrophenols, substituted amides, carbamates, ureas, nitrogen heterocycles, and quaternary nitrogen compounds (see e. g. *Crafts* 1961 and *Audus* 1964).

The application of phenoxy acids as herbicides followed the important discovery of *Slade, Templeman & Sexton* in 1940 (reported in 1945) that certain synthetic plant hormone (auxin) analogues were toxic at very low concentrations to certain broad-leaved plants without causing damage to cereals. The subsequent intensive search for other compounds exerting a selective phytotoxic action resulted in the introduction of 2,4-dichloro- and 2-methyl-4-chlorophenoxyacetic acids (commonly abbreviated as

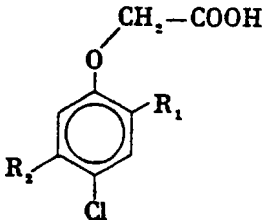
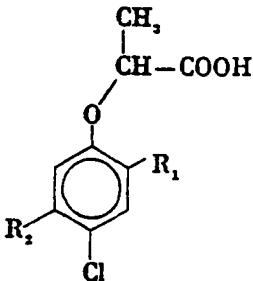
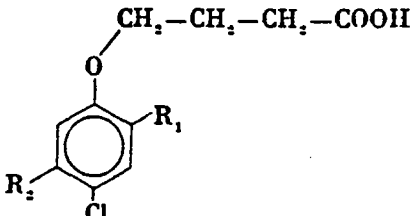
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2,4-D and MCPA, respectively) as highly potent selective herbicides, vastly superior to all other weed killers previously used. Their effect is preferentially directed against dicotyledonous plants, monocotyledons usually being more resistant. Somewhat later, the merits of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), particularly in combatting ligniform vegetation were discovered. In recent years, derivatives of other phenoxyaliphatic acids, such as 2-phenoxypropionic and 4-phenoxybutyric acids have been introduced as highly selective herbicides. The butyric acid analogues derive their activity from a metabolic conversion within the plant to the corresponding phenoxyacetic acids.

The structural formulae and the common names of the phenoxy acids referred to above are listed in Table 1.

Table 1. Phenoxyaliphatic acids of importance as herbicides.

Chemical name	Common name	Structural formula
Phenoxyacetic acids		
2,4-Dichlorophenoxyacetic acid ($R_1 = \text{Cl}, R_2 = \text{H}$)	2,4-D	
2-Methyl-4-chlorophenoxyacetic acid ($R_1 = \text{CH}_3, R_2 = \text{H}$)	MCPA, 4K-2M	
2,4,5-Trichlorophenoxyacetic acid ($R_1 = \text{Cl}, R_2 = \text{Cl}$)	2,4,5-T	
Phenoxypropionic acids		
2-(2,4-Dichlorophenoxy)-propionic acid ($R_1 = \text{Cl}, R_2 = \text{H}$)	2,4-DP, Dichlorprop.	
2-(2-Methyl-4-chlorophenoxy)-propionic acid ($R_1 = \text{CH}_3, R_2 = \text{H}$)	MCPP Mecuprop	
2-(2,4,5-Trichlorophenoxy)-propionic acid ($R_1 = \text{Cl}, R_2 = \text{Cl}$)	2,4,5-TP, Fenoprop, Silvex	
Phenoxybutyric acids		
4-(2,4-Dichlorophenoxy) butyric acid ($R_1 = \text{Cl}, R_2 = \text{H}$)	2,4-DB	
4-(2-Methyl-4-chlorophenoxy)-butyric acid ($R_1 = \text{CH}_3, R_2 = \text{H}$)	2,4,5-TB	
4-(2,4,5-Trichlorophenoxy)-butyric acid ($R_1 = \text{Cl}, R_2 = \text{Cl}$)	MCPB	

The marketed herbicide preparations contain either water-soluble amine or alkali salts, or oil-soluble esters, of the various phenoxy acids.

After being applied to the plant, the phenoxy herbicides may be taken up from the foliage and translocated within the plant, thus exerting a systemic action. Uptake from the roots is also possible. Within the plant the phenoxy acids are mainly transported along with the assimilation products to the meristem and young growing tissues and also to the seeds and other storage organs.

The persistence of the phenoxy acids in vegetative tissues is usually not very high, being of the order of a few weeks. In storage organs, however, they may persist for considerable periods of time (*Aamissepp* 1961). It should be remarked that also in plant tissues killed by phenoxy herbicides the chemicals may show a remarkable stability: *e. g.* in the bark and leading shoots of aspen plants exposed to toxic concentrations of 2,4-D, the herbicidal activity remained apparently unaltered for more than a year (report from Dr. L. Eliasson, the Royal College of Forestry, Stockholm).

At low dosage levels the phenoxy herbicides elicit physiological effects similar to those of the natural growth hormones; at higher dosage they induce an excessive, uncontrolled growth, ultimately leading to death of the plant. The effect upon plant metabolism is complex. Metabolic processes shown to be affected include respiration, nitrogen and phosphorus metabolism, and salt uptake, as well as formation of carbohydrates, RNA, and indoleacetic acid (see *e. g.* *Audus* 1964). Some of the physiological effects of phenoxy herbicides seem to be associated with binding of SH-groups. Despite intensive research efforts, however, the specific biochemical mechanism underlying their action has not been definitely established.

The toxicity of herbicidal phenoxy compounds to terrestrial animals appears to be moderate. Published acute oral LD₅₀ values for 2,4-D and 2,4,5-T range between approximately 300 and 1000 mg/kg body weight in several laboratory animals; the acute toxicity in cattle and sheep apparently is of the same order. Dogs seem to be more susceptible, the acute oral LD₅₀ being about 100 mg/kg. The results of feeding experiments with various domestic animals suggest that acute or subacute poisoning is unlikely to occur after ingesting crops sprayed with phenoxy herbicides at a recommended rate (see reviews by *Rowe & Hymas* 1954 and by *Dalgaard-Mikkelsen & Poulsen* 1962). In contrast, the piscicidal activity of phenoxy herbicides may be appreciable. With 2,4-D, a water concentration of 10 p.p.m. has proved fatal for various species of fish, on 24 hours' exposure, and with 2,4,5-T ester, lethal effects have been observed at levels as low as 1—2 p.p.m. (*Bauer* 1961; *Andersson & Berzins* 1961; *Davis & Hughes* 1963).

In assessing the possible hazards involved in the use of phenoxy herbicides, attention should also be paid to indirect hazards. For instance, interference of the herbicides with plant metabolism may lead to an accumulation in the plant of endogenous toxic metabolites, such as nitrate and cyanide, to levels as high as to endanger the health of animals feeding on the treated herbage (Stahler & Whitehead 1950; Swanson & Shaw 1954; Frank & Grigsby 1957).

In the last few years, several reports of fatal phenoxy herbicide poisoning in human subjects have been published, some of the cases being due to accidental exposure (Goldstein, Jones & Brown 1959; Monarca & Di Vito 1961; Todd 1962; Berkley & Magee 1963), and others apparently to intentional ingestion, in suicidal purpose, of undiluted commercial preparations (Curry 1962; Herbach & Machata 1963; Popham & Davies 1964; Nielsen, Kaempe & Jensen-Holm 1965; Johnson & Koumides 1965; Geldmacher-v. Mallinckrodt & Lautenbach 1966).

Cases of accidental, at times fatal, phenoxy herbicide poisoning in cattle have been investigated by, or come to the knowledge of, the National Veterinary Institute at several occasions during recent years. According to available information, the affected animals mostly have had access to toxic amounts of the herbicide, *e. g.* in the form of spraying solutions or formulated products. Unintentional contamination with herbicides of water or feed supplies has also been observed repeatedly, sometimes associated with toxic effects in animals. In addition, there have been rather frequent reports to the Institute of cases of damage to domestic animals and wildlife suspected of being caused by acute or chronic phenoxy herbicide poisoning. Although in certain of these cases phenoxy acid residues, indicative of exposure, have been detected in the tissues, the aetiological role of the herbicides has not often been convincingly established.

Undoubtedly, the wide-spread use of phenoxy herbicides provides ample possibilities for the direct or indirect exposure of human beings and animals to these materials, and this, together with the growing concern in wide circles for environmental hazards, has accentuated the need for monitoring our environment for herbicide residues, and for a better understanding of the physiological significance of prolonged exposure to these residues.

The purpose of the investigations under review was:

to develop methods for isolating, detecting and quantitatively measuring small amounts of phenoxy herbicides in various biological materials, suitable for diagnosing cases of poisoning and for residue analysis,

to study the tissue distribution, elimination and—to some extent—the metabolism of these compounds in experimental animals, and

to study the toxicological effects of phenoxy herbicides in animal feeding experiments.

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Development of the analytical method

A prerequisite for the successful analysis of a given compound in a complex mixture is its quantitative isolation from the bulk of the sample material, and its separation from soluble sample constituents which might interfere in the subsequent detection and determination of the compound. In toxicological and pesticide residue analysis, the isolation and separation may involve considerable difficulties because of the complexity of the sample material, and the usually small amount present of the compound for which one is searching. A possible biotransformation or postmortal degradation of the compound may also contribute to the analytical difficulties. Consequently, procedures for toxicological and pesticide residue analysis must meet high demands for separating efficiency, as well as for selectivity and sensitivity.

Studies on the isolation of phenoxy acids from various biological materials, and their separation from extractives were reported in papers I and II.

EXTRACTION OF THE SAMPLE

Animal tissues were minced and acidified with sulphuric acid and then subjected to alternative extraction techniques, including extraction by shaking of a sample—sodium sulphate mixture, Soxhlet extraction of a sample—sodium sulphate, or sample—celite mixture, direct homogenizing of sample with organic solvent in a mixer, and homogenizing with aqueous buffer. Of these techniques, homogenizing with organic solvent showed most promise, being effective, rapid, and convenient. Applying this technique to tissues with and without added phenoxy acid, solvents of varying polarity were screened for selectivity and extracting efficiency as revealed by thin-layer chromatography, emulsification-tendency and photometric determination of the extracts. The results obtained indicated 2-propanol to be the solvent of choice for animal tissues. The course of the extraction was followed by homogenizing liver tissue, fortified with 2,4-D, with successive portions of fresh propanol and determining the amount of phenoxy acid extracted in each fraction. As seen from paper II, Fig. 1 (lower curve), the cumulative extraction curve at first rose but then levelled off completely

after the second extraction, the plateau corresponding to an overall recovery of approximately 95 per cent of added 2, 4-D (if corrected for losses in the post-extraction steps). From the upper curve of the figure is seen that 2, 4-D, when incorporated in tissues under physiological conditions, may be equally effectively extracted.

With other types of sample material, conditions for quantitative and selective extraction were elaborated in an analogous way. The homogenizing technique using 2-propanol as a solvent, proved eminently satisfactory for extracting most animal tissues, ingesta, faeces, and a variety of vegetable materials. From vegetable materials of a low moisture content and from other dried-in sample materials the phenoxy acids may be less easily extractable, unless the sample is rehydrated before extraction. A similar experience has been reported by *Bevenue, Zweig & Nash (1962)*. Most likely, the added water serves, at least in part, as a desorptive agent, since the effect is most striking with slightly polar solvents.

Also for soil the rehydration proved helpful. In this case, extraction is preferably performed by shaking with 2-propanol.

With sample materials rich in lipids, analytical difficulties may arise when using propanol, because of co-extraction of emulsion-promoting sample constituents. In such instances, aqueous acetone proved more suitable, and was applied successfully to materials as different as brain tissue, eggs, and bees.

Lipid-containing body fluids such as blood and milk were conveniently extracted with acetone.

In order to split possible phenoxy esters, which, if present in the sample, would be extracted along with the acids, an alkaline hydrolysis step was inserted in the analytical procedure. Experiments proved the butyl and propyleneglycolbutylether esters of 2,4-D to be quantitatively hydrolyzed under the experimental conditions. This hydrolysis step was also expected to cleave possible carbohydrate esters and amino acid conjugates of phenoxy acids which might be formed in growing vegetable tissues (see *e. g. Andreue & Good 1957* and *Kläncht 1961*) and are likely to be extracted with aqueous 2-propanol. Extracts of animal tissues would not be expected to contain stable conjugates of chlorinated phenoxy acids, since these acids have been found to be excreted largely unchanged in several animal species (compare *Metabolism*, below). Possible protein complexes of the phenoxy acids, which might be present both in animal and vegetable materials, should not interfere with the extraction of the acids because of the generally reversible nature of such complexes, and of the denaturing action of the solvent and the added mineral acid.

PURIFICATION OF THE EXTRACT

Owing to the relatively high acid strength of the phenoxyacetic acids (the pK_a values being of the order of 3), an extensive separation of these acids from co-extracted neutral or weakly acidic materials should be attainable by partitioning the extract between an organic solvent and an aqueous phase of relatively low pH. This principle has been applied also by *Marquardt & Luce* (1951, 1955).

Accordingly, the distribution of phenoxyacetic, as well as analogous phenoxypropionic and phenoxybutyric acids, in various systems and at various pH values was studied using ultraviolet spectrophotometry. The partition ratio or apparent partition coefficient, D , was calculated as the ratio of total concentration of the acid in the organic phase to total concentration of the acid in the aqueous phase, and then the partition coefficients of the undissociated acids (K_D) were calculated by means of the following equation

$$\log K_D = \log D + \text{pH} + \log [(h) + K_a] \quad (1)$$

In deriving this equation, the association of the acids in the organic phase was neglected. Since the dimerization constants of carboxylic acids in organic solvents such as benzene and chloroform usually are of the order of 10^3 mole⁻¹ (see *e. g.* *Brown & Mathieson* (1954)), it can be shown that dimerization will not appreciably influence the partition coefficient at concentrations below 10^{-4} M. In the present experiments, the overall concentration was in the range 10^{-5} to 10^{-4} M. Therefore, D will be defined by

$$D = \frac{[HA_{org}]}{[HA] + [A]} = \frac{[HA_{org}]}{[HA]} \cdot \frac{1}{1 + \frac{[A]}{[HA]}} \quad (2)$$

where $[HA_{org}]$ denotes the concentration of undissociated acid in the organic phase, $[HA]$ the concentration of undissociated acid in the aqueous phase, and $[A]$ the concentration of ionized acid in the aqueous phase.

After substituting K_D for $\frac{[HA_{org}]}{[HA]}$ and $\frac{K_a}{(h)}$ for $\frac{[A]}{[HA]}$,

where K_a is the apparent dissociation constant of the acid and (h) is the hydrogen ion activity of the aqueous phase, equation (2) can be reduced to

$$D = \frac{K_D \cdot (h)}{(h) + K_a} \quad (3)$$

By transposing into a logarithmic form and rearranging, equation (1) will be obtained which is better suited for calculations than is equation (3).

Table 2. Partition coefficients (logarithmic) of phenoxy acids between organic solvents and aqueous buffers.

Compound	Organic solvent	Log K_D (m $\pm$ s)
2,4-D	Benzene	0.9 $\pm$ 0.06
"	Dichloromethane	1.4 $\pm$ 0.1
"	Chloroform	1.3 $\pm$ 0.1
"	Chloroform—ethyl ether (3:1)	2.1 $\pm$ 0.1
"	Ethyl ether	2.5 $\pm$ 0.1
MCPA	Chloroform	1.5 $\pm$ 0.1
2,4,5-T	Chloroform	2.0 $\pm$ 0.1
MCPP	Chloroform	c. 2.2
MCPB	Chloroform	c. 5

The partition coefficients thus calculated are summarized in Table 2.

Conditions for quantitative extraction of the phenoxy acids into the different organic phases and back into aqueous phase were calculated, and a "clean-up" procedure was developed, which involves extractive transfer of the acids into benzene at pH 2, then into aqueous buffer at pH 6.2, and finally into chloroform at pH 2. In model experiments, this procedure yielded quantitative recoveries with the phenoxyacetic acids and also with phenoxypropionic acids. The 4-phenoxybutyric acids, being weaker acids (pK_a values around 5), would require a higher pH for a quantitative extraction into aqueous phase. Extraction at pH 9 proved useful for isolating these, as well as other, weak acids, *e. g.* the dinitrophenol herbicides. Admittedly, however, the selectivity in the buffer extraction step will be reduced at the higher pH values.

CHROMATOGRAPHIC SEPARATION

With the object of separating the phenoxy acids from extractives passing the partitioning step, and at the same time of obtaining evidence as to their identity, a paper chromatographic system was developed (see paper I). The procedure employs development by the circular technique, *sec*-butanol, saturated with M aqueous ammonia, serving as the solvent. This technique is rapid, and it proved to function excellently with extracts low in extractives such as those from natural waters and many body fluids. The R_F values

of 2,4-D, MCPA and 2,4,5-T fall in the range 0.6—0.7, the R_D values (movement relative to 2,4-D) being 1.00, 0.97 and 1.10, respectively.

More heavily contaminated extracts require a system of higher tolerance against overloading, and therefore a thin-layer chromatographic method was subsequently developed. The system, silica gel G: kieselguhr G (3:2)/ethyl acetate: n-hexane: formic acid (20:80:0.3 by volume), showed a good tolerance for extractives, and proved to effect a clean separation with extracts of most biological materials. Usually, extract aliquots equivalent to 5 g of sample, or more, could be applied without overloading the chromatogram. In rare instances, as with extracts of heavily decomposed animal materials, resolution was unsatisfactory. The separation often could be improved, however, by repeating the development with the same solvent. The R_F values of the phenoxyacetic acids in this system (with a double development) are in the region 0.3—0.4, the R_D values for 2,4-D, MCPA and 2,4,5-T being 1.00, 1.25 and 1.31, respectively (compare paper II, Table 1.)

For detecting the phenoxy acids on the chromatograms, the silver nitrate—phenoxyethanol reagent of *Mitchell* (1958) proved eminently suitable, the chlorinated acids appearing as dark spots against a pale background. The limit of detection with this reagent is about 0.1 μ g of phenoxy acid on thin-layer plates and about 0.3 μ g on paper chromatograms.

A rough estimate of the amount present may be based on measurement of the spot area, but obviously, elution of the spots and quantitative determination by an independent method will be more reliable and, in addition, provide confirmatory evidence as to the identity. The silver reagent cannot be used for locating the spots to be eluted, but experiments proved the fluorescent reagent 4-methylumbelliferone to be suitable for this purpose.

Elution experiments proved the phenoxy acids to be effectively and rapidly extracted from paper, as well as siliceous adsorbents, by ammoniacal methanol.

DETERMINATION

The quantitative determination of the isolated phenoxyacetic acids was studied in experiments reported in paper I. A photometric technique based on the colour reaction with chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulphonic acid) in sulphuric acid, first described by *Freed* (1948), was developed.

The critical factors of the reaction were assessed by systematically varying the experimental conditions (temperature and time of reaction,

and composition of reagent). From the results, as presented in paper I, Figs. 2—5, may be concluded that the colour development will be completed within 15—20 minutes, if the sample is heated in an air oven at 140°C with a reagent of a water content below 5 per cent w/w. At a chromotropic acid concentration of 0.4 per cent w/w, Beer's law will be valid for concentrations up to at least 0.5 μ mol of phenoxyacetic acid (approximately 110 μ g of 2,4-D) per 5 ml of reagent (paper I, Table 1).

The absorption spectrum of the coloured product (identical for all the phenoxyacetic acids) has maxima at 480 and 580 m μ and a minimum at 504 m μ . The 580-m μ maximum was selected for photometric measurement.

It was found that some irregularities in the results could be attributed to an uncontrollable degradation of the reagent during heating, giving rise to a variable background absorption. In order to minimize this error, the measurement was supplemented with a geometric three-point correction, which proved to enhance the precision of the procedure considerably. The corrected molar absorptivities at 580 m μ of 2,4-D, MCPA, and 2,4,5-T were all near to $1.3 \cdot 10^4$ litre mole⁻¹ cm⁻¹.

ACCURACY AND PRECISION

The reliability of the method was checked in recovery experiments. Thus, after adding 2,4-D in concentrations down to 0.15 p.p.m. to natural waters, recoveries of 84—102 per cent of the added amount were obtained with the paper chromatographic method (paper I, Table 2). Similarly, 2,4-D, MCPA, and 2,4,5-T, when added to a variety of biological materials at levels of 4 p.p.m., or above, were recovered to approximately 70—90 per cent, using the thin-layer chromatographic version of the method (paper II, Tables 3 and 4). In both sets of experiments the standard deviation was of the order of 4—5 per cent and the coefficient of variation approximately 5—7 per cent.

SENSITIVITY

The sensitivity of the silver reaction, when applied in paper chromatography, is about 0.3 μ g of chlorophenoxy acid, whereas on thin-layer plates amounts as small as 0.05—0.1 μ g can be detected. Hence, using thin-layer chromatography and applying the equivalent of 3 g of sample, concentrations

of 0.1 p.p.m. in the sample should be detectable. With extracts low in extraneous matter the limit of detection can be suppressed by increasing the aliquots taken to chromatography. Thus, in natural waters, concentrations as low as 0.0002 p.p.m. may be detected.

The lower limit of determination of the method should be defined in terms of probability of differentiating a residue from a random variation of the sample blank value. The blanks obtained, when control samples supposedly free of phenoxy acids were carried through the full procedure, correspond to 0.2 μ g of apparent 2,4-D (range 0—0.5), or a concentration of 0.07 p.p.m. (range 0—0.17) (compare paper II, Table 3). Therefore, the practical lower limit of determination was set at 1 μ g of phenoxyacetic acid, or 0.3 p.p.m. (calculated on a 3-g sample aliquot).

SELECTIVITY

Particular care was devoted to checking the selectivity of the individual steps of the analytical procedure (see paper I).

In a comprehensive study of the scope of the chromotropic acid reaction, it was found, in corroboration of earlier results (*Le Tourneau & Krog* 1952), that the reaction is restricted to compounds capable of splitting off a one-carbon fragment equivalent to formaldehyde, under the experimental conditions. Further, the violet-red chromogen proved to be identical in spectral characteristics in each case and to be formed in stoichiometric proportions. As appears from paper I, Table 4, positively reacting compounds include, in addition to phenoxyacetic acid derivatives, glycolic acid, 3-indoleacetic acid and complexones. Only acidic compounds have been considered here, since others will not be present in the purified sample extract. Faint red colours were obtained also with alloxan, tryptophan, and dialuric, hippuric and uric acids, probably due to impurities in the test compounds. Interestingly, several potentially reactive compounds, such as the haloacetic acids, oxalic acid, and glycine, did not react under the conditions of test. In contrast, precursors of a few other aldehydes were found to give coloured products, although the absorption maxima of the products were definitely displaced, when compared with the formaldehyde chromogen, and the colour intensity was mostly low.

Experiments further proved that all of the chromotropic acid-reactive compounds were effectively separated from the chlorophenoxyacetic group in the paper chromatographic step—if not removed at an earlier stage in the analytical procedure. Complementary chromatographic studies with a

wide variety of organic acidic compounds which might be present in presumptive sample materials (or processed extracts thereof), also showed that only a few had R_f values close to those of the phenoxyacetic acids (see Paper I, Table 3.). Of these, only halogen-containing compounds reacted with the silver reagent to form the characteristic, dark-coloured spots. None of the compounds resembling phenoxyacetic acids in both chromatographic mobility and silver reactivity proved to react with chromotropic acid.

The selectivity of the thin-layer chromatographic system, when tested in a similar way, proved to be excellent.

From the experiments reviewed may be concluded that an analytical procedure involving a partition "clean up" of the extract, as described above, in conjunction with a paper or thin-layer chromatographic separation, and a photometric determination, will afford an adequate degree of selectivity.

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Distribution and elimination

Studies of the distribution of phenoxyacetic acids in, and their elimination from, the animal organism are reported in paper III.

PLASMA LEVELS

After giving 2,4-D amine as single oral doses to calves, pigs, rats, and chickens, blood samples were withdrawn at intervals, and plasma was analyzed for 2,4-D using the chromatographic—photometric method described in the foregoing section. Some of the animals were sacrificed at intervals and tissues analyzed (see below). Plasma concentration was plotted against time to give curves of the type shown in Fig. 1 A (compare paper III. Figs 1—4).

The initial steep rise, apparent on all curves, indicates the phenoxy acid to be fairly rapidly absorbed from the alimentary canals of the species studied. Maximum plasma concentrations, ranging between 100 and 250 $\mu\text{g}/\text{ml}$ for a dose of 100 mg/kg, were usually attained within 2—7 hours after dosing. The declining portion of the curves proved to be exponential; when plotted on a semi-logarithmic scale the graphs approximate to linearity, as is shown in Fig. 1 B, thus indicating a first-order rate of disappearance of 2,4-D from plasma. From the slope of the semi-logarithmic plots the biological half-lives of 2,4-D in plasma were calculated (Table 3).

As seen, the half-lives differ among species, the lowest values being found in rats (about 3 hours for 2,4-D amine) and the highest in pigs (about 12 hours), calves and chickens taking intermediate positions.

Similar experiments were performed with an alkali salt of 2,4-D and with 2,4,5-T amine and 2,4-D butyl ester. The similarity of the plasma concentration—time curves obtained with the different 2,4-D salts indicates, as would be expected, that the cation of the salts does not appreciably influence the absorption, distribution, or elimination of the phenoxy acid. Further, a comparison of the 2,4-D and 2,4,5-T data does not suggest the physiological properties of 2,4-D to be fundamentally altered by introduction of another chlorine atom in the molecule. The 2,4-D ester differed strikingly from the other compounds tested in giving only low and variable plasma

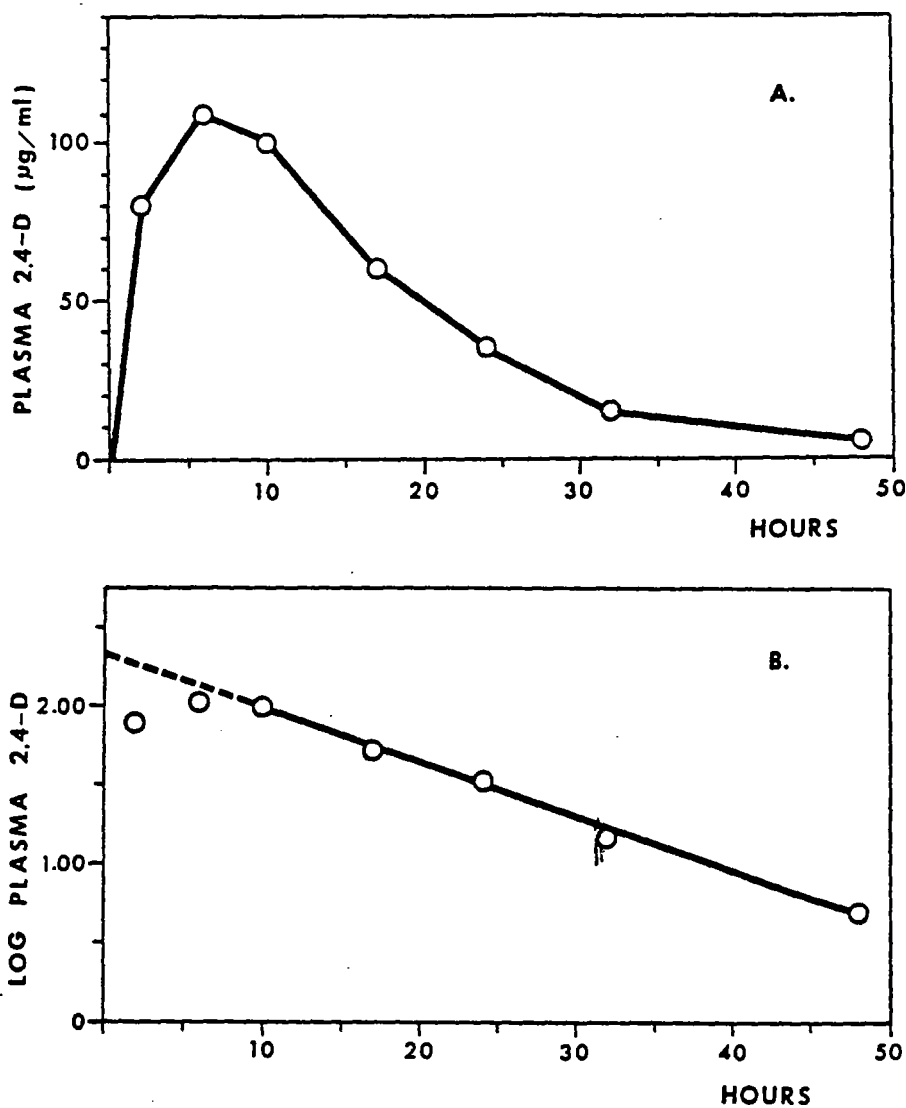


Fig. 1. A. Plasma levels of 2,4-D in a pig given 2,4-D antine (50 mg/kg b. w.) as a single oral dose. B. Semi-logarithmic plot of the same curve.

levels of 2,4-D, the ester not being detectable in plasma at any time after dosing. The results may be attributed to a low absorption or hydrolysis rate of the ester (see Metabolism, below).

As shown by the data given for pigs and calves in Table 3, the plasma

to zero time. From the extrapolated "zero time plasma levels" obtained, the apparent distribution volume, V_D , expressed in per cent of the body weight, was calculated by means of the formula

$$V_D = \frac{A \cdot 100}{c_0} \quad (4)$$

where A is the dose administered, in mg/kg body weight, and c_0 is the "zero time plasma concentration", in $\mu\text{g/ml}$. These calculations are based on the assumptions that the oral dose given is completely absorbed, which does not seem unreasonable in view of the relatively small residues found in faeces of orally dosed pigs, and that diffusion equilibrium will be attained relatively rapidly, which seems to be borne out by the comparatively small differences between plasma and tissue half-lives. The V_D values thus calculated, were found to range between 35 and 60 per cent of the body weight, depending on the species. However approximate, these values clearly exceed the extracellular fluid volume (ranging between 15 and 25 per cent of the body weight for most species), and therefore suggest some intracellular distribution of the phenoxy acids.

More direct evidence of the phenoxy acids penetrating cellular membranes was obtained from analysis of blood cells. In model experiments 2,4-D was added to whole blood, and the 2,4-D levels in blood cells and plasma determined separately on aliquots withdrawn after various times. The blood cells were washed with saline before analysis, but only once and for a few minutes, in order to avoid excessive leaching of possible intracellular phenoxy acid. Blood cells and plasma obtained from experimental animals receiving 2,4-D were analyzed in the same way. Invariably a substantial fraction of the total 2,4-D, usually 10—30 per cent, appeared in the blood cells (cf. paper III, Tables 3 and 4). Analysis of whole blood gave concordant results.

TISSUE LEVELS

Pigs, rats, and chickens were given single oral doses of 2,4-D amine, and the animals were sacrificed at intervals and specimens of tissues taken to analysis for 2,4-D. (Dead and killed animals were examined histopathologically; see Toxicity, below.)

Representative results obtained with a pig are shown graphically in Fig. 2.

Table J. Plasma half-life values of phenoxyacetic acids in different animal species after a single oral dose.

Species	Material	Dose mg/kg b.w.	Plasma half-life, hours ($m \pm s$)
Calf	2,4-D amine	50	7 $\pm$ 1.5
"	" "	100	8.5 $\pm$ 1
"	" "	200	7 $\pm$ 1
"	" K-Na salt	100	8 $\pm$ 1
"	" ester	100	10 $\pm$ 1
Pig	2,4-D amine	50	10 $\pm$ 1
"	" "	100	12 $\pm$ 1.5
"	" ester	100	11 $\pm$ 2
Rat, female	2,4-D amine	100	3.3 $\pm$ 0.5
" male	" "	100	2.9 $\pm$ 0.5
" "	" K-Na salt	100	3.5 $\pm$ 0.5
" "	" ester	100	6 $\pm$ 2
" "	2,4,5-T amine	100	3.0 $\pm$ 0.5
Chicken	2,4-D amine	100	7 $\pm$ 1

disappearance rate of 2,4-D does not seem to be significantly influenced by dosage, a result which is reconcilable with the feeble binding of the phenoxy acid to plasma proteins demonstrated in paper IV (see Metabolism, below).

In experiments with repeated administration of 2,4-D, the plasma disappearance rate was seen to increase gradually in some of the animals. In a typical case a pig was given 2,4-D amine orally as daily doses of 50 mg/kg. No clinical effects were seen, but the plasma level of 2,4-D (at 24 hours after dosing) was found to decrease during the first 8 days from 50 to 8 μ g/ml, and then to remain at the lower level. Since a concomitant increase in urinary output of 2,4-D was noted, and there was no indication of a metabolic transformation being involved, the finding most likely reflects an enhanced renal excretion.

Conversely, a reduced disappearance rate was usually encountered after doses sufficiently high to produce toxic symptoms, an effect which probably was due to an impaired excretion. Conceivably, also a delayed absorption might have influenced the result.

An attempt was made to estimate the apparent distribution volume of phenoxy acids from plasma levels. The terminal linear portion of the log plasma concentration—time plots (Fig. 1 B) was extrapolated backwards

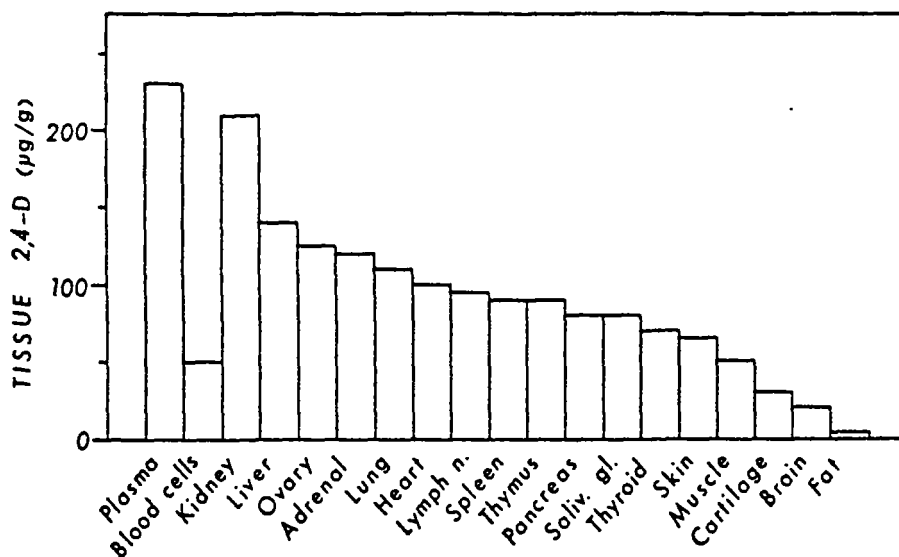


Fig. 2. Tissue levels of 2,4-D in a pig (at 6 hours after dosing) given 2,4-D amine (100 mg/kg) as a single oral dose.

As seen, a rapid and high uptake of 2,4-D occurred in the excretory organs, the liver and kidney levels relative to plasma (at 6 hours after dosing) amounting to 70 and 85 per cent, respectively. Rather high levels (60—70 per cent) were also rapidly attained in the lungs, adrenals, myocardium, and ovaries. Somewhat lower, yet comparatively high, levels (30—50 per cent) were equally rapidly attained in a variety of tissues, including the lymph nodes, spleen, thymus, thyroid, pancreas, and salivary glands. Apparently, the phenoxy acid distributed itself fairly evenly over the body water. The relative concentrations found in skin, skeletal muscle and cartilage were moderate (20—30 per cent), and those in adipose tissue and brain low (5—10 per cent). The low brain levels observed suggest a limited rate of transfer across the blood-brain barrier. This would not be unexpected in view of the currently accepted lipid-partition mechanism of drug transfer across body membranes (see *e. g.* Schanker 1962). Because of the extensive ionization of phenoxyacetic acids at plasma pH, only a minute fraction (less than 1/10,000) of the acids will be present in the undissociated form, the concentration of which determines the rate of diffusion across the lipid membrane. It should be noted, however, that in cases of acute or subacute poisoning, rather high concentrations, in an absolute sense, may be attained in the brain (up to 65 µg/g), as evidenced by the results summarized in paper III, Table 9. Also in cases of

fatal phenoxy herbicide poisoning in human subjects, moderate to high phenoxy acid levels in central nervous tissue have been reported (*Herbich & Machata 1963; Nielsen et al. 1965; Geldmacher—v. Mullinckrodt & Lautenbach 1966*).

As judged from the rapid appearance and the high concentration of 2,4-D in the urine and kidneys of pigs, renal excretion seems to be a major route of elimination of 2,4-D in this species. The acid apparently is excreted in an unchanged form (see Metabolism, below). Both phenomena may be attributed to the low lipid membrane permeability of the phenoxy acid, at normal physiological pH. This would be expected to impede not only the penetration into the microsomal system of liver cells responsible for detoxication processes, but also the renal tubular reabsorption of the glomerularly filtered phenoxy acids (*cf. Brodie, Cosmides & Rall 1965*). Moreover, a tubular secretion of phenoxy acids does not seem unlikely, in view of the demonstration in various animal species of a transport mechanism of low specificity (the hippurate transport mechanism) capable of secreting a variety of acidic compounds, including aromatic and arylsubstituted carboxylic acids (see review by *Sperber 1959*).

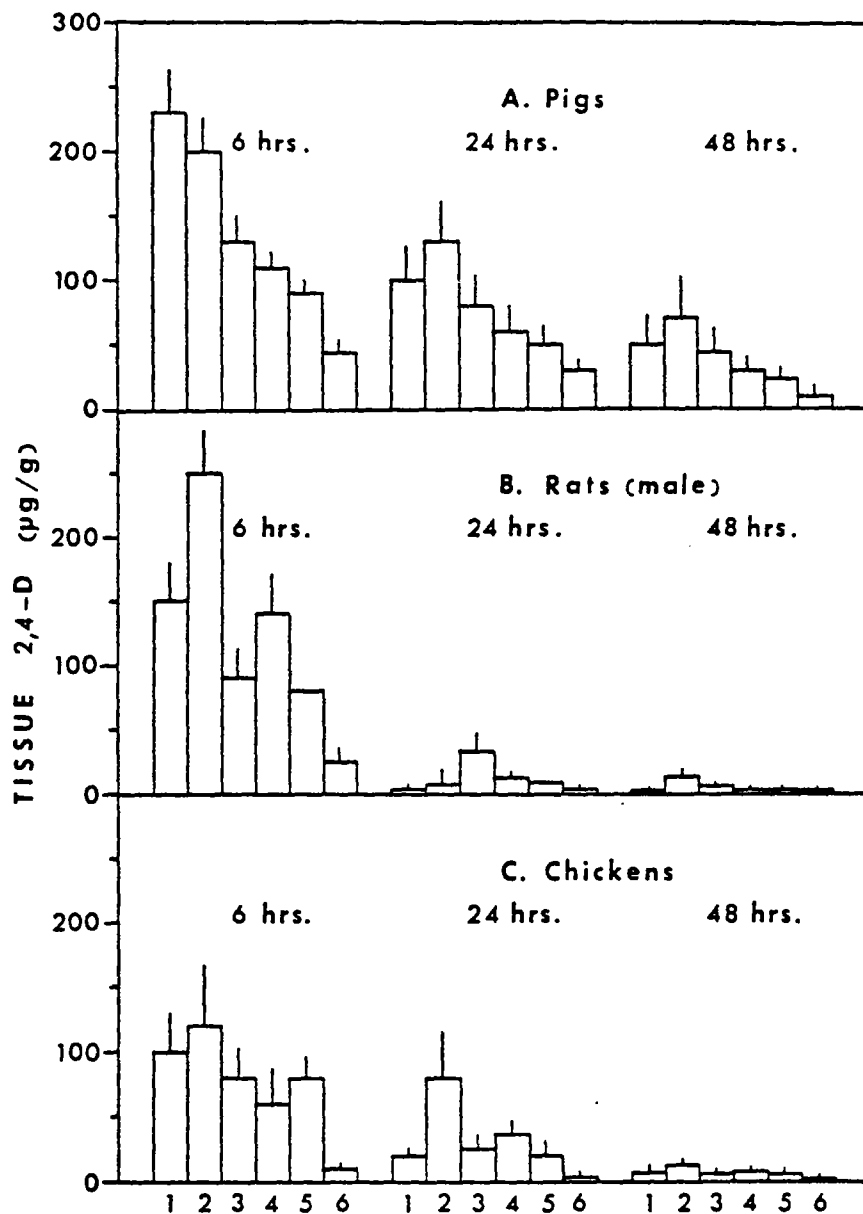
The observed biliary excretion of 2,4-D lends some support to the postulated tubular secretion of phenoxyacetic acids, since tubular and biliary secretion of many organic anions seem to be governed by principally similar mechanisms (*Sperber 1959*).

The distribution of 2,4-D in representative tissues of pigs, rats, and chickens after a single oral dose is illustrated in Figs. 3 A, B, and C. The diagrams are based largely on results presented in paper II, Tables 5—7, but data obtained subsequently have also been included.

As seen, the relative kidney levels were higher in rats and chickens. This result, which is consistent with the high plasma disappearance rate observed in these species, may be an indication of an effective renal excretion. Otherwise, no striking species differences in distribution pattern were apparent.

The rate of disappearance of 2,4-D from tissues after being given in subtoxic doses was fairly high in all species examined, the peak levels always being attained in less than 24 hours, and the levels thereafter usually decreasing to a few $\mu\text{g/g}$ within 2—4 days. The tissue half-lives ranged between 5 and 10 hours in rats and between 10 and 30 hours in pigs and chickens.

In order to study the effects of prolonged exposure to phenoxy acids, 2,4-D amine was administered in the diet to pigs and in the drinking water to rats and chickens, and the animals sacrificed after varying times of exposure (see Toxicity, below). Plasma and tissues were analyzed for 2,4-D (Fig. 4).



Figs. 3 A, B and C. Average tissue levels of 2,4-D, at different times after dosing, in different species given a single oral dose of 2,4-D amine (100 mg/kg). 1: plasma, 2: kidney, 3: liver, 4: lung, 5: spleen, 6: skeletal muscle. Vertical bars denote standard deviation.

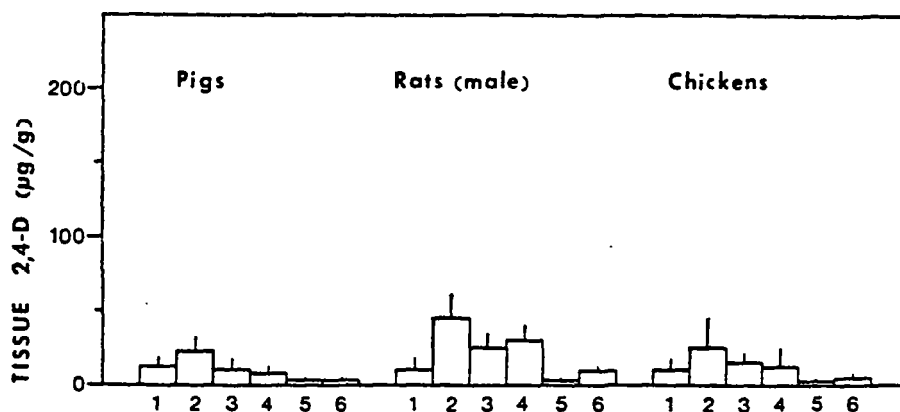


Fig. 4. Average tissue levels of 2,4-D in different species after repeated oral administration of 2,4-D amine. 1: plasma, 2: kidney, 3: liver, 4: lung, 5: brain, 6: skeletal muscle. Vertical bars denote standard deviation.

A comparison of Fig. 4 with Figs. 3 A, B, and C does not reveal any marked change in distribution pattern of 2,4-D with repeated dosage, except for some elevation of the tissue levels relative to plasma in the latter case, reflecting the slightly lower elimination rate of phenoxy acid from tissues. Absolute tissue levels, however, were not seen to increase during these experiments. Therefore, prolonged ingestion of phenoxy acids, at the dose levels tested (corresponding to daily intakes between 25 and 100 mg/kg body weight), would not be expected to involve any hazard of accumulation in the species studied.

It can be noted that laying hens evidently are able to excrete some 2,4-D with the eggs. Residues of up to 2 µg/g were found in the yolk, the albumen containing only traces below 0.4 µg/g.

In another long-term study, a sow was given 2,4-D amine during the gestation period (see Toxicity, below). On parturition fifteen piglets were born, ten out of which died within 24 hours. These piglets did not ingest 2,4-D-containing milk or feed before death. On analysis of their tissues, appreciable concentrations of 2,4-D were found, ranging from 80 to 300 per cent, relative to plasma of the dam, which seems to indicate a fairly unhindered passage of the phenoxy acid across the placental barrier of swine. Considering the apparently restricted penetration into the brain and the observed urinary and biliary excretion of phenoxyacetic acids, suggestive of a limited membrane permeability at plasma pH, a low rate of placental transfer would be expected.

Metabolism

As reported in paper IV, some experiments were performed in order to study the possible biotransformation of phenoxyacetic acids in the animal organism.

In preliminary *in vitro*-studies using the gel-filtration technique, the mobility of 2,4-D in Sephadex gel was found to increase in the presence of plasma proteins. The result, which must be interpreted as a definite, though weak, interaction of 2,4-D with the proteins, is in line with the data obtained by *Teresi & Luck (1948)*, in dialysis experiments with unsubstituted phenoxyacetic acid and serum albumin. Several findings reported in the previous section of this review, such as the moderate plasma levels of 2,4-D attained in various species after oral dosing (suggesting a large distribution volume) and the high plasma disappearance rate of 2,4-D, also point to a slight plasma protein-binding of 2,4-D. If the plasma protein-binding were extensive, high and persistent plasma levels of 2,4-D would be anticipated. Further, the plasma disappearance rate would be expected to increase at high plasma levels, owing to the protein binding capacity being exceeded. This would be accompanied by a marked increase in unbound phenoxy acid in plasma, and thence by an enhanced rate of diffusion from plasma. From the results of *Teresi & Luck*, the concentration of unsubstituted phenoxyacetic acid corresponding to saturation of plasma albumin can be estimated to approximately 3 mM (or about 450 μ g/ml). For the more bulky chlorinated derivatives, lower values would be expected.

As judged from differential hydrolysis studies, urinary conjugation 2,4-D in pigs was not extensive. Acid hydrolysis of thin-layer chromatographically separated fractions of urine extracts was found to release 2,4-D-like material in amounts not exceeding 10 per cent of total phenoxy acid. Likewise, biliary 2,4-D was shown to be essentially unconjugated.

The failure to detect sizable amounts of conjugates or other metabolites of 2,4-D by the author, as well as by other workers (*Lisk et al. 1963; Bache et al. 1964; St. John et al. 1964; Clark et al. 1964*), is consistent with other findings, mentioned above and suggesting a low membrane permeability of phenoxyacetic acids at plasma pH.

Oral administration of a 2,4-D ester to various animal species resulted in only trace amounts of ester appearing in plasma, tissues, or urine. In contrast,

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2,4-D acid was present in body fluids and tissues, although at relatively low levels. It may be concluded that the ester is either slowly absorbed and rapidly hydrolyzed, or slowly hydrolyzed, and the liberated 2,4-D acid rapidly absorbed. The alternative possibility that the ester is rapidly absorbed and then slowly hydrolyzed seems remote, since in that case intact ester should be detectable in plasma.

LOW

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Toxicity

The acute, subacute, and chronic toxicities of phenoxyacetic acids were studied in animal experiments reported in paper V.

ACUTE TOXICITY

In acute toxicity experiments carried out in connection with the distribution studies, phenoxyacetic acid derivatives were given orally by stomach tube to calves, pigs, rats, and chickens (Table 4). Blood samples were withdrawn at intervals for phenoxy acid determination.

As seen, definite, although reversible, toxic effects from 2,4-D were observed in calves at a single dose of 200 mg/kg body weight. Main symptoms of poisoning were anorexia, tympanites, muscular incoordination and dysphagia.

In pigs, adverse effects appeared at a single dose of 100 mg 2,4-D/kg; the clinical signs included anorexia, diarrhoea, a stilted gait and, in severe cases, vomiting, marked muscular weakness and general depression. On autopsy, signs of gastro-intestinal irritation and pulmonary lesions were the main findings.

Rats and chickens apparently tolerated 2,4-D as single oral doses of up to 100 and 300 mg/kg, respectively, without clinical and morphological effects.

In accordance with results of other workers (*Hill & Carlisle 1947; Rowe & Hymas 1954*), no difference in toxicity between different salt formulations of 2,4-D was observed. Likewise, the effects of 2,4,5-T, as an amine salt, apparently were similar, both qualitatively and quantitatively, to those of 2,4-D. An ester formulation of 2,4-D, however, proved to be less acutely toxic by the oral route than the water-soluble salts, probably owing to a low rate of absorption or hydrolysis.

SUBACUTE TOXICITY

Repeated daily oral doses of 2,4-D amine or ester were given to pigs and chickens, the dosage schedule and main results being summarized in Table 5. Dead, as well as surviving, animals were autopsied and tissues

Table 4. Acute toxicity of phenoxyacetic acid derivatives.

Species	Material	Number treated	Dosage mg/kg	Clinical effects	Autopsy findings
Calf	2,4-D amine	2	50	Transient dysphagia in one animal	—
"	" "	2	100	Anorexia; bloating in one animal	—
"	" "	2	200	Anorexia, thirst, muscular weakness, subsiding in 3—4 days	—
"	" K-Na salt	2	100	Transient dysphagia in one animal	—
"	" butyl ester	2	100	Unaffected	—
Pig	2,4-D amine	2	50	Anorexia in one animal	Gastro-enteritis
"	" "	2	100	Anorexia, stilted gait, transient depression	" "
"	" "	2	500	Vomiting, severe muscular weakness, coma ^{a)}	Gastro-enteritis, renal and hepatic congestion
"	" "	2	1000	Vomiting, severe muscular weakness, coma ^{a)}	Gastro-enteritis, renal and hepatic congestion
"	" butyl ester	2	100	Unaffected	Slight gastro-enteritis
"	2,4,5-T amine	2	100	Anorexia, vomiting, diarrhoea locomotory disturbances	Gastro-enteritis
Rat	2,4-D amine	35	100	Unaffected	No significant gross changes
"	" K-Na salt	10	100	"	" " " "
"	" butyl ester	14	100	"	" " " "
"	2,4,5-T amine	10	100	"	" " " "
Chicken	2,4-D amine	3	100	"	" " " "
"	" "	6	200	"	" " " "
"	" "	2	200	"	Proventriculitis in one bird

a) Killed in a moribund state at 24 hours after dosing.

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Table 5. Subacute toxicity of 2,4-D amine and ester.

Species	Material	Dosage mg/kg/day	Number of doses given	Clinical effects	Autopsy findings
Pig	2,4-D amine	50	3	Unaffected	No significant gross changes
"	" "	50	8	Anorexia, diarrhoea, general depression	Gastric ulcer, catarrhal enteritis
"	" "	50	10	Unaffected	No significant gross changes
"	" "	50	15	"	Bronchial lymph node hyperplasia, renal fatty degeneration
"	" "	50	51	Anorexia, retarded growth	Catarrhal enteritis
"	" "	100	3	Vomiting, general depression	Gastric ulcer, catarrhal colitis
"	" "	100	7	Vomiting, diarrhoea, muscular weakness	Gastric ulcer, catarrhal enteritis, pneumonia, renal fatty degeneration
"	" "	300	2	General depression	Renal fatty degeneration
"	" ester	50	5	Unaffected	No significant gross changes
"	" "	50	7	Anorexia, diarrhoea, muscular weakness	Catarrhal gastro-enteritis, pneumonia
"	" "	50	12	Unaffected	No significant gross changes
"	" "	50	23	Muscular weakness	Bronchial lymph node hyperplasia, kidney enlargement
"	" "	300	3	Anorexia, diarrhoea, muscular weakness, general depression	Gastric ulcer, catarrhal enteritis, pneumonia
Chicken	" amine	300	5	Dead on fifth day	Renal and visceral gout
"	" "	300	12	Unaffected	Slight kidney enlargement
"	" "	300	24	"	" " "

examined microscopically. 2,4-D levels were determined in plasma and representative tissues (paper III).

As will be seen, both 2,4-D amine and 2,4-D ester evoked toxic effects in pigs at the low dosage level of 50 mg/kg/day. The lesions were observed mainly in the digestive, respiratory, and excretory organs. It may be noted that the gastric ulcerations were consistently localized in the fundus rather than the cardiac region of the stomach, thus differing from the lesions seen in spontaneous cases of gastric ulcer in pigs. In instances of macroscopic pneumonia, the histopathologic picture was dominated by alveolar cell proliferation. The renal fatty degeneration involved varying tubular sections and Henle's loop.

The chickens seemingly tolerated 300 mg/kg/day of 2,4-D amine without clinical effects or gross morphological changes.

The results of these studies seem to suggest a correlation between the appearance of clinical symptoms and the plasma level of phenoxy acid. Thus, in pigs, plasma 2,4-D apparently has to exceed a threshold value of about 200—300 μ g/ml for the signs of acute poisoning to appear. There seems to be a similar threshold level in calves.

CHRONIC TOXICITY

The effect of prolonged exposure to orally administered 2,4-D was investigated in pigs, rats, and chickens.

Pigs. A group of five 2 months-old pigs (litter-mates) was maintained on an experimental diet containing 2,4-D amine at a level of 500 p.p.m. for up to 12 months, two other litter mates serving as controls. This dose level was calculated to correspond to a daily intake of about 25—50 mg/kg body weight. In all animals depression of the growth rate, not associated with a reduced feed intake, was evident. Locomotory disturbances were common, and two animals exhibited a unique elongation of the anterior lateral hoofs. Other significant clinical effects were not noted. Haematological and biochemical examination revealed a moderate hyperchromic anaemia, slightly elevated plasma GOT, slight hypoalbuminaemia and moderate albuminuria. The animals were killed at intervals between 2 and 12 months. No characteristic gross changes were observed, but microscopically, parenchymatous degeneration of the liver and slight degenerative changes in the kidneys were detected.

The reduced feed efficiency and the clinical-chemical and histopathological evidence point to a mainly hepatotoxic action of the phenoxy acid in