# The Excretion of *N*-(6-chloropicolinoyl)-glycine by the Dog Fed 2-Chloro-6-(trichloromethyl)pyridine

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A dog was fed carbon-14–labeled 2-chloro-6-(trichloromethyl)pyridine and its excreta were collected. Most of the radioactivity appeared in the urine as a compound different from either 2-chloro-6-(trichloromethyl)pyridine or any simple hydrolysis product. Isolation of the radioactive compound permitted melting point, ultraviolet absorption spectrum, and infrared absorption spectrum to be determined. All agreed with synthetically prepared *N*-(6-chloropicolinoyl)glycine.

Published reports describe the detoxication of such compounds as hexachloroethane (*3*) and DDT (*1*, *4*, *6*, *10*) by the mammal, but little has been written concerning the metabolism and detoxication of compounds containing trichloromethyl groups attached directly to aromatic ring systems.

Dogs, rabbits, and frogs excrete picolinuric acid after being fed 2-picoline (*11*), but this process involves an intermediate oxidation of the methyl group to a carboxyl, rather than a hydrolysis of a trichloromethyl group.

The study described in the following paper attempts to show the fate of ingested 2 - chloro - 6 - (trichloromethyl)-pyridine in the dog. 2-Chloro-6-(trichloromethyl)pyridine is the active ingredient in *N*-Serve (The Dow Chemical Co.) nitrogen conservor.

## Materials

2 - Chloro - 6 - (trichloromethyl) - $C^{14}$-pyridine. The 2-chloro-6-(trichloromethyl)-$C^{14}$-pyridine was prepared by the method described in a previous publication (*8*).

6-Chloropicolinic acid. 6-Chloropicolinic acid was prepared by the hydrolysis of 2-chloro-6-(trichloromethyl)pyridine (*8*).

6-Hydroxypicolinic acid. 6-Hydroxypicolinic acid was prepared as described by Fischer, Hess, and Stahlschmidt (*2*).

*N*-(6-chloropicolinoyl)glycine. Glycine (2.08 grams, 0.0278 mole) was dissolved in 30 ml. of water contained in a 250-ml., four-necked flask equipped with two dropping funnels and a thermometer.

An aqueous solution of sodium hydroxide (2.72 grams in 15 ml. of water) and a solution of 6-chloropicolinyl chloride (6.0 grams, 0.34 mole) in 25 ml. of dimethoxyethane were added, simultaneously, to the glycine solution over a 1-hour period. The temperature was kept below 30° C. At the end of the addition, the mixture was stirred for 0.5 hour and acidified with a slight excess of hydrochloric acid. The resulting product was filtered off and recrystallized first from methanol and then from water to yield 3.9 grams of colorless needles melting at 205° to 206° C.

Calcd. for $C_8H_7ClN_2O_3$: C, 44.7%; H, 3.30%; Cl, 16.55%; N, 13.10%.

Found: C, 45.0%; H, 3.43%; Cl, 16.44%; N, 13.16%.

## Preparation of Feed

A stock solution of 480 mg. of 2-chloro - 6 - (trichloromethyl) - $C^{14}$ - pyridine (0.1 mc. per mmole) in 100 ml. of acetone was prepared. One milliliter of this solution was added to each of the 120-gram portions of Purina Dog Chow which constituted a single feeding. In order to minimize volatilization losses, the feed was kept in individual 120-gram portions in tightly closed screw cap jars until immediately before feeding. A similar procedure was employed for the two feedings of compound having a specific activity of 5 mc. per mmole.

## Methods

**Feeding Procedure.** A female beagle weighing 10 kg. was fed Purina Dog Chow containing 40 p.p.m. of untagged 2 - chloro - 6 - (trichloromethyl)pyridine for 3 weeks before the beginning of the tagged feeding and for 1 week after its end. During the tagged feeding, 120-gram portions containing 40 p.p.m. of 2-chloro-6-(trichloromethyl)-$C^{14}$-pyridine were hand-fed at 8:15 A.M. and at 4:15 P.M. A separate day's feeding of 5 mc. per mmole specific activity was made later to obtain a more highly radioactive urine for paper chromatography.

**Collection and Counting of Excreta.** Excreta were collected twice daily. Urine and feces were saved separately, weighed, and stored with toluene as a preservative at 4° C. Both urine and feces were counted in infinite thickness samples. Daily residue excretion was estimated from the product of weight times infinite thickness count rate.

**Paper Chromatography.** Samples of the most radioactive urine voided after the 5 mc. per mmole feeding were applied to 1-inch wide strips of Whatman's No. 1 filter paper. One strip was developed with 1-butanol saturated with 1.5*N* ammonium hydroxide. Another was developed with 1-butanol, triethylamine, and water, 5:2:1 v./v. A third was developed with benzene, propionic acid, and water, 2:2:1 v./v. The strips were counted after they had air-dried at room temperature.

A similar procedure was used for the acid hydrolyzates of the metabolic product but with one additional solvent system. This was the *tert*-butyl alcohol (2-methyl-2 propanol) 2-butanone, diethylamine, and water, 40:40:4:20 of Redfield (*9*).

**Compound Location.** The distribution of radioactivity on the paper strip chromatograms was measured with a Forro flow counter. The position of untagged reference compounds was determined by ultraviolet absorption printing, as described by Markham and Smith for purines and pyrimidines (*5*). Glycine was located with ninhydrin spray reagent.

**Isolation of Urinary Metabolite.** Urine (3090 grams) was vacuum evaporated to 450 ml. and adjusted to pH 11 with ammonia. Count rate and weight data showed no loss of radioactivity during this evaporation. The precipitate was separated by centrifugation, and the remaining paste was freed further from mother liquors by vacuum filtration.

The pH of the combined supernatant and filtrate was adjusted to 3 with 6*N* hydrochloric acid and cooled to 4° C. for 16 hours. The crystals were filtered off and washed with 15 ml. of ethanol. The combined washings and filtrate were extracted continuously with ethyl ether for 48 hours.

The ether extract was decanted from the crystals which had formed during extraction. The crystals were stirred with 10 ml. of ethanol, cooled to 0° C., and filtered. The washing was added to the decanted ether solution and evaporated to dryness to yield 2.5 grams of sirup (I) containing slightly more than one half of the radioactivity initially present. Substance (I), 2.4 grams, was dissolved in 3 ml. of ether and placed on a 9 × 1 inch column of Celite 545 impregnated with 0.5*N* sulfuric acid according to the procedure of Phares and

Mosback (7). Development with ether followed.

Most of the radioactivity appeared in the first 100 ml. of effluent. On evaporation to dryness, this portion left 600 mg. of radioactive residue. This residue was transferred to 10 4-inch wide strips of Whatman's No. 3 filter paper and developed for 16 hours with 1-butanol saturated with 1.5$N$ ammonia.

The radioactive regions were cut out from the chromatograms, combined, and eluted with two 35-ml. portions of water. The eluates were filtered off, combined, and vacuum evaporated to dryness to yield 200 mg. of sirup.

When acidified with 3.2 ml. of 0.4$N$ hydrochloric acid and cooled to 4° C. for several hours, the sirup deposited 40 mg. of tan crystals (II).

Substance (II), 15.7 mg., was dissolved in 0.2 ml. of 1$M$ potassium phosphate pH 5.6 buffer and adsorbed on 1 gram of Dicalite. The resulting mixture was placed at the top of a 3/4-inch i.d. chromatographic adsorption tube packed with 7 inches of pH 5.6 buffered Dicalite. Development with ethyl acetate saturated with the pH 5.6 buffer followed.

Eight-milliliter portions of effluent were collected and counted. The highest concentration of radioactive material was in the seventh portion. On evaporation to dryness under a current of air, the ethyl acetate from the seventh portion deposited 1.5 mg. of colorless crystals melting at 204° to 206° C.

**Acid Hydrolysis of Metabolite.** One-tenth milligram of the metabolite was heated with 1 ml. of concentrated hydrochloric acid at 100° C. for 16 hours. The hydrolyzate was vacuum-evaporated to dryness prior to chromatographic examination.

## Results

Count rate and weight data demonstrated that at least 80% of the ingested 2 - chloro - 6 - (trichloromethyl) pyridine appeared in the urine. Paper chromatography indicated the presence of one chief compound. The $R_f$ values for this compound and for the products of acid hydrolysis are summarized in Table I.

**Table I. $R_f$ Values for Paper Strip Chromatograms**

| Compound | $R_f$ in: 1-Butanol saturated with 1.5N $NH_4OH$ | 1-Butanol, triethylamine, water, 5:1:2 v./v. | Benzene, propionic acid, water 2:2:1 v./v. | tert-Butyl alcohol, 2-butanone, diethylamine, water 40:40:4:20 v./v. |
|---|---|---|---|---|
| Urinary excretion product | 0.46 | 0.56 | 0.73 | ... |
| Hydrolysis products of same | 0.32,[a] 0.18[a] 0.03 | 0.53,[a] 0.30[a] 0.04 | ... | 0.21 |
| 2-Chloro-6-(trichloromethyl)pyridine | 0.94 | 0.88 | 0.99 | ... |
| 6-Chloropicolinic acid | 0.36 | 0.48 | 0.74 | ... |
| 6-Hydroxypicolinic acid | 0.16 | 0.28 | 0.76 | ... |
| Glycine | 0.03 | 0.04 | ... | 0.21 |

[a] Radioactive products of hydrolysis.

Apparently, the excretion product can be hydrolyzed to glycine, 6-chloropicolinic acid, and minor amounts of 6-hydroxypicolinic acid. Because the conditions employed will form 6-hydroxypicolinic acid in small yields from 6-chloropicolinic acid, the authors feel that the 6-hydroxypicolinic acid was not a structural unit of the compound from urine.

$N$-(6-chloropicolinoyl)glycine should give rise to the behavior summarized in Table I. The isolated metabolic product failed to depress the melting point of a synthetic sample of $N$-(6-chloropicolinoyl)glycine. Isolated and synthetic materials showed identical $R_f$ values on paper chromatography. Both possessed an $\epsilon_{max.}$ of $5.9 \times 10^3$ at 275 mμ in $10^{-4}M$ ethyl acetate solution.

Figure 1 shows a comparison between the infrared absorption spectrum of the urinary product and that of synthetic $N$-(6-chloropicolinoyl)glycine. Both samples were prepared in potassium bromide pellets. The agreement is close.

## Discussion

One must conclude that the dog excreted much of ingested 2-chloro-6-(trichloromethyl)pyridine in its urine as $N$ - (6 - chloropicolinoyl)glycine. Failure of the metabolic processes to effect $N$-alkylation or opening of the pyridine ring is probably due to the fact that neither 2-chloro-6-(trichloromethyl)pyridine nor 6-chloropicolinic acid possesses basic properties in aqueous solution. In its chemical behavior, 6-chloropicolinic acid resembles benzoic acid more closely than picolinic acid.

A possible mechanism for the conver-

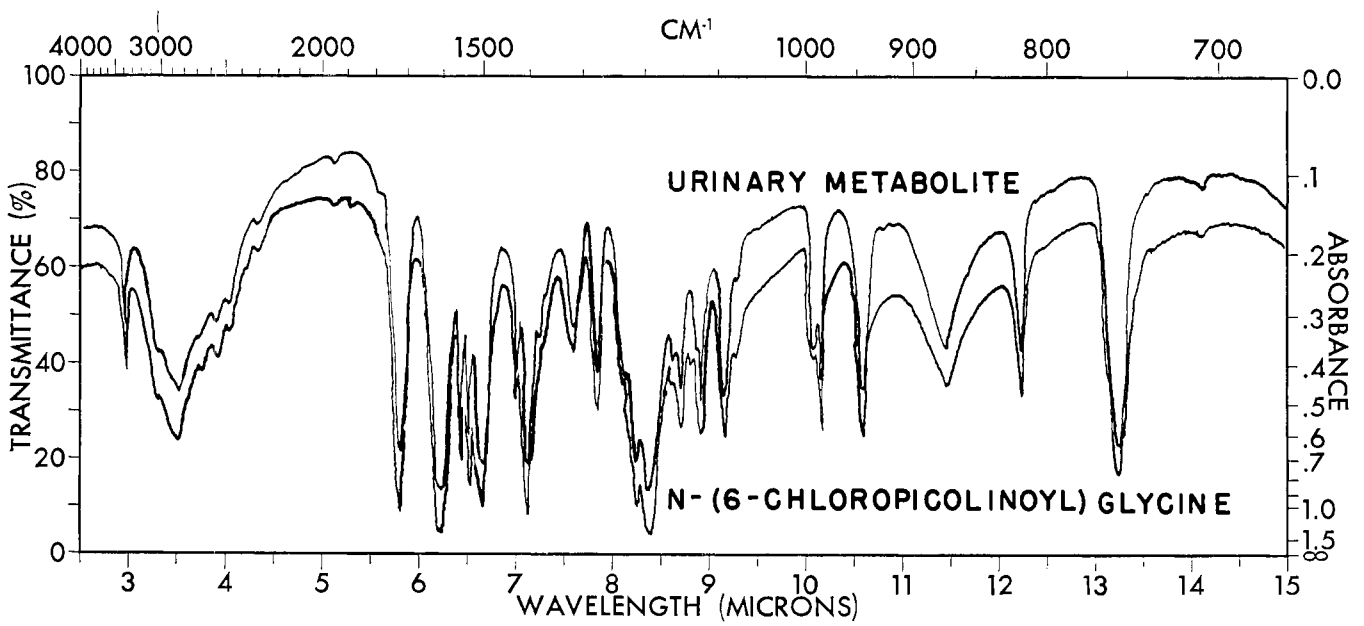


Figure 1. Infrared absorption spectra of product isolated from dog urine and of N-(6-chloropicolinoyl)glycine

sion of 2-chloro-6-(trichloromethyl)-pyridine to *N*-(6-chloropicolinoyl)-glycine involves the in vivo hydrolysis of 2-chloro-6-(trichloromethyl)pyridine to 6-chloropicolinic acid and subsequent conjugation with glycine. Kinetic studies completed recently in the authors' laboratory, support this hypothesis.

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**METABOLISM**

# The Metabolism of Naphthaleneacetic Acid-1-$C^{14}$ in Rats


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The metabolic fate of naphthaleneacetic acid (NAA) was investigated in rats following single oral doses ranging from 0.1 mg. to 250 mg. From 71 to 90% of the $C^{14}$ was excreted in the urine, and from 3 to 20% was present in the feces in the period of 0 to 3 days. In addition to a small amount of unchanged NAA, four metabolites were found in the urine. The percentages of the two major metabolites naphthaceturic acid and naphthacetylglucosiduronic acid varied with the dose level. Studies of rats with cannulated bile ducts indicated that, with a 0.1-mg. dose, from 5 to 12% of the administered $C^{14}$ appeared in the bile at 2 to 6 hours and 21 to 53% was present in the urine. The excretion pattern was reversed after a 100-mg. dose; 4 to 21% appeared in the bile and only 1 to 7% appeared in the urine.


NAPHTHALENEACETIC ACID (NAA) is used in numerous ways for modifying the growth and development of plants. The premature dropping of apples is delayed by spraying the trees with a dilute solution shortly before harvest (*5*). The methyl ester of NAA is used to prevent the sprouting of potatoes after harvest (*8*). During the storage of potatoes, the ester is hydrolyzed to the free acid (*15*) to which humans might be exposed when consuming treated fruits and vegetables.

The widespread use of this compound raises the question as to the manner in which an animal can metabolize and eliminate the material from the body. Bernhard and Caflisch-Weill (*3*) investigated the fate of NAA in several animal species at only one dose level. Since the excreted metabolites formed from certain compounds vary quantitatively with the dose administered (*14*), the authors have studied the metabolism of NAA given orally at several levels ranging from 0.1 mg. to 250 mg. per rat; the highest level is the $LD_{50}$ in a 250-gram rat (*11*).

The lowest dose corresponds to a human dose of 25 mg., which is much higher than would be expected through the consumption of treated foods (tolerance had been set at 1 p.p.m.). However, the series of results presents some basis for extrapolation to even lower doses.

### Materials and Methods

Carboxyl-labeled naphthaleneacetic acid-$C^{14}$ was obtained from Tracerlab (Waltham, Mass.). The radiochemical purity was established by paper chromatography. The specific activity was 0.89 mc. per mmole. This material was diluted with nonradioactive NAA to obtain a compound of suitable activity (not more than 5 $\mu$c. per rat) for the metabolism study. The metabolite naphthaceturic acid (m.p. 154° C.) was prepared from naphthylacetyl chloride and glycine (*10*). The naphthylacetyl chloride (b.p. 188° C. per 23 mm.) was prepared from NAA and thionyl chloride (*4*).

**Series 1: Urine and Feces Collection.** Male Osborne-Mendel rats (250 to 280 grams) were given NAA-$C^{14}$ by stomach tube using solutions neutralized with sodium hydroxide. The dosages of 0.1 to 250 mg. contained from 0.5 to 5.0 $\mu$c. of $C^{14}$. The syringes were rinsed with water, and the amount of NAA in the rinsings was determined by radioactivity measurement. These quantities of NAA were deducted from the initial doses so that the actual doses consumed could be calculated. The animals were placed in metabolism cages which permitted separate collections of urine and feces.