AN ABSTRACT OF THE THESIS OF

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Title The Effect of 2,4-Dichlorophenoxyacetic Acid on the In Vivo

Metabolism of Acetate in Rats

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Normal adult rats absorbed and metabolized C^{14}-labeled acetate

rapidly. The greatest percentage of the administered radioactivity

was recovered in the expired CO_2, however small amounts of radio-

activity were also found in the urine and feces. There were two rates

of $C^{14}O_2$ elimination in normal adult rats. The small amount of

radioactivity recovered in the urine was found to consist of several

different metabolites; their relative abundance was dependent upon

the sex of the rat and the C^{14}-labeled acetate used.

Orally administered 2,4-D was found to effect the secondary

rate of $C^{14}O_2$ elimination, and to reduce the percentage of radio-

activity recovered. The presence of 2,4-D altered the pattern of

radioactive metabolites found in the urine. In females, the effect

of 2,4-D was greater on the metabolism of the methyl carbon than on

the carboxyl carbon of acetate.

The presence of 2,4-D was found to have a similar effect on

the rate of $C^{14}O_2$ elimination over a wide range of dosages. At lower

dosages, five milligrams or less per rat, the effect was reduced. The

accumulative recovery of radioactivity was increased as the dosage of 2,4-D was decreased. When the time between 2,4-D treatment and acetate administration was increased, up to 48 hours, a similar effect on the $C^{14}O_2$ elimination rate was observed. However, there was a trend toward greater recovery of the radioactivity. With a pretreatment time of one week, 2,4-D showed no effect on the acetate metabolism.

Large amounts of radioactivity were found in the stomach of rats given 2,4-D and acetate-1-C^{14} orally. From this it was concluded that 2,4-D was affecting the absorption of acetate. This conclusion was further verified when 2,4-D was given orally, and acetate was injected into the peritoneal cavity. In this work, 2,4-D did not alter the $C^{14}O_2$ elimination pattern, although there was a slight reduction in the percentage of the radioactivity recovered.

THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID
ON THE IN VIVO METABOLISM OF ACETATE
IN RATS

by

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Introduction

In the past 23 years that 2,4-D and other chlorinated phenoxy-

acetic acids have been used as herbicides, there has been considerable

investigation, attempting to elucidate the effect of these compounds

on various plant metabolic pathways. Several workers (5,10,17) have

looked at the effect of 2,4-D on glucose metabolism in plants. In

low concentrations, 2,4-D inhibits glucose absorption. It also

inhibits the reactions of glycolysis and the pentose cycle. However,

the herbicidal activity of 2,4-D has not been ascribed to any effect

that it might have on the absorption and metabolism of glucose in

plants. Under the influence of 2,4-D, it has been found that there

are gross changes in the chemical composition of the proteins of

the leaves, stems and roots of red kidney bean plants (25,33). Akers

and Fang (1) have found that the amount of aspartic acid and glutamic

acid is decreased in bean plants treated with 2,4-D. The effect of

2,4-D on acetate metabolism has been studied in bean leaves and stems

(11) and in pea roots (27). Results indicated that 2,4-D increased

acetate absorption and catabolism in bean stems. However, in leaf

tissue, low concentrations of 2,4-D decreased the catabolism while

increasing the synthetic activity, and high concentration appeared to

increase both catabolic and synthetic activities. In pea roots,

2,4-D as well as several non-herbicidal phenoxyacetic acids were found

to inhibit acetate absorption. It was therefore concluded that the

herbicidal activity of 2,4-D was not due to its inhibition of acetate

uptake and subsequent metabolism.

The effects of 2,4-D and its analogues in several metabolic

pathways have been quite extensively studied, but the literature is

devoid of information concerning the effects of these compounds on

the metabolic systems in mammals. To date, very little information

is available on the effect of 2,4-D in mammals. The LD_{50} of 2,4-D

for rats is 300 to 1000 mg/kg body weight (19). Khanna, Rao and Fang

(18) have shown that 78% of an 80 mg dose of 2,4-D is excreted as

2,4-D in the urine of rats within 48 hours. Of the urinary excretory

products, some 0.25% of the originally administered 2,4-D was found

as metabolites of 2,4-D. This work clearly shows that the bulk of the

administered 2,4-D is eliminated from the rat as unchanged residue

in a relatively short time. Florsheim and Velcoff (12) have studied

some of the effects of 2,4-D on the thyroid function in male rats.

Using subcutaneous injections of sodium 2,4-D, they found that 80

mg/kg per day did not affect the growth rate, nor did it affect thy-

roid, pituitary, adrenal or testicular weight, even though the 2,4-D

was given daily for several weeks. However, when 100 mg/kg of 2,4-D

were injected, both the thyroid and body weights decreased. In the

lower doses of 2,4-D, it was found that there was an increase in

I^{131} uptake by the thyroid. It was found that the 2,4-D was not

affecting the pituitary thyrotrophic hormone, but was acting directly

on the peripheral iodine pool. Of interest is the fact that 2,4-D

was seen to affect the iodine uptake only in rats with normal, healthy

thyroids. The effect was not observed in hypophysectomized or

iodine-depleted animals.

In work with rat liver mitochondria, Brody (5) has shown that 2,4-D and several other plant growth regulators uncouple oxidation from phosphorylation. The same observation has been made by Switzer (28) in his work with soybean mitochondria. Wort (36) has compiled some literature that shows 2,4-D to be effective in inhibiting the action of a wide variety of enzymes.

The research efforts presented here were initiated in an attempt to determine if 2,4-D would have any effect on the metabolism of the biosynthetically and catabolically important intermediate, acetate. Initially, it was necessary to establish a control metabolic pattern for both acetate-1-C^{14} and -2-C^{14}. The excretion pattern for expired CO_2 has been well documented by several workers (15,31,32), however very little information could be found concerning the metabolites of acetate, as excreted in the urine (14,20,32) and feces of rats. Once a control for the excretion of the catabolic products of acetate had been established, it was then possible to proceed directly with the work which has ultimately shown both the respiratory CO_2 pattern and the urinary metabolites of acetate-C^{14} to be affected by the oral administration of 2,4-D. The following presents in detail the results of this work.

Materials

For this work adult white rats of an inbred Oregon State Wistar
strain were used. They were fed Purina Chow, at liberty. The rats
were five to nine months old when used for experimentation. The
weights of female rats ranged from 250-280 g, while the male rats
weighted 350-400 g.

All of the chemicals used in this research were of the highest
quality available. The herbicide 2,4-dichlorophenoxyacetic acid,
2,4-D, was obtained in the acid form from Nutritional Biochemicals
Corporation. The 2,4-D was actually used as the potassium salt and
was prepared by dissolving equal amounts of 2,4-D and K_3PO_4 in aqueous
solution.

Crystalline sodium acetate-1-C^{14} and -2-C^{14} were obtained from
either New England Nuclear Corporation or Volk Chemicals. The so-
lutions of acetate-C^{14} used for oral administration were prepared such
that the specific activity was about 5×10^7 dpm per milliliter. The
labeled acetate was diluted with unlabeled acetate to give a con-
centration of 14.5 μmoles per ml. This allowed the administration
of 29 μmoles of acetate, since, with few exceptions, two milliliters
of the acetate solution were given to each rat. For injection of
acetate-1-C^{14} into the peritoneal cavity, a special solution was pre-
pared. This solution was prepared in 0.9% NaCl, and contained about
2×10^8 dpm per ml. The concentration of acetate was 58 μmoles per
milliliter. Since only 0.5 ml of this solution was injected, the
quantity of acetate given was comparable to that present in the orally

administered acetate-C^{14} solution.

Benzoic-C^{14} acid was purchased from Packard Instrument Corporation and was used as a standard source compound for internal standaridization of samples counted in a semi-automatic Packard Tricarb liquid scintillation counter, series 314 E. For scintillation counting, Liquifluor was purchased from Pilot Chemicals. Liquifluor contains 1.25 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 100 g of 2,5-diphenyloxazole in one liter of toluene. The actual solvent used for counting consisted of four milliliters each of the following two solvents: A. toluene containing 42 ml of Liquifluor per liter; and B. redistilled methylcellsolve containing 50 g of naphthalene per liter.

For the vacuum filtration of BaCO$_3$ precipitates, Whatman Glass Fiber GF-83, weighing 55 g/m^2 and containing ultra-fine fibers, was used.

Methods

Oral Administration of Chemicals

The oral administration of the various materials was accomplished
by means of a syringe and a thin Teflon tubing, 0.022 inches I.D. and
3.5 inches long. The rat was held vertically, the mouth held open
with large forceps, and the tube was gently inserted into the oral
cavity and down into the stomach. Care was taken to prevent the tube
from going into the lungs.

In work with male rats, it was necessary to lightly anesthetize
the animals with ethyl ether fumes. Immediately after the dosing, an
air stream was passed over the rats in order to aid the recovery from
the anesthesia.

Intraperitoneal Injection of Acetate

The acetate-1-C^{14} solution for injection was placed into vials
which had been sterilized 15 minutes by steam in a Shampaine Sterilizer.
One-half milliliter of the solution for injection was pipetted into a
vial which was then sealed by melting the opening. For injection, a
one milliliter syringe, a one inch #22 needle and the vial containing
the acetate solution were sterilized 15 minutes in steam. At the time
of injection, the vial containing the acetate-1-C^{14} was broken open
and its contents immediately drawn up into the sterilized syringe.
The acetate was injected into the peritoneal cavity, taking care not
to inject the solution into the intestine or any of the internal

organs. From this point on, the CO_2, urine and feces were collected and analyzed exactly as that from rats receiving the oral administration of acetate-C^{14}.

Collection and Analysis of CO_2, Urine and Feces

Immediately after the acetate-C^{14} was administered, the rat was placed into a Delmar metabolism cage and the CO_2, urine and feces were continuously collected. Room air was pulled through a column of Drierite to remove moisture, then through a column of NaOH pellets to remove CO_2. From there, it passed through the metabolism cage from top to bottom and into a column of NaOH solution which trapped the expired CO_2 as Na_2CO_3. The air was pulled through the system by means of an aspirator. During the entire experiment, both food and water were available to the rats. The urine separator provided a means of separating the urine and feces, though there was frequent contamination of the feces with urine.

Sodium hydroxide from the CO_2 trap was drained from the absorber and the absorber was rinsed with distilled water. The NaOH was then made up to a convenient volume, 100 ml or 200 ml. An aliquot of the NaOH solution was pipetted into a 22 x 150 mm culture tube. Distilled water was used to rinse the sides of the tube. Excess 10% $BaCl_2$ was added and the tubes were again rinsed with water. The $BaCO_3$ precipitation was allowed to continue for about 30 minutes, and the precipitate was filtered through a pre-weighed glass filter. The $BaCO_3$ was rinsed from the culture tube with additional distilled water. The precipitate was then washed with five to ten milliliters of 50%

ethanol, followed by five to ten milliliters of reagent grade acetone.

The precipitate on the filter disc was then placed under a heating

lamp for 20 minutes to ensure dryness. The discs were then reweighed

on a Mettler, Gram-atic Balance and the radioactivity was counted.

The actual radioactivity measured was corrected to zero thickness of

the precipitate. In all cases, the analyses were carried out in

duplicate or triplicate, and if the weights of the samples varied more

than one milligram, the precipitation was repeated.

The urine samples were collected and centrifuged at low speed in

a Phillips-Drucker L-708 centrifuge to remove small particles of food.

Aliquots of urine were pipetted directly into scintillation vials

and the radioactivity was counted.

The radioactivity in the feces was obtained by first crushing

the individual fecal pellets and extracting with 15 ml of 50% ethanol.

The solid materials were centrifuged out, as in the procedure for urine.

An aliquot of this supernatant was then counted by means of the liquid

scintillation counter.

Measurement of Radioactivity

The $BaCO_3$ precipitates were counted by means of a thin-window

G. M. tube, utilizing a Tracerlab 64 Scaler.

For liquid scintillation counting, a four and four mixture of

the counting solvents was used. The radioactivity was measured by

means of a Packard Tricarb liquid scintillation counter. The actual

dpm of radioactivity was determined by using an internal standard

of benzoic-C^{14} acid in toluene.

The radioactivity of the one dimensional chromatograms was scanned on a Forro strip scanner with a Tracerlab Precision Ratemeter and an Esterline-Angus graphic ammeter recorder.

Tissue samples were counted by means of a Tracerlab SC-16 windowless gas flow counter, utilizing a Raychronix 64 scaler. The counting gas mixture was 1.3% butane and 98.7% helium. For this work, no attempt was made to convert the observed counts into dpm, since the thickness and uniformity of the tissues varied considerably.

Paper Chromatography and Radioautography

The urine obtained 12 hours after the administration of the acetate-C^{14} was chromatographed one dimensionally on one by $18\frac{1}{2}$-inch strips and two dimensionally on ten by ten inch sheets of Whatman number one chromatography paper. The urine was spotted directly and developed in either of the following solvents: BAW (n-butanol; acetic acid; water, 12:3:5) (26, p. 151) or EAW (95% ethanol; ammonium hydroxide; water, 18:1:1) (26, p. 273). For the two dimensional chromatograms, it was found best to use the BAW solvent first, followed by EAW. The chromatographic solvents were freshly prepared each time they were used.

Radioautograms of the two dimensional sheets and selected one dimensional strips were prepared by laying Kodak No-Screen, medical, X-ray film over the chromatograms for a period of four to eight weeks. The radioautograms were developed in a solution of Kodak D-19 at room temperature for about three to four minutes. Development was arrested by rinsing the films in a one percent solution of acetic acid. The

films were then placed in Kodak Fixer at room temperature until they were cleared. Following this treatment, the chromatograms were rinsed in cold running water for two to three hours and then air dried. Fresh solutions were prepared each time a series of films was developed.

A Photovolt electronic densitometer, model 525, was used to estimate the percent of the various radioactive metabolites in the urine. The light source had a 1.5 mm opening to the photoreceptor. The zero density was checked at several places on the radioautogram. Ten readings were taken for each spot on the radioautogram; these values were then averaged. The outline of each spot was traced on heavy paper, cut out and weighed on the analytical balance. The relative amount of radioactivity present was calculated by multiplying the average density of each spot by its weight. This method of estimating relative radioactivity was found to give results within about five percent error, even when different individuals made the determinations.

Column Chromatography

In addition to using paper chromatography for the separation of the urinary metabolites, the technique of using column chromatography was employed. A column of 25 cm by 2.5 cm was filled with 15 g of Whatman Standard Grade cellulose powder. A slury of the cellulose was prepared in 95% ethanol. The column was gravity packed and washed with about 400 ml of 95% ethanol. Two methods were employed in placing the urine on the column.

1. The urine was placed directly onto the column.

2. The urine was freeze-dried overnight, taken up in a minimal volume of water and placed onto the column. This method decreased the quantity of water being placed on the column.

After the addition of urine, approximately 200 ml of 95% ethanol was run through the column. Then, a continually increasing concentration of 20% acetic acid and water into ethanol was added until the volume of eluate totaled 1100 to 1200 ml. Ten milliliter fractions were collected by means of a Rinco automatic fraction collector.

In order to determine the elution pattern of labeled metabolites from this column, 0.5 ml of every second or third fraction was plated on planchets, air dried and the radioactivity counted. The fractions which contained radioactivity for each metabolite were combined, air dried and stored for later use.

Characterization and Identification of the Urinary Metabolites

Determination of the Volatile Components of Rat Urine. In order to determine if the urine contained any volatile radioactive components, the following procedure was followed. A five milliliter sample of urine was adjusted either to a pH 1-2 with 0.1 N H_2SO_4 or pH 9-10 with 0.1 N $Ca(OH)_2$. The total volume was then made up to 60 ml. This mixture was distilled, and five milliliter fractions were collected. The fractions were individually assayed for radioactivity by means of the liquid scintillation counter.

Protein in Urine. An attempt was made to determine if any of the radioactivity in the urine was due to the presence of protein. For this, equal volumes of urine and 10% trichloroacetic acid were mixed

and heated in boiling water for five minutes. The solution was then
cooled and filtered through a pre-weighed glass filter. The glass disc
containing protein was dried under a heat lamp and the radioactivity
was assayed in the liquid scintillation counter.

Acetone Bodies in Urine. Because it seemed logical that acetate
might be metabolized to yield acetone, β-hydroxybutyric acid and
acetoacetic acid, a determination was carried out for the amount of
radioactivity in the acetone bodies. The method used was essentially
that of Van Slyke (16), except that smaller volumes were employed. In
order to ensure that some acetone could be detected, carrier acetone
was added to the analysis.

Briefly, the method of analysis followed this procedure: to
2.5 ml of urine in a 25 ml volumetric flask, ten milliliters of water
and five milliliters of a 20% $CuSO_4$ solution were added. The mixture
was shaken, and five milliliters of a $Ca(OH)_2$ suspension were added.
The mixture was then diluted with water to 25 ml and set aside for
30 minutes. After this time, the solution was filtered.

Once the glucose in the urine had been removed by the above
procedure, 12.5 ml of the filtrate, 100 ml of 17 N H_2SO_4, and 35 ml
of 10% mercuric sulfate were added to a 250 ml flask. The flask was
then connected to a reflux condenser and heated to boiling. After the
boiling had begun, five milliliters of five percent potassium dichro-
mate solution were added through the condenser. The solution was then
boiled gently for 90 minutes and allowed to cool. The crystals were
collected by filtration through a glass filter. The crystals on the
disc were placed in a liquid scintillation vial, and the radioactivity

was counted.

Colorimetric Tests on Paper Chromatograms. In an attempt to
identify or characterize the radioactive metabolites found in the
urine, various colorimetric tests were carried out on paper chromato-
grams. Before any of the following colorimetric reactions were
employed, the chromatograms were steamed in an autoclave, without
pressure, for 30 minutes. This procedure removed any residual solvent
that might interfere with the tests.

Two methods were used to determine the presence of organic acids.
In the first method, an acid-base indicator was prepared according to
Aronoff (3, p. 119) in which 62.5 mg of methyl yellow and 187.5 mg of
bromophenol blue were dissolved in 25 ml of 95% ethanol. This stock
solution was then made just basic with 2 N NaOH. It was diluted 1:20
with ethanol and used as a dip. The color reaction varied from yellow
to red depending upon the concentration or the acidity of the material
on the chromatogram. The background color was green-blue, and the
colors were quite stable.

In the second method, the Altman reagent (26, p. 280,298) was used
to detect organic acids. This reagent was useful in locating many of
the Kreb's citric acid cycle intermediates, phenolic acids and aroyl-
glycines. The reagent was prepared by mixing one volume of five per-
cent p-dimethylaminobenzaldehyde in acetic anhydride with four volumes
of acetone that had been dried over $CaSO_4$. The chromatograms were
dipped rapidly through the reagent and then left to air dry. Colors
began to appear within 20 minutes, however color development was
allowed to continue over night. Heating for two to three minutes at

130°C intensified the colors.

The Ehrlich reagent (26, p. 193) was used in the detection of compounds containing the indole moiety. This reagent was prepared by making a ten percent solution of p-dimethylaminobenzaldehyde in concentrated HCl. Immediately before use, one volume of the above was added to four volumes of acetone. The chromatograms were dipped rapidly and the acetone blown off. Note was taken of the initial colors, because the colors changed over a period of time.

Sugars in the urine were detected by means of the aniline-diphenylamine reagent (26, p. 251). Equal volumes of one percent analine in acetone and one percent diphenylamine in acetone were combined. Ten volumes of this mixture were added to one volume of 85% phosphoric acid. The chromatograms were dipped and heated to 95 to 100°C for two to three minutes. Since the reagent contained concentrated phosphoric acid, care was taken not to over heat the chromatograms. This reagent was very useful because of the wide range of colors obtained with the different sugars.

A single reagent (26, p. 357) was used to analyze for fatty acids and cholesterol. The chromatograms were first immersed for 20 minutes in 100 ml of a 0.2% solution of aqueous copper acetate. The excess copper acetate was removed by washing the chromatograms in running water for 20 minutes. After complete drying, the copper salts were detected by dipping the chromatograms in a 0.03% solution of dithiooxamide in 95% ethanol. The fatty acids and cholesterol appeared as green spots on a lighter green background.

Ninhydrin reagent was used to detect the presence of amines,

amino acids, peptides and proteins that might be present in the urine.

The reagent was prepared (26, p. 15) by making a 0.2% solution of

ninhydrin in acetone. The chromatograms were dipped through the so-

lution and the acetone allowed to evaporate. Alpha-amino acids

reacted without heating, however two to three minutes heating at 105°C

allowed the reaction of many other amino compounds.

Isotope Dilution of Urea. Urea was identified as one of the

urinary metabolites of acetate by the following method. The first

peak of radioactivity from the column chromatography was combined with

500 mg of crystalline urea. These crystals were then taken-up in a

minimal volume of hot methanol. Excess ethyl ether was added, and the

crystals of urea precipitated. These crystals were dried and weighed.

Some were counted for radioactivity; the remaining crystals were re-

crystalized several times.

Solvent Extraction of Spot II. Since spot II on the paper

chromatograms was not identified, its solubility characteristics were

determined. Spot II was found to correspond with peak D from column

chromatography; for this reason, the combined fractions of the column

eluate were used to determine its solubility in various solvents.

After the combined fractions were air dried, they were taken-up in

distilled water. A volume of the redissolved metabolite was added

to a 60 ml separatory funnel. The pH was found to be pH 5-6. The

solution was then extracted with one of four solvents; ethyl ether,

t-amyl alcohol, ethyl acetate or carbon tetrachloride. After the

layers of solvent had separated, the aqueous layer was removed and the

procedure repeated. The two organic extractions were combined and

analyzed for radioactivity. The remaining aqueous fraction was made

basic to pH 10-11 by using 0.1 N NaOH. The solvent extraction was then

repeated, as above.

Tissue Analysis

In order to determine if there was any specific tissue which

acquired a high concentration of radioactivity under the influence of

2,4-D, rats were killed at a specified time and selected tissues were

analyzed. For this work, the rats were killed by CCl_4 vapors and were

immediately devicerated. The organs and tissues collected were as

follows:

blood	liver
brain	lungs
fat (from the abdomen)	muscle (from the abdomen)
heart	spleen
intestine	stomach
kidney	

Once the tissues were excised, they were frozen and then freeze-

dried. The dried tissues were weighed, cut into very small pieces and

ground to a fine powder by means of a mortar and pestal. One tenth

gram of each tissue preparation was weighed into a separate planchet

and smoothed to a nearly uniform layer.

At various times, both the urine and selected tissues were

freeze-dried. The freeze-drying process was carried out in an ap-

paratus manufactured by the Virtis Company. The samples were placed

in glass beakers or plastic vials and then frozen over night at -10 to

-15°C. The freeze-drying apparatus was cooled by an isopropanol-

dryice bath. The samples were then placed on the rotating platform and

the system was sealed. The pressure in the vacuum chamber was reduced

to 0.05 mm of Hg. After the maximum vacuum was attained, a 250 watt

infrared heat lamp, held 20 to 24 inches away, was used to speed the

freeze-drying process. The freeze-drying process was continued for

18 to 24 hours. The samples were removed and stored in a desiccator

for further use.

Results and Discussion

I. The Metabolism of Orally Administered C^{14}-Labeled Acetate in

    Normal Adult Rats.

Even though many workers (8,14,15,20,30,32,35) have reported data
concerning the _in vivo_ metabolism of acetate in rats, it was necessary
to establish the normal metabolic pattern of acetate-C^{14} for both
adult male and female rats. It was found that the greatest percentage
of exogenously supplied acetate is metabolized in the body and excreted
in the form of CO_2. Small amounts of acetate metabolites were found
in the urine and feces.

Incorporation of Radioactivity into the Expired CO_2

The rats were given an oral dose of acetate-C^{14}, and their ex-
pired CO_2 was collected and analyzed for radioactivity as described
under Methods. Figure 1 shows the accumulative percent recovery of
radioactivity in the expired CO_2 from normal adult rats. This figure
clearly shows that from 92 to 97% of the administered radioactivity
was recovered in the expired CO_2 within 36 hours. This percent re-
covery compares favorably with that found by other workers using rats
(24,30) and guinea pigs (4).

The specific activity, as a function of time, for the expired CO_2
is shown in Figure 2. This graph shows that acetate-C^{14} was very
rapidly metabolized and eliminated in the CO_2. Tolbert, Kirk and
Baker (30) found, with continuous analysis of the expired $C^{14}O_2$, that

Figure 1. The Accumulative Percentage of Administered Radioactivity
Recovered in the Expired CO_2 of Normal Adult Rats Given
C^{14}-Labeled Acetate.
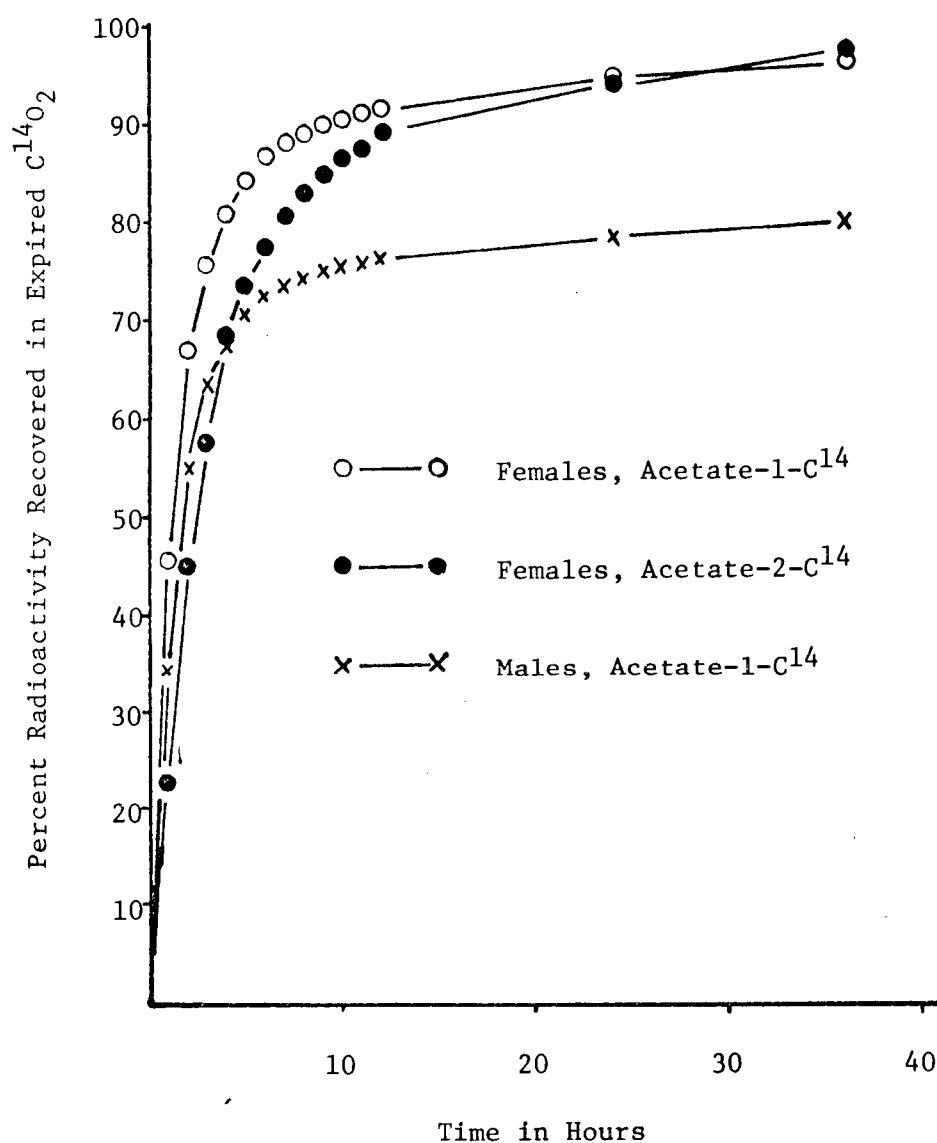
Figure 2. The Specific Activity per Hour of the Expired CO_2 from Normal Adult Rats Given C^{14}-Labeled Acetate.
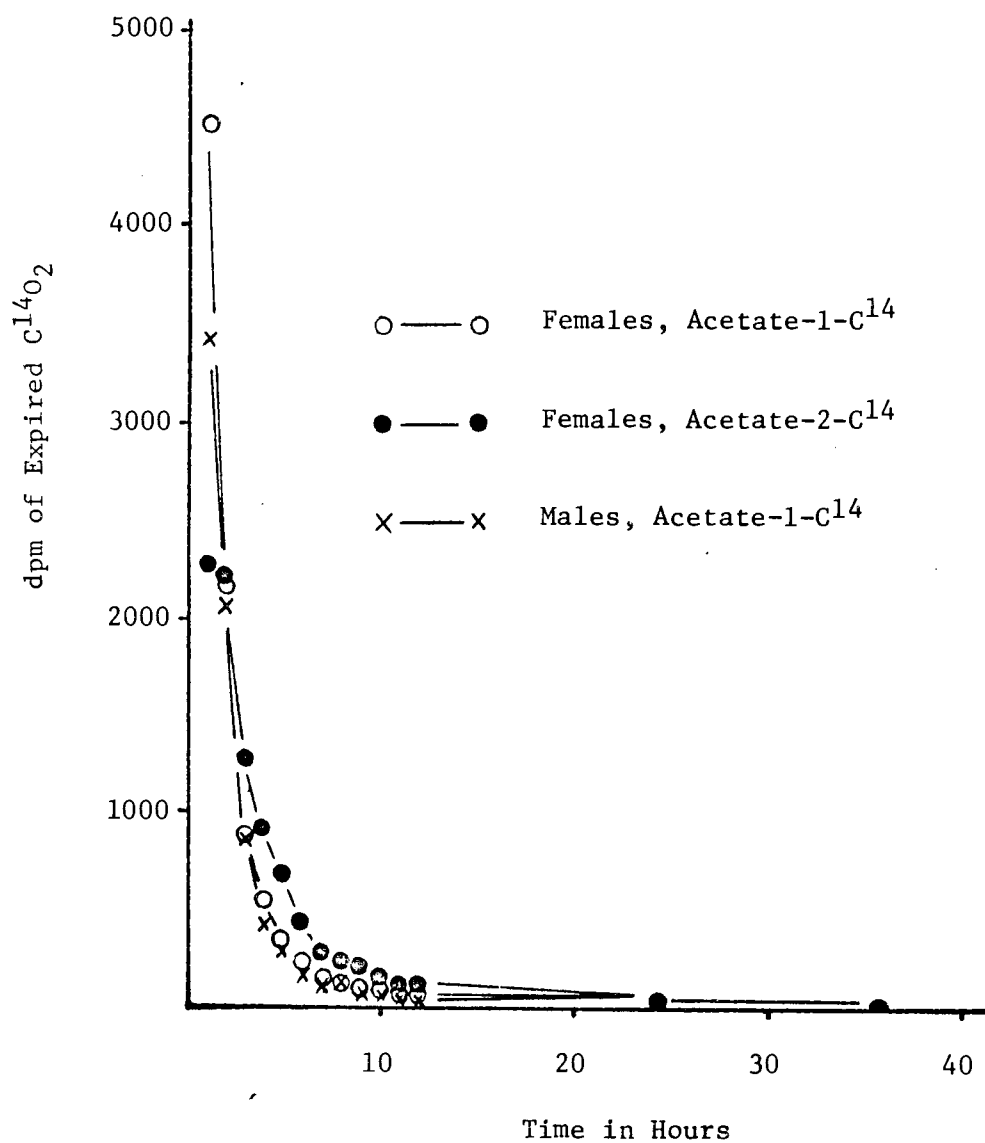
the maximum specific activity in the CO_2 occurred at about 30 minutes. More recent work, using a smaller metabolism cage with a shorter turnover time of the air in the system (24), has shown that the maximum specific activity of the expired CO_2 occurs within ten to fifteen minutes after the administration of acetate-C^{14}.

A kinetic plot of the $C^{14}O_2$ expired from normal adult rats is shown in Figure 3. The numerical data with the standard deviations are shown in Table 1. It is readily seen that there were two separate rates for the elimination of $C^{14}O_2$. The initial rate was very rapid and had a biological half-life of four to six hours. The second rate of elimination was much slower and continued for an extended period, having a biological half-life of 20 to 25 hours.

Table 1. The Mean Radioactivity and Standard Deviations Per Hour of Expired $C^{14}O_2$ from Normal Adult Rats Given C^{14}-Labeled Acetate.

Hours After Acetate Administration	Radioactivity Recovered in Expired $C^{14}O_2$					
	Females				Males	
	Acetate-1-C^{14} [a]		Acetate-2-C^{14} [b]		Acetate-1-C^{14} [c]	
	dpm	±deviation	dpm	±deviation	dpm	±deviation
1	4523	997	2271	281	3435	959
2	2173	208	2229	159	2067	314
3	879	202	1278	118	848	347
4	540	108	903	164	430	255
5	341	67	659	110	279	121
6	223	29	443	87	166	61
7	140	25	285	38	124	60
8	100	14	244	42	106	23
9	80	14	201	44	77	11
10	72	10	149	7	51	14
11	64	10	126	11	41	10
12	55	13	132	17	35	12
24	26	4	41	3	20	4
36	16	4	29	5	12	3

[a] five samples
[b] three samples
[c] four samples

Figure 3. Kinetic Plot of the Expired $C^{14}O_2$ from Normal Adult
Rats Given C^{14}-Labeled Acetate.
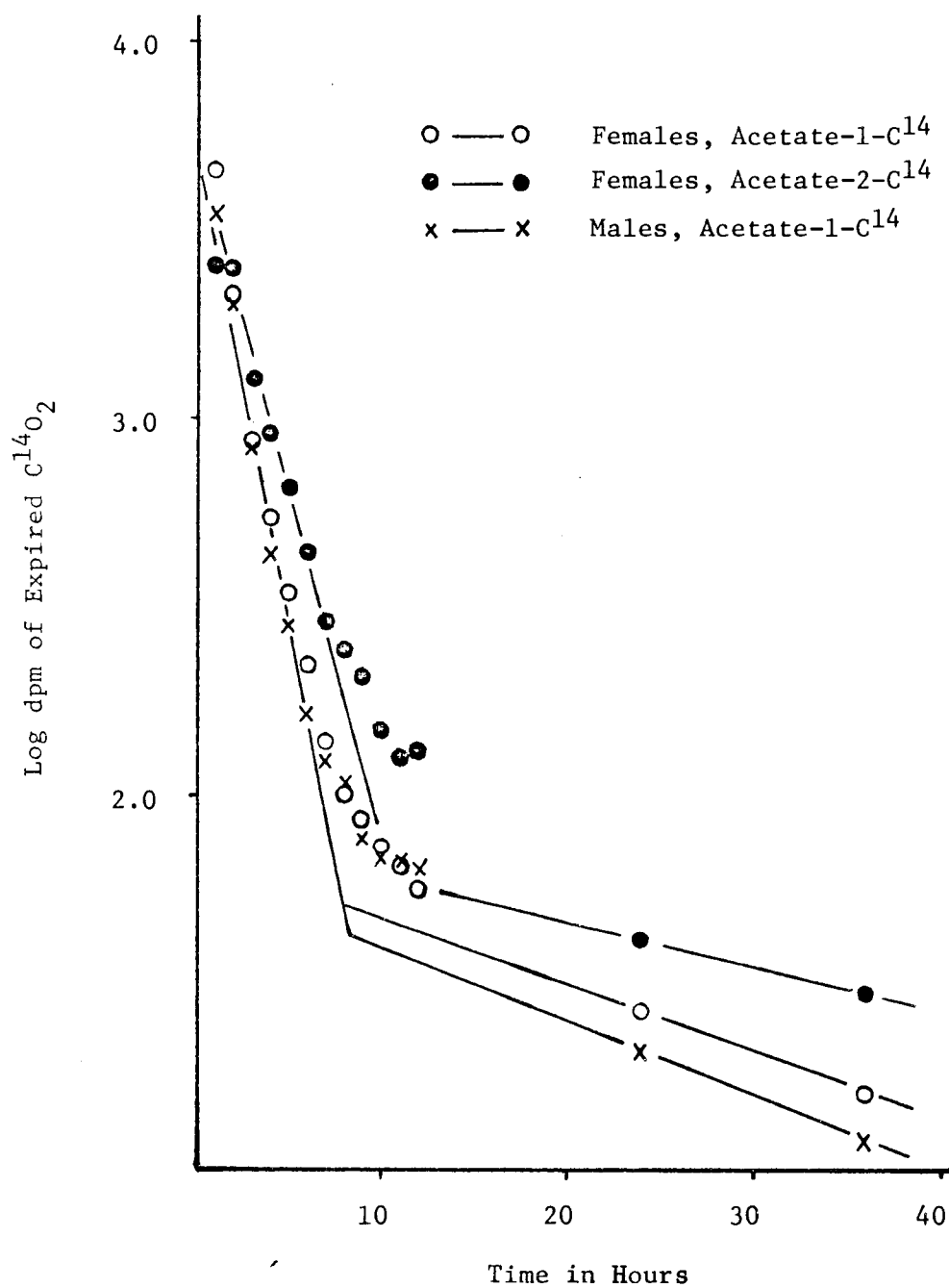
The appearance of two separate rates of $C^{14}O_2$ elimination has been explained as representing two major routes of metabolism for the acetate carbons.[1] The initial, rapid rate of elimination is thought to be due to the direct reaction of acetate with CoA-SH to form acetyl-CoA, which is subsequently oxidized to CO_2 through the Kreb's citric acid cycle. In the intact animal, the CO_2 from the citric acid cycle would be present in the form of bicarbonate which is circulated through out the blood. As the blood bicarbonate is turned over, there is a subsequent release of CO_2 from the lungs. This scheme can be represented as follows:

$$\begin{matrix} \text{Acetate} & & \text{Citric} & & & & & \\ + & \rightarrow & \text{Acid} & \rightarrow & (CO_2) & \rightarrow & \text{Blood Bicarbonate} & \rightarrow \text{Expired } CO_2 \\ \text{CoA-SH} & & \text{Cycle} & & & & & \end{matrix}$$
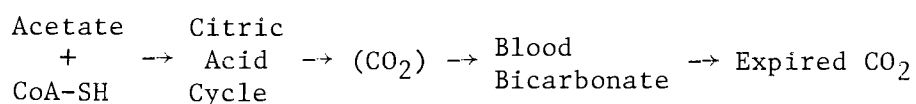
The slower, secondary rate of elimination is thought to be due to the circulation of the acetate carbons through several of the body pools such as fatty acids and amino acids. Subsequently, these compounds are slowly catabolized, much as the initial acetate was eliminated.

In Figure 3, it is seen that the slopes for the $C^{14}O_2$ elimination of acetate-1-C^{14} and -2-C^{14} are not identical, especially for the initial rate. This observation is not alarming, since it would indicate that the two carbons of acetate are not metabolized in exactly the same manner.

When 2,4-D was used for experimentation, it was administered in the form of the potassium salt. To determine if salt would effect the metabolism of acetate in adult rats, 100 mg of K_3PO_4 in aqueous

solution was orally administered to adult female rats, and one hour later, acetate-2-C^{14} was given. Results showed that there was no significant difference between the metabolism in salt fed rats and those rats which had been given only acetate-2-C^{14}. Therefore, it was decided that the feeding of acetate-C^{14} alone would suffice as a control for each rat that was used.

Excretion of Radioactivity in the Urine and Feces

Table 2 shows that only a small percentage of the administered radioactivity was recovered in the urine and feces of normal adult rats given acetate-C^{14}. The radioactivity recovered from the feces constituted a very small fraction of the administered dose. Due to the type of apparatus used for these experiments, complete separation of the urine and feces was not always possible. Thus, the small amount of radioactivity that was present in the feces was questionable as to its actual source.

When rats were injected intraveneously with acetate-1-C^{14}, Gordon (14) found that from 1.0 to 1.5% of the administered dose was recovered in the urine. The present work is in good agreement with Gordon's findings, in that 1.2 to 2.5% of the orally administered acetate-1-C^{14} was found in the 24 hour urine. Bergman et al. (4) found 0.14 to 1.51% of the radioactivity in the urine of guinea pigs which had been intraperitoneally injected with acetate-2-C^{14}. Bergman analyzed the urine for a period of six hours. Had he collected and analyzed for a longer period of time, the urine radioactivity may have reached the 2.8% reported here for the 24 hour urine.

Table 2. Percentage of Administer Radioactivity Recovered in the
          Excreta of Normal Adult Rats Given C^{14}-Labeled Acetate.

Percent Radioactivity Recovered in 24 Hours

	Females		Males
	Acetate-1-C^{14}	Acetate-2-C^{14}	Acetate-1-C^{14}
CO_2	95.3	89.4	79.2
Urine	2.0	2.8	1.2
Feces	0.5	0.5	0.3
Total	97.8	92.7	80.7

Total Percent Radioactivity Recovered

	Females		Males
	Acetate-1-C^{14}	Acetate-2-C^{14}	Acetate-1-C^{14}
CO_2	96.9	92.2	80.6
Urine	2.5	3.1	1.5
Feces	0.5	1.3	0.3
Total	99.9	96.6	82.4

Analysis of Urine from Normal Adult Rats

Although the percentage of the administered radioactivity re-
covered in the urine was quite low, the urine collected 12 hours after
the acetate-C^{14} administration was subjected to further analyses in
an attempt to identify and characterize the radioactivity.

Volatile Components in the Urine. In an attempt to determine if
any of the urinary radioactivity was present in the form of volatile
components, the urine was made either acidic or basic and then steam
distilled. As described under Methods, fractions were collected and
analyzed for radioactivity. In neither the acidic distillate nor the
basic distillate were any radioactive components found.

Trichloroacetic Acid Precipitation of Urine. Trichloroacetic acid and heat treatment were used to precipitate the urinary proteins described under Methods. Initially, fresh urine was used to carry out the precipitation. It was found that there was very little precipitate, and the radioactivity was only about 0.04% of the total urine radioactivity. Since urine does not normally contain large amounts of protein, 12 hour urine that had been freeze-dried was taken up in a small volume of water and the precipitation carried out. From this sample, the amount of material precipitated was greater, however the radioactivity accounted for was only about 0.03% of the total urine radioactivity.

Since the amount of radioactivity precipitated by trichloroacetic acid was almost insignificant, it is possible that the radioactivity present in the precipitate was due to contamination.

Urinary Acetone Bodies. It seemed possible that some of the radioactivity present in the urine was in the form of acetone bodies; acetone, acetoacetic acid and β-hydroxybutyric acid. The method of Van Slyke (16) was used to precipitate the acetone bodies in the urine. A known amount of acetone was added to the urine to ensure an accurate determination. However, in every case that an acetone body determination was made, no radioactivity was found. MacKay et al. (22) have found acetone bodies to be $-C^{14}$ labeled in the urine of fasted rats given acetic acid-C^{14}. In their work, acetic acid was given in much higher dosages than was the acetate used in the present study.

Paper Chromatography of Normal Adult Rat Urine. In an attempt to further characterize and identify the radioactivity in the 12 hour

urine, both one dimensional and two dimensional paper chromatograms
were prepared. An acidic solvent, BAW, and a basic solvent, EAW,
were employed for chromatographic separations.

Table 3 gives a summary of the chromatographic separations from
the two dimensional chromatograms. The Rf values and the percent of
the total urine radioactivity of each spot were determined from
several chromatograms. Normal females given acetate-1-C^{14} showed five
radioactive spots, including the origin. Spots I and II constituted
some 96% of the total urine radioactivity. In normal females given
acetate-2-C^{14}, there were six radioactive spots found on chromatograms
of the urine. Again, spots I and II composed over 96% of the urine
radioactivity. Spot I constituted about the same percentage of the
urine radioactivity for normal adult females treated either with
acetate-1-C^{14} or -2-C^{14}. This observation was also true for spot II.

Normal adult males given acetate-1-C^{14} showed only three radio-
active spots in the 12 hour urine. Spots I and II made up about 98.5%
of the urine radioactivity. Spot I comprised a far greater percentage
of the total urine radioactivity for males than for the females. Spot
II constituted a smaller percentage in the urine of males than in the
urine of females.

Table 3 also gives evidence that the metabolism of acetate-1-C^{14}
and -2-C^{14} was not entirely the same in normal female rats. This
table shows that the amounts of the minor metabolites varied, depend-
ing upon the position of the C^{14} label. It is readily obvious that the
female rats had several minor metabolites present which were not pre-
sent in the urine of males. However, it is of importance to note that

Table 3. The Rf Values and Relative Abundance of the 12
Hour Urinary Metabolites of Acetate-C^{14} from
Adult Rats Treated and Not Treated With 2,4-D.

Percent of Total Urine Radioactivity

Spot Number	Rf Values BAW	EAW	Females Acetate-1-C^{14} Control	2,4-D Treated	Acetate-2-C^{14} Control	2,4-D Treated	Males Acetate-1-C^{14} Control	2,4-D Treated
1 (urea)	0.56	0.51	74.1	78.5	72.1	44.5	91.5	71.4
2	0.28	0.01	21.8	14.0	23.3	30.8	7.1	23.1
3	0.86	0.73	----	2.2	2.0	24.3	----	----
4	0.48	0.25	0.8	----	1.2	----	----	----
5	0.54	0.07	----	----	0.8	----	----	----
6	0.47	0.03	----	2.9	----	----	----	3.9
7	0.37	0.04	----	2.0	----	----	----	----
8	0.81	0.44	2.7	----	----	----	----	----
9 (origin)	0.00	0.00	0.6	0.4	0.6	0.4	1.4	1.6

the major metabolites were present under all circumstances, though the relative percentages varied.

Column Chromatography of Normal Adult Rat Urine. In order to obtain rather large quantities of the radioactive metabolites in relatively pure form, a method was developed for using column chromatography. As described under Methods, standard grade cellulose was used as the column packing. Figure 4 shows a typical elution pattern of the urine from a rat treated with acetate-2-C^{14}. This method provided very effective separation of the major urinary metabolites. For the female rats, 70 to 75% of the radioactivity applied to the column was found to be present in the first peak, A. The second major peak, D, typically constituted about 20% of the original radioactivity. The two minor peaks, B and C, usually represented one to two percent of the original urine radioactivity.

The various fractions were combined and allowed to air dry. When spoted for paper chromatography, peak A was found to correspond to spot I; peak D corresponded to spot II. It is of interest that both peaks B and C when chromatographed are identical to spot V. This observation is difficult to explain, since both peaks were consistently separate on the column. However, in the two chromatography solvents they appeared to be the same thing.

In column chromatography of urine from female rats which had been treated with acetate-1-C^{14}, only two radioactive peaks were found, and these corresponded to spots I and II. The recovery of the original urinary radioactivity from such rat urines was usually from 90 to 97%. Column chromatography of male rat urine yielded only two radioactive

Figure 4. Column Chromatographic Separation of Radioactive
Metabolites from the Urine of a Control Female Rat
Fed Acetate-2-C^{14}. The Elution was Accomplished
on a Cellulose Column with 95% Ethanol and an In-
creasing Gradient of 20% Acetic Acid.
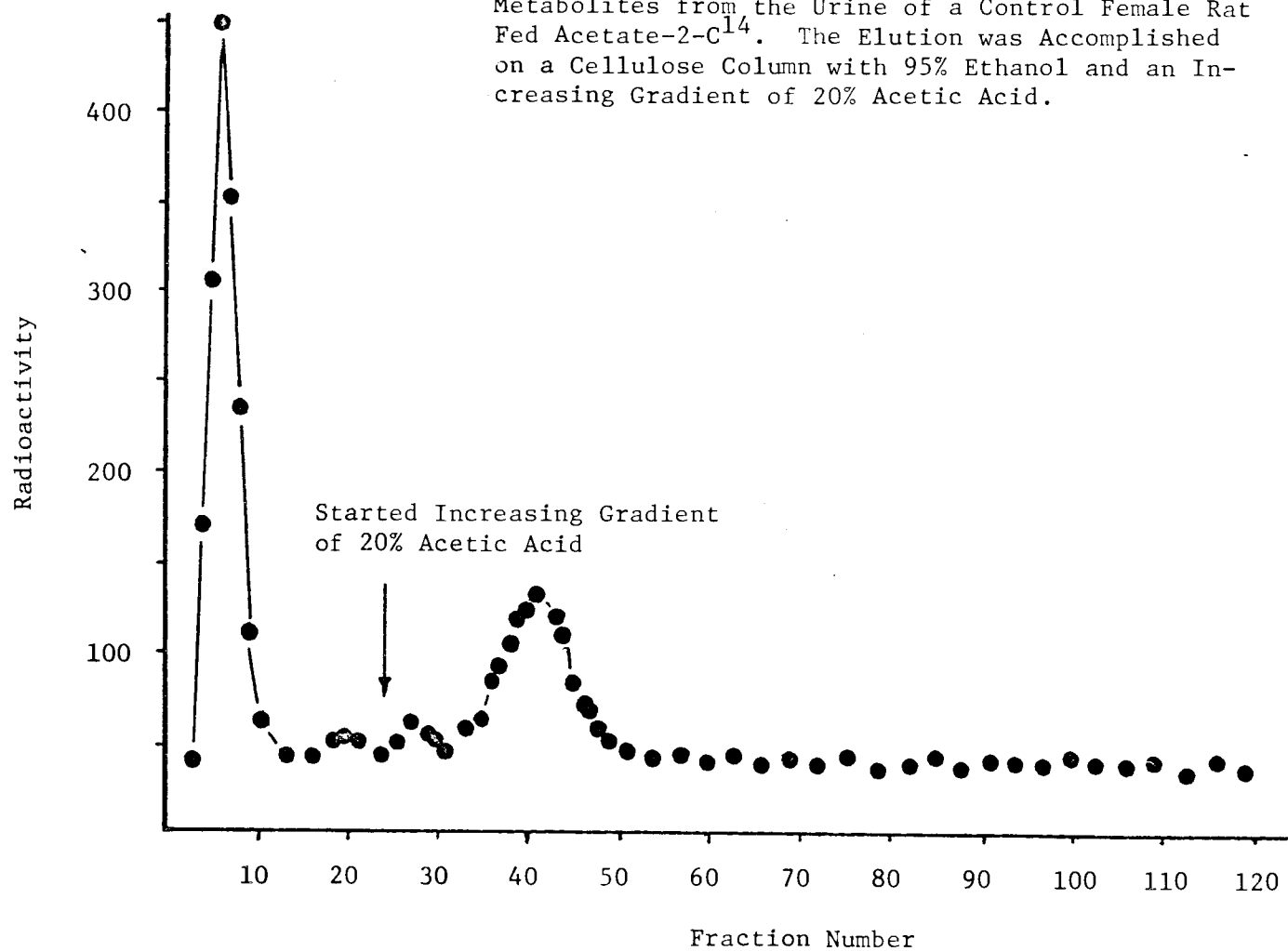
peaks, as with the females given acetate-1-C^{14}. The recovery of

urinary radioactivity for males was found to be 95 to 98%.

Characteristics of the Urinary Metabolites of Acetate-C^{14} from

Normal Adult Rats. The Rf values of the various urinary metabolites

for the two solvent systems used in this work are given in Table 3.

The various colorimetric reactions and procedures mentioned below are

discussed under Methods.

A). Spot I

Spot I gave a very strong color reaction with the Ehrlich reagent

and chromatographed the same as unlabeled urea in the two solvents.

For this reason, unlabeled urea and spot I were co-crystalized. It

was found that after only one recrystalization a constant specific

activity was obtained. The specific activity of the crystals was not

altered by further recrystalizations. This strongly indicated that

spot I was urea.

B). Spot II

Spot II was separated from the other urinary radioactivity by

means of column chromatography. Many different colorimetric reactions

were carried out on this spot. A weakly positive reaction was given

with the acid-base indicator. This reaction was observed for both of

the chromatographic solvents. The Altman test for organic acids, es-

pecially those from the Kreb's citric acid cycle and phenolic acids,

gave a very weak reaction only on chromatograms run in the EAW solvent.

No such reaction was observed with the BAW solvent. This difference

in colorimetric reactions observed in the two solvent systems may have

resulted from the diffusion of Spot II in the BAW solvent. As seen

in Table 3, spot II remains close to the origin in EAW, but moves some

distance in BAW. Spot II gave negative colorimetric tests for sugars,

indoles, fatty acids and amino acids and peptides.

A further attempt was made to characterize spot II by extracting

with various solvents. A summary of these results is given in Table

4. These results indicated that spot II is preferentially water so-

luble, possibly due to some acidic characteristic. Spot II may also

have some aromatic characteristics, as indicated by the slight solu-

bility in acidic ethyl ether. As mentioned above, in the EAW solvent,

a weakly positive reaction was obtained with Altman reagent which is

positive for phenolic acids.

Table 4. Solvent Extraction of Radioactive Spot II from the Urine
of Adult Rats Given C^{14}-Labeled Acetate.

| | Radioactivity Extracted, cpm [a] | | |
| | Solvent Layer | | |
Extracting Solvent	Acidic	Basic	Aqueous Layer
Ethyl Ether	600	0	1600
t-Amyl Alcohol	74	290	1680
Ethyl Acetate	0	60	1400
Carbon Tetrachloride	0	0	2400

[a] initially, 2500 cpm were used

Allantoin, an oxidative product of uric acid, chromatographed

similarly to spot II, and since allantoin was known to be present in

rather large quantities in rat urine (9, p. 147), it was suspected

that spot II was allantoin. However, when an attempt was made to co-

crystalize spot II with allantoin, the radioactivity was lost during

the first recrystalization. Creatine and creatinine were also found

to chromatograph similarly to spot II. However, when spot II was co-crystalized with creatine and creatinine, the radioactivity was lost in the first recrystalization. Thus, spot II remained unidentified.

C). Spot V, peaks B and C from the column.

Spot V was found to give a weakly positive test with both the acid-base indicator and the Altman reagent. Because insufficient material was present, no further characterization was possible.

D). Other Urinary Metabolites of Acetate-C^{14}

Because it was not possible to isolate any of the other urinary radioactive spots, all remained unidentified.

It is of interest to note that very little work has been published on the urinary metabolites of acetate-C^{14}. Gordon (14), in his work with citrate metabolism, found that citrate was labeled in the urine of rats treated with acetate-1-C^{14}. In the work presented here, the presence of citrate was specifically investigated. From the colorimetric reaction provided by the Altman reagent, citrate was detected in the urine, however its chromatographic location did not correspond to the locations of any of the radioactive metabolites.

Lee and Lifson (20) studied the accumulation of succinate in rat urine after acetate administration. Their work showed that succinate accumulated in the urine only after malonate administration. The presence of malonate brought about the urinary excretion of succinate, α-ketoglutarate and citrate, presumably because malonate inhibited the succinic dehydrogenase. The metabolism of fluoroacetate may not be the same as acetate, however it is interesting to note that Gal et al. (13) found five metabolites of fluoroacetic acid-2-C^{14}. One of the

metabolites was found to be fluorocitrate; the others remained uni-
dentified.

II. The Effect of an Oral Dose of 2,4-D on Acetate Metabolism in
     Adult Rats.

Elimination Pattern of Radioactivity in Expired CO_2 of 2,4-D Treated
Rats

Female Rats. In many experiments 2,4-D,was given orally to adult
female rats, and one hour later acetate-C^{14} was orally administered.
Several examples of the results are shown in Figures 5,7,8,9. There
appeared to be considerable variation in the excretory patterns ob-
served. The initial rate of elimination was rapid, however at about
hour six to eight the rate changed, and there was an increase in the
rate of $C^{14}O_2$ expired. This increase in the specific activity of the
CO_2 was not accompanied by a corresponding increase in the amount of
CO_2 expired. Within the error attributed to the normal variations in
the activity of the rat, the rate of CO_2 expired remained constant.

In Figure 5 is shown the $C^{14}O_2$ expiratory pattern of a single
female rat treated with 100 mg of 2,4-D followed one hour later by
acetate-1-C^{14}. This example is presented as being representative of
several rats. The results showed that an oral dose of 2,4-D affected
the excretory pattern of the labeled CO_2. The initial amount of
radioactivity expired was considerably lower for the treated rat than
for the control animal, and there were not the two discrete rates of
$C^{14}O_2$ elimination. When compared to Figure 3, the initial rate of

Figure 5. Rate of Administered Radioactivity Recovered in the Expired $C^{14}O_2$ of an Adult Female Rat Treated with 100 mg of 2,4-D and One Hour Later Given Acetate-1-C^{14}.
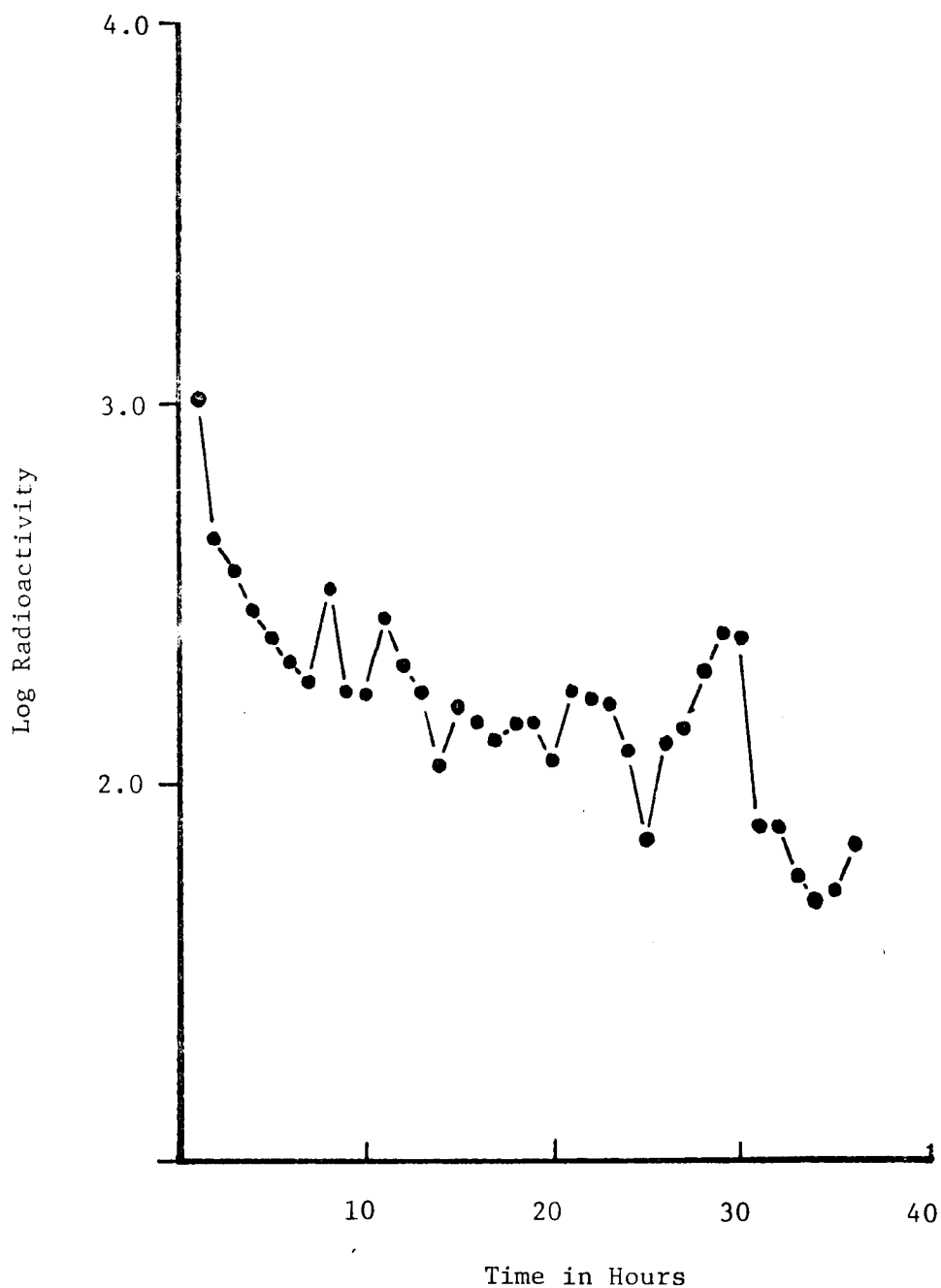
$C^{14}O_2$ elimination did not appear to be affected for the first few hours. However, from the seventh hour until the end of the 36 hour CO_2 collection, the rate of $C^{14}O_2$ expiration became very erratic. The curve shown here is quite typical; from hour six to 36 there were usually four to six peaks, or increases in the rate of $C^{14}O_2$ elimination.

Table 5 shows that female rats treated with 100 mg of 2,4-D had a lower percent recovery of radioactivity in the expired CO_2 than did control rats, Table 2. Rats treated with 2,4-D expired from 63.5 to 66.5% of the administered radioactivity in 24 hours and 65.8 to 82.1% in 36 hours. This data clearly shows that 2,4-D not only interfered with the $C^{14}O_2$ elimination pattern of acetate-C^{14}, but also prevented the acetate from being oxidized and eliminated in the form of $C^{14}O_2$.

Male Rats. Male rats were treated similarly to the females mentioned above. They were orally administered 160 mg of 2,4-D, thus making the mg/kg dosage comparable to that given the females. One of several such experiments has been selected as being typical in its $C^{14}O_2$ excretory pattern. Data collected during this experiment showed that the initial rate of elimination of $C^{14}O_2$ decreased rapidly, as shown in Figure 6, while later elimination took place at an erratic rate.

The $C^{14}O_2$ elimination pattern of males was quite similar to that of the females. The percent recovery of the administered radioactivity was somewhat less for the males, Table 5, and the percent recovery of $C^{14}O_2$ radioactivity in the 2,4-D treated males was considerably less than for the controls which show 79.2% recovered in 24 hours and

Figure 6. Rate of Administered Radioactivity Recovered in the Expired $C^{14}O_2$ from an Adult Male Rat Treated With 160 mg of 2,4-D and One Hour Later Given Acetate-1-C^{14}.
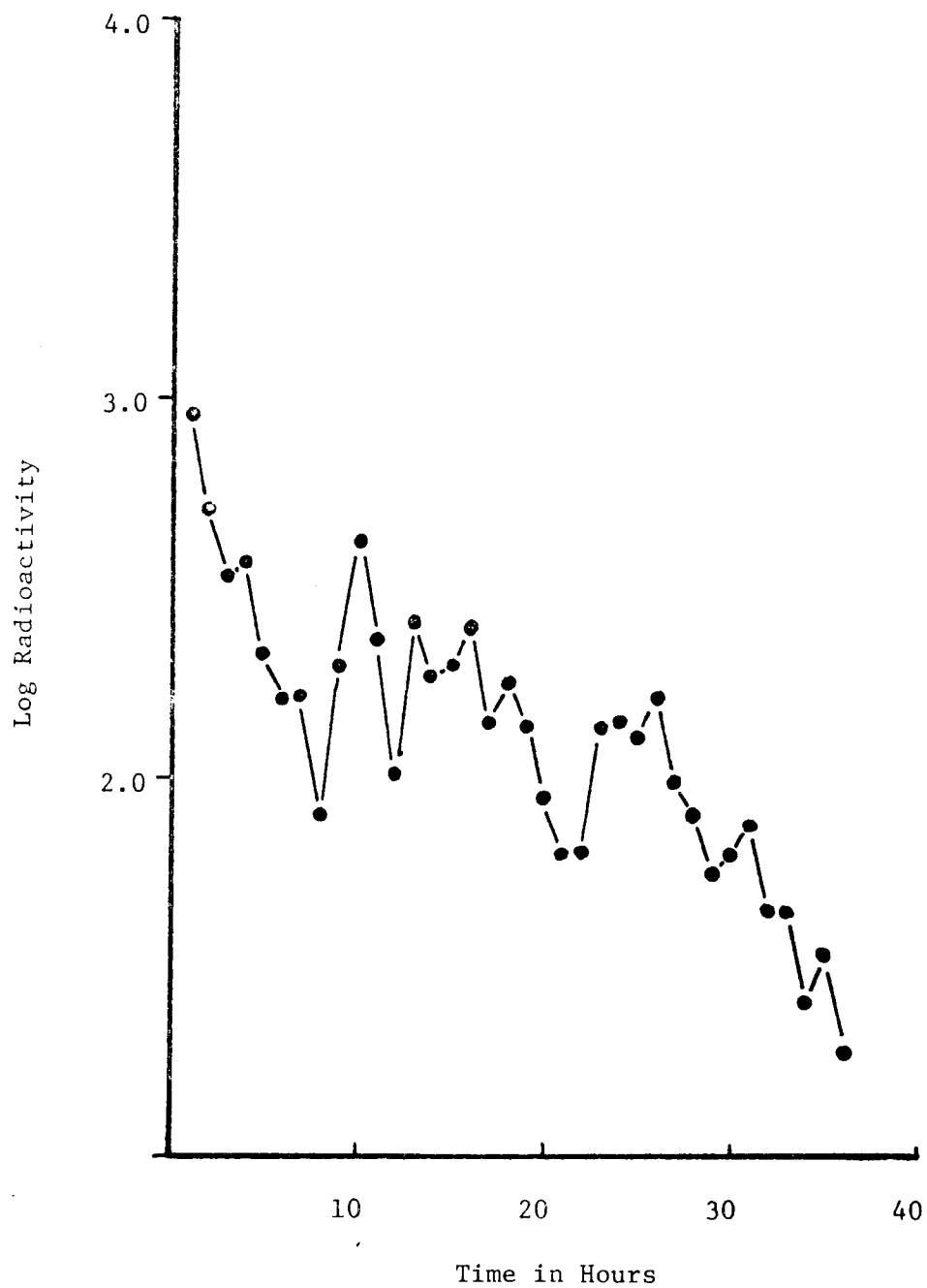
80.6% recovered in 36 hours.

Table 5. Percentage of Administered Radioactivity Recovered in the
Excreta of Adult Rats Treated with 2,4-D and One Hour Later
Given Acetate-C^{14}.

Percent Radioactivity Recovered in 24 Hours

| | Females | | Males |
	Acetate-1-C^{14}	Acetate-2-C^{14}	Acetate-1-C^{14}
CO_2	66.5	63.5	55.9
Urine	2.3	1.1	1.0
Feces	0.1	0.7	0.1
Total	68.9	65.3	57.0

Total Percent Radioactivity Recovered

| | Females | | Males |
	Acetate-1-C^{14}	Acetate-2-C^{14}	Acetate-1-C^{14}
CO_2	85.1	65.8	68.8
Urine	2.8	1.8	1.3
Feces	0.1	1.2	0.1
Total	88.0	68.8	70.2

Elimination of Radioactivity in the Urine and Feces

As with the control animals, the radioactivity recovered in the
urine and feces of 2,4-D treated rats was quite small. Table 5 shows
the percentage of radioactivity recovered in the urine and feces of
both males and females treated with 2,4-D and acetate-C^{14}. Typically,
the percent recovery was lower for the 2,4-D treated rats than for the
control animals, Table 2. In the exceptions to this observation, the
percent recovery was only slightly higher for the 2,4-D treated than
for the control animals. The 2,4-D did not appear to be altering the
metabolism of acetate in such a way as to produce large amounts of

urinary metabolites. It did prolong the turn over of acetate in the body. The 2,4-D could have been preventing absorption of the acetate, or a large percentage of the administered acetate may have been used for biosynthesis of body materials. Morris and Freed (23) have recently found that in plant tissues 2,4-D seems to be promoting an increase in the production of certain nucleoproteins. To date, no information of this general nature is available concerning animals, however it would prove of interest to investigate such matters in animals and animal tissues.

Urine Analysis of Rats Treated with 2,4-D and Acetate C^{14}

The radioactive metabolites of normal adult rat urine have already been discussed. Work with the urine of male and female rats given 2,4-D and acetate-C^{14} showed that there were no volatile radioactive components, just as in the controls. The percentage of radioactivity recovered from trichloroacetic acid precipitation showed no variation from that of the control animals, and the acetone bodies were found to be devoid of all radioactivity.

Results from paper chromatography of 12 hour urine are shown in Table 3. Females given 2,4-D and acetate-1-C^{14} showed six radioactive spots, while there were only four spots from acetate-2-C^{14} fed rats. The males given 2,4-D and acetate-1-C^{14} also showed only four radioactive spots in the urine. As the data is presented in Table 3, it is apparent that some of the radioactive spots present in the 2,4-D treated rats were not present in the controls. Also, some of the radioactive spots present in the controls were absent in the urine of 2,4-D

treated rats.

Besides comparing the fluctuation of the minor metabolites, it is also of special interest to compare the 2,4-D treated rats with the controls in other respects. In 2,4-D treated female rats given acetate-1-C^{14}, the percentage of labeling in the urine actually decreased over that of the control. This incerease in the amount of incorporation in urea of some 4.5% is thought to be significant, because the values were taken from work with several different rats and many experiments, and in every case this slight increase was noted. Spot II decreased by about 30% under the influence of 2,4-D. Spots III, VI and VII appeared under 2,4-D stress, while spot IV of the control was lost.

Females given 2,4-D and acetate-2-C^{14} also differed from the controls. Urea decreased by almost 40% in the 2,4-D treated rats, and spot II increased by over 30%. The greatest increase for any single spot was noted for III, which increased over 12 times that found in the control. Spots IV and V of the control were not found in the 2,4-D treated rats.

In comparing the urinary metabolites of acetate-1-C^{14} and -2-C^{14}, the two carbons of acetate were metabolized in part by the same pathways and partially by separate pathways. In the acetate-1-C^{14} and -2-C^{14} controls, spot I constituted approximately the same percentage of the radioactivity found in the urine. This was also true for spot II. In acetate-1-C^{14} fed rats under the stress of 2,4-D, the percentage of total urinary radioactivity found as urea increased while the percentage of radioactivity as spot II decreased. Since

urea has only one carbon, the evidence indicated that the two carbons
of acetate were split, possibly involving a decarboxylation reaction,
and that the presence of 2,4-D had a greater effect on the further
metabolism of the methyl carbon than on the carboxyl carbon. Of spec-
ial interest is the observation that 2,4-D increased the amount of
spot III. Spot III was entirely absent in the acetate-1-C^{14} control,
however under the influence of 2,4-D it was present, though only as a
minor metabolite. Spot III is present in the acetate-2-C^{14} control as
a minor metabolite, and under the influence of 2,4-D it increased such
that it was considered a major metabolite, along with urea and spot II.

In the male rats, it was interesting to note that urea decreased
in abundance under the influence of 2,4-D. Spot II increased about
three times. Spot VI, present with the 2,4-D treated acetate-1-C^{14}
females, was again present under the same conditions with the males.
Spot III which showed such interesting results with the females was
not to be found in the male urine.

Clearly, these data showed that acetate was metabolized different-
ly by the two sexes and that the carbons of acetate did not follow
identical pathways in their metabolism. The influence of 2,4-D was
found to greatly affect the urinary metabolites of acetate-C^{14}.

As with the control animals, the only identified urinary meta-
bolite of acetate was urea. Spot II for the controls showed the same
colorimetric characteristics as spot II for the 2,4-D treated animals.
Other than the determination of the Rf values listed in Table 3, there
has been no further characterization of metabolites.

III. The Effect of 2,4-D on Acetate Metabolism in Adult Rats As a
Function of Dosage

Because 100 mg of 2,4-D were shown to affect the rate of $C^{14}O_2$ elimination from rats given both acetate-1-C^{14} and -2-C^{14}, it was of importance to determine what effect several different dosage levels of 2,4-D, ranging from 2.5 mg to 150 mg per animal, would have on acetate-C^{14} metabolism. For this work, only female rats were used, however both acetate-1-C^{14} and -2-C^{14} were employed. A dose of 2,4-D was orally administered, and one hour later the acetate-C^{14} was given. Results of five different doses are given for acetate-1-C^{14}, Figure 7, while results of six different doses are given for acetate-2-C^{14}, Figure 8.

In these two figures, it is clearly shown that 2,4-D had a pronounced effect on the $C^{14}O_2$ elimination pattern over a wide range of dosages. Ten to 100 mg of 2,4-D appeared to have a similar effect on the CO_2 elimination from acetate-1-C^{14}. The increase of ten to 100 mg of 2,4-D showed no increased effect on the $C^{14}O_2$ elimination pattern. Only at a dosage level of five milligrams of 2,4-D did the effect on the elimination pattern seem to be decreased. For acetate-2-C^{14} metabolism, 2,4-D in dosages of ten to 150 mg per rat appeared to have approximately the same effect on the $C^{14}O_2$ elimination pattern. At a dosage of five milligrams of 2,4-D per rat, the observed effect on the $C^{14}O_2$ elimination was just barely apparent.

It is of interest to note that even though the oral LD_{50} of 2,4-D for rats, mice, guinea pigs, and rabbits has been found to be as

100 mg   50 mg   25 mg   10 mg   5 mg   Control

3.5

3.0

Log Radioactivity

2.5

2.0

Time in Hours

Figure 7. Rate of Administered Radioactivity Recovered in the Expired $C^{14}O_2$ of Adult Female Rats, as a Function of 2,4-D Dosage. One Hour after the Oral Dose of 2,4-D, the Rats Were Orally Given Acetate-1-C^{14}.
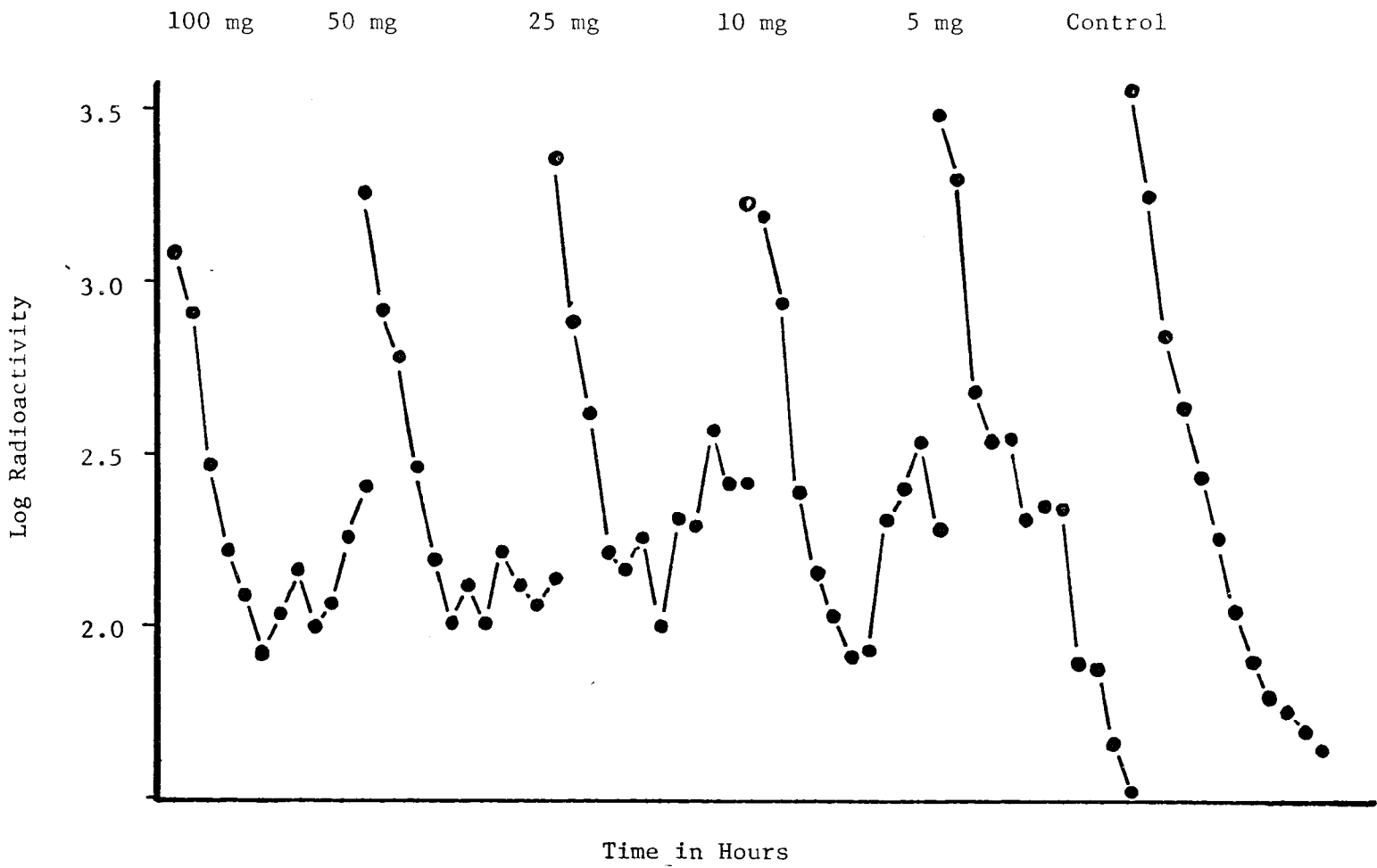
Figure 8. Rate of Administered Radioactivity Recovered in the Expired $C^{14}O_2$ of Adult Female Rats, as a Function of 2,4-D Dosage. One Hour after the Oral Dose of 2,4-D, the Rats Were Given Acetate-2-C^{14}.
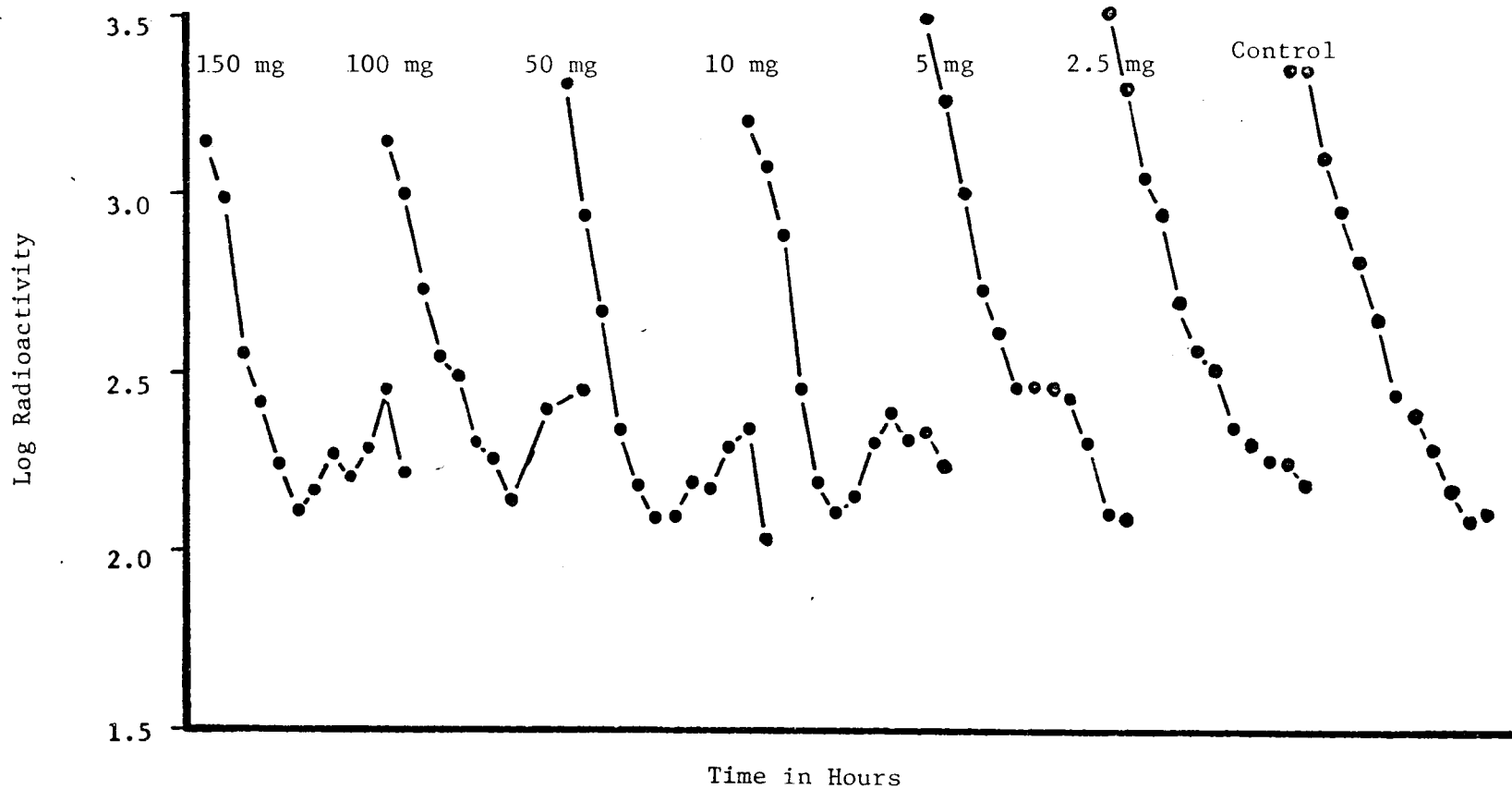
high as 300 to 1000 mg/kg body weight (19), 2 5 mg of 2,4-D for a 250 g rat appeared to alter the elimination pattern of $C^{14}O_2$ for the metabolism of acetate-2-C^{14}. Among the many workers interested in the toxicology of 2,4-D, Thomssen (29) has found that poisoning of domestic animals with the normal amount of 2,4-D used in commercial sprays and dusts is very improbable. The work presented in the present research bears out the fact that rats can readily recover from very large doses of orally administered 2,4-D. This work also showed that even though 2,4-D may not be extremely toxic, it does effect the metabolism of a rat, even in small doses.

For each dosage of 2,4-D, an analysis was carried out for the gross activity in both the urine and feces, as described under Methods. The results, along with the percent radioactivity recovered in the CO_2, are shown in Tables 6 and 7, for acetate-1-C^{14} and -2-C^{14} respectively. These tables give the percent radioactivity recovered from the $C^{14}O_2$ collected for 36 hours, and the percent recovery recorded for the urine and feces which were collected for 72 hours following the administration of acetate-C^{14}. Initial work showed that control experiments resulted in the recovery of essentially 100% of the administered radioactivity. The percentages given in Tables 6 and 7 are calculated as the percent recovery of the radioactivity administered to the rats.

There was no apparent variation in the patterns of $C^{14}O_2$ elimination when the dosage of 2,4-D was varied from ten to 100 mg per rat, Figures 7 and 8. However, as seen by comparing the various dosages of 2,4-D used with both acetate-1-C^{14}, Table 6, and -2-C^{14}, Table 7, there did appear to be a gradual increase in the percentage

of radioactivity recovered in the CO_2 as the dosage of 2,4-D was de-
creased.

Table 6. Percentage of Administered Radioactivity Recovered in the
Excreta of Adult Female Rats Given Varying Doses of 2,4-D
and One Hour Later Given Acetate-1-C^{14}.

Percent Radioactivity Recovered in 24 Hours
mg Dose of 2,4-D per Rat

	100	50	25	10	5	NONE
CO_2	66.5	72.4	80.0	75.2	90.8	95.3
Urine	2.3	1.7	2.6	0.9	2.4	2.0
Feces	0.1	0.2	0.4	0.8	0.3	0.5
Total	68.9	74.3	83.0	76.9	93.5	97.8

Total Percent Radioactivity Recovered
mg Dose of 2,4-D per Rat

	100	50	25	10	5	NONE
CO_2	85.1	82.1	82.7	77.1	92.5	96.9
Urine	2.8	2.0	3.0	0.9	2.6	2.5
Feces	0.1	0.5	0.5	1.1	0.7	0.5
Total	88.0	84.6	86.2	79.1	95.8	99.9

The percentage of radioactivity found in the gross urine analysis
did not show any significant trend. Only a very small percentage of
the administered radioactivity was found in the urine   Again, the
radioactivity in the feces was extremely low, and due to the poor
separation of urine from feces, the radioactivity present in the feces
could usually be attributed to contamination of the feces by urine.
Thus, the radioactivity present in the feces was somewhat question-
able.

Table 7. Percentage of Administered Radioactivity Recovered in the Excreta of Adult Female Rats Given Doses of 2,4-D and One Hour Later Given Acetate-2-C^{14}.

Percent Radioactivity Recovered in 24 Hours
mg Dose of 2,4-D per Rat

	150	100	50	10	5	2.5	NONE
CO_2	53.4	63.5	62.5	60.9	77.6	86.6	89.4
Urine	1.9	1.1	1.2	1.1	2.4	2.8	2.8
Feces	0.4	0.7	0.4	0.4	0.7	0.3	0.5
Total	55.7	65.3	64.1	62.4	80.7	89.7	92.7

Total Percent Radioactivity Recovered
mg Dose of 2,4-D per Rat

	150	100	50	10	5	2.5	NONE
CO_2	58.3	65.8	68.6	65.3	81.4	89.0	92.2
Urine	3.1	1.8	1.7	1.4	2.8	3.2	3.1
Feces	0.8	1.2	0.7	0.6	1.1	0.6	1.3
Total	62.2	68.8	71.0	67.3	85.3	92.8	96.6

IV. The Effect of 2,4-D on Acetate Metabolism in Adult Rats as a Function of the Time After 2,4-D Treatment.

Since 2,4-D was found to have a demonstrable effect on the in vivo metabolism of acetate-C^{14}, and the effect was dependent upon the dosage of 2,4-D, it was decided to see what effect there would be if the time between 2,4-D and acetate-C^{14} administration was varied. Seven different time intervals and a constant 100 mg per rat dosage of 2,4-D were employed for this work. The results of this series of experiments are shown in Figure 9. From this figure, it can be seen that 2,4-D affected the metabolism of acetate, even when the 2,4-D was given 48 hours prior to the administration of the acetate. As
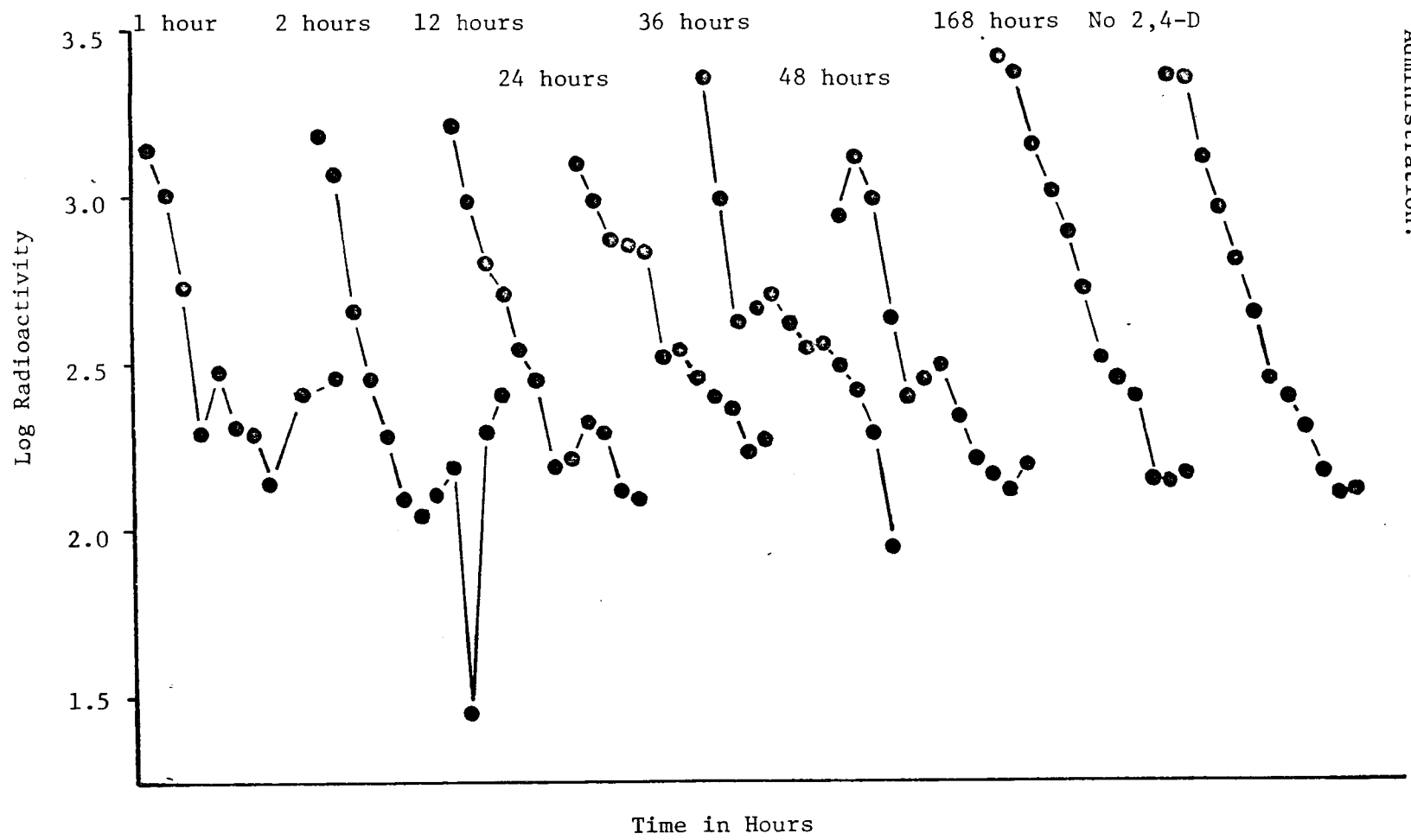
Figure 9. Rate of Administered Radioactivity Recovered in the Expired $C^{14}O_2$ of Adult Female Rats, as a Function of Time Between 2,4-D and Acetate-2-C^{14} Administration.

shown in Figure 9, the effect of the 2,4-D on the $C^{14}O_2$ elimination rate did not seem to be appreciably less than for the shorter time periods. Of special interest is the fact that the effect of the 2,4-D on acetate metabolism did not last for an entire week. The significance of this observation lies in the fact that although the effect of 2,4-D on acetate metabolism was quite dramatic for the short time intervals studied, the effect of 2,4-D was not long lasting. Thus, an organism that has received an oral dose of 2,4-D can be expected to recover in a relatively short period of time, if the dose of 2,4-D was not exceptionally large.

A comparison of the percentages of the administered radioactivity recovered in the CO_2, urine and feces for this series of experiments is shown in Table 8. There was a gradual trend toward greater percent recovery as the time between 2,4-D and acetate administration was increased. As mentioned for the series of experiments employing varying dosages of 2,4-D, the percentage of administered radioactivity recovered in the urine and feces was quite low.

Of interest is the recent observation by Clark et al. (7) in which they found that 90% of the 2,4-D orally administered to sheep was recovered in the urine within 28 hours. Using paper chromatography and paper electrophoresis, they established that the radioactivity in the urine was identical to that of the 2,4-D. Similar observations have been made by Lisk et al. (21) in which steers were given 2,4-D, and the radioactivity was found as 2,4-D in the urine. These findings seem quite reasonable since phenoxyacetic acids are primarily eliminated from the body, intact (34), not requiring

Table 8. Percentage of Administered Radioactivity Recovered in the
Excreta of Adult Female Rats as a Function of the Time
Between Administration of 100 mg of 2,4-D and Acetate-2-
C^{14}.

Percent Radioactivity Recovered in 24 Hours
Hours Between 2,4-D and Acetate Administration

	1	2	12	24	36	48	168	No 2,4-D
CO_2	63.5	63.4	62.7	71.4	75.4	62.7	85.8	89.4
Urine	1.1	3.1	2.7	3.2	2.2	1.2	3.2	2.8
Feces	0.7	0.5	0.4	0.3	0.1	0.6	0.4	0.5
Total	65.3	67.0	65.8	74.9	77.1	64.5	89.4	92.7

Total Percent Radioactivity Recovered
Hours Between 2,4-D and Acetate Administration

	1	2	12	24	36	48	168	No 2,4-D
CO_2	65.8	78.1	65.0	74.5	80.6	64.5	89.0	92.2
Urine	1.8	3.2	2.9	3.5	2.4	1.5	3.8	3.1
Feces	1.2	1.0	0.6	0.6	0.3	0.9	0.5	1.3
Total	68.8	82.3	68.5	78.6	83.3	66.9	93.3	96.6

any detoxication mechanism. Very recently Khanna, Rao and Fang (18)
have shown that 78% of an 80 mg per rat dose of 2,4-D is excreted in
the urine of rats within 48 hours. These workers have also shown that
the actual rate of 2,4-D elimination is dependent upon the dose admin-
istered. The elimination of 2,4-D is much faster when smaller doses
are given. These observations (7,18,21) on the elimination rate of
2,4-D may well explain why there was a decrease in the effect of 2,4-D
on acetate-C^{14} metabolism. As the time between 2,4-D administration
and acetate feeding was increased, the actual amount of 2,4-D in the
rat's stomach and other organs decreased. As the amount of 2,4-D in
the body decreased, the effect of the 2,4-D on acetate metabolism di-
minished. Since there was no observed effect of 2,4-D on acetate
metabolism when the 2,4-D was given one week prior to the acetate, it
can be assumed that in the period of one week the 2,4-D had been re-
moved from the site at which it affects the metabolism; more possibly
the 2,4-D had been entirely eliminated from the body of the animals.

V. Comparative Accumulation of Radioactivity in Rat Tissues after
   Acetate Administration, With and Without 2,4-D Treatment

Several experiments were run in an attempt to determine if there
was any particular tissue which accumulated radioactivity under the
influence of 2,4-D treatment. For this work, female rats were given
either an oral dose of acetate-1-C^{14} or an oral dose of 100 mg of
2,4-D followed one hour later by acetate-1-C^{14}. After a time lapse of
six hours, the rats were sacrificed, and selected tissues and organs
were freeze-dried and analyzed for radioactivity as described under

Methods. Results of this work are shown in Table 9.

Table 9. Comparison of the Radioactivity Remaining in the Tissues of
Adult Female Rats Given Acetate-1-C^{14}, Treated or Not Treated
with 100 mg of 2,4-D. The Rats were Sacrificed Six Hours
after the Acetate Administration.

cpm per 0.1 g of Tissue

| | No 2,4-D | | 2,4-D Treated | |
	Rat #1	Rat #2	Rat #1	Rat #2
Blood	1563	1543	1844	1917
Brain	1054	1093	1017	1165
Fat	950	984	499	712
Heart	1345	1363	1332	1362
Intestine	3099	3195	1323	1313
Kidney	1419	1451	1328	1203
Liver	2709	2754	1805	2173
Lungs	2796	2533	2686	2509
Muscle	753	919	858	956
Spleen	1955	2048	714	698
Stomach	2492	2432	16941	18043

From the counting data, it was apparent that the radioactivity

in the individual tissues of the 2,4-D treated rats differed signifi-

cantly from the corresponding tissues in the control rats. Of the

11 tissues analyzed, the brain, heart, kidney, lungs and muscle had

the same amount of radioactivity in 2,4-D treated and control rats.

The fat, liver, intestine and spleen all showed reduced radioactivity

under the influence of 2,4-D. However, the blood and especially the

stomach of 2,4-D treated animals were found to contain more radio-

activity than those of the controls. The exceptionally large amount

of radioactivity in the stomach of 2,4-D treated animals was rather

surprising since acetate is normally rapidly absorbed and metabolized.

These results indicated that 2,4-D was affecting the absorption and

transportation of acetate and/or its metabolites. The radioactivity

was about seven times higher in the stomach of 2,4-D treated rats than

in the stomach of control rats. This would indicate that the absorpt-

ion of acetate was being inhibited. The fact that four of the tissues

showed lower radioactivity in 2,4-D treated rats may mean that the

2,4-D was inhibiting the transportation and incorporation of the radio-

activity into these tissues. It may also mean that the 2,4-D was

slowing the synthetic activity of these tissues, or was possibly

increasing the catabolic activity. If the latter possibility is

assumed, it may account for the increased radioactivity in the blood

of the 2,4-D treated rats.

Armstrong, Schubert and Lindenbaum (2) gave $Na_2C^{14}O_3$ and $CaC^{14}O_3$

to rats and made a very extensive investigation as to the location of

the radioactivity in the tissues. Radioactivity was found in all of

the body tissues, including the bones and teeth. Since only gross

information was sought, Table 9, only a few of the organs and tissues

were studied.

VI. Comparative Study of 2,4-D Effect on the Labeling Pattern of

    Excreta from Rats Receiving Acetate by Intraperitoneal Injection

    or Oral Administration.

Comparisons of the tissue analyses of the rats treated and those

not treated with 2,4-D suggested that one of the actions of 2,4-D was

the inhibition of the absorption of acetate across the stomach wall.

If this is truly the case, then if 2,4-D were orally administered and

acetate were injected, the 2,4-D would have no effect. In this

series of experiments, 2,4-D was orally administered and one hour later

the acetate-1-C^{14} was injected into the peritoneal cavity.

Figure 10 shows the accumulative percent recovery of the expired $C^{14}O_2$ from treated and non-treated rats. From this figure, it is immediately apparent that the percent recovery of the administered radioactivity was reduced in the 2,4-D treated rat. However, the two curves appear to be very much the same. In Figure 11 the kinetic plot of the expired $C^{14}O_2$ shows that there are two distinctly different rates for the elimination of the $C^{14}O_2$. The most interesting observation here is that the 2,4-D treated rat showed just two rates of $C^{14}O_2$ elimination. In rats given both 2,4-D and acetate-C^{14} orally, the secondary, slower rate was not single as in the controls, rather it consisted of several fluctuating rates. Thus, the kinetic plot of the $C^{14}O_2$ expired from rats which had been orally administered 2,4-D and injected with acetate-1-C^{14} appeared to be very much like that for the acetate-1-C^{14} injected control. Again, this evidence lends support to the observation that 2,4-D was preventing the rapid and complete absorption of acetate from the stomach.

In Table 10 are compiled data showing the percent of the administered radioactivity recovered in the CO_2, urine and feces. From this table, one can see that rats given 2,4-D orally and the acetate-1-C^{14} intraperitoneally excreted slightly less radioactivity in the $C^{14}O_2$ than did the controls. The urine radioactivity was slightly greater for the 2,4-D treated rats.

Figure 10. Accumulative Percentage of Administered Radio-
activity Recovered in the Expired CO_2 of Adult
Female Rats Orally Treated or Not Treated with
100 mg of 2,4-D and One Hour Later Intra-
peritoneally Injected with Acetate-1-C^{14}.

Y-axis: Percent Radioactivity Recovered in Expired $C^{14}O_2$

●——● Control
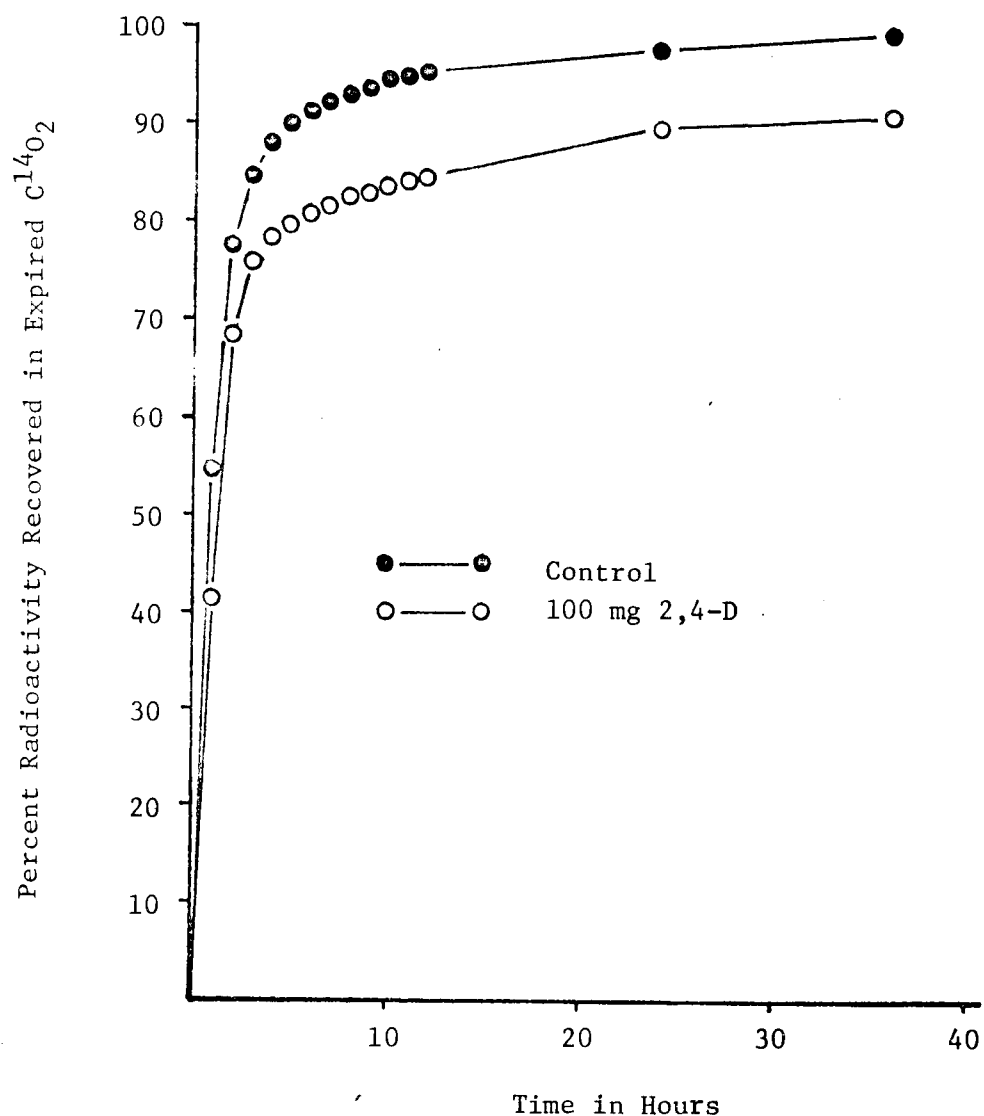○——○ 100 mg 2,4-D

Time in Hours

Figure 11. Kinetic Plot of the Expired $C^{14}O_2$ from Adult Female
Rats Orally Treated or Not Treated With 100 mg of
2,4-D; Receiving an Intraperitoneal Injection of
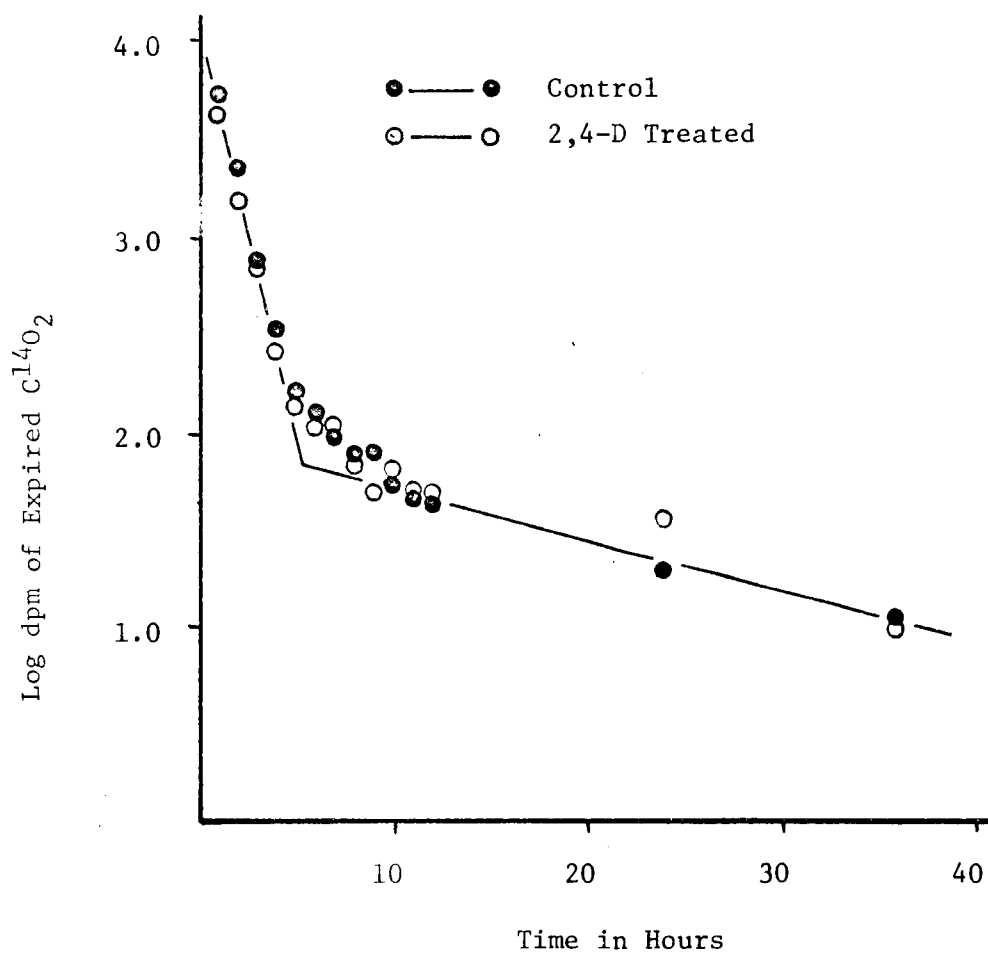Acetate-1-C^{14}.

Table 10. Comparison of the Percent of Administered Radioactivity
Recovered in the Excreta of Adult Female Rats, Treated
or Not Treated with 100 mg of 2,4-D, and Oral or Intra-
peritoneal Administration of Acetate-1-C^{14}.

Percent Radioactivity Recovered in 24 Hours

	No 2,4-D		Oral 2,4-D	
	Oral Acetate	Injected Acetate	Oral Acetate	Injected Acetate
CO_2	95.3	95.2	66.5	88.7
Urine	2.0	0.6	2.3	1.1
Feces	0.5	0.4	0.1	0.2
Total	97.8	96.2	68.9	90.0

Total Percent Radioactivity Recovered

	No 2,4-D		Oral 2,4-D	
	Oral Acetate	Injected Acetate	Oral Acetate	Injected Acetate
CO_2	96.9	97.6	85.1	90.0
Urine	2.5	0.7	2.8	1.2
Feces	0.5	0.5	0.1	0.3
Total	99.9	99.8	88.0	91.5

Table 10 shows an interesting comparison of the two methods of
acetate-1-C^{14} administration for both 2,4-D treated and non-treated
rats. The two types of control animals expired about the same percent-
age of the administered radioactivity in the form of $C^{14}O_2$. The orally
administered acetate appeared to lead to a greater percent recovery
of radioactivity in the urine, although, in both cases, the radio-
activity was quite low. The radioactivity from rats injected with
acetate-1-C^{14} was recovered much faster and more completely than
radioactivity from the rats given both 2,4-D and acetate orally. In
some very early work on acetate metabolism, Williams and Van Bruggen

(35) found that in the body tissues, the radioactive label depended

upon the route of tracer administration, either intravenous or intra-

peritoneal. No report was found in the literature comparing oral and

intraperitoneal administration. However, it is reasonable to assume

that the two different means of administration could lead to different

metabolic pathways.

Summary and Conclusions

The aim of this research was to determine the control metabolism of C^{14}-labeled acetate in adult rats, and then to determine if the herbicide 2,4-D would affect the normal metabolic pattern in the intact adult rat. The following is a summary of the important observations from this work.

Normal adult rats absorbed and metabolized C^{14}-labeled acetate rapidly. Although the greatest percentage of the administered radioactivity was recovered in the expired CO_2, small amounts of radioactivity were found in the urine and feces.

There were two rates of $C^{14}O_2$ elimination in the normal adult rat. The first rate was very rapid and had a biological half-life of four to six hours. The secondary rate was much slower and had a biological half-life of 20 to 25 hours.

The small amount of radioactivity recovered in the urine of normal rats was found to consist of several metabolites. In the urine of females treated with acetate-1-C^{14} or -2-C^{14} and males treated with acetate-1-C^{14}, were found two major metabolites. One of these metabolites was identified as urea. The second major metabolite, spot II, was thought to be a phenolic acid, but was not positively identified. Several minor metabolites of acetate-C^{14} were found, and their abundance was seen to be dependent upon the sex of the rat and the position of C^{14}-labeled acetate used.

Orally administered 2,4-D was found to have an effect on the slower rate of $C^{14}O_2$ elimination. Also, the presence of 2,4-D was

found to significantly reduce the percent recovery of the administered radioactivity.

Orally administered 2,4-D affected the pattern of radioactive metabolites found in the urine. In female rats given 2,4-D and acetate-1-C^{14}, the percentage of C^{14}-labeled urea increased while spot II decreased. However, in female rats given 2,4-D and acetate-2-C^{14}, the amount of urea decreased considerably while spot II increased. Male rats given 2,4-D and acetate-1-C^{14} showed a decrease in urea and an increase in the amount of spot II.

Of special interest was the fact that the quantities of the minor urinary metabolites fluctuated. The presence of 2,4-D resulted in the disappearance of some of the minor metabolites, and the appearance of others. Spot III, present as a minor metabolite in the acetate-2-C^{14} control, increased some 12 times under the influence of 2,4-D treatment. The presence of 2,4-D also resulted in the appearance of spot III with the administration of acetate-1-C^{14}, although it was absent in the control.

Since the rates for the elimination of $C^{14}O_2$ were different for acetate-1-C^{14} and -2-C^{14}, it was concluded that the two carbons of acetate did not follow identical pathways in their metabolism. Also, it was noted that 2,4-D had a greater effect on the further metabolism of the methyl carbon than on the metabolism of the carboxyl carbon of acetate.

Similar effects on the rate of $C^{14}O_2$ elimination were observed for ten to 150 mg of 2,4-D per rat. At five milligrams of 2,4-D or less per rat, the effect on the $C^{14}O_2$ elimination rate was decreased.

However, there was an increasing percentage of the administered radioactivity recovered as the dose of 2,4-D was decreased. There was no significant change in the radioactivity recovered in the urine and feces over the range of dosages used.

The time between 2,4-D treatment and acetate administration was varied from one hour to one week. No decrease in the effect of the 2,4-D on the $C^{14}O_2$ elimination rate was seen at any of the pretreatment time intervals from one to 48 hours. However, when the 2,4-D was administered one week before the acetate, no effect was seen on the acetate metabolism. As the pretreatment time increased, there was a corresponding increase in the percentage of the radioactivity recovered. It was concluded that with increased pretreatment time the 2,4-D had sufficient time to be eliminated from the body of the rat.

When the rats were killed six hours after acetate-1-C^{14} administration, several individual tissues were found to contain the same amount of radioactivity in the controls as their corresponding tissues in the 2,4-D treated rats. Some tissues had less radioactivity, while other, such as the blood and stomach, possessed much more radioactivity under the influence of 2,4-D than they possessed in the control animals. It was concluded that 2,4-D was inhibiting the absorption of acetate.

When rats were orally administered 2,4-D and intraperitoneally injected with acetate-1-C^{14}, there was no observed effect of the 2,4-D on the rate of $C^{14}O_2$ elimination. This observation supports the conclusion that 2,4-D inhibited the absorption of acetate. There

was noted a slight decrease in the percentage of radioactivity re-

covered from the rat. This decrease in the recovery of radioactivity

was probably due to the effect of the 2,4-D on the actual metabolism

of acetate.

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