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BODY BURDEN DETERMINATION AND METABOLITE  
IDENTIFICATION OF DIPHACINONE  
IN THE MOUSE

by  
William Patrick Cahill, Jr.

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A Dissertation Submitted to the Faculty of the  
COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements  
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College  
THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my  
direction by William P. Cahill, Jr.  
entitled Body Burden Determination and Metabolite  
Identification of Diphacinone in the Mouse  
be accepted as fulfilling the dissertation requirement for the  
degree of Doctor of Philosophy

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## ABSTRACT

This research was conducted in two parts. The first consisted of two studies investigating the tissue distribution and excretion of 1-<sup>14</sup>C-diphacinone in male and female mice as a function of time. The second part investigated the composition of diphacinone metabolites in mouse excreta.

The first body burden study tested 13 tissues plus excreta to establish which tissues contained the highest rodenticide concentrations. This study utilized tissue digestion prior to counting. The second study tested plasma and whole blood and those tissues containing the highest diphacinone concentrations. The tissues and excreta were prepared for counting in a commercial sample oxidizer.

The two studies revealed that the liver had the highest concentration of all tissues tested followed by the fallopian tubes, plasma, whole blood and lungs. Adipose tissue contained the lowest concentration.

Sixty-one and 65 per cent of the radioactivity was excreted in feces of male and female mice respectively by 48 hours during the first study. Accountability of administered dose was 59 per cent at 24 hours, 71 per cent at 48 hours and

75 per cent at 192 hours. Recoveries for the second experiment never exceeded 74 per cent.

The concentration of radioactivity was found in the second study to increase in the male liver up to 7.5 hours. The concentration within the female livers peaked around hour 3. These differences in liver concentrations were the only ones significantly different between the sexes.

The second part of this research was conducted using 1-<sup>14</sup>C and 2'-<sup>14</sup>C-labeled preparations of diphacinone. A number of suspected metabolites of diphacinone in mouse urine were shown to be absent by comparing retention times of these suspected metabolites and those of radioactive labeled metabolites from mouse urine determined on a HPLC adsorption and reverse phase column. Thus benzilic acid, diphenylacetic acid, diphacinone, p-hydroxybenzophenone, p-methoxybenzophenone, benzophenone, benzhydrol, dimethoxyphenylacetic acid, 1,3-indandione, 1,2,3-indantrione and 2-acetylbenzoic acid were not observed in mouse urine. In addition, highly suspected iron-diphacinone chelates were not detected in the urine. The sodium salt of diphacinone was not eliminated as a component of mouse urine by the comparison of retention times and  $R_f$  values. However, if this compound was present in mouse urine, it represented an extremely small per cent of the total activity.

The presence of suspected metabolites as conjugates in urine was checked for after acid hydrolysis. Evidence was obtained indicating the presence of a conjugate of diphenylacetic acid. Hydrolysis with acid and heat resulted in the disappearance of a major peak of the pre-hydrolysis elution profile and the appearance of a new radioactivity maximum at 42 minutes with a prominent shoulder at 40 minutes. The 40 minute maximum is in near agreement with the retention time of diphenylacetic acid ( $39\frac{1}{2}$  minutes). However, because of retention time sensitivity to slight changes in pH of the eluant or hydrolysate, the larger peak at 42 minutes may represent diphenylacetic acid.

The largest percentage of radioactivity found in mouse urine did not elute except with very polar eluants. Acid hydrolysis had no apparent effect on this material since an increase in radioactivity or appearance of new maxima were not observed during the first portions of profiles from this activity after hydrolysis.

Nearly all of the radioactivity found in fecal extract eluted only with polar eluants. No metabolites could be identified in this extract. Also, neither diphenylquinone nor its sodium salt was found in the feces.

## REVIEW OF LITERATURE

Diphacinone (2-diphenylacetyl-1,3-indandione; Figure 1A) was first described in a patent (The Upjohn Company 1954) wherein its synthesis, physical properties and anticoagulant property were described. Studies thereafter showed diphacinone to have unsuspected potency as an anticoagulant (Correll et al. 1952; Field et al. 1952; Saunders et al. 1955; and Heisey, Saunders and Olson 1956). For example, 0.05 mg/kg diphacinone was about as effective as 10 mg/kg dicumarol (3,3'-methylenebis(4-hydroxy-1,2-benzopyrone); Figure 1B) for producing prothrombinemia in rabbits (Correll et al. 1952). Diphacinone was also found to be more potent than warfarin (3-alpha-phenyl-beta-acetylethyl-4-hydroxycoumarin; Figure 1C) in rabbits after a single dose, as were the iron, copper, and sodium salts of this compound (Saunders et al. 1955). The potassium, nickel, zinc, manganese, and magnesium salts and salts formed from ammonia, lower alkylamines and lower alkanolamines also showed the same order of toxicity as the parent compound (The Upjohn Company 1956).

A second patent described an improved procedure for the synthesis and mentioned both the anticoagulant and rodenticidal properties of this compound (The Upjohn Company 1958). Subsequent papers established the importance of

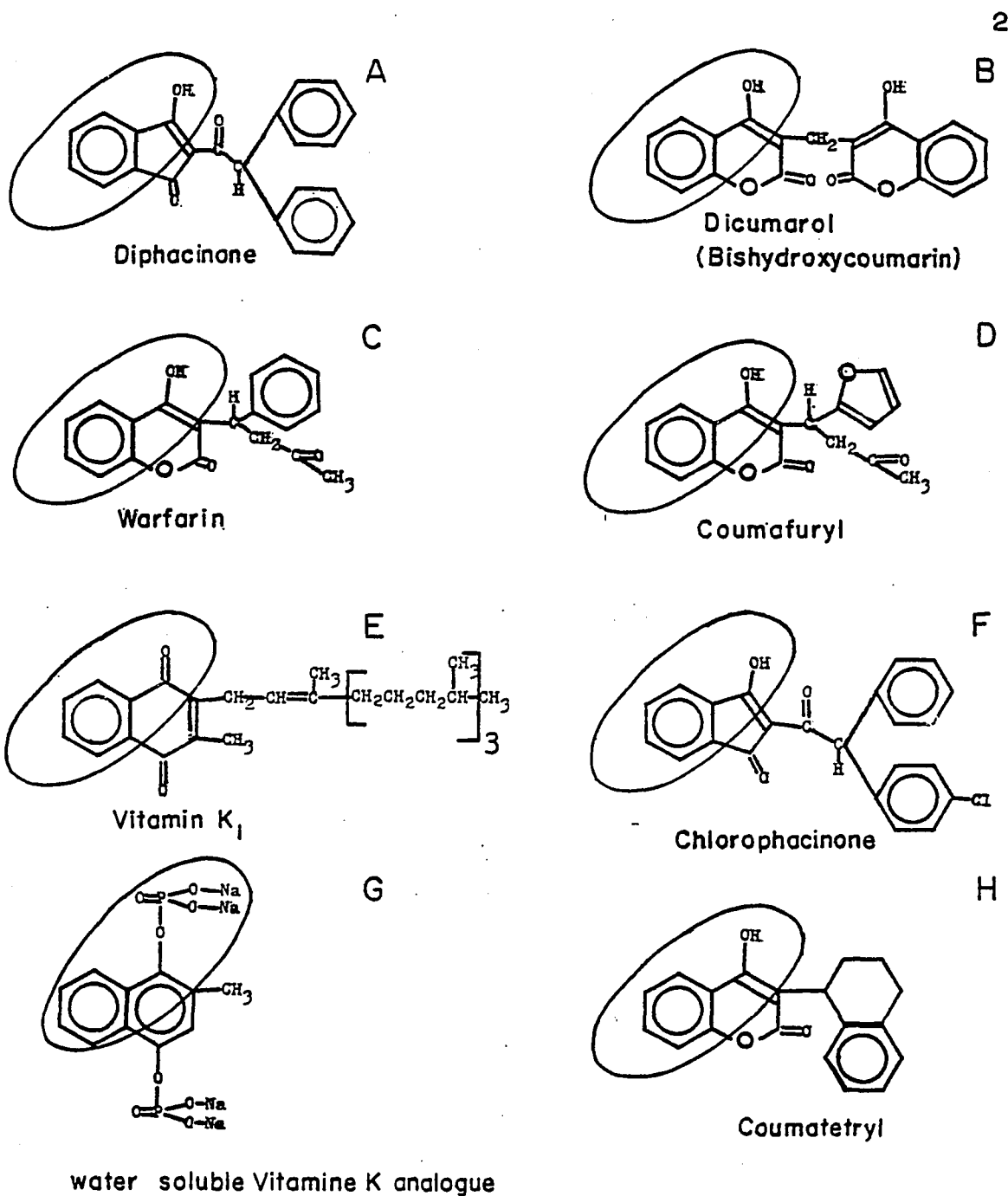


Figure 1. Formulas of Common Anticoagulant Rodenticides and Vitamin K Showing Proposed Active Site on Molecule.

this material and its preparations as a rat control agent in agricultural situations (Bently and Larthe 1959, Rowe and Redfern 1968, and Teshima 1970), urban areas (Barbehenn 1970, and Field 1972) and in industrial areas ("Perimeter Baiting" 1973).

A third patent pertained solely to the use of this compound and its salts as a rodenticide, citing numerous different mortality studies (The Upjohn Company 1956).

Studies which compared diphacinone with other common rodenticides were also made. Bently and Larthe (1959) found that diphacinone was effective against the black rat (Rattus rattus) at a concentration of 0.0125 per cent. Warfarin and coumafuryl (3-alpha-(2-furyl)-beta-acetylethyl-4-hydroxy-coumarin; Figure 1D) both used at 0.025 per cent were less effective. Rowe and Redfern (1968) reported that 0.0125 per cent diphacinone was approximately as toxic and acceptable to the house mouse (Mus musculus) as 0.025 per cent warfarin. Likewise, Hayes and Gaines (1959) found in laboratory trials using dosed bait, in which plain bait was available, that diphacinone was about as good as warfarin against the Norway rat (Rattus norvegicus). Table 1 summarizes the dosage (concentration in bait) levels currently recommended by the United States Public Health Service (1962).

In addition to controlling rodents, the compound has had excellent success in controlling the vampire bat

Table 1. Dosage Levels Recommended by the U. S. Public Health Service in 1962 for Anticoagulant Rodenticides.

Anticoagulant	Concentration in Bait (%)		
	<u>M. musculus</u>	<u>R. rattus</u>	<u>R. norvegicus</u>
Diphacinone	0.0125-0.025	0.005-0.01	0.005-0.01
Coumafuryl, Pindone	0.025-0.5	0.025	0.025
Warfarin	0.025-0.05	0.025	0.005-0.025

(Deamodus rotundus) in Central and South America (Thompson, Mitchell and Burns 1972; "Vampire Bat, A New Idea Works" 1972). One technique involved systemic administration of diphacinone at levels that were non-toxic to cattle, yet fatal to bats which fed upon these cattle. Another involved smearing a captured bat with petroleum jelly impregnated with the substance, and allowing the bat to return to its colony. Total colonies have been known to be eradicated by these methods. The compound was used for control of the deer mouse (Peromyscus spp.) in order to prevent destruction of conifer seed in California (Radwan 1970).

It is necessary at this point to discuss the very close physiological activity between coumarin and indandione derivatives used as rodenticides, since inference to the nature of the indandiones will be made from the more extensively studied coumarin compounds. Prior to the use of indandiones as rodenticides, these compounds along with various coumarins were used in medicine as prophylactic and therapeutic agents against thromboembolism. Poller (1962) in his book on the medical uses of these compounds made no distinction between these compounds regarding mode of action. He stated that vitamin K (Figure 1E) was antidotal for both classes of compounds, and that human patients that have developed resistance towards one class of compounds through extensive use were also resistant towards the other class

even though they have not used them in therapy. He further stated that both classes of compounds depress the production, in the liver, of at least 4 clotting factors. More recently, rats resistant to coumarin anticoagulants were shown to be resistant to the indandione type also, even though these animals or their predecessors had never been exposed to these compounds (Lund 1972). Additional insight into this similarity can be gained by inspection of Figure 1, p. 2.

The representative structures of the anticoagulant types are in part similar to that of vitamin K. If the active site of the enzymes responsible for synthesizing the clotting factors required the encircled portions of these structures, then the addition of any group on the benzene ring could destroy the activity of the compound. Overman et al. (1944) found that hydroxy groups, carbon residues, or halogens on the benzene ring of coumarin either reduced or eliminated the activity of these compounds.

The mode of action of diphacinone as an anti-coagulant was studied. Administration of the compound resulted in rapid decrease in factor VII and resulted in prothrombinemia (Vercillo and Moreo 1960). The extent and duration of such changes was dose dependent. Typically, administration of 15 - 45 mg of diphacinone in a single dose to rabbits resulted in a decrease of factor VII from 100 per cent to 10 - 35 per cent in 24 hours. Return to normal

occurred in 72 hours. Prothrombinemia was detected 18 - 72 hours after administration.

Other studies showed that diphacinone, in addition to causing a decrease in prothrombin, also diminished the prothrombin-proconvertin complex concentration and produced a significant lengthening in the blood coagulation time (Vanags and Selmens 1957, and Konyukhov 1964). Additional effects reported were decline in blood pressure concomitant with decreased respiration rate and depth. These effects were not attributed to a direct action on the heart nor did they appear to be centrally located (Rowe and Redfern 1968).

As with humans, vitamin K<sub>1</sub> (2-methyl-3-phytyl-1,4-naphthoquinone; Figure 1E) was found to be an effective antidote against these anticoagulants for the rat (Correll et al. 1952; Field et al. 1952; Heisey, Saunders and Olson 1956; and Vercillo and Moreo 1960). Typically, 20 mg of natural vitamin K neutralized the effect of a therapeutic dose of diphacinone in less than 24 hours. However, the water soluble vitamin K analogue (tetrasodium-2-methyl-1,4-naphthohydroquinone(diphosphate); Figure 1G) showed no antidotal activity (Correll et al. 1952).

Considerable evidence confirmed the occurrence of resistance towards anticoagulant rodenticides (Lund 1972). When resistance first appeared, certain investigators felt

that mutant strains had developed the ability to utilize vitamin K more efficiently. Mineral oil was thus tested in baits for these rats to prevent vitamin K absorption and found to be useful in overcoming their resistance to the rodenticide. However, when tested in the field this additive has little effect on the rat populations. Apparently the mineral oil prevented uptake of vitamin K from the bait but not from other sources (Drummond and Wilson 1968).

Antibacterial agents such as sulfaquinoxaline were also incorporated into baits to eliminate production of vitamin K by microbial action in the intestine. This was no more effective than non-treated bait (Lund 1964).

Thierry and Suttie (1969) studied the whole-body and subcellular distribution of carbon-14 vitamin K in resistant and normal rats. They observed minor differences in these distributions but concluded that these were insufficient to explain the increased vitamin requirement of resistant rats.

Mutation towards increased capability of serum protein to bind the anticoagulant was suspected and tested. Agents such as phenylbutazone were found to be without effect. Ethionine was also incorporated into baits to inhibit an increase in enzyme activity that might be responsible for

the rapid and excessive breakdown of warfarin, but was found to be unsuccessful (Lund 1972).

Pool et al. (1968) found no significant difference in the response of resistant rats to injected or oral doses of warfarin. It was therefore considered likely that the resistance was not due to the rapid excretion of warfarin or to its conversion to a non-toxic substance in the intestine.

Although Greaves and Ayres (1969) and Pool et al. (1968) demonstrated that the resistance to warfarin in Rattus norvegicus, found in Wales, was heritable by an autosomal dominant gene, Hermodson, Suttie and Link (1969) were the first to formulate a theory consistent with all known facts. They showed that normal and resistant rats metabolized carbon-14-warfarin at the same rate. On the other hand, vitamin K depleted, resistant rats, fed for 3 days on a low vitamin K diet and thereafter a measured quantity of vitamin K<sub>3</sub>, required much larger amounts of the vitamin to restore the prothrombin level of the blood than normal rats. They also found that while heterozygous resistant rats required about twice as much vitamin K<sub>3</sub> as susceptible rats, homozygous ones needed about 20 times as much as normal rats. This increased vitamin K requirement in resistant rats was the first significant differences observed between normal and warfarin resistant rats in any metabolic aspect other

than their resistance to the coumarin anticoagulants. These authors explained their data by assuming that vitamin K and the anticoagulant compete for a site on a protein necessary for the synthesis of vitamin K dependent clotting factors. They postulated that in a mutant rat the affinity of that site for the vitamin is lowered and its affinity for the anticoagulants is lowered to a much greater extent. In addition they found that the protein in question is located in liver cell ribosomes. They were able to show that ribosomes from normal rats were able to bind 2 - 3 times as much radioactive warfarin as those from resistant rats.

Lund (1972) provided additional data in support of the above hypothesis. Rats from the areas of resistance or offspring of crosses of these rats may die suddenly with internal bleedings, although they have never or have not for several months, been exposed to an anticoagulant. This is explained by their decreased ability to utilize vitamin K in the food. Although most of this resistance occurred in countries where only coumarin type compounds were used, resistance towards indandione compounds was known even though rodent populations had never been exposed to the latter compounds.

Matschiner et al. (1970) showed that in the presence of warfarin the epoxide of vitamin K, which is recycled by reduction to vitamin K in normal animals, accumulated and

appeared to be a competitive inhibitor of vitamin K in the synthesis of blood clotting factors. Zimmerman and Matschiner (1972) in preliminary studies showed that less vitamin K oxide accumulated in the liver of warfarin-resistant rats and proposed that the reductase was less sensitive to inhibition.

Lowenthal and MacFarlane (1964) determined that in the vitamin K deficient rats, the increase of the plasma level of factor VII by an optimal dose of vitamin K<sub>1</sub> (0.5 µg/100gm) can be partly or completely inhibited by the simultaneous administration of an indirect anticoagulant. When a ratio of vitamin K<sub>1</sub> to anticoagulant which produced partial inhibition was kept constant but the doses were increased, the inhibition at first increased or became complete, but when the doses were increased further, the inhibition began to decrease and finally disappeared completely. However, in anticoagulant pretreated animals, the simultaneous administration of additional anticoagulant had no effect on the response of the clotting factor production to vitamin K. They proposed that in addition to the normal site of action larger doses of vitamin K can act at an alternate site or by another mechanism. Indirect anticoagulants inhibited the action of vitamin K at the normal site by an antagonism which was not of the competitive type while larger

doses of the vitamin reversed the inhibition by acting at the alternate site, which was anticoagulant insensitive.

Lowenthal, MacFarlane, and McDonald (1960) and Lowenthal and MacFarlane (1967) further showed that the 2-chloro analog of vitamin K (2-chloro-3-phytyl-1,4-naphthoquinone) competitively antagonized the effect of vitamin K at the secondary site but had no effect on the coumarin sensitive site.

Martin (1973) determined that rats resistant to chlorophacinone [2-((p-chlorophenyl)phenylacetyl)-1,3-indandione; Figure 1E] or to warfarin and coumatetryl (3-(alpha-teteryl)-4-hydroxycoumarin; Figure 1H) required 30 times more vitamin K than normal rats to bring about a recovery in the coagulation activity to 50 per cent of its normal value after it was depressed by vitamin K free diets. They also showed that the amounts of these rodenticides required to reduce the coagulation activity to 50 per cent of normal was 100 times greater for the resistant than for the susceptible rats for warfarin and chlorophacinone, but only 30 times for coumatetryl. These results were in agreement with the work of Hermadsen, Suttie and Link (1969) and were compatible with the hypothesis for the presence of a protein receptor with a decreased binding affinity for these anticoagulants, although the binding affinity at this receptor site was not identical for all 3 compounds. In

contrast, they verified the Lowenthal and MacFarlane work in 1967 that showed that the 2-chloro analog of vitamin K had virtually the same anticoagulant potency in both warfarin susceptible and resistant strains of rat. They conjectured that this receptor site, which was identical to the vitamin K coumarin insensitive receptor, remains unaltered in the warfarin resistant rat and was therefore equally available for occupation by 2-chloro vitamin K or any other competitive inhibitor of vitamin K.

An interesting sidelight to the problem of rodent control is the problem of palatability of the compound. Although the parent compounds are very unpalatable and therefore must be used at extremely low levels in baits, a recent new problem has arisen in this area. Since the patents have elapsed for certain anticoagulants, especially warfarin, new manufacturers are apparently more lax in their quality control and produce a product containing unpalatable side products. This was determined not only by chemical analysis, but also by feeding tests comparing old with newer batches. Recently newer batches of diphacinone have also been shown to be less palatable than older batches (Bowerman and Brooks 1972).

Ikeda, Conney, and Burns (1968) showed in in vitro studies with carbon-14 warfarin that it was hydroxylated in positions 6, 7, and 8 and that this occurred in the liver

microsomes and required NADPH and oxygen. Another unidentified metabolite was formed in the soluble fraction of liver and required NAD but not oxygen. In addition, they showed that dosing the rats for 4 days with phenobarbital, chlordane or DDT prior to isolating the livers increased the LD<sub>50</sub> value 10 fold and caused a 3 to 5 fold increase in the 3 hydroxylation reactions. They concluded that pretreatment of rats with these 3 chemicals stimulated the metabolism of warfarin in vivo and decreased its toxicity.

Barker, Hermodson and Link (1970) fed <sup>14</sup>C-warfarin to rats and used paper chromatography to resolve the activity in rat urine extracts into six spots, several of which were double headed peaks. Thin layer chromatographic techniques were used to resolve these peaks into separate components. The identified metabolites found were: 6-hydroxywarfarin, 7-hydroxywarferin, 8-hydroxywarfarin as were found by Ikeda, Conney and Burns (1968) in vitro and in addition 4'-hydroxywarfarin and 2,3-dihydro-2-methyl-4-phenyl-5-oxo-gamma-pyrano(3,2-c)(1) benzopyran. Upon enzymatic hydrolysis with glucuronidase, additional 7-hydroxywarfarin was formed with the loss of 1 of the peaks. This metabolite was assumed to be the glucuronate of the 7-hydroxy compound. They also found the same metabolites in extracts of rat feces. However, the relative quantities of the metabolites were somewhat

different. Most of the radioactivity was excreted in the first 2 days.

A great many investigations have been made, mostly with the coumarin type compounds, on the different aspects of anticoagulants. Comparable investigations of the indandiones are lacking. In particular the body or organ distribution and metabolic fate of diphacinone has never been described. It was for this reason, therefore, that the present study was undertaken.

PRELIMINARY EXPERIMENTS AND  
BODY BURDEN STUDIES

Introduction

The increasing importance of diphacinone for cattle protection from the vampire bat (pp. 3, 5 ) has heightened the demand for basic chemical and toxicological information on this compound. The physiological response elicited by diphacinone, its mechanism of action and effective antidotes are known. However, data required as part of the legal registration requirements were lacking on the chemical stability and body burden studies for diphacinone. This dissertation describes the results of such studies. In addition, an LD<sub>50</sub> study was conducted because of the large disparity existing between the literature values for diphacinone.

Diphacinone

The non-labeled and 1-<sup>14</sup>C-diphacinone preparations used in these studies were synthesized by modifications of the procedure described in a patent (The Upjohn Company 1954). The procedures were furnished by the Velsicol Chemical Corporation. The millimolar specific activity of the 1-<sup>14</sup>C-diphacinone was 6.61 mCi/millimole. Purity of this carbon-14 preparation, as confirmed by thin layer

chromatography and autoradiographic techniques, was greater than 98 per cent. Only 1 minor impurity was detected.

Benzhydryl labeled diphacinone was obtained from New England Nuclear, courtesy of the Velsicol Chemical Corporation. An extremely small amount of this material was available and was therefore utilized only during the metabolite identification study. This product was greater than 99 per cent pure.

To date no numbering system has been observed in the literature to designate those carbon atoms comprising the acetyl portion of the diphacinone molecule. The numbering system shown in Figure 2 is therefore suggested and will be used on occasion throughout this work.

#### Chemical Stability of Diphacinone

The chemical stability of diphacinone was determined in buffered solutions ranging in pH from 2 to 12 at 24°, 50° and 80°C over a period of 17 days. Buffers were prepared as described on p. D-79 of the CRS Handbook of Chemistry and Physics (1966-1967). The pH of each buffer was checked using a pH meter and found to be within  $\pm 0.15$  pH unit of that desired. The buffer recipes used are listed in Table 2 since more than one choice is possible for each pH.

A standard solution of 1-<sup>14</sup>C-diphacinone, containing 10 mg (194.4  $\mu$ Ci) in 2 ml distilled acetone was prepared from which 125  $\mu$ l (equivalent to 0.625 mg or  $2.7 \times 10^6$  dpm)

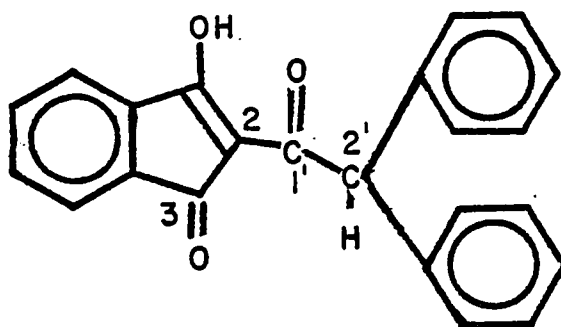


Figure 2. Proposed Numbering System for Diphacinone.

Table 2. Composition of Buffers for Hydrolysis Studies of Diphacinone.

pH	Composition of Buffer
2	75 ml 0.2M KCl, 19.5 ml 0.2M HCl
4	100 ml 0.1M KH Phthalate, 0.2 ml 0.1M HCl
6	100 ml 0.1M $\text{KH}_2\text{PO}_4$ , 11.2 ml 0.1M NaOH
8	50 ml 0.1M $\text{KH}_2\text{PO}_4$ , 46.1 ml 0.1M NaOH
10	100 ml 0.05M $\text{NaHCO}_3$ , 21.4 ml 0.1M NaOH
12	50 ml 0.05M $\text{Na}_2\text{HPO}_4$ , 26.9 ml 0.1M NaOH

was measured into each of 18 glass scintillation vials. The acetone was removed by evaporation using an air stream. Five ml of the appropriate buffer was added, and the solution was swirled and agitated using a "Vari Whirl" for 5 minutes. Six vials were capped and stored in the dark at room temperature while 6 vials each were placed in thermostatically controlled cabinets at 50° and 80°C.

Aliquots were removed after 1, 3, 5, 7, 15, and 17 days. The solutions at pH 2 and 4 showed turbidity upon addition and agitation of the buffer while the other solutions remained clear. Ten  $\mu$ l of each buffer was spotted directly on a 2.0 mm silica gel thin-layer chromatography plate and allowed to dry under the slight heat of a hair dryer. The plates were then developed with benzene: methanol: triethylamine (7:2:1) and the radioactive degradation products were detected by autoradiography. A common black ink containing enough radioactivity to darken the X-ray film upon 2 day exposure was placed on each of 3 corners of the developed TLC plate prior to autoradiography to provide a means of realigning the film on the plate after development. Kodak Blue Brand medical X-ray film BB-54 fast screen type was used and developed with Kodak liquid X-ray developer followed by Kodak Rapid Fixer.

## Results and Discussion of Chemical Stability Study

Evaluation of the X-ray films used to monitor TLC plates spotted with the various buffered solutions showed no decomposition at room temperature or 50°C through 17 days. No degradation was observed through day 3 at 80°C for any pH. The following remarks refer only to the 80°C mixtures. Slight degradation was detected on day 7 only at pH 6. By day 10 an increased decomposition in terms of the number and intensity of the spots on the films was apparent. This could be determined in a semi-quantitative manner relative to another time due to uniformity of aliquot size spotted and time of exposure of film. Six components not detectable in the diphacinone standard were observed after 10 and 13 days at pH 6. At pH 10 or 12 neither degradation nor the impurity present in the diphacinone standard which migrated near the solvent front was observed. This component, the red impurity of all diphacinone syntheses, was not completely removed during the purification procedure. Apparently strong alkali alters or destroys and prevents its formation. On the other hand, this compound appears more intense on days 10 and 13 at 80°C for pH 2-8. The spot is particularly intense for pH 2 on day 13. All other degradation products observed at 80°C after 10 and 13 days appeared as faint spots relative to the parent compound or the red impurity. The  $R_f$

values for the components formed in pH 6 buffer are listed in Table 3.

Table 3.  $R_f$  Values of Radioactive Decomposition Products of 1- $^{14}\text{C}$ -Diphacinone, pH 6 at 80°C.

$R_f$	Relative Intensity
0.047	dark --- diphacinone
0.11	medium
0.25	medium
0.45	faint
0.51	medium
0.61	medium
0.93	dark --- red impurity

#### Summary of Chemical Stability Study

Diphacinone was found to be stable at pH 2 to 12, at room temperature and at 50°C for 17 days. No decomposition was noted through day 3 at 80°C. Very slight decomposition was observed on day 7 for pH 6. Decomposition increased with time, but never appeared to exceed 10 - 20 per cent by visual estimation. The compound was resistant to degradation by alkali at all temperatures studied and least stable at pH 6 at 80°C. Some decomposition occurs in acid pH at 80°C upon prolonged exposure. The concentration of

the red impurity appeared to increase with decreased pH at 80°C.

#### Experimental Animals

Four to 5 week old mice, CD-1 Swiss variety, were obtained from the Charles River Breeding Laboratory, Inc., Wilmington, Mass. Upon arrival the mice were housed by sex in translucent polypropylene and transparent polystyrene cages fitted with wire grid covers with integral feeder and bottle holders. Mice were fed Wayne Laboratory Animal Diet "Lab Blox" for rats, mice and hamsters. Bedding material consisted of 3 parts pine chips to 1 part cedar chips. Twelve mice were housed in the 8" by 16½" (0.92 ft<sup>2</sup>) cages and 8 mice in the 11" by 7" (0.54 ft<sup>2</sup>) cages. Cages were cleaned at least once a week. Mice were allowed to acclimate to their surroundings for at least 1 week prior to experimentation.

#### LD<sub>50</sub> Study

The toxicological literature on diphacinone reveals a wide range for the oral LD<sub>50</sub> for mice. The Upjohn Company (1971), for example, found that for deaths up to 6 days, the LD<sub>50</sub> value was 343 mg/kg. Fontaine et al. (1965) found in a similar study that the LD<sub>50</sub> value was 750 mg/kg. Because of the large range of LD<sub>50</sub> values, the following study was conducted to establish a narrower range of these values and

to determine whether a sex difference response could be demonstrated for this compound.

#### Dosing Solution

A stock dosing solution was prepared by dissolving 600 mg of non-labeled diphacinone in 24 ml of corn oil (Mazola). The mixture was heated in boiling water to facilitate the dissolving process, during which time the volumetric flask was inverted numerous times to assure homogeneity. The solution was cooled slowly until the level of the corn oil lowered to the meniscus, thus giving a solution of 600 mg per 25 ml. Since this concentration slightly exceeds the solubility of diphacinone at room temperature, the initial dilution was made while the solution was warm. The various dosing solutions were prepared by serial dilution of this stock solution. The scheme used for the preparation of the dosing solutions is shown in Table 4.

#### Dosing Procedure

The mice were dosed with a 1 cc tuberculin syringe adapted with a feeding tip. Five mice of each sex were dosed at each level shown in Table 4. The average time at which each cage was dosed was recorded. Control mice were given 0.60 ml of corn oil.

Table 4. Preparation of Dosing Solutions for LD<sub>50</sub> Study.

Dosing Solution	Preparation	Dosing Volume (ml)	Animal Burden (mg/kg)
Stock	600 mg / 25 ml	0.75	900
Stock	600 mg / 25 ml	0.50	600
A	5 ml stock to 10 ml	0.50	300
B	5 ml A to 10 ml	0.67	200
C	5 ml B to 10 ml	0.80	120
D	5 ml C to 10 ml	0.65	48.8

### Results of LD<sub>50</sub> Study

The difference in susceptibility to diphacinone between the sexes is best observed for the 300 mg/kg doses (Table 5). Five female mice compared to 3 males were dead within 21 hours after dosing at the 300 mg/kg level with no deaths during this time for female controls. The fourth and fifth males died after 59 and 77 hours.

The lethality of doses greater than 300 mg/kg was apparently not due to the anticoagulant effect of this compound. Mice dosed at this or higher levels showed signs of nervousness and muscle twitching progressing to spasms prior to death. Only occasional slight external bleeding was observed for these higher doses compared to the lower doses, where frequent bleeding was observed from the feet, eyes, ears, mouth and anus.

No difference was observed between male mice dosed at 48 mg/kg and the male controls (Table 6). Beyond 111 hours, at this dose, a difference was observed between dosed and control females. The data for 120 and 200 mg/kg doses are inconclusive with respect to male versus female mortality. At no level of dose does the data indicate that male mice were more susceptible to diphacinone than female mice.

The mortality data was adjusted using Abbott's formula and analyzed on probit logarithm paper (Figure 3). The 72 hour mortality for the male mice indicated an LD<sub>50</sub>

Table 5. LD<sub>50</sub> Determination at 300 to 900 Dose Equivalents for Male and Female Swiss CD-1 Mice.

Dose Equivalent mg/kg	Sex	Hour After Dosing						
		4	7	9 $\frac{1}{2}$	11 $\frac{1}{2}$	21 $\frac{1}{2}$	76 $\frac{1}{2}$	108
900	Female	4 <sup>a</sup>	-	5	-	-	-	-
	Male	3	-	4	-	5	-	-
600	Female	5	-	-	-	-	-	-
	Male	-	1	-	4	5	-	-
300	Female	-	2	-	3	5	-	-
	Male	-	1	-	1	3	4	5
Control	Female	0	0	0	0	0	2	2
	Male	0	0	0	0	0	1	1

<sup>a</sup>Cumulative mortality.

Table 6. LD<sub>50</sub> Determination at 48 to 200 Dose Equivalents for Male and Female Swiss CD-1 Mice.

Dose Equivalent mg/kg	Sex	Hour After Dosing												
		9 $\frac{1}{2}$	27 $\frac{1}{2}$	46	60	76	81	103	108	117	129	170	189	216
200	Female	1 <sup>a</sup>	2	-	-	-	3	-	5	-	-	-	-	-
	Male	-	-	1	-	-	3	5	-	-	-	-	-	-
120	Female	-	-	-	-	-	-	-	3	-	4	-	5	-
	Male	-	-	-	-	-	-	-	3	-	-	4	-	-
48	Female	-	-	-	1	-	-	-	3	-	4	5	-	-
	Male	-	-	-	1	-	-	-	-	-	-	-	-	-
Control	Female	-	1	-	2	-	-	-	-	3	-	-	-	-
	Male	-	-	1	-	-	-	-	-	-	-	-	-	-

<sup>a</sup>Cumulative mortality.

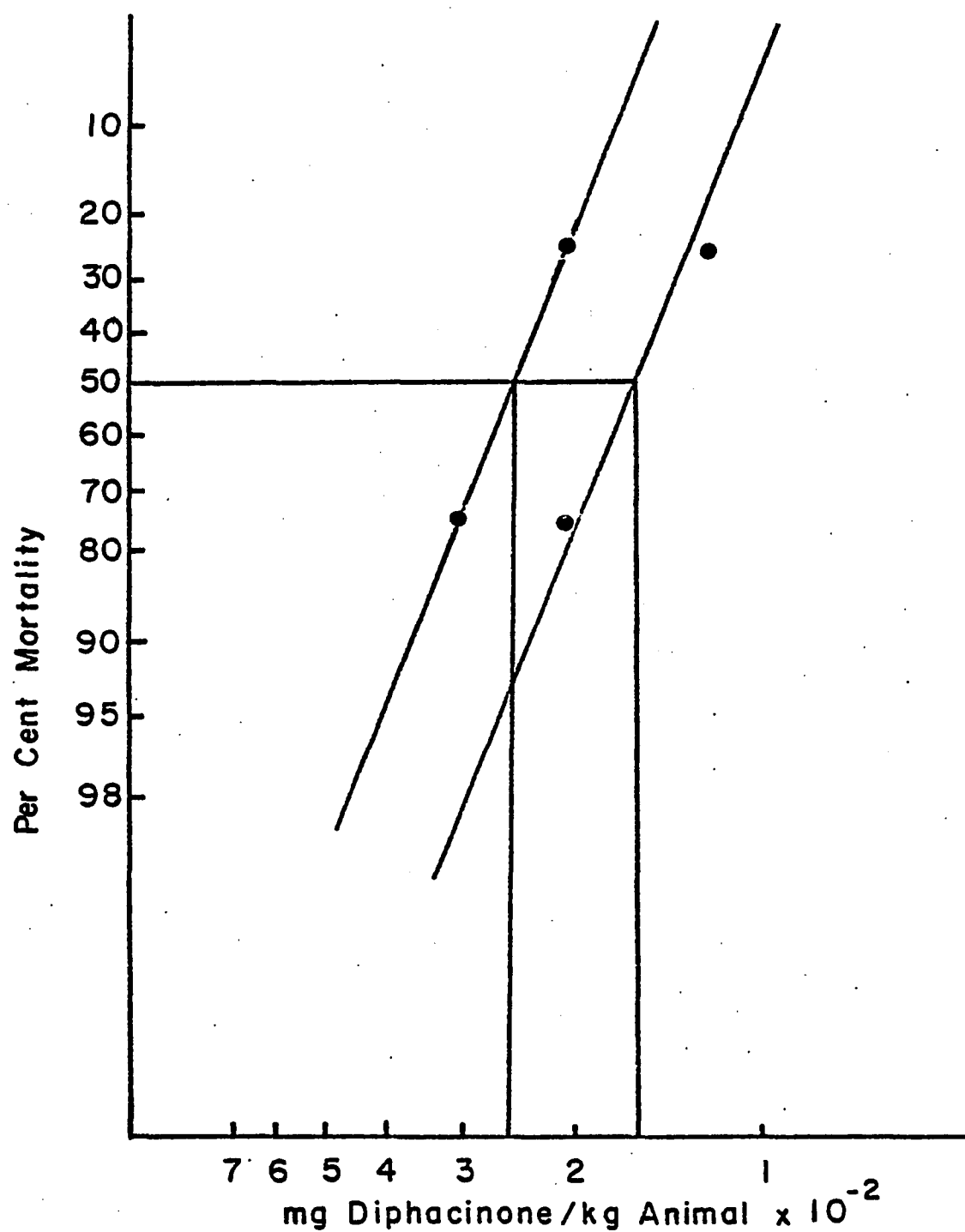


Figure 3. Plot of Per Cent Mortality versus Quantity of Diphacinone for LD<sub>50</sub> Determination.

of approximately 245 mg/kg while the 96 hour data indicated an LD<sub>50</sub> of approximately 155. Due to the limited number of mice per dose level and the relatively high mortality of the controls, these data can only approximate the LD<sub>50</sub> but do narrow the range for these values. It is seen that these rough values are in closer agreement with those determined by the Upjohn Company (1971) than those determined by Fontaine et al. (1965). In particular, at the value of 750 mg/kg given by Fontaine, all mice would have died within 21 hours in this study.

#### Calibration of Scintillation Counter

The Nuclear-Chicago Corporation Scintillation Counter, Model 6833, used in these studies, was equipped with dual scaler/timer, 100 sample changer, external standard (Radium 226), data calculator/listner and matched pair of multiplier phototubes. The settings for the calibration of the counter for counting efficiency were attenuation 16.1 with the upper discriminator at 9.9 v and the lower at 0.5 v (integral counting). These same settings were used for counting standards in which no quench occurred. Optimum counts with a normal background were attained with these settings.

### First Method

Carbon-14 labeled toluene used for this calibration was obtained from Packard Instrument Company, Inc. as a non-sealed source, specific activity of  $5.69 \times 10^5$  dpm/g  $\pm$  3%. The toluene was cooled at 0°C at which temperature its density is 0.884 g/ml.

To a glass counting vial was added 10 ml Aquasol<sup>R</sup> and 25  $\mu$ l of the cooled toluene (22.1 mg) equivalent to  $12.6 \times 10^3$  dpm. The sample was dark adapted for 10 minutes and counted 12 times for 1 minute each. The ratio of averaged to theoretical dpm was 9,124:12,575 for an efficiency of 72.6 per cent.

### Second Method

This procedure was similar to the first method except that a sealed source of <sup>14</sup>C-toluene was used. The toluene standard obtained from Amersham-Searle (activity of 32,000 dpm) was contained in a standard glass scintillation vial and sealed to protect against loss by volatilization. The sample was counted 10 times for 5 minutes each. The average dpm was 26,404 which corresponds to a machine efficiency of 82 per cent.

Determination of Specific Activity  
of 1-<sup>14</sup>C-Diphacinone

Carbonyl-<sup>14</sup>C-dimethyl phthalate, obtained from New England Nuclear through the Velsicol Chemical Corporation, possessed an activity of 22.8 mCi/0.669 g (34.1 mCi/g), equivalent to 6.61 mCi/millimole. The diphacinone synthesized from this material should therefore have the same molar activity of 6.61 mCi/millimole equivalent to 19.4  $\mu$ Ci/mg. One ml of a 50 ml toluene solution containing 3.4 mg of 1-<sup>14</sup>C-diphacinone (66.1  $\mu$ Ci) was counted in 10 ml of Aquasol<sup>R</sup> cocktail. The average dpm of 2 - 1 ml aliquots, each counted for 10 - 1 minute periods and corrected for background (45 cpm), was  $2.27 \times 10^6$  dpm. After correction for machine efficiency (72.6 per cent first method), the corrected value was  $3.12 \times 10^6$  dpm. The theoretical value for the millimolar specific activity of 1-<sup>14</sup>C-diphacinone was  $2.93 \times 10^6$  dpm. This difference is within the experimental error of the original weighing (assuming 0.2 mg as balance sensitivity).

Using the value for the counting efficiency as determined by the second method, the calculated dpm for the 0.068 mg diphacinone was  $2.78 \times 10^6$  dpm, again within the experimental error of the original weighing.

Carbon Dioxide Respiratory  
Study

Methods and Materials

The carbon dioxide respiratory studies were conducted in an all glass Roth metabolism cage (Roth et al. 1948), Delmar Science Laboratory, Chicago, Ill. In each study, 3 mice were each administered 0.4 ml (5.3  $\mu$ Ci) of the dosing solution containing 1- $^{14}$ C-diphacinone equivalent to 13.6 mg/kg. The metabolism cage was lubricated at all joints to produce an air tight system into which a deliberate small leak was produced by insertion of a stopcock into 1 of the joints. Air was pulled through the chamber and then through 100 ml of ethanolamine to absorb the carbon dioxide. In the first study the trapping solution was replaced every 24 hours for 4 days, while in the second study a single trapping solution was analyzed only at the end of 5 days. During the second study excreta were not removed in order to determine whether any volatile activity might be detected as the result of microbial degradation.

One ml aliquots of the trapping solution, the ethanolamine quenched counting standards and background standards quenched with 1 ml of ethanolamine, were counted for 4 minutes using standard counting procedures. The trapping solution cpm were corrected for a slightly increased quench over that of a counting standard containing 1 ml of the stock ethanolamine.

## Results and Discussion

No significant difference in dpm was found between 1 ml aliquots of the trapping solution and the background standard in the first study, when counted using the channel ratio method. The results for the second study showed a small but significant difference between the 1 ml aliquot of the trapping solution and the background standards. The average value for the 1 ml aliquot of trapping solution, counted 3 times for 4 minutes each, was 37 dpm while the average for the background standard was 30 dpm. This difference of 7 dpm is equal to 700 dpm for the 100 ml of trapping solution or less than  $2 \times 10^{-3}$  per cent of the total administered dose.

It is very unlikely that any labeled carbon dioxide could be evolved from the 1- $^{14}\text{C}$ -diphacinone administered to the mice for these 2 studies because of the difficulty in breaking the benzene ring-carbonyl carbon bond required to form the product. It cannot be concluded from this study, however, that carbon dioxide is not liberated during the metabolism of diphacinone. Evidence that carbon dioxide was also not released from the carbonyl group adjacent to the benzhydryl carbon is furnished in the metabolite identification section by conclusively showing that benzophenone, benzhydrol, and some of their hydroxylation products were not detected.

### Calibration of Microapplicator

A modified ISCO-Model M microapplicator was used for dosing in the body burden studies. The modification allowed sufficiently large doses to be delivered in less than 30 seconds and still have the desired accuracy. The needle of the syringe was replaced with a length of intramedic polyethylene tubing, the end of which was fitted with approximately an inch of the tip of a feeding syringe.

The device was calibrated by weighing the corn oil delivered by a single drive, which included waiting for 10 seconds after the drive was completed, and touching the tip to a tared beaker. No further corn oil exuded after this time interval. The range and standard deviation for 30 deliveries, over the range of the syringe were as follows: range, 0.141 to 0.149 g; standard deviation, 0.0023.

### Introduction to Body Burden Studies

A body burden study of an ingested substance measures the distribution of the compound and its metabolites within the organs and tissues of the animal and the rate and extent of excretion. These quantitative relationships suggest likely interactions not implicitly studied. For example, the site of the mechanism of action of the chemical or its biotransformation to metabolites is suggested by preferential uptake or affinity of the compound by an organ

or tissue. In the case of a labeled compound, the partitioning of the radioactivity between the urine, feces and carbon dioxide suggests the nature of the metabolites and therefore the type of detoxification occurring within the animal. Such studies then become the basis for an inquiry into the nature of the subcellular interactions of the chemical. In addition, these studies are required by law in order for a compound to obtain registration for certain uses. The necessity of these studies thus transcends the scientific and extends into the practical as well.

#### Body Burden Study I-- Methods and Materials

##### Dosing Solution

A stock solution of 1-<sup>14</sup>C-diphacinone was prepared by dissolving 25.8 mg of the anticoagulant (specific activity 19.4  $\mu$ Ci/mg) in 25 ml of corn oil (Mazola). This was diluted 1 : 1 with corn oil, forming the dosing solution (1.61  $\mu$ Ci/0.0825 mg/0.16 ml) used for both the male and female studies.

##### Dosing Procedure

Thirty male and 18 female mice were dosed using the modified microapplicator, with 0.16 ml of the diphacinone dosing solution (4.13 mg/kg). Just prior to dosing the tip of the feeding syringe was touched to an adsorbent tissue to remove adhering solution. After the 6 second drive phase

of the applicator, the feeding syringe tip was left within the mouse's stomach for an additional 20 seconds to permit the pressure head generated by the drive phase to dissipate and deliver the desired volume. Three mice of the same sex were placed in a cage resulting in 10 cages with males and 6 with females. The average time of dosing was recorded for each cage to establish collection times for urine and feces and eventual sacrifice.

#### Sampling Procedure

The sampling schedule for male mice was 3, 6, 12, 24, 48, 72, 96, and 192 hours. Feces and urine were collected at the time of sacrifice or every 24 hours and composited for each cage of mice sacrificed beyond 24 hours. The mice were sacrificed individually with chloroform. One cage of 3 mice at each sampling period (3 replicates) and 3 cages at hour 192 (9 replicates) were sacrificed. The female schedule was 6, 24, 48, and 192 hours. The same number of animals was sacrificed as for the corresponding hours in the male study.

In addition to urine and feces, epididymis, fallopian tubes, heart, kidney, lipid, liver, lung, skeletal muscle, small intestine, spleen, testes and visceral muscle were analyzed for radioactivity. The entire organ or sufficient tissue was placed in 1-ounce preweighed opaque storage bottles and frozen immediately. The visceral muscle, transversus abdominis, which lines the abdominal cavity was easily

removed in long strips. The skeletal muscle was taken from the muscle masses of the hind leg. The large mass of yellowish adipose tissue surrounding the ovaries or testes was taken unless absent, in which case adipose tissue was removed from the wall of the abdominal cavity. Only sufficient small intestine, randomly chosen with respect to proximity to the stomach, was taken to insure 3 subsamples.

The urine-feces separators were constructed from 5 gallon water bottles after the method of Halladay (1973). The feces were placed in 1-ounce sample bottles. The urine was stored in graduated centrifuge tubes. All excreta were frozen until time of assay.

#### Tissue Preparation for Radioassay

Each organ was subsampled (replicated) 3 times. The size of the subsample was selected to insure a counting efficiency of at least 30 per cent. Occasionally an efficiency below this value was encountered, especially for the feces. The range of subsample sizes for the various tissues and the lowest counting efficiency corresponding to the largest subsample for a particular tissue are listed in Table 7.

Subsamples taken from the frozen organs were weighed into polyethylene vials and digested with 1-ml of NCS<sup>R</sup> (Amersham Searle Corporation) on a hot plate adjusted not to exceed 50°C. The digestion, although usually complete within

Table 7. Tissue Subsample Sizes Required for Suitable Counting Efficiencies.

Tissue	Subsample Size	Lowest Counting Efficiency
Liver	15-35 mg	32%
Adipose	100-350 mg	30%
Brain	30-100 mg	40%
Spleen	15-35 mg	30%
Small Intestine	50-100 mg	30%
Fallopian Tubes	50-150 mg	40%
Urine	1 ml	58%
Feces	15-20 mg	20%
Epididymis	40-120 mg	34%
Testes	30-100 mg	49%
Muscle	15-60 mg	30%
Kidney	15-50 mg	30%

4 hours, was terminated when the digest was clear. Occasionally an additional ml of NCS<sup>R</sup> was added and the sample heated overnight at 35 - 30°C. If the tissue was still not completely digested, the sample was nevertheless counted, since only small amounts of a thin connective membrane remained. Upon complete digestion, the solutions were brown to yellow depending on the tissue. After cooling to room temperature, 15 ml of Aquasol<sup>R</sup>-2 (New England Nuclear) was added to the vial and the contents gently mixed.

Digestion of feces proved difficult, since not only were small subsamples required, but also the digestion was always incomplete. This was observed during Body Burden Study II where approximately 10 per cent of the feces could not be oxidized to carbon dioxide and water but formed an inorganic "clinker" not easily decomposed by strong acid or base. The resulting fecal digests were darkly colored. Treatment with 20 - 30 mg benzoyl peroxide per vial bleached these digests to a pale yellow color. This condition only lasted for a few hours. It was followed by the formation of a purple color in many cases. The fecal digests were not successfully bleached and were counted at low efficiencies (20 - 40 per cent).

#### Counting Procedure

Quench curves were prepared by plotting per cent efficiency of counting versus channel ratio. Quench

standards were prepared for each specific tissue by adding varying amounts of the tissue digest to a series of scintillation vials containing known amounts of 1-<sup>14</sup>C-diphacinone. The quench standards were also used to establish discriminator and attenuator settings which permitted maximum dpm detection. These settings were found to be essentially identical for each tissue. The discriminator settings were 0.5 - 9.9 v for channel A and 0.8 - 9.9 v for channel B, fixing a ratio B/A of about 0.75 for non-quenched standards. The attenuator setting used throughout this study was E - 100. The equations used to calculate specific activity of the tissues and the per cent of the administered dose found in tissues are discussed in Appendix A.

#### Body Burden Study I-- Results

The tissue uptake of diphacinone in males in order of decreasing specific activity was: liver > lung > intestine > spleen > kidney > visceral muscle > epididymus > heart > testes > skeletal muscle > adipose tissue (Table 8). In females the sequence was the same except the spleen followed the heart. Furthermore, unlike the male reproductive organs, the fallopian tubes were found to contain a relatively high concentration of diphacinone, being second only to the liver.

Table 8. Specific Activity ( $\mu\text{Ci} \times 100/\text{g}$ ) of Male and Female Mice Tissues at Each Sampling Period, Body Burden Study I.

Identifications: M = male, F = female.

Tissue	Hour	3	6		12	24		48		72	96	192	
	Sex	M	M	F	M	M	F	M	F	M	M	M	F
Liver		25.5	14.9	26.0	16.5	7.9	13.0	2.2	2.9	2.0	1.6	0.79	1.1
Heart		2.9	1.9	3.7	2.1	0.81	4.5	0.16	0.25	0.26	0.28	0.20	0.21
Kidney		3.2	2.4	4.0	2.2	1.1	2.5	0.14	0.36	0.25	0.08	0.31	0.43
Spleen		3.2	1.1	2.4	1.4	0.55	2.0	0.31	0.42	0.27	0.20	0.15	0.17
Adipose		1.1	1.0	1.4	1.6	0.39	0.98	0.056	0.076	0.045	0.047	0.038	0.038
Intestine		6.1	2.2	4.8	4.7	1.1	6.1	0.32	0.51	0.19	0.088	0.079	0.097
Skeletal Muscle		1.3	0.76	1.4	0.59	0.18	0.85	0.039	0.073	0.050	0.049	0.033	0.036
Lung		7.9	2.9	6.5	3.2	0.85	3.2	0.60	0.46	1.61	0.10	0.42	1.5
Visceral Muscle		3.2	1.0	2.0	2.4	0.44	2.1	0.076	0.26	0.062	0.049	0.036	0.032
Epididymus		3.1	1.1	---	2.3	0.89	---	0.37	---	0.22	0.33	0.095	---
Testis		1.7	1.2	---	1.6	0.54	---	---	---	0.26	0.15	0.087	---
Fallopian Tubes		---	---	9.0	---	---	---	---	---	---	---	---	0.53

The decrease in specific activity for male tissues at hour 6 (Table 8) may be due to a dosing error rather than to genetic or true animal variation. Means for the 3 subsamples of the 2 individual male livers at hour 6 were 21.6 and 8.1  $\mu\text{Ci/g}$ . (One mouse was dead at time of sacrifice.) If the higher value is considered to be representative for liver at hour 6, the maximum diphacinone concentration in most tissues still occurred before hour 6. Adipose tissue, however, showed a higher concentration at 12 than at 3 hours. This delayed build-up was followed by a rapid 30 fold decrease by 48 hours, whereas levels for most tissues declined by 10 and 20 times over 189 hours, except visceral muscle which declined similarly to adipose tissue.

Tissue concentrations of diphacinone in females probably peaked before 6 hours, except for the heart, intestine and visceral muscle, which showed higher concentrations at 24 than at 6 hours (Table 8). Differences in replicate analyses were small for the heart and visceral muscle lending credence to this observation.

Specific activities of excreta between the sexes were remarkably similar considering that these values were dependent upon the amount of food and water consumed. These values were slightly higher for females than males which may reflect in part the higher dose per unit weight for the smaller females (Table 9). The specific activity increased

Table 9. Specific Activity ( $\mu\text{Ci} \times 100/\text{g}$ ) of Male and Female Mice  
Excreta at Each Sampling Period, Body Burden Study I.

Excreta	Hours After Dosing								
	3	6	12	24	48	72	96	144	192
Urine (male)	0.16	0.043	1.7	1.5	0.38	0.038	0.015	0.015	0.0085
Urine (female)	---	0.075	---	1.1	0.39	0.036	0.017	0.017	0.009
Feces (male)	1.8	12.0	133.0	140.0	26.0	2.9	0.95	0.67	0.64
Feces (female)	---	44.0	---	138.0	37.0	3.7	1.4	0.87	0.64

abruptly from 6 to 12 hours, leveled off, and dropped sharply after 24 hours.

The amount of the dose excreted by the males as feces varied somewhat symmetrically about the peak at hour 24 (Table 10). Excretion of activity by the females in the first 6 hours was 6 times that excreted by the males (Table 10), while by hour 24 the females excreted 46 and the males 50.0 per cent of the dose.

Percentages of the administered dose present in individual tissues and organs are listed in Table 11. The corresponding percentages for excreta are found in Table 10. Accountability of the administered dose was determined after 24, 48 and 192 hours for both sexes (Table 12). These data were obtained by summing the mean per cent of doses for urine and feces by cage from time zero to the desired hour, and the per cent of dose for each tissue sampled at that hour. Generally, the recovery of the administered dose increased with time (Table 12), which paralleled the larger proportion of activity present in feces. This trend was also observed in Body Burden Study II suggesting that radioactivity accountability was greater for feces or excreta in general than animal tissue. Nearly 1.5 times as much activity remained in the female tissues than in the males 24 hours after dosing. Beyond hour 24, however, this difference was not observed.

Table 10. Average Cage Means of Per Cent of Administered Dose in Male and Female Mice Excreta at Each Sampling Period, Body Burden Study I.

Excreta	Hours After Dosing								
	3	6	12	24	48	72	96	144	192
Urine (male)	0.37	0.15	3.9	4.0	1.1	0.10	0.044	0.040	0.021
Urine (female)	---	0.18	---	2.9	0.10	0.094	0.048	0.060	0.023
Feces (male)	0.050	0.39	15.0	46.0	15.0	2.1	0.92	1.6	0.97
Feces (female)	---	2.7	---	43.0	22.0	2.8	1.1	2.2	0.58

Table 11. Per Cent of Administered Dose in Male and Female Mice Tissue  
Per Sampling Period, Body Burden Study I.

Identifications: M = male, F = female.

Tissue	Hour	3		6		12		24		48		72		96		192	
	Sex	M		M	F	M		M	F	M	F	M		M		M	F
Liver		23.1		17.8	21.0	18.6		8.1	11.0	3.0	3.0	2.6		2.0		1.2	1.3
Heart		0.24		0.16	0.35	0.30		0.09	0.51	0.02	0.026	0.026		0.035		0.023	0.019
Kidney		0.83		0.62	0.84	0.58		0.35	0.57	0.052	0.099	0.087		0.029		0.12	0.11
Spleen		0.16		0.083	0.12	0.071		0.029	0.11	0.016	0.028	0.017		0.013		0.009	0.011
Adipose		0.18		0.13	0.18	0.18		0.05	0.07	0.018	0.014	0.010		0.012		0.011	0.0061
Intestine		1.5		0.43	0.92	0.87		0.21	1.5	0.066	0.078	0.61		0.024		0.022	0.024
Skeletal Muscle		0.18		0.081	0.22	0.13		0.031	0.25	0.015	0.015	0.012		0.016		0.022	0.013
Lung		1.05		0.46	0.86	0.55		0.12	0.40	0.086	0.069	0.078		0.014		0.071	0.22
Visceral Muscle		0.29		0.18	0.12	0.24		0.043	0.20	0.016	0.044	0.013		0.011		0.014	0.009
Epididymus		0.16		0.063	---	0.12		0.43	---	0.025	---	0.011		0.017		0.011	---
Testis		0.19		0.15	---	0.16		0.05	---	0.034	---	0.025		0.015		0.010	---
Fallopian Tubes		---		---	0.44	---		---	0.21	---	0.029	---		---		---	0.029

Table 12. Per Cent Recovery of Administered Dose for Hours 24, 48 and 192, Body Burden Study 1.

Hour	Tissue	Male % Recovery	Female % Recovery
24	Urine	4.00	2.90
	Feces	46.00	43.00
	Tissue <sup>a</sup>	<u>9.02</u>	<u>14.60</u>
	Total	59.02	60.50
48	Urine	5.10	3.00
	Feces	61.00	65.00
	Tissue <sup>a</sup>	<u>3.36</u>	<u>3.41</u>
	Total	69.46	71.41
192	Urine	5.30	3.20
	Feces	66.50	71.60
	Tissue <sup>a</sup>	<u>1.43</u>	<u>1.37</u>
	Total	73.23	76.17

<sup>a</sup> Excludes Blood, Brain, Hide and Remaining Organs.

Nearly all of the radioactivity was accounted for in the excreta after 192 hours in both sexes.

Body Burden Study II--  
Methods and Materials

#### Dosing Solution

The dosing solution was prepared as described in Study I except that 7.9 mg of the anticoagulant was dissolved in 25 ml of corn oil without subsequent dilution. The resulting solution was 0.99  $\mu$ Ci/0.051 mg/0.16 ml.

#### Dosing Procedure

Thirty-three male and 30 female mice, 3 mice per cage, were dosed with 0.16 ml of the diphacinone solution (2.56 mg/kg). The same dosing procedure described in Study I was employed.

#### Sampling Procedure

The sampling schedule was 1½, 3, 4½, 6, 7½, 9, 12, 24, 48, 72, and 96 hours after dosing. The 12 hour sample was eliminated in the female study. The animals were sacrificed with chloroform, using a minimum exposure. This permitted the heart to remain beating to facilitate blood collection.

Each mouse was immediately weighed and decapitated. The first 3 - 4 drops of blood were collected in pre-weighed tissue oxidation combustion thimbles packed with a single

combusto-pad. Additional blood was collected in a 5 ml heparinized centrifuge tube. The heart was removed and cut several times to allow occluded blood to drain and then it was dabbed on Kimwipe tissue. The entire heart, lung, liver, brain and both kidneys were removed. The liver was divided among 3 or more combustion thimbles while both kidneys were combined into 1 sample. All tissues from organs were placed in pre-weighed thimbles and weighed immediately. Whole blood was fractionated using a bench model clinical centrifuge (International Equipment Co.) at setting 7 for 30 minutes. Plasma was removed, using a tuberculin syringe fitted with a 22 gauge spinal tap needle, and injected onto pre-weighed combusto-pads packed within combustion thimbles and weighed.

Feces and urine collections began at hour 6. The entire fecal sample was divided among as many as 5 combustion thimbles for the 24 hour sampling periods. Each subsample was combusted, radioassayed individually and the dpm for each summed. The urine was transferred into thimbles packed with combusto-pads. Up to 1.6 ml of urine was added to a thimble containing 3 pads. As many as 4 thimbles plus pads were required for some of the 24 hour periods. Each thimble was combusted after drying thoroughly for several days, counted individually and the dpm summed for a given sample.

Excreta were collected from each cage at the time of sacrifice or at each 24 hour period. In addition, several extended urine bladders obtained during organ removal were included as part of the urine collection for that hour.

#### Tissue Preparation for Radioassay

All tissues, whole blood, plasma and excreta samples were quantitatively oxidized to carbon dioxide, water and inorganic substances with a Packard-306 tissue oxidizer. The accuracy of the technique was determined by combustion of a cold tissue impregnated with a known amount of  $^{14}\text{C}$ -activity and determining the per cent of radioactivity recovered. The  $^{14}\text{C}$ -standard was supplied by Packard Instrument Co., Inc. with an activity of  $7.77 \times 10^5$  dpm/ml  $\pm$  3 per cent.

The times required for a quantitative tissue oxidation were predetermined for each tissue. Representative quantities and times are shown in Table 13. In a few instances where the pre-set times were insufficient, the combustion time was extended manually without interruption of the run until oxidation was complete. In all cases, the samples were combusted at least 10 seconds beyond a visual indication of total combustion. A standard was run every 20 samples in order to evaluate the efficiency of combustion. These standards were also used to determine the overall counting efficiency. The oxidized samples did not produce a visible ash except the fecal samples, which had a residue

Table 13. Time Required for Quantitative Oxidation of Tissue Sample Sizes Used in Body Burden Study II.

Tissue	Quantity	Time
Dry Feces	0.8 g	90 sec
Entire Heart (wet)	0.11 g	30 sec
Entire Lung (wet)	0.15 g	30 sec
Entire Liver (wet)	0.8 g	90 sec
Two Kidneys (wet)	0.4 g	70 sec
Entire Brain (wet)	0.45 g	60 sec
Urine (evaporated)	1 ml	30 sec
Whole Blood (evaporated)	4 drops	30 sec
Plasma (evaporated)	0.1 ml	30 sec

of about 10 per cent. This ash did not contain activity and was insoluble in water, strong alkali and aqua regia. After each sample oxidation, a "memory blank" was run which consisted of oxidizing a combustion thimble to determine retention of any radioactivity from a previous combustion. Initial studies with these blanks showed that even after samples containing as much as  $10^5$  dpm, the average amount of radioactivity detected in these blanks was on the order of 12 cpm above background.

The ratio of Carbo-Sorb<sup>R</sup> to Permaflor V<sup>R</sup> (Packard Instrument Co., Inc.), the carbon dioxide absorbent and scintillation cocktail respectively, was 9 : 12. This ratio was recommended by the instrument manual for the size of samples combusted during this study and was verified experimentally. The quench was found to be independent of sample size within the ranges previously mentioned.

As part of this body burden study, carcasses for hour 6, 9, 48, 72, and 96 were assayed to determine total dose accountability. The frozen carcasses were thawed, skinned and weighed and the remaining organs and viscera were removed from the muscle-bone mass. The organs, viscera and muscle bone mass were minced separately with scissors into 250 ml beakers and digested with 30 ml of concentrated nitric acid at low heat (50°C) for several hours until digestion was essentially complete. The digest remained

overnight at room temperature before preparation for scintillation counting. During this time a layer of solidified mass plus lipid covered the solution. This was removed as a disc and discarded. The digests were adjusted to 30 ml with distilled water and a 2 ml aliquot of each was placed into centrifuge tubes. The aliquots were neutralized with strong alkali to pH 5 - 7 and diluted to 5 ml. One ml of the neutralized digests was analyzed using the standard liquid scintillation counting procedures previously described. Triplicate subsamples of the hides, each approximately 400 mg, were combusted. The mean value was used to calculate the total in each hide.

#### Counting Procedure

Samples, standards and blanks were counted as described under counting procedures for study I. Increased quenching produced by the ethanalamine in the Carbo-Sorb<sup>R</sup> required a lower attenuation setting (B-500) to maximize the cpm. The average overall efficiency of combustion, trapping, the scintillation solution processes and the counter, was determined to be 70.2 per cent, with a range of 66 - 72. All sample cpm were corrected with the 70.2 efficiency value.

Body Burden Study II--  
Results

Specific activities for each tissue for both sexes are listed in Table 14. Uptake of 1-<sup>14</sup>C-diphacinone by tissues as measured by specific activity was: liver > plasma > whole blood > lung > kidney > heart > brain for both sexes. This sequence was the same for tissues common to both body burden studies.

High concentrations of activity were present in male tissues as early as 1.5 hours after dosing, with a further slight increase through 7.5 hours, followed by an abrupt decrease and somewhat slower decline through hour 96. This trend was most obvious for liver, but could also be seen in the other tissues.

Peaking of radioactivity concentrations in female tissues occurred at shorter times than for male tissues. Most female tissues reached maximum concentrations between 1.5 and 4.5 hours. The brain and heart peaked before 3 hours, while these tissues peaked between 6 and 9 hours for males. There was a significant difference between male and female brain and heart at 7.5 hours. Other significant differences occurred at 48 hours for the heart and at 7.5 hours for the liver. All other observable differences were insignificant at the 0.05 confidence level (Appendix C). Significant differences in specific activity for a given tissue were found over all sampling periods (Appendix B).

Table 14. Specific Activity ( $\mu\text{Ci} \times 100/\text{g}$ ) of Male and Female Tissues by Hour, Body Burden Study II.

Tissues	Hours After Dosing										
	1.5	3.0	4.5	6.0	7.5	9.0	12	24	48	72	96
Liver											
Male	16.5	14.9	15.9	17.3	20.1	10.6	11.0	4.0	1.60	1.60	1.10
Female	16.2	17.4	15.8	12.2	11.5	11.0	---	4.65	2.57	1.70	1.29
Heart											
Male	1.30	1.20	1.50	1.30	1.50	0.93	0.65	0.27	0.16	0.10	0.079
Female	1.39	1.22	1.04	0.74	0.82	0.69	---	0.31	0.094	---	0.067
Kidney											
Male	1.80	2.06	2.32	1.93	2.32	1.52	1.22	0.64	0.24	0.19	0.12
Female	1.84	2.01	2.53	3.50	1.87	1.64	---	0.61	0.31	---	0.18
Lung											
Male	2.24	1.92	2.41	2.28	2.48	1.60	---	0.55	0.22	0.19	0.16
Female	2.35	2.34	1.98	1.42	1.68	1.37	---	0.57	0.24	---	0.15
Brain											
Male	---	0.23	0.37	0.31	0.35	0.23	0.19	0.11	0.11	0.042	0.028
Female	0.30	0.24	0.24	0.17	0.20	0.18	---	0.094	0.050	0.032	0.034
Whole Blood											
Male	3.45	3.03	3.84	3.31	3.77	2.42	1.70	0.52	0.15	0.12	0.070
Female	4.02	3.66	2.76	1.96	2.16	1.76	---	0.53	0.12	---	0.060
Plasma											
Male	5.84	5.37	6.62	5.06	4.15	3.54	3.27	0.67	0.21	0.11	0.15
Female	5.59	5.34	4.79	2.93	3.93	3.02	---	0.66	---	0.079	0.060

The percentages of the dose in tissues of both sexes are listed in Table 15. Generally, the amounts were higher in male than in female tissues. The specific activity of the female liver correlated with the per cent of dose recovered from that tissue. However, the comparable trends for the male liver differed. Per cent dose peaked early and declined extremely slowly through hour 7.5 when it dropped markedly, whereas the specific activity did not peak until this time.

All per cent doses for female tissues declined gradually from hour 1.5 or 3, except for the kidney which peaked at hour 6. This was also observed for the kidney specific activity.

Most of the per cent dose for male tissues peaked about hour 4.5 which was similar to the trends for specific activity of these tissues. The notable exception was the brain where the per cent dose peaked before hour 3.

#### Discussion of Body Burden Studies

##### Study I

An examination of tissue specific activities (Table 8) showed preferential uptake of diphacinone by only the liver and lung. The high concentration of activity in the small intestine early in the experiment was most likely due to unabsorbed diphacinone. The next 5 most active

Table 15. Per Cent of Administered Dose in Male and Female  
Tissues by Hour, Body Burden Study II.

Tissue	Hours After Dosing										
	1.5	3.0	4.5	6.0	7.5	9.0	12	24	48	72	96
<b>Liver</b>											
Male	25.2	16.9	22.8	21.1	19.1	13.2	10.5	7.1	2.4	2.0	1.6
Female	19.9	20.5	19.9	13.0	13.5	12.3	---	4.9	3.1	1.9	1.5
<b>Heart</b>											
Male	0.115	0.129	0.180	0.123	0.134	0.099	0.067	0.027	0.017	0.012	0.0089
Female	0.15	0.13	0.12	0.070	0.080	0.083	---	0.031	0.011	0.012	0.0071
<b>Kidney</b>											
Male	0.649	0.665	0.869	0.661	0.729	0.581	0.436	0.241	0.089	0.077	0.052
Female	0.54	0.62	0.63	0.89	0.56	0.47	---	0.18	0.093	0.058	0.060
<b>Lung</b>											
Male	0.38	0.308	0.440	0.432	0.411	0.271	0.225	0.092	0.038	0.032	0.026
Female	0.37	0.40	0.32	0.23	0.27	0.22	---	0.087	0.037	0.021	0.024
<b>Brain</b>											
Male	---	0.093	0.144	0.125	0.152	0.087	0.064	0.043	0.023	0.017	0.013
Female	0.13	0.11	0.12	0.10	0.091	0.082	---	0.037	0.022	0.013	0.011

tissues contained similar concentrations of activity as did the skeletal muscle and adipose tissue. Although the muscle mass exceeded that of the liver by a factor of 5 in a 20 gram mouse, the ratio of 51 : 13 of the quantities ( $\mu\text{Ci/g}$  times weight of tissue in grams) in liver and muscle respectively emphasizes the preferential uptake of diphacinone by the liver.

Although the concentration of anticoagulant was relatively high in the fallopian tubes, no report could be found to support any effect by this compound on the fecundity in mice. Diphacinone was found to have no effect on fertility in humans (Upjohn Company 1971).

The concentration of diphacinone in all tissues declined to less than 10 per cent of the 3 hour concentration by 48 hours, except the epididymus which retained 12 per cent (Table 8). Thus diphacinone was found not to be retained or stored preferentially by any organs or tissues, including the liver which showed an appreciable initial affinity for the compound. The data also showed that adipose tissue had the least preference for diphacinone. This was somewhat surprising since its low water solubility (15 ppm) suggested lipophilic properties. On the other hand, its low solubility in corn oil (2.4 per cent at  $80^{\circ}\text{C}$ ) does not represent a high degree of lipophilicity. Nevertheless, the ratio of these solubilities strongly favors partitioning

into adipose tissue. Diphacinone is probably transformed to the sodium salt of the enolate form in body fluids which imparts much greater water solubility to the compound.

More than 66.1 and 68.0 per cent of the administered activity was excreted by hour 48 for the male and female mice respectively during this study (Table 10). This was in agreement with the 90 per cent decline in the tissue concentrations for the same period. Twice as much activity was excreted in the urine by males than by females through hour 48.

Per cent recovery of the administered dose is shown in Table 12. The totals do not include activities from the blood, bone, brain and hide. Reasonable estimates of these percentages can be obtained from Body Burden Study II (Table 16). The values for these tissues at hours 48 and 192, however, comprised a very small part of the total per cent administered dose. Addition of reasonable extrapolated values for per cent of dose in these tissues for hours 48 and 192 would place the total recovery between 90 and 100 per cent.

## Study II

Near maximum levels of activity were present in all tissues 1.5 hours after dosing (Table 14). However, most male tissues showed additional increases in specific activity through hour 7.5. This trend was most pronounced

Table 16. Per Cent Recovery of Applied Dose at Selected Times.

Cage	Hour	Organs	Excreta	Hide	Carcass	Remaining Organ & Visceral Tissue	Total % of Dose Recovered
4	6	23.8	0.42	7.45	1.84	12.2	45.7
6	9	15.1	2.93	6.22	2.09	13.0	39.3
9	48	2.62	48.4	5.26	1.42	6.76	64.5
10	72	2.27	35.1	4.43	1.13	5.49	48.4
11	96	1.71	63.1	3.68	1.05	4.34	73.9

for the liver although definitely discernible in the other tissues.

The low level of activity found in the brain may suggest that the blood brain barrier is operative against this compound, or these low values could be a function of the low lipid solubility of diphacinone. The specific activity for male whole blood and plasma peaked around 4.5 hours followed by a gradual decline. These overall trends suggested the following relationships and equilibria:

1. Diphacinone was rapidly absorbed by the blood from the intestine.
2. It was rapidly and preferentially taken up by the liver from the blood.
3. Blood concentrations of diphacinone increased slightly paralleling tissue increases during the first 7.5 hours after dosing. This indicated that adsorption was occurring from the intestine during this period.
4. For the female, adsorption from the intestine was complete shortly after 1.5 hours.
5. Beyond 7.5 hours, adsorption became insignificant with a simultaneous decline in tissue concentration.

The slower decline in blood activity may reflect the transport of metabolites from tissues to the kidneys and the hepatic-bile systems and efficient removal as indicated by increased urine and fecal radioactivity beyond 9 hours,

thus preventing a secondary buildup of activity in the blood (Table 17). This partitioning of activity during the course of the study is shown in Figure 4.

The accountability of total activity for 5 representative cages of male mice is listed in Table 16. In general this increases with time, during which a progressively larger proportion is attributable to the excreta, especially the feces. This points out the likelihood that activity within the tissues, especially the carcass, is more difficult to account for by the nitric acid digestion method than in the feces by the tissue oxidation technique. If the suspected 25 per cent loss of urine radioactivity due to the large surface of the urine-feces separator is taken into account for cage 11, for example, the total dose accountability increases from 73.7 to 75.9 per cent.

Variability between sexes by tissue and hour is shown in Appendix C. No significant differences for the plasma at the 90 per cent confidence level, or the plasma, lung, whole blood and kidney at the 95 per cent confidence level were observed. At this latter level only 4 of 67 tissues by hour cells showed significant variation. Persistent significant differences were not revealed.

The results of the analysis of variance by tissue over all hours are shown in Appendix B. For all cases significant differences exist.

Table 17. Per Cent Dose in Excreta by Sex for Cage to Time of Sacrifice.

Cage	Hour After Dosing	Male % of Dose			Female % of Dose		
		Feces	Urine	Total	Feces	Urine	Total
1	1.5	-	-	-	0.09	-	0.09
2	3	-	-	-	0.32	-	0.32
3	4.5	-	0.34	0.34	0.80	-	0.80
4	6	0.04	0.38	0.42	3.51	0.41	3.92
5	7.5	1.21	0.41	1.62	0.31	0.11	0.42
6	9	2.00	0.93	2.93	2.50	0.43	2.93
7	12	15.1	2.45	17.6	-	-	-
8	24	28.7	2.07	30.8	46.0	3.38	49.4
9	48	45.3	3.13	48.4	38.9	2.73	41.6
10	72	30.1	5.03	35.1	50.0	1.11	51.1
11	96	54.2	8.89	63.1	41.2	2.36	43.6

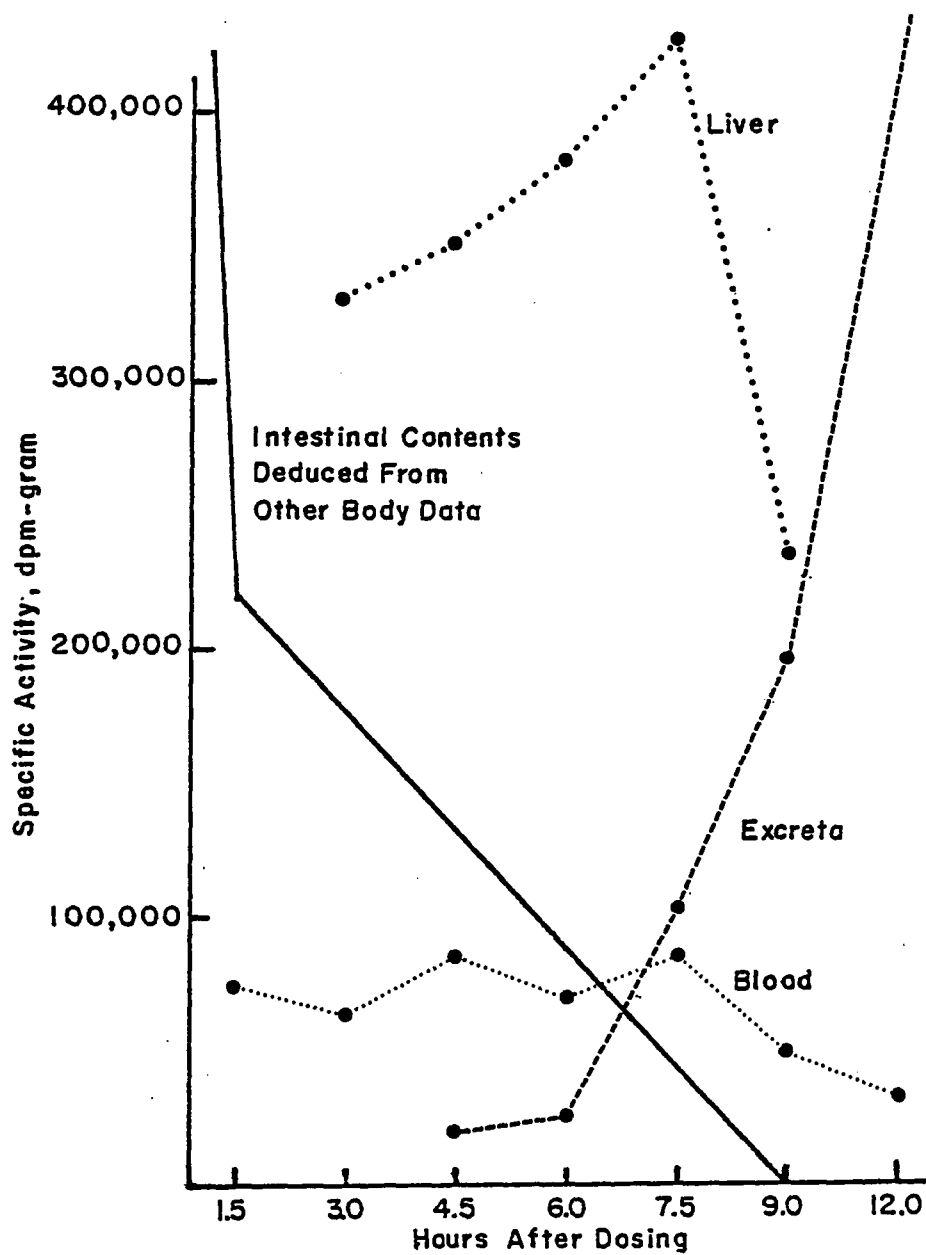


Figure 4. Partitioning of Radioactivity among the Intestinal Contents, Blood, Liver and Excreta, Body Burden Study II.

## Discussion of Both Body Burden Studies

The administered dose for the first and second body burden studies was 1.6 and 1.0  $\mu\text{Ci}/\text{mouse}$ , respectively, regardless of size or sex. Table 18 shows the comparison between the adjusted specific activities for the tissues and times in common between the 2 studies for male and female mice. The values for the first study (tissues digested) were adjusted by dividing the obtained values by 1.6 in order to normalize tissue levels for comparison between studies.

Results of these adjusted activities appear comparable for male mice (Table 18). Two major exceptions are lung tissue at hour 3 and liver tissue at hour 6. Adjusted activities of female tissues for the digestion technique are significantly higher through hour 24.

The gradual increase in specific activity for male tissues from 1.5 to about 7.5 hours in Study II was not observed in the first study. This may be due to the probably dosing error at hour 6 in Study I and the lack of additional sampling periods during this time.

Slower adsorption of diphacinone by male mice may account for the difference in susceptibility noted in the  $\text{LD}_{50}$  study. The faster rate of adsorption from the female mouse intestine would effectively produce a more lethal concentration of the anticoagulant in the tissues, especially

Table 18. Comparison of Adjusted Specific Activity ( $\mu\text{Ci} \times 100/\text{g}$ ) for Male and Female Tissues between Body Burden Studies I and II.

Identifications: M = male, F = female, I Tiss. Dig. = Study I Tissue Digested, II Tiss. Ox. = Study II Tissue Oxidized.

Tissue \ Hour	3	6		12	24		48		72	96
	Sex M	M	F	M	M	F	M	F	M	M
Liver										
I Tiss. Dig.	16.0	9.3	16.4	10.3	4.9	8.1	1.4	1.8	1.3	1.0
II Tiss. Ox.	14.9	17.3	12.2	11.0	4.0	4.7	1.6	2.6	1.6	1.1
Heart										
I Tiss. Dig.	1.8	1.2	2.32	1.3	0.50	2.84	0.10	0.16	0.16	0.18
II Tiss. Ox.	1.2	1.3	0.74	0.65	0.27	0.31	0.16	0.09	0.10	0.08
Kidney										
I Tiss. Dig.	2.00	1.5	2.50	1.4	0.68	1.56	0.09	0.23	0.16	0.05
II Tiss. Ox.	2.06	1.9	---	1.2	0.64	0.61	0.24	0.31	0.19	0.12
Lung										
I Tiss. Dig.	4.9	1.8	4.04	2.0	0.53	1.98	0.38	0.29	1.00	0.06
II Tiss. Ox.	1.9	2.3	1.42	1.3	0.55	0.51	0.22	0.24	0.19	0.16

the liver, during the initial hours after dosing. This is consistent with the tissue analytical data, and is also supported by the higher initial excretion of activity at hours 6 and 24 for Body Burden Study I (Table 10) and during the first 9 hours of Study II (Table 16).

The per cent dose, although a useful measure for ready accountability of the total dose or for following the quantity of toxicant and its distribution, is a function of both size (weight) and concentration within the organ or tissue and is not as useful as specific activity, a concentration term, for evaluating the relative toxic situations within organs or tissues at a given time. For example, in Table 14 the specific activities are approximately the same for the male and female livers at 1.5 hours while the corresponding per cents of dose are 25.2 and 19.9, respectively. This does not represent a more toxic situation to the male, but actually a less toxic condition when identical doses are administered to the 2 sexes. This difference in values was due to the relatively larger male liver, the average male/female liver ratio being 1.53/1.22. In the animals sacrificed at hour 3 the female livers were larger, the male/female ratio being 1.05/1.18. The same ratio for animals sacrificed at 4.5 hours is 1.43/1.20.

Per cent dose for the liver on the other hand, or the liver weight along with the specific activity, could be

considered as a measure of detoxification. Thus a larger liver or higher per cent of the dose in tissues with similar specific activities would imply a more rapid route of detoxification. The consistently higher liver contents of diphacinone and lower specific activity up to 4.5 hours for the male mice suggest an initial higher rate of detoxification of diphacinone and predicts a lesser susceptibility of male than female mice for the anticoagulant. This is in accord with the results of the LD<sub>50</sub> study described earlier on pages 26 to 30.

The urine-feces separator used in these studies had too large a surface area over which the urine flowed. Although this did not affect the fecal assay, it is probable that a considerable amount of the activity in the urine was not collected, remaining as a film on the glass. This loss may have been as high as 25 per cent due to the small volume of urine collected during a 24 hour period (3 - 5 ml per cage of 3 mice). This amount would elevate the total per cent recovery very little, but would alter the ratio of activity in feces and urine to a greater extent.

## METABOLITE IDENTIFICATION STUDY

### Introduction

The fate of a compound ingested by an animal is generally construed to mean not only the elimination of that chemical from the animal, but also the metabolic fate, if any, of the substance. This last process referred to as biotransformation, effects the storage, elimination, inactivation and potentiation of the chemical.

Knowledge of metabolite identity permits evaluation of toxicity of these compounds and allows development of analytical procedures for their specific determination. Metabolite identification also reveals specific types of deactivation such as hydroxylation with or without subsequent conjugation and molecular fragmentation. These products of biotransformation are useful in elucidation of metabolic pathways. These studies along with body burden studies provide data on the elimination and detoxification of a compound.

### Methods and Materials

The high pressure liquid chromatograph (HPLC) used was a Tracor model 990 Isochromatographic Pump with a Model 970 Variable Wave Length detector. The injection port was adapted with a 20  $\mu$ l sample loop. The columns used were Partisil<sup>TM</sup>-10-ODS reverse phase and Partisil<sup>TM</sup>-10 adsorption

columns. Both had identical dimensions of 25 cm x 4.6 mm I.D. ( $\frac{1}{4}$  in O.D.). The column packing was 10 - 25  $\mu$ m in diameter. The active groups for these packings were the octadecyl (C-18) to silica and the Si-OH respectively.

The distilled water in the reverse phase column eluents was degassed before mixing with the alcoholic components. This was accomplished by vigorous shaking of the water under a vacuum of approximately 10 mm Hg for several minutes. Use of a degassed eluent prevented formation of air locks in the high pressure pump thus avoiding interruption of the separation procedure. This treatment was required for predominantly aqueous eluents only. Slight degassing occurred after this treatment, but was insufficient to produce an air lock.

All HPL chromatographic studies were made with a flow rate of 1 ml/min. The working solutions of suspected metabolites were prepared by appropriate dilution of stock solutions until the elution maximum monitored at 253 nm remained on scale at attenuation 4. The retention times of suspected metabolites were determined by dividing the distance in inches the chart paper traveled from the point of injection to the elution maximum by the recorder speed in inches per minute.

In order to place adequate activity on the HPLC column, large volumes of urine or urine extract, relative to

the 20  $\mu$ l sample loop, were injected onto the reverse phase or adsorption columns respectively, using a stop-flow technique. Fifteen  $\mu$ l of sample were injected into the loop, the flow started and permitted to continue for several seconds. The flow was stopped, followed by injection of another 15  $\mu$ l. This procedure was repeated until the desired activity had been injected onto the column. The time of flow during this procedure was measured and allowed for during collection of the first 1 ml fraction, such that all subsequent 1 ml collections corresponded to the appropriate minute after injection. The retention times of labeled urinary metabolites were determined by collecting 1 ml fractions in scintillation vials. Solvent was removed from the adsorption column eluate with an air stream, after which 4 ml of scintillation cocktail was added. Ten ml of Aquasol cocktail solution was added to 1 ml of the eluate from the reverse phase column. The vials were counted and the dpm plotted versus time to produce an elution profile from which the retention times were read. For profiles longer than 2 hours, 5 ml fractions were collected through low activity regions and treated similarly to the 1 ml fractions. The actual quantity of activity injected onto the column was determined in each case by counting a known volume of the sample independently, establishing the dpm per  $\mu$ l. The corrected cpm for each 1 ml or 5 ml fraction was summed

over the entire elution profile to determine the per cent recovery of the injected activity.

#### Extraction of Mouse Urine

Male mice were dosed with either 5  $\mu$ Ci of 1- $^{14}$ C-diphacinone or 2'- $^{14}$ C-diphacinone in corn oil at 4 day intervals for several weeks. The feces and urine were collected as described in Body Burden Study I, p. 37. The metabolites from the 2 labeled preparations are called "carbonyl labeled metabolites" and "benzhydryl labeled metabolites".

Aliquots of mouse urine (2 ml) containing 547,000 dpm were extracted with either 5 ml of ethyl ether or ethyl acetate 5 times each. The extracts were reduced to 5 ml and 1 ml aliquots counted. The ether extracted 43.0 and the ethyl acetate 71.7 per cent of the radioactivity in the tissue. The ether extract was clear and faintly colored while the ethyl acetate was cloudy and colored a strong yellow. Upon standing, some solid and water settled out leaving a clear solution. Reduction of this volume resulted in additional precipitation of solid.

#### Acid Hydrolysis of Mouse Urine

Hydrolysis of Carbonyl Labeled Conjugates. The ethyl acetate was evaporated by an air stream and the residue digested in 2 ml of 6M HCl for 24 hours at 85°C. After

evaporation of the acid solution, the residue was heated for an additional 12 hours at 85°C resulting in blackening of the residue. It was then extracted with benzene and centrifuged at moderate speed for several minutes. The supernatant was transferred to another centrifuge tube and spun down again. A 67  $\mu$ l aliquot (48,800 dpm) was chromatographed on the adsorption column with 10 per cent ethanol/hexane for 121 minutes and then with methanol.

Hydrolysis of Benzhydryl Labeled Conjugates. Male mouse urine (1 ml) was hydrolyzed with 1 ml of 12M HCl at 85°C for 18 hours. The hydrolysate was neutralized to pH 6 and 16.4  $\mu$ l (2867 dpm) were injected onto the reverse phase HPLC column using stop-flow technique.

#### Microfiltration of Urine Extract

The ethyl acetate containing metabolites from 1-<sup>14</sup>C-diphacinone was evaporated with air leaving a small volume of water in the tube. This was extracted in situ with benzene, since ethyl acetate was not recommended for use with the millipore filter, and passed through a 0.22  $\mu$  millipore filter. A 55  $\mu$ l aliquot (70,425 dpm) was chromatographed on the adsorption column with the 10 per cent ethanol/hexane eluent. A 100 per cent methanol eluent was introduced at minute 120.

### Methylation of Urine Extract

A portion of an ethyl acetate extract containing the urinary benzhydryl labeled metabolites was stripped of solvent by an air stream and methylated with diazomethane in ethyl ether. The ether was removed and the residue redissolved in methanol.

### Comparison of Toluene and 95% Ethanol as Fecal Extractants for Radioactive Metabolites of Diphacinone

The following procedure was used:

1. A 0.5 g portion of feces was pulverized in the presence of small amounts of sodium sulfate with a mortar and pestle.
2. The mixture was extracted for 8 hours in a soxhlet apparatus with 100 ml of solvent at a turnover rate of 3 extractions per hour.
3. A portion of the extracted feces (100 mg) was digested with 5 ml of concentrated  $\text{HNO}_3$ .
4. After 24 hours, the acid digest was neutralized to pH 2 - 3 with  $\text{Na}_2\text{CO}_3$ , diluted 1 : 1 with distilled water and 0.5 ml was counted. The channel ratio method was used to determine counting efficiency.
5. One ml of each extract was counted in duplicate.

## Preparation of Iron-Diphacinone Complexes

Preparation of Iron-59-Diphacinone Complex. One ml of ferric chloride solution containing 13  $\mu$ Ci of iron-59 was adjusted to pH 3 with 0.1M NaOH. This solution was then agitated with a 1 ml solution of diphacinone in methylene chloride followed by successive additions of solid diphacinone until the pale red color was well developed. The methylene chloride solution was stored under the aqueous layer throughout the study. A 4.5  $\mu$ l aliquot of the methylene chloride solution contained 3,785 dpm. A 16.4  $\mu$ l aliquot containing 20,861 dpm was chromatographed on the adsorption column with 10 per cent ethanol/hexane.

Preparation of Iron-14C-Diphacinone Complex. The preparation was made as above except that 5 mg of 1-<sup>14</sup>C-diphacinone in methylene chloride was first agitated with a ferric nitrate solution at pH 3 followed by additional non-labeled diphacinone until an orange-red color developed. The final solution was diluted to 25 ml with methylene chloride. A 23  $\mu$ l aliquot was chromatographed on the adsorption column with 10 per cent ethanol/hexane eluent.

Acid Stability of an Iron-Diphacinone Preparation. A non-labeled iron-diphacinone preparation was made as described under preparation of the iron-59-diphacinone preparation. The organic and aqueous layers were separated and the methylene chloride evaporated leaving a red-brown residue

on the walls of the centrifuge tube. Two ml of 6M HCl was added and the mixture heated at 80 - 85°C for 18 hours. During the digestion, the colored residue remained on the walls of the glass tube thus decreasing contact between the acid and the iron complexes. The acid solution was then decanted, the tube dried under an air stream and the contents redissolved in methylene chloride. Nearly all of the residue dissolved, forming a deep red colored solution again. A 5  $\mu$ l aliquot was injected onto the adsorption column and monitored for 40 minutes. During this time 2 components eluted with retention times of 7 and 25 minutes.

#### Procedure for Testing Iron-Diphacinone Complex Formation as a Function of pH

Solutions of ferric nitrate were adjusted to pH 3, 6, and 8 using pH paper as indicator. The solutions at pH 6 and 8 precipitated a considerable quantity of ferric oxide. These mixtures were agitated prior to sampling. A saturated solution of diphacinone in methanol was prepared for these tests.

First Method. Several drops of each iron solution were spotted on a neutral silica gel TLC plate (pH 6.8 - 7.9) to which several drops of the diphacinone solution were added. The formation of a red color was accepted as evidence that a complex formed. Silica gel TLC plates were used because of their neutral pH and white background.

Second Method. This procedure was similar to that for the first method except that a band of the diphacinone solution was applied to the TLC plate below the iron spots and was eluted over the iron spots with methanol. This removed the red complex from the brown background produced by ferric oxide.

#### Fecal Extracts

Each of 4 male mice was dosed with 5  $\mu$ Ci of 1-<sup>14</sup>C-diphacinone twice during a 4-day period. The feces were collected for 1 week beyond the initial dosing and then extracted for 8 hours with 100 ml of a 95 per cent ethanol solution in a soxhlet apparatus. Fifty-one  $\mu$ l of the extract, equivalent to 42,000 dpm, was injected onto the adsorption column using the stop-flow method. One ml fractions were collected in scintillation vials as for the other elution profiles.

#### Results of Metabolite Identification Study

Elution Profiles of Ethyl Acetate Extracts  
of Mouse Urine Determined on an Adsorption  
Column Using a 10% Ethanol/Hexane Eluent

#### Elution Profiles Containing Carbonyl Labeled Metabolites.

Non-hydrolyzed Urine Extract: A typical elution profile of mouse urine containing carbonyl labeled metabolites is shown in Figure 5. A 54.7  $\mu$ l aliquot (36,900 dpm) was

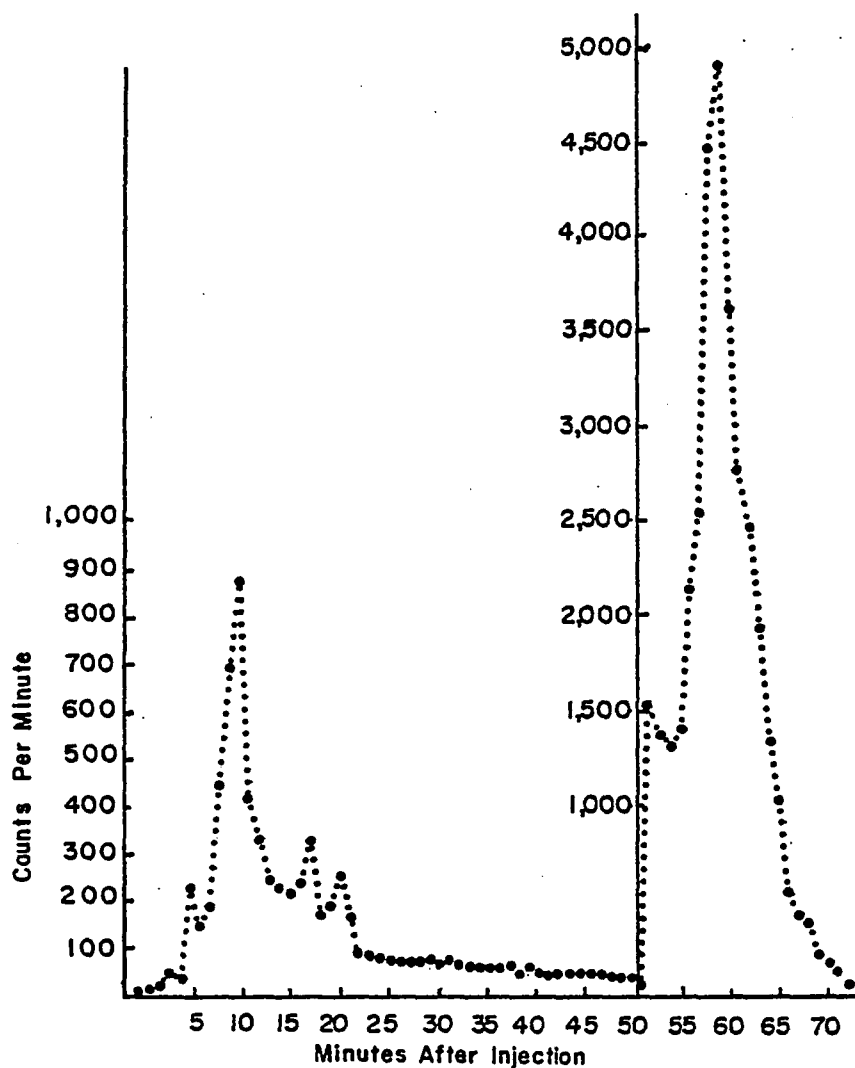


Figure 5. Elution Profile of Ethyl Acetate Extract of Urine Containing Carbonyl Labeled Metabolites.

Column: Adsorption 10/25  $\mu$ m.  
Eluent: 10% v/v ethanol/hexane switched to  
100% methanol at minute 45,  
breakthrough at minute 51.  
Flow Rate: 1 ml/min.

injected onto the HPLC adsorption column. The profile was developed with a 10 per cent ethanol/hexane eluant at a flow rate of 1 ml per minute through minute 44. The eluent was then switched to methanol with breakthrough at 50 minutes (vial 50).

The activity maximum at 10 minutes corresponded to 9 per cent of the total injected activity. The total activity accounted for through minute 49 was 13 per cent. The remaining activity was eluted with methanol. The total detected activity was 36,675 dpm or approximately 100 per cent.

Acid-hydrolyzed Urine Extract: The elution profile of an acid-hydrolyzed ethyl acetate extract of urine containing carbonyl labeled metabolites is shown in Figure 6. Methanol breakthrough occurred at minute 126. The recovery was approximately 45,500 dpm or 93 per cent. Evidence for only 1 compound was observed beyond 126 minutes. The components with retention times of 17 and 20 minutes present before hydrolysis were not apparent in this profile.

Microfiltered Urine Extract: The elution profile of a microfiltered ethyl acetate extract of carbonyl labeled metabolites from urine is shown in Figure 7. The polar eluent was introduced at minute 120 with breakthrough at minute 126. Approximately 65,000 dpm or 92 per cent of the total injected activity was accounted for. As with the elution profile of the acid hydrolyzed profile, the peaks at

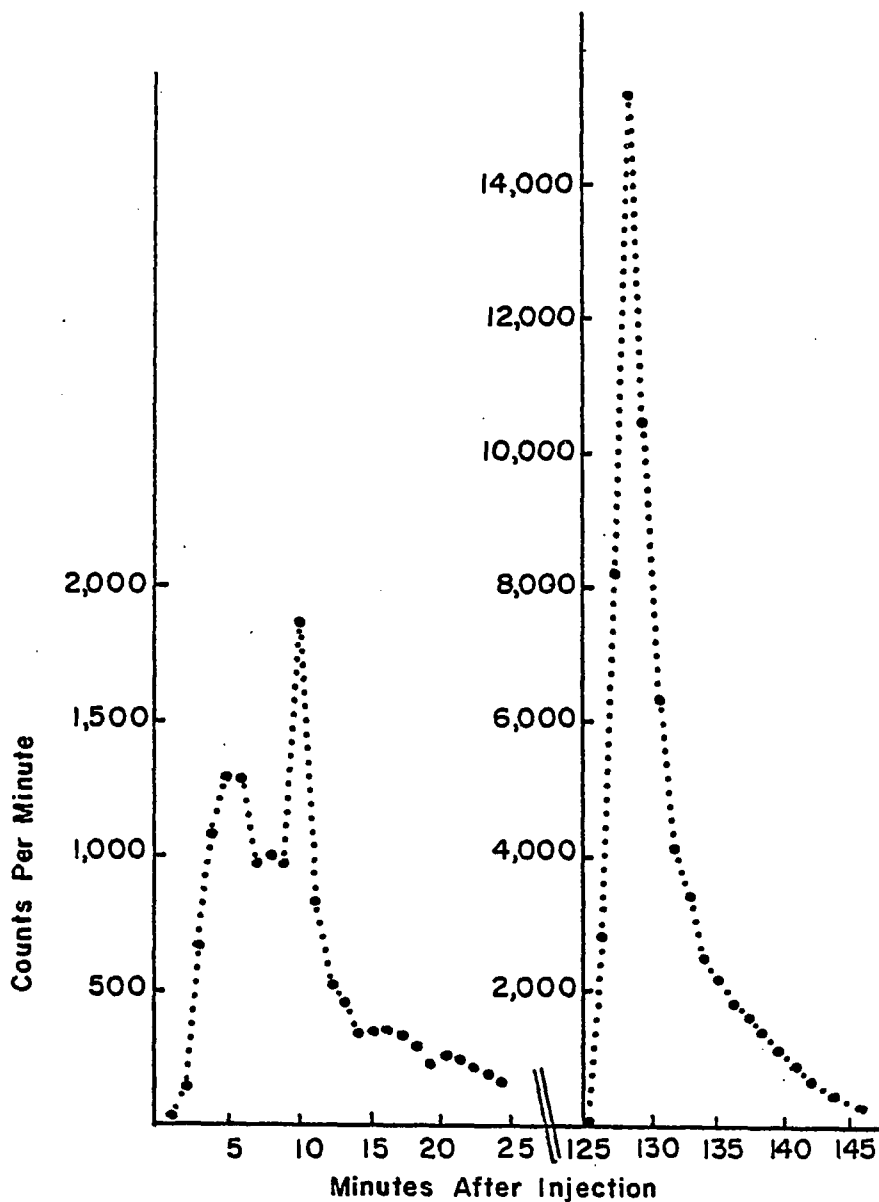


Figure 6. Elution Profile of Acid Hydrolyzed, Charred Urine Containing Carbonyl Labeled Metabolites.

Column: Adsorption 10/25  $\mu$ m.  
Eluent: 10% v/v ethanol/hexane switched to  
100% methanol at minute 120,  
breakthrough at minute 128.  
Flow Rate: 1 ml/min.

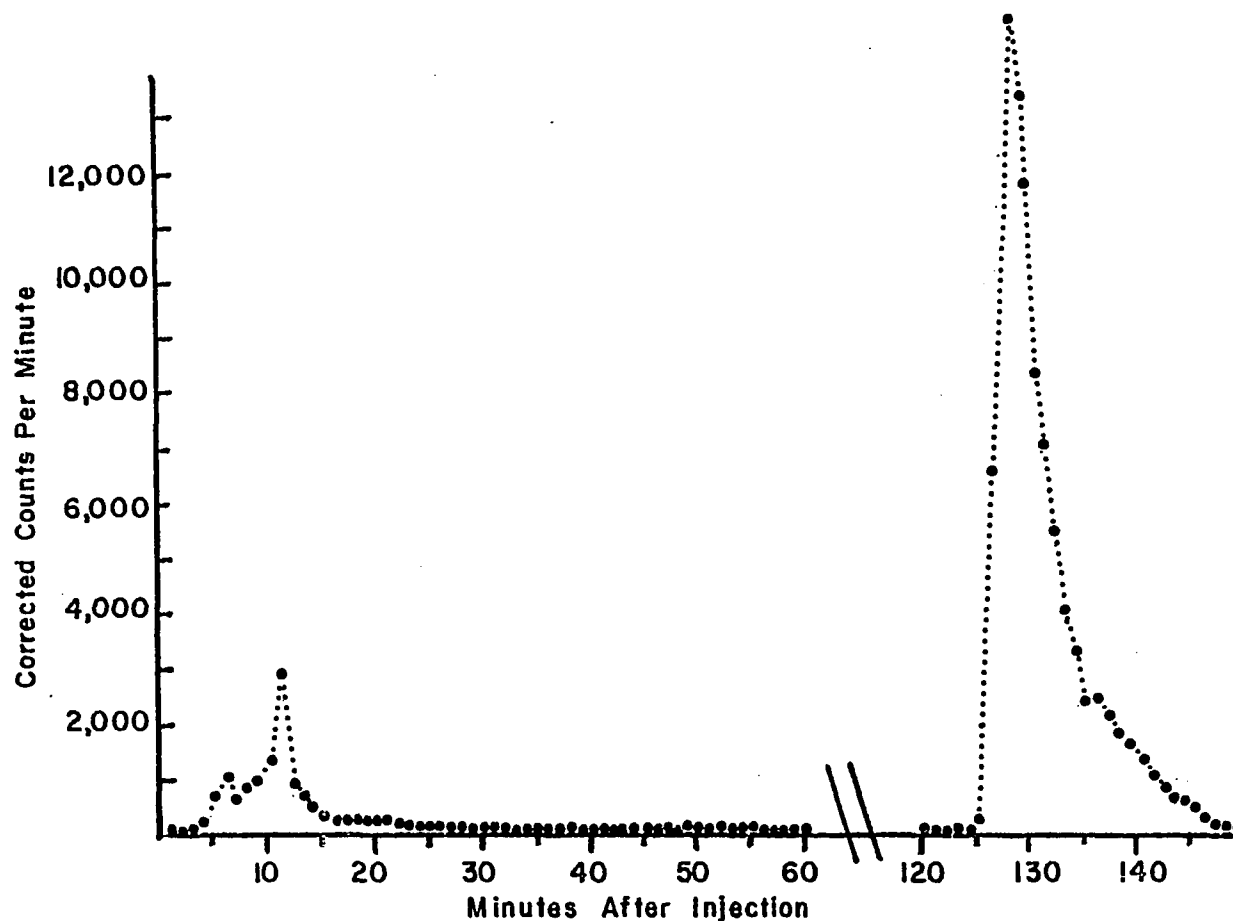


Figure 7. Elution Profile of Ethyl Acetate Extract of Mouse Urine Containing  $1\text{-}^{14}\text{C}$ -carbonyl Metabolites after Microfiltration.

Column: HPLC Adsorption.

Eluent: 10% v/v ethanol/hexane to minute 120, 100% methanol  
from minute 120-148, breakthrough at minute 125.

Flow Rate: 1 ml/min.

17 and 20 minutes were absent. Evidence for only 1 component was found after the methanol breakthrough.

Elution Profiles Containing Benzhydryl Labeled Metabolites.

Non-hydrolyzed Urine Extract: A representative elution profile of a non-hydrolyzed ethyl acetate extract of mouse urine containing benzhydryl-<sup>14</sup>C-labeled metabolites on the adsorption column is shown in Figure 8. A total of 39.8  $\mu$ l (35,195 dpm) of the concentrated extract was injected by the stop-flow method. The usual 10 per cent eluent was used through minute 29 when the change to methanol was made. The methanol breakthrough occurred during minute 34 (vial 34). The total recovery was 33,000 dpm, equivalent to 95 per cent of the injected radioactivity.

The activity maximum at 6 minutes corresponded to 3 per cent of the total injected onto the adsorption column. The total activity through minute 33 represented 4.4 per cent of the total. The breakthrough of the 100 per cent methanol eluent was accompanied by a large increase of activity in the eluate. The elution data for the latter eluent indicated at least 2 components in a relative concentration of about 1 : 2.

Methylated Urine Extract: The peak at minute 6 in Figure 8 shifted to minute 4 (Figure 9). The smaller peak at minute 11 may represent a methylated product of 1 of the

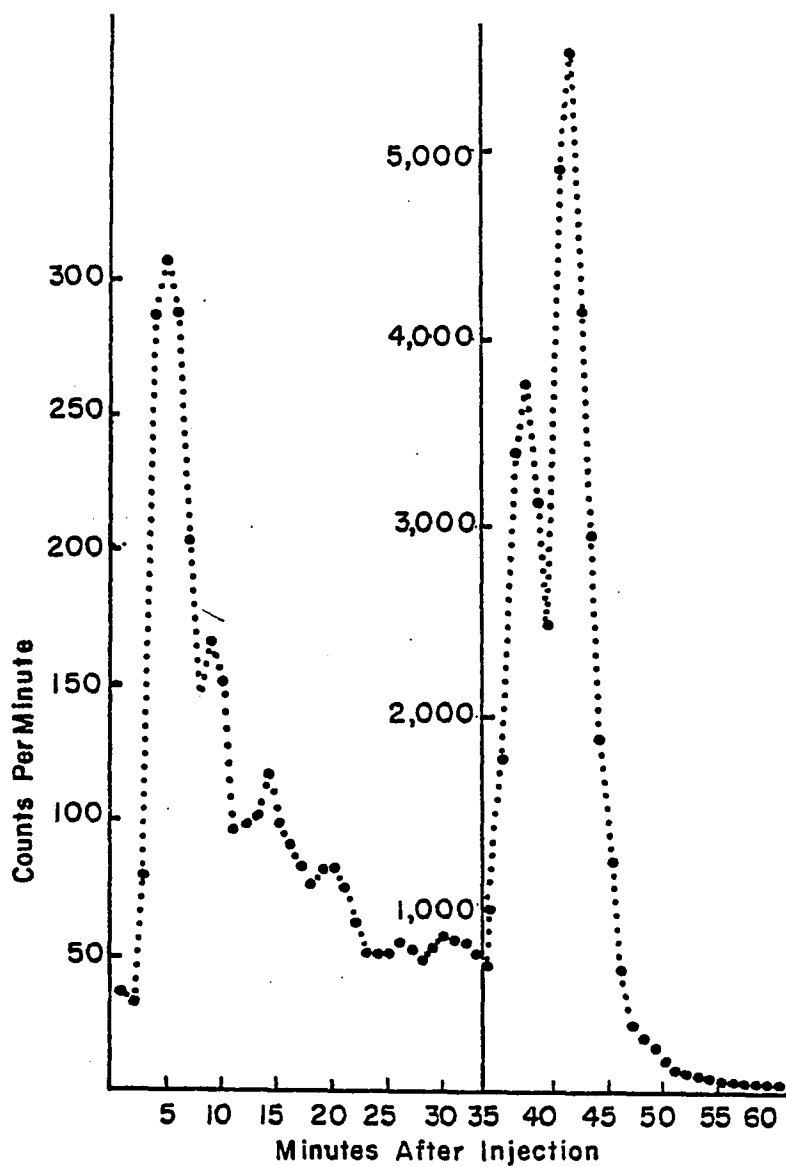


Figure 8. Elution Profile of Ethyl Acetate Extract of Urine Containing Benzhydryl Labeled Metabolites.

Column: Adsorption 10/25  $\mu$ m, length 22 cm,  
o.d.  $\frac{1}{4}$ ".

Eluent: 10% v/v ethanol/hexane switched to 100%  
methanol at minute 30, breakthrough  
at minute 35.

Flow Rate: 1 ml/min.

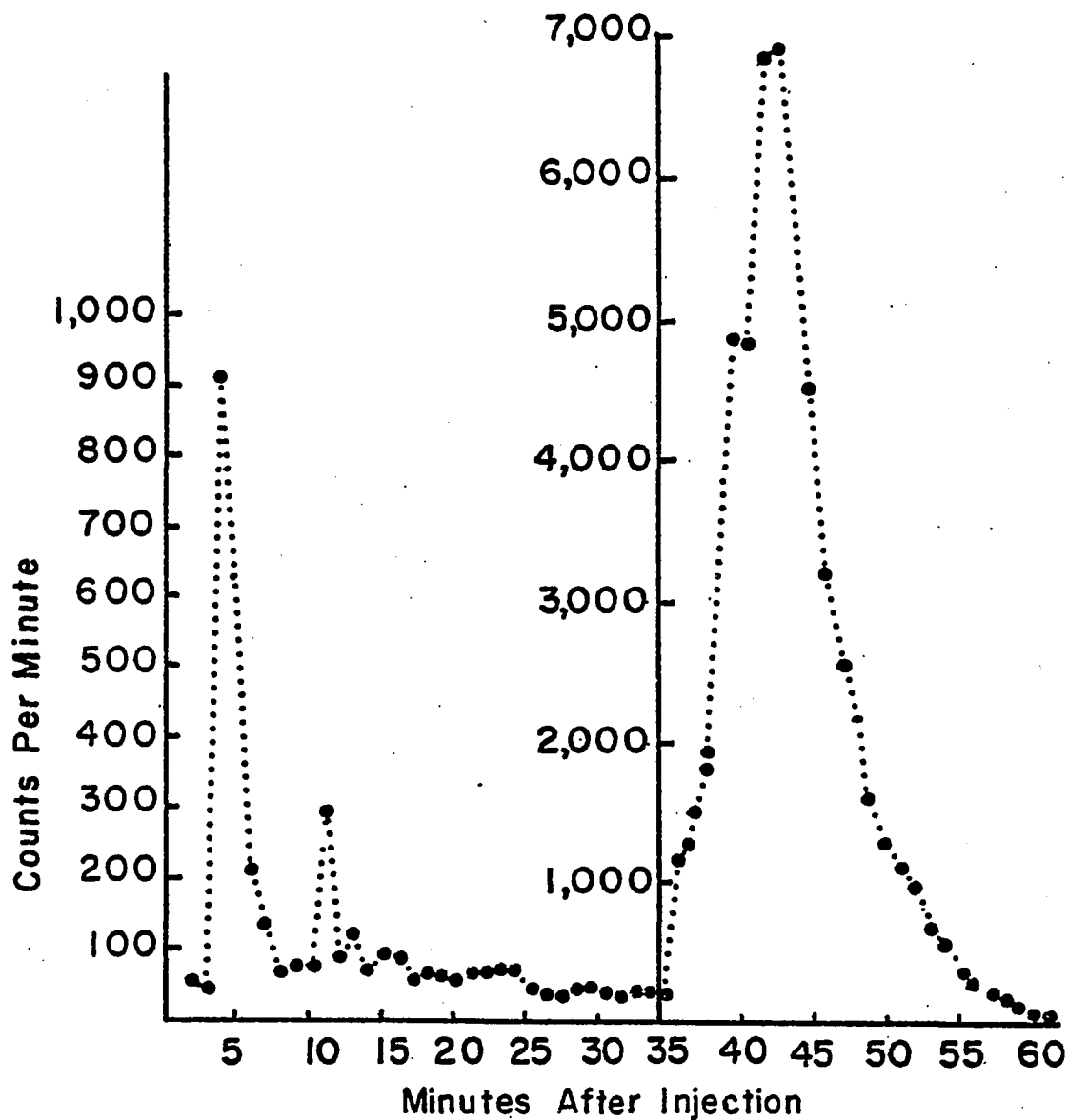


Figure 9. Elution Profile of Methylated Ethyl Acetate Extract of Urine Containing Benzhydryl Labeled Metabolites.

Column: Adsorption 10/25  $\mu$ m.  
Eluent: 10% v/v ethanol/hexane switched to 100% methanol at minute 30, breakthrough at minute 36.  
Flow Rate: 1 ml/min.

minor components. One component eluted with the methanol eluent. No quantitative studies were made with this profile.

Elution Profiles of Mouse Urine Determined  
on a Reverse Phase HPLC Column with 5%  
Methanol in Degassed Distilled Water

Carbonyl Labeled Metabolites.

Hydrolyzed and Non-hydrolyzed Urine: The profiles for both raw and hydrolyzed urine containing carbonyl labeled metabolites were determined on a reverse phase column using 5 per cent methanol-distilled water with 1 drop 8.5M glacial acetic acid per 400 ml eluent (Figure 10). There was no difference between these profiles through minute 30 and at minute 60. Both profiles exhibited a prominent maximum at minute 5 and a much smaller one at minute 8. These were the only components observed. These peaks represented approximately 5 per cent of the total radioactivity. Strict quantification was not determined for these studies.

Benzhydryl Labeled Metabolites.

Non-hydrolyzed Urine: The elution profile for raw mouse urine containing benzhydryl labeled metabolites is shown in Figure 11. An aliquot of 41.9  $\mu$ l (53,875 dpm) of urine was developed on the reverse phase column at a flow rate of 1 ml per minute. The eluent was 5 per cent methanol-degassed, distilled water to which 1 drop of 8.5M glacial acetic acid per 400 ml distilled water was used. After 51 minutes the eluent was switched to 1 : 1 acetone : water, which emerged

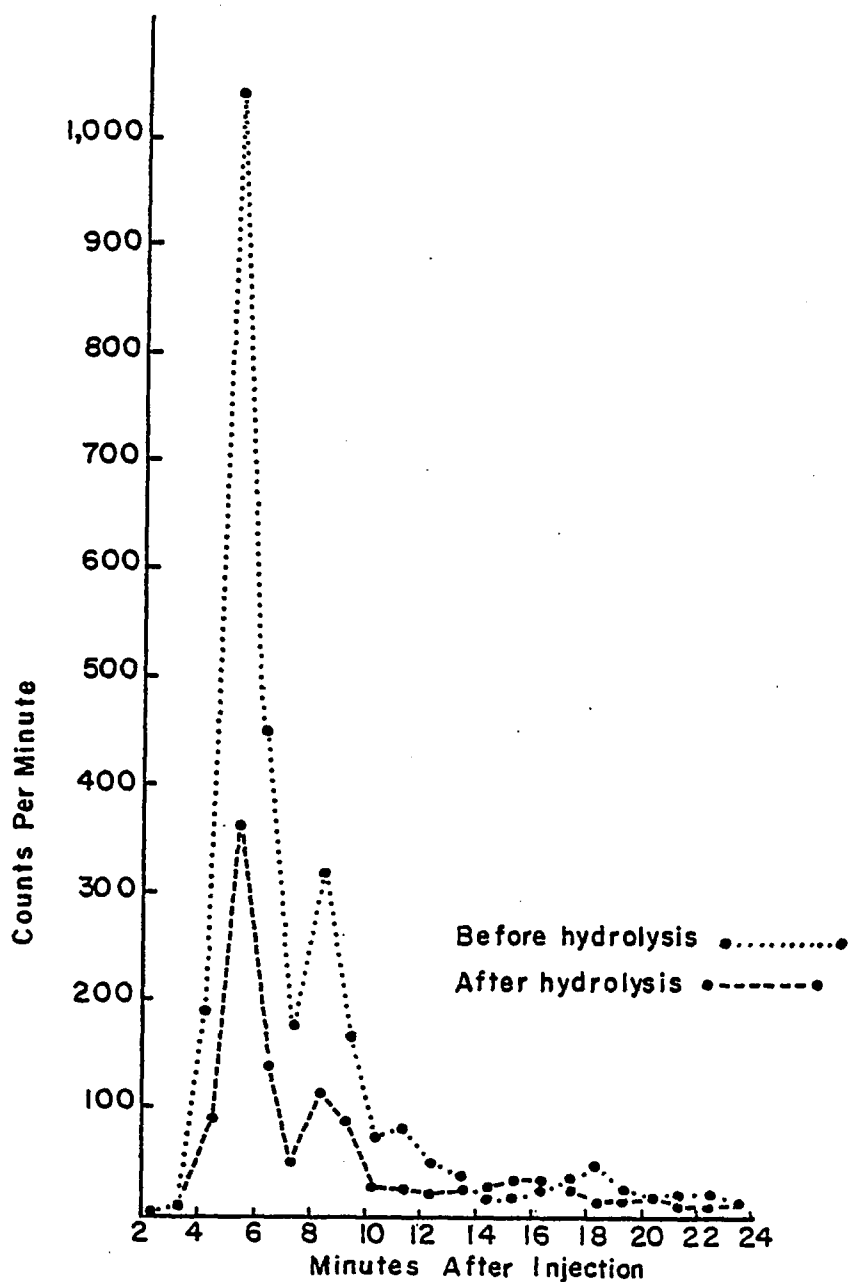


Figure 10. Elution Profile of Urine  $^{14}\text{C}$ -carbonyl-Diphacinone Metabolites before and after Acid Hydrolysis.

Column: ODS reverse phase.  
Eluent: 5% v/v methanol/water.  
Flow Rate: 1 ml/min.

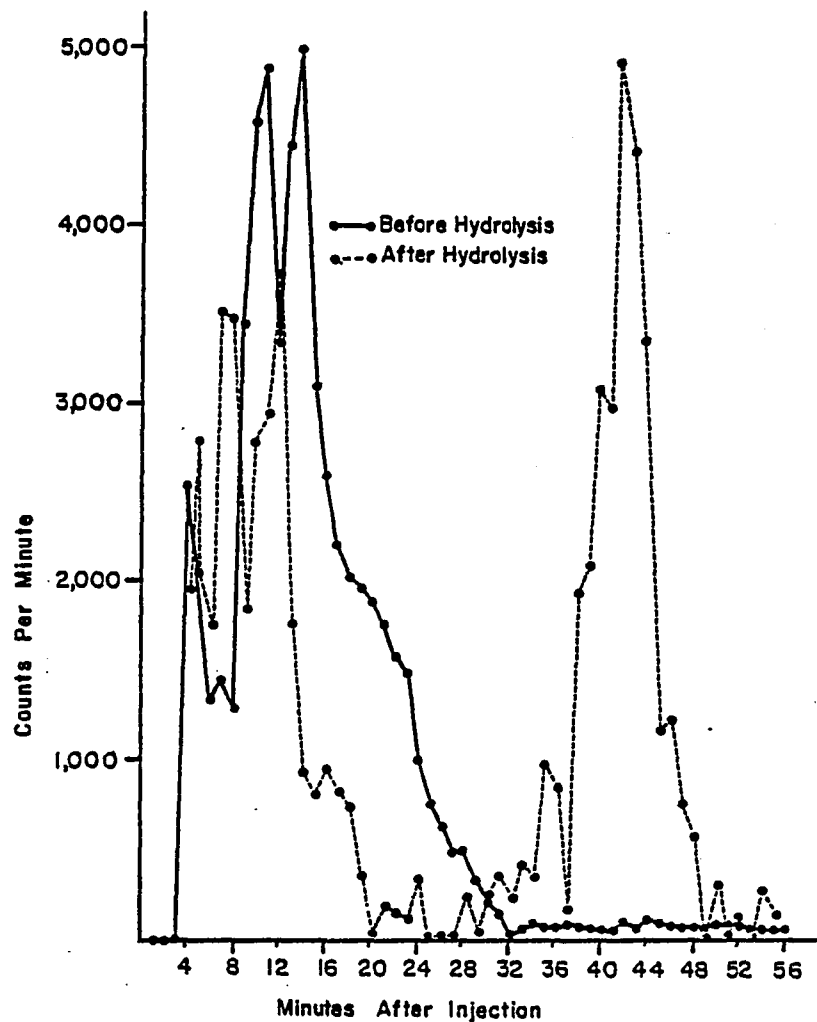


Figure 11. Elution Profile from a High Pressure Liquid Chromatograph, C-14-activity of Metabolites of Benzhydryl Labeled Diphacinone.

Column: Partisil reverse phase, length  
22 cm, o.d.  $\frac{1}{4}$ ".  
Eluent: 5% methanol/water  
Flow Rate: 1 ml/min.

at 57 minutes. This peak is not shown on Figure 10. The remaining activity eluted within 6 minutes after the 1:1 acetone:water breakthrough. Approximately 27 per cent of the injected activity was accounted for by minute 32. The 3 major activity maxima prior to 30 minutes represented, in increasing time of elution, 2.8, 8.1, and 10.5 per cent of the total injected activity. Ninety-five per cent of the total injected activity was accounted for.

Hydrolyzed Urine: The elution profile for acid hydrolyzed urine containing benzhydryl labeled metabolites is shown in Figure 11. Approximately 2375 dpm, or about 82 per cent, were accounted for. The maxima, about 42 minutes, accounted for 13 per cent while the activity between 3 and 20 minutes accounted for 13.5 per cent of the total. A 1:1 acetone:degassed-distilled water was begun at minute 64 with breakthrough at minute 69. No evidence was found for more than 1 component beyond minute 69. The low amount of activity injected onto the column resulted from the dilution of the urine both by the added acid and sodium hydroxide solutions. In addition, only 16.4  $\mu$ l of the hydrolysate was used for the profile.

#### Elution Profile of a Fecal Extract on the Adsorption Column

The elution profile of an aliquot (41,283 dpm) of the 95 per cent ethanol extract of feces obtained from mice

dosed with the 1-<sup>14</sup>C-diphacinone (Figure 12) showed 1 minor radioactivity maximum at minute 7, corresponding to less than 1 per cent of the total activity injected onto the column. The eluent was changed from 10 per cent methanol/hexane to methanol at minute 40 with breakthrough occurring near the end of minute 46. A broad maximum then eluted, peaking at minute 49 - 50. The recovery was 36,742 dpm or 89 per cent. Three per cent eluted prior to the methanol breakthrough.

#### Comparison of Toluene and Ethanol as Extractants for Feces

The total activity extracted with toluene and 95 per cent ethanol was 19,580 and 133,100 dpm respectively. The total activity found remaining in a 100 mg portion of feces extracted with 95 per cent ethanol was 8,870 dpm or 44,380 dpm per 500 mg of feces. Seventy-five per cent of the activity was extracted by the 95 per cent ethanol extractant or 6.8 times as much as by the toluene.

#### Discussion of Results

The formal structure of diphacinone with likely points of cleavage by either biological or chemical agents is shown in Figure 13. If disruption of the bond between the carbon atoms indicated by position 1 occurred, diphenylacetic acid would be formed. If prior oxidation at the benzhydryl carbon was followed by cleavage at 1, benzilic acid would result. Oxidation at this same carbon atom, with

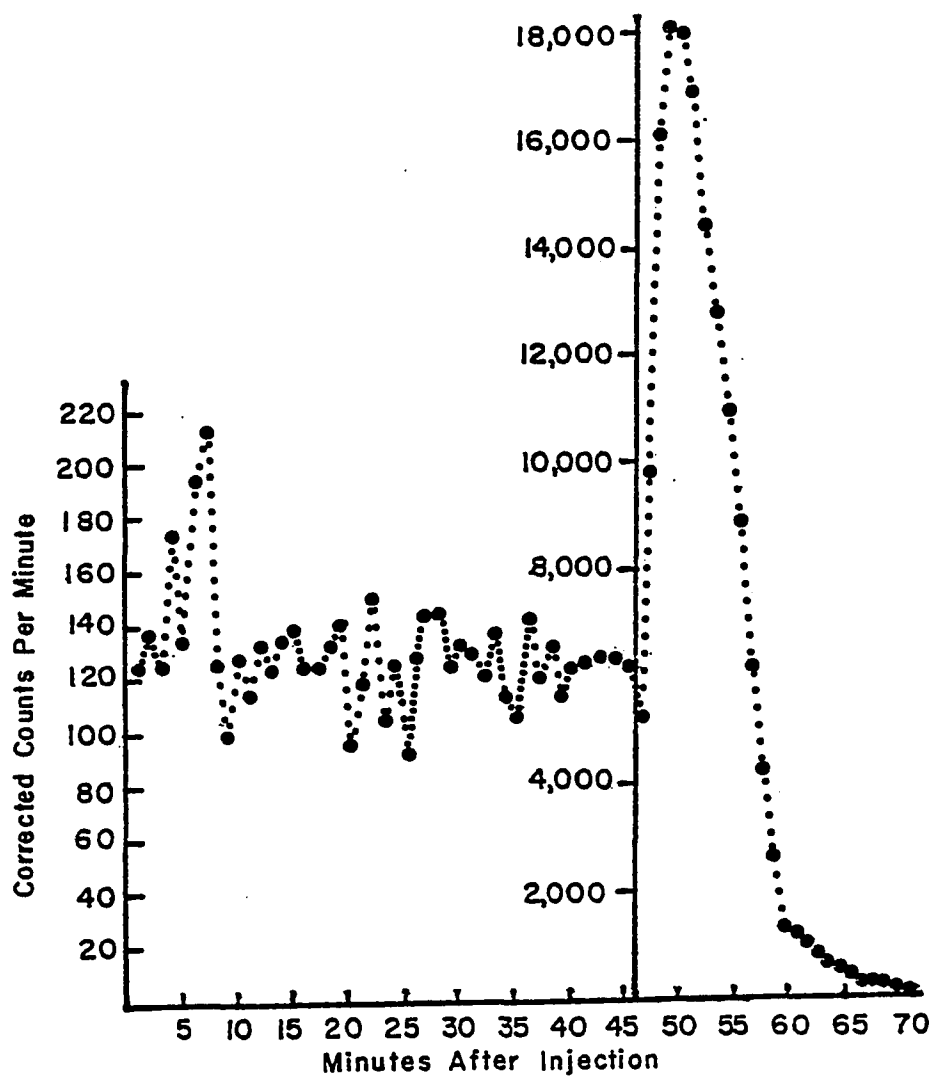


Figure 12. Elution Profile of 95% Ethanol Fecal Extract Containing  $^{14}\text{C}$ -carbonyl Metabolites.

Column: HPLC Adsorption  
Eluent: 10% v/v ethanol/hexane through minute 39, 100% methanol from 39-70 minutes, breakthrough at minute 46.  
Flow Rate: 1 ml/min.

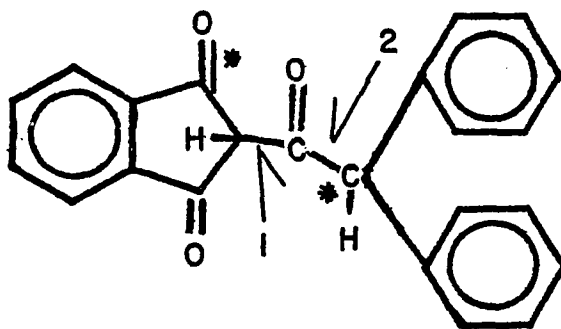


Figure 13. Formal Structure of Diphacinone Showing Points of Cleavage and Labeled Carbon Atoms.

cleavage at position 2, would produce benzophenone or benzhydrol. Hydroxylation or methoxylation on either of the geminal aromatic rings, in addition to the mentioned oxidations and cleavages, would result in additional possible metabolites.

The forementioned fragmentation products contain the benzhydryl carbon. However, compounds containing the carbonyl groups, such as 1,3-indandione would also be formed by cleavage at 1, while oxidation and cleavage at 1 could result in the formation of 1,2,3-indantrione (ninhydrin) or possibly 2-acetylbenzoic acid. In addition, another series of compounds would be formed by hydroxylation (methoxylation) of these products on the aromatic ring of the indandione moiety. This latter path may well be the most effective route of detoxification of diphacinone (p. 6).

Initial investigations concerning the nature of the urinary metabolites of diphacinone were conducted by comparing the retention times of radioactivity maxima, both before and after acid hydrolysis, with those of suspected metabolites determined with a high pressure liquid chromatograph. To aid in this study preparations of diphacinone labeled with carbon-14 at the 1-carbonyl and benzhydryl positions were utilized separately. Figure 13 shows the location of the 2 carbon atom labels.

Tables 19 and 20 list the retention times of a number of compounds suspected to be metabolites of diphacinone and radioactivity maxima obtained from elution profiles on HPLC columns. In addition,  $R_f$  values of these compounds and the labeled urinary metabolites obtained using analytical TLC are shown in Table 21. Table 22 shows representative retention times on the Partisil-10 adsorption column with 5 different 10 per cent ethanol/hexane eluants using ultraviolet adsorption to detect elution maxima. The largest deviation from the mean retention time was 14 seconds and the largest range was 20 seconds.

For a radioactivity maximum to be collected in vial 6 (plotted at minute 6) the peak activity for that component eluted between minute 5 and 6. This same variability in the determination of retention times for the radioactivity maxima was assumed as for the known compounds and was taken into account when comparisons of these values were made. Symmetry considerations of the elution maxima were also taken into account to better estimate the true retention time for the urinary components. For example, the peak at minute 10 in Figure 5 is skewed, suggesting that the maximum is nearer minute 9 than 10. If the maximum eluted at 9'15", considering the variability in the measurements of retention times, its range of retention time could be from 8'55" to 9'35". The peak at minute 6 in Figure 8 is symmetrical, an indication

Table 19. Retention Times of Suspect Metabolites of Diphacinone Determined on a Partisil Adsorption Column with a 10% Ethanol/Hexane Eluent at a Flow Rate of 1 ml per Minute.

Suspect Metabolite	Retention Time	Observed Radioactive Maxima from Labeled Diphacinone
<b>Benzhydryl Label</b>		
Benzilic acid	3' 30"	6'
Benzophenone	3' 28"	10'
Diphenylacetone	3' 30"	14'
p-Methoxybenzophenone	4' 15"	20'
Benzhydrol	4' 29"	
Diphacinone	5' 08"	
Diphenylacetic acid	5' 30"	
Sodium diphacinone	6' 00"	
p-Hydroxybenzophenone	6' 07"	
Dimethoxyphenylacetic acid	7' 15"	
<b>Carbonyl Label</b>		
1,3-Indandione	5' 15"	5'
1,2,3-Indantrione	7' 30"	10'
2-Acetylbenzoic acid	8' 00"	17'
Iron-tridiphacinone <sup>a</sup>	8' 00"	20'
Iron-didiphacinone <sup>a</sup>	27' 00"	
Iron-mono-diphacinone <sup>a</sup>	142' 00"	

<sup>a</sup>Presumed chemical composition.

Table 20. Retention Times of Suspect and Labeled Metabolites Determined on a High Pressure Liquid Chromatographic Reverse Phase Column with a 5% Methanol-Water Plus 1 Drop of 8.5M Glacial Acetic Acid Eluent at a Flow Rate of 1 ml per Minute.

Suspect Metabolite	Retention Time	Retention Times of Urinary Labeled Metabolites	
		Before Hydrolysis	After Hydrolysis
Carbonyl Label			
1,2,3-Indantrione	10' 15"	5'	5'
2-Acetylbenzoic Acid	11' 15"	8'	8'
1,3-Indandione	26' 30"	18'	
Benzhydryl Label			
Benzilic Acid	11' 0"	4'	5'
4,4-Dimethoxy-benzhydrol	31'	11'	7-8'
p-Hydroxybenzo-phenone	32'	14'	12'
Diphenylacetic Acid	38' 30" 41', <sup>a</sup>		16' 35'
Benzhydrol	45' 15"		40'
Benzophenone	90'		42'
Sodium Diphacinone	90'		47'
p-Methoxybenzo-phenone	90'		50'
Diphacinone	90'		54'

<sup>a</sup> Determined with a 5% methanol-water plus 3 drops 8.5M glacial acetic acid per 400 ml eluant.

Table 21.  $R_f$  Values for Labeled Urinary Metabolites and Certain Suspected Metabolites as Determined on 2 mm Silica Gel Preparative TLC Plates Developed with 30% Ethanol/Hexane Eluent.

Carbonyl Label	Benzhydryl Label
0.19	0.19
0.25	0.31
0.38	(0.46) Na diphacinone
0.44	(0.54) Diphacinone
(0.46) Na diphacinone	(0.56) Diphenylacetic acid
0.50	0.74
(0.54) Diphacinone	
0.61	
0.66	
0.74	

Table 22. Repeat Retention Times Obtained from an HPLC Chromatograph.

Compound	Retention Times (minutes-seconds)					Average
Benzophenone	3'20"	3'25"	3'30"	3'30"	3'35"	3'28"
Benzhydrol	4'15"	4'30"	4'30"	4'35"	4'35"	4'29"
Diphacinone	5'00"	5'20"	5'15"	5'15"	5'15"	5'11"
Na-Diphacinone	5'50"	6'00"	6'06"	6'10"	5'55"	6'00"

that the peak maximum is near 5'30". Therefore, based on this information, compounds with retention times between about 5'10" and 5'50" could be considered as possible metabolites. However, those compounds with a retention time just outside of these ranges were also considered.

Examination of Table 19 shows that 4 suspected metabolites containing the benzhydryl carbon fall within or just outside the 5'10" to 5'50" range for the 6 minute urinary component discussed above. Three of the suspected metabolites are just outside this range, but must be considered because of the approximate manner in determining this range. These suspect metabolites referred to above are diphacinone, diphenylacetic acid, sodium diphacinone and p-hydroxybenzophenone. Table 19 also shows that diphacinone and 1,3-indandione have retention times close to that for the carbonyl labeled urinary metabolite with a 5 minute retention time. This metabolite, however, is an extremely minor component of mouse urine (Figure 5). The other suspected metabolites with the 1-carbonyl carbon have retention times sufficiently different from the labeled metabolites to establish the absence of these compounds in the urine of mice fed diphacinone.

By comparison of retention times between suspected metabolites and carbonyl labeled metabolites (Table 20) determined on the reverse phase column, it was found that

none of the suspected 1-carbonyl metabolites formed by cleavage of diphacinone have retention times which correspond to those for the labeled metabolites. Comparison of retention times determined on the reverse phase column for labeled metabolites and the 4 suspected metabolites, established as tentative metabolites by comparison of retention times on the adsorption column, shows that p-hydroxybenzophenone and diphenylacetic acid do not occur free in mouse urine. The retention time for diphacinone and its sodium salt were too large on this column to be useful for establishing the absence or presence of these compounds as urinary metabolites.

Comparison of  $R_f$  values of these 2 compounds with those obtained for radioactive metabolites on silica gel with 30 per cent ethanol/hexane (Table 21) indicate that diphacinone is not present in the urine. The confirmation of the presence of sodium diphacinone is inconclusive due to the closeness of the  $R_f$  values.

The compounds listed in Table 19 with retention times less than 5 minutes are sufficiently far removed from the 6 minute maximum of the benzhydryl component to eliminate them as possible metabolites of diphacinone.

The presence of urinary conjugates containing diphacinone derived radioactivity was tested for by comparison of the elution profiles of urine and its ethyl acetate extracts before and after acid hydrolysis. The retention time of

any fragment released by hydrolysis was compared with those of suspected metabolites as previously described. An extract of urine from mice fed carbonyl labeled diphacinone was evaporated and hydrolyzed with 6M HCl at 80 - 85°C for 24 hours. The solution was again evaporated, the residue charred, extracted with benzene and eluted on the adsorption column. The portion of the profile before the polar eluent broke through (Figure 5), although slightly different in appearance from that of the non-hydrolyzed urine extract (Figure 4), represented the same per cent of the total activity injected onto the column. The most important feature of the profile after hydrolysis was the lack of an increase in the percentage of the activity or the presence of additional activity maxima eluted during the early portion. This indicated the absence of relatively simple metabolites liberated during the hydrolysis and implies that the carbonyl labeled activity not readily eluted from the adsorption column was not subject to hydrolysis.

The partial elution profile of raw mouse urine containing benzhydryl labeled metabolites, before and after hydrolysis with strong acid and heat and subsequent neutralization to pH 5-6, is shown in Figure 10. The peak at minute 14 has nearly disappeared from the profile of the hydrolyzed urine with the appearance of a new activity maximum at minute 42. Hydrolysis of conjugates would lengthen the retention

time of the aglycone on the reverse phase column. This retention time was fairly close to that of diphenylacetic acid ( $39\frac{1}{2}'$ ), the only suspected metabolite to elute near this activity maximum on this column. It was found, however, that the retention time of acidic components was fairly sensitive to small changes in pH as would be expected. For example, the retention time for diphenylacetic acid with 5 per cent methanol-water and 0.05 ml 8.5M acetic acid per 400 ml of eluent is changed by an additional 0.1 ml of 8.5M acetic acid from  $38\frac{1}{2}$  to 41 minutes (Table 19). Due to the sensitivity in retention time of this acid to small changes in pH, the pH difference between the neutralized urine hydrolysate and the acidified 5 per cent methanol-water eluent would cause a difference in the retention time of diphenylacetic acid in the urine compared to the control.

A portion of the activity maximum at minute 11 also shifted after hydrolysis (Figure 11). Benzoic acid had a retention time of 11 minutes on the reverse phase column with this eluent. However, its retention time on the adsorption column eliminated this compound as an observed metabolite. A significant amount of activity under the 11 minute peak was not shifted, thus suggesting coincidental retention times for several labeled components. For example, of the approximately 8 per cent activity associated with the minute 11 peak, less than half appeared to shift after hydrolysis.

The location of this shifted activity may be the maximum indicated by the shoulder at minute 40. The retention time of this shoulder corresponds very closely to that for diphenylacetic acid. The per cent activity associated with the peak at minute 14 plus the partial activity shifted from the peak at minute 11 very nearly equaled that located about the peak at 40-42 minutes. Since the 2 major maxima in the non-hydrolyzed profile were but 3 minutes different in retention time, it is probable that upon hydrolysis they would elute similarly if the 2 diphacinone derived compounds were related other than by hydroxylation of the aromatic ring. It is apparent that diphenylacetic acid, although not found in free form in the urine as a metabolite of diphacinone, may have been excreted as a conjugate. The percentages of activity which eluted prior to minute 50, before and after hydrolysis, were also similar, 27 to 26.5 per cent respectively. The nearness of these percentages indicates that these early eluting, water soluble components, upon acid hydrolysis, liberate components which are also inherently water soluble. This also suggests that the late eluting activity was not hydrolyzed to materials eluting within 56 minutes.

The urine extract containing benzhydryl labeled metabolites used for the profile in Figure 8 was methylated with diazomethane in ethyl ether. The profile of the methylated extract is shown in Figure 8. The peak at minute 6

shifted to minute 4, revealing the smaller peak at minute 11 which appeared as a shoulder in Figure 9. Since this reagent effects only acids, enols and phenols under the conditions of the reaction, the component which shifted to minute 4 possessed such a group.

The profile after the polar eluent breakthrough indicated the presence of at least 2 components in the late eluting fraction similar to that of the profile prior to methylation. No additional activity was observed in the elution profile during the first 35 minutes. However, conjugates and iron complexes of diphacinone would not be effected since sugar hydroxyl groups are not effected by this reagent and the enolic groups of diphacinone in these complexes are bound by the iron atom.

The partial elution profiles for both hydrolyzed and non-hydrolyzed raw mouse urine, containing the carbonyl label, are shown in Figure 10. The profiles are nearly identical in both the position of radioactivity maxima and the per cent of the total activity injected appearing during the first 25 minutes (3 per cent). This lack of appreciable activity both before and after hydrolysis, compared to the 27 per cent for the benzhydryl label, which eluted during this same period, indicates that the moieties bearing the carbonyl label are less water soluble and form fewer acid sensitive conjugates than those bearing the benzhydryl label.

This is further verified by the results from the elution profile for the hydrolysis of ethyl acetate extraction of urine containing 1-<sup>14</sup>C-diphacinone metabolites on the adsorption column. Both studies revealed that hydrolysis with strong acid and heat produced no significant alteration of the corresponding pre-hydrolysis elution profile.

Up to now, most of the discussion centered on the early portions of the elution profiles, that is, on that portion where activity eluted before the polar eluent was incorporated. This corresponded to about 13 and 3 per cent for the carbonyl and 5 and 27 per cent for the benzhydryl label on the adsorption and reverse phase respectively. Thus greater than 85 per cent for the carbonyl and 95 per cent of the benzhydryl labeled moieties remained on the adsorption column with the 10 per cent ethanol/hexane through at least 34 minutes although there was evidence that this activity remained on the column at least 2 hours (Figures 6 and 7). At least 70 per cent of the activity with the benzhydryl and 95 per cent for the carbonyl label remained on the reverse phase column through 2 hours.

That portion of the diphacinone derived activity excreted in mouse urine which eluted early on both types of columns has been described. Twenty-seven per cent of the radioactivity for the benzhydryl label was found to be water soluble. Approximately half was acid unstable and yielded

compounds upon hydrolysis very similar in elution behavior to diphenylacetic acid. In addition, many suspected metabolites were found to be absent both free and as acid sensitive conjugate forms of mouse urine.

The properties of the radioactive metabolites of diphacinone that elute only with methanol from the adsorption column can be summarized as follows:

1. They represent the major proportion of diphacinone derived metabolites injected onto either column.
2. They are apparently resistant to acid hydrolysis.
3. They are not relatively simple organic molecules.
4. They are readily removed from both columns with polar eluents.
5. Upon elution with polar eluents, the evidence indicates that they are composed of at least 2 components.
6. Methylation with diazomethane produced no observable effect upon the eluting characteristics of these compounds.

In order to determine if any type of common organic compound would not elute on the adsorption column with the 10 per cent eluent, the elution characteristics of many different types of compounds were tested. Aromatic aldehydes, ketones, amines, carboxylic acids, phenols, amides and naphthalene derivatives were all found to have retention

times less than 10 minutes. Phenolphthalein, the most complex of these related compounds, was found to have a retention time of 20 minutes. This represents the longest retention time for any compound tested on the adsorption column except the iron complexes listed in Table 19.

To eliminate the possibility that this activity was occluded within intact cells or adhering to cellular debris, the following study was undertaken even though the strong acid used for conjugate hydrolysis should have broken up this type of complex. The urine extract containing carbonyl labeled metabolites was passed through a 0.22  $\mu$  millipore filter after the ethyl acetate solvent was replaced with benzene. Figure 8 shows the elution profile determined on the adsorption column. This profile was quite similar to that for the non-filtered, non-hydrolyzed urine, except that the minor components at 17 and 20 minutes were absent. The profile is nearly identical to that of the acid hydrolyzed urine. Its features through hour 2, together with the percent recovery of the activity, indicate that cellular debris larger than 0.22  $\mu$  was not responsible for this retained activity.

Since diphacinone is a  $\beta$ -triketone, a special case of a  $\beta$ -diketone, this compound should exhibit the classical ligand properties of these compounds and form complexes with certain metal cations. Possibly this can occur within the

animal to form iron-diphacinone complexes. These compounds readily form in acid medium and were shown to be stable during acid hydrolysis, comparable to that for splitting conjugates.

These complexes readily formed at pH 1 and 3. Relatively little complex formed at pH 6 presumably due to the non-availability of ferric ion in ferric oxide. Complex formation was not observed at pH 8. The range of pH over which these complexes can form is encountered in the stomach, parts of the small intestine and often in the urine. The iron circulating in the blood and in the tissues is not present as an insoluble oxide, but is complexed with carrier proteins and would probably be more available for complex formation. This evidence and supposition thus placed these compounds on the list of suspected metabolites.

Elution profiles on the adsorption column with 10 per cent ethanol/hexane were determined for 3 different iron-diphacinone preparations. The first was of a non-labeled preparation. Three peaks or components with retention times of 7, 27, and 148 minutes were detected using ultraviolet adsorption detection during a 5 hour elution. These components represented 2.4, 16.1, and 81.5 per cent of the total area under the 3 peaks.

The elution profiles of both the  $^{59}\text{Fe}$ -diphacinone and an iron- $^{14}\text{C}$ -diphacinone preparation were determined

(Figure 14). The radioactivity maximum observed at minute 7 in both profiles contained 21.4 and 40.0 per cent of the total activity injected onto the column respectively. The high value for the minute 7 peak for the iron- $^{14}\text{C}$ -diphacinone preparation was probably due to some uncombined diphacinone since it would also elute at this time. After 1 hour, acetone, a better solvent for these complexes than methanol, was employed in both cases to desorb the late eluting complexes. During the next 25 minutes, less than 1 per cent of the remaining activity on the column was eluted. The same result was obtained during a previous profile with this  $^{59}\text{Fe}$  preparation, using methanol as the eluent. This is in contrast to the high recovery of the metabolites derived from labeled diphacinone recovered from the columns with these polar eluents. The discrepancy in the elution characteristics between these labeled complexes and the late eluting urinary metabolites established the absence of these complexes in the urine. No explanation is available for the discrepancy between the elution profile of the non-labeled iron-diphacinone preparation monitored by ultraviolet adsorption and the labeled iron-diphacinone preparation monitored by scintillation counting.

The elution profile of the fecal extract as determined on the adsorption column was similar to those of urine extracts (Figure 12). Very little activity is present before

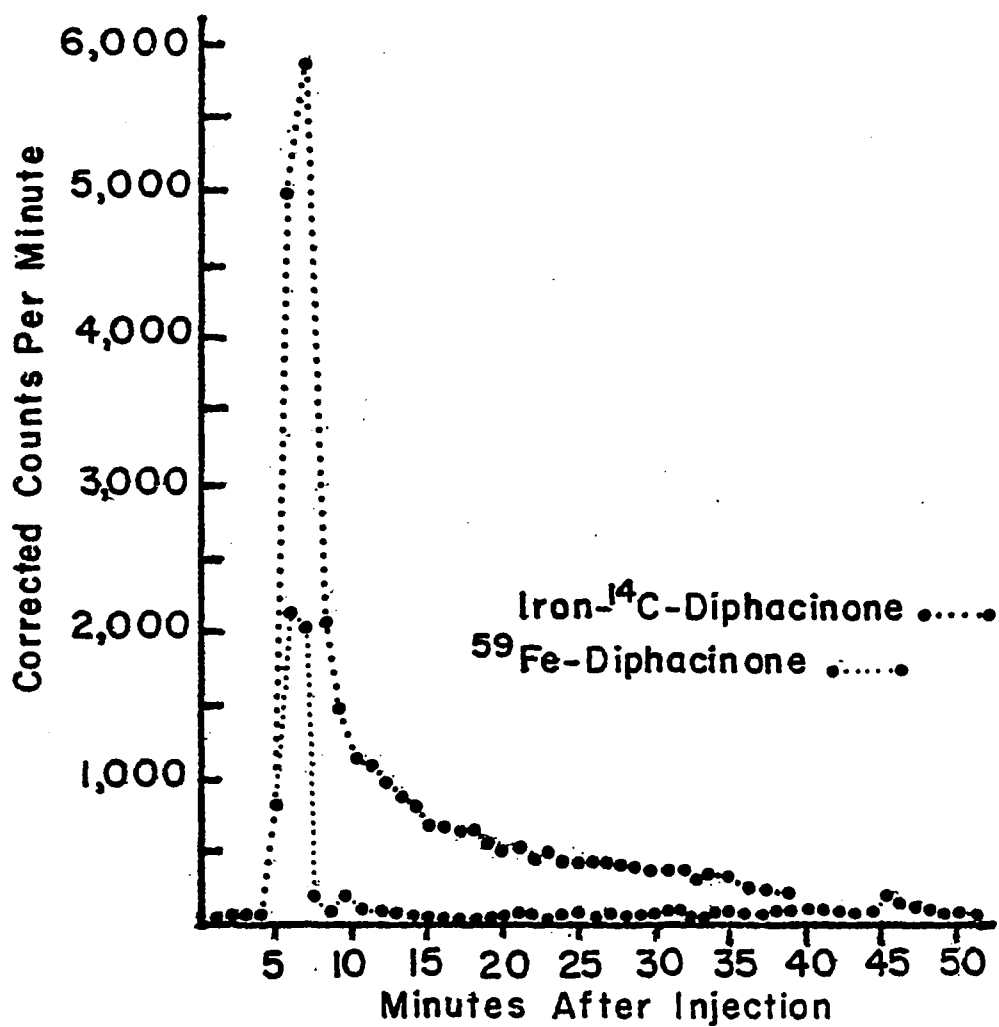


Figure 14. Elution Profile of  $^{59}\text{Fe}$ -Diphacinone and  $\text{Iron-}^{14}\text{C}$ -Diphacinone Preparations.

Column: HPLC Adsorption  
Eluent: 10% v/v ethanol/hexane to minute 39,  
100% methanol from minute 39-50,  
breakthrough at minute 44.  
Flow Rate: 1 ml/min.

the breakthrough of the methanol eluent. The peak at minute 7 corresponded to less than 1 per cent of the injected activity. The broad elution peak at minute 49 - 50 revealed only 1 component eluting with the polar eluent. The time required to elute this activity indicated a polar fecal component.

The profile revealed the absence of relatively simple suspected metabolites in the feces. Worthy of note is the absence of diphacinone in the feces, an indication that this compound, at the level administered, is efficiently absorbed from the gastrointestinal tract or modified if not absorbed.

#### Summary

A number of suspected metabolites of diphacinone in mouse urine were shown to be absent by comparing the retention times of authentic samples and those of radioactive labeled metabolites from mouse urine determined on an HPLC adsorption and reverse phase column. Thus benzoic acid, diphenylacetic acid, diphacinone, p-hydroxybenzophenone, and p-methoxybenzophenone were not observed in mouse urine. In addition the highly suspected iron-diphacinone chelates were not detected in the urine.

The sodium salt of diphacinone was not eliminated as a component of mouse urine by the comparison of retention times and  $R_f$  values. However, if this compound was present

in the urine of mice, it represented an extremely small per cent of the total activity in the urine.

The presence of the suspected metabolites or model compounds as conjugates in urine was checked for after acid hydrolysis. Evidence was obtained indicating the presence of a conjugate of diphenylacetic acid. Hydrolysis with acid and heat resulted in the disappearance of a major peak of the per-hydrolysis elution profile and the appearance of a new radioactivity maximum at 42 minutes with a prominent shoulder at 40 minutes. The 40 minute maximum is in near agreement with the retention time of diphenylacetic acid ( $39\frac{1}{2}$  minutes). However, because of retention time sensitivity to slight changes in pH of the eluent or hydrolysate, the larger peak at 42 minutes may represent diphenylacetic acid.

The largest percentage of the radioactivity found in the mouse urine did not elute except with very polar eluents such as 100 per cent methanol or acetone. Acid hydrolysis had no apparent effect on this material since an increase in radioactivity or appearance of new maxima was not observed during the first portions of the profiles from this activity after hydrolysis.

Nearly all of the radioactivity found in the fecal extract eluted only with polar eluents. No suspected metabolites were identified in this extract.

## APPENDIX A

### FORMULAS FOR CALCULATION OF SPECIFIC ACTIVITY AND PER CENT OF DOSE

All data for Body Burden Study I were processed through the College of Agriculture Numerical Analysis Center. Experimentally determined uncorrected cpm for both channels, subsample weights and total organ weight were coded by sex, hour, tissue, subsample code and cage. Channel ratio and efficiency data for construction of the regression curves were determined using each corresponding tissue digest as the source of quench. The sample channel ratio and overall efficiency were determined by computer program to calculate specific activity and per cent dose using the following equations:

$$\text{Specific Activity } \mu\text{Ci/g} = \frac{(\text{cpmu} - \text{background cpm})}{(0.82)(2.22 \times 10^6)(\text{subsample wt. gm})(\text{quench correction})}$$

The cpmu and background are the uncorrected counts per minute for the sample and background counts per minute, respectively. The constant 0.82 represents the average machine counting efficiency determined by counting a sealed source standard of carbon-14. The constant 2.22

$\times 10^6$  is the number of disintegrations per minute per microcurie of radioactive substance. The subsample weight in grams represents that weight of the entire organ taken during the replication of that organ. The quench correction term represents the efficiency of counting, not including machine efficiency, produced by quench due to the presence of NCS and degraded tissue pigments.

$$\text{Per Cent of Dose} = \frac{(\text{Specific Activity})(\text{Organ Weight})(100)}{1.60}$$

The constant, 1.60 represents the total activity in microcuries of carbon-14 diphacinone administered to the animal.

APPENDIX B

ANOVA BY TISSUE OVER ALL HOURS --  
BODY BURDEN STUDY II

Tissue	Male			Female		
	Degrees of Freedom	F Value	Probability	Degrees of Freedom	F Value	Probability
Liver	10-21	54.2	0.01	9-20	39.0	0.01
Brain	9-20	34.3	0.01	9-19	36.2	0.01
Plasma	10-20	5.2	0.01	8-17	12.1	0.01
Blood	10-20	119	0.01	8-17	46.4	0.01
Heart	10-22	516	0.01	8-16	34.5	0.01
Lung	10-22	122	0.01	8-17	36.5	0.01
Kidney	10-22	68.7	0.01	8-17	39.9	0.01

APPENDIX C

COMPARISON OF LIKE TISSUES  
BY HOUR BETWEEN SEXES  
IN BODY BURDEN STUDY II

Identifications: t = t test value, P = probability, ns = not significant at the 90% confidence level.

Hour	Heart		Lung		Kidney		Liver		Blood		Plasma		Brain	
	t	P	t	P	t	P	t	P	t	P	t	P	t	P
1.5	0.30	ns	0.15	ns	0.12	ns	0.17	ns	0.49	ns	0.045	ns	---	--
3.0	0.005	ns	0.85	ns	0.11	ns	0.78	ns	0.60	ns	0.016	ns	0.205	ns
4.5	1.76	8 <sup>a</sup>	1.10	ns	0.74	ns	0.11	ns	1.3	ns	0.42	ns	1.6	ns
6.0	0.96	ns	0.87	ns	0.91	ns	1.81	7.5 <sup>a</sup>	0.74	ns	0.25	ns	1.4	ns
7.5	2.57	1.5 <sup>a</sup>	1.94	6 <sup>a</sup>	1.00	ns	2.3	3 <sup>a</sup>	2.0	5 <sup>a</sup>	0.04	ns	2.3	3.5 <sup>a</sup>
9.0	1.20	ns	0.66	ns	0.38	ns	0.34	ns	1.0	ns	0.13	ns	0.87	ns
24.0	0.70	ns	0.33	ns	0.11	ns	0.87	ns	0.27	ns	0.002	ns	0.42	ns
48.0	2.3	3.5 <sup>a</sup>	0.32	ns	1.17	ns	1.90	6 <sup>a</sup>	0.92	ns	---	--	1.6	ns
72.0	1.3	ns	0.63	ns	0.098	ns	0.28	ns	---	--	0.37	ns	1.1	ns
96.0	0.63	ns	0.39	ns	1.8	8 <sup>a</sup>	0.71	ns	0.56	ns	0.23	ns	0.78	ns

<sup>a</sup> Not significant at the (100 - a) confidence level.

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