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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Received for review March 3, 1966. Accepted June 29, 1966. Division of Agricultural and Food Chemistry, Winter Meeting, ACS, Phoenix, Ariz., January 1966.

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 μc. 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 μc. 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.

The collection vessels were immersed in ice throughout the experiment in order to minimize decomposition of the metabolites without the addition of a preservative. Food and water were provided *ad libitum*. At the end of the daily collection periods the cages were washed with water, and these rinsings routinely were added to the urine for counting.

Series 2: Urine and Bile Withdrawal. In this series, male Osborne-Mendel rats (350 to 435 grams) were anesthetized with pentobarbital. The bile ducts were cannulated by inserting a polyethylene tube in the common bile duct, and the urinary bladders were ligated at the urethra. Dosages of 0.1 and 100.0 mg. were then administered by stomach tube. There were two collection periods for each group (2 and 6 hours after administration). The urine was withdrawn from the bladder with a syringe. A small quantity of water then was injected into the bladder for a rinse which was added to the collected samples.

Radioactivity Measurements. All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Aliquots of 0.1 to 0.2 ml. of urine, bile, extract of feces, and fractions of these from DEAE cellulose columns were counted directly in vials containing 18 ml. of 30% absolute methanol in toluene (v./v.) with 3 grams per liter of 2,5-diphenyloxazole (PPO) and 100 mg. per liter of 1.4-bis-2-(5-phenyloxazoyl)-benzene (POPOP) (*6*). An internal standard of C^{14}-labeled toluene was added to one of a pair of duplicate vials to correct for quenching and efficiency of counting. The results were expressed as disintegrations per minute (d.p.m.). Samples were corrected for background and counted until 10,000 counts accumulated or at $^1/_2$ hour, whichever was shorter, to give a probable error of 1%.

The feces were triturated with an equal quantity of water, and concentrated hydrochloric acid was added to produce a concentration of 2*N*. The mixture was heated for 2 hours, cooled, and then extracted 3 times with 2 volumes of a 1:1 mixture of ethanol and ethyl ether. The extract was filtered through glass wool and aliquots were placed in counting vials.

Isolation and Characterization of the Excretion Products. Aliquots (5.0 ml. each) of the first day's urine from each rat in series 1 was fractionated on 10 grams of DEAE-cellulose (formate form) in a column 22 × 2.2 cm. After the urine was applied, the column was washed with 200 ml. of water. Elution was then continued with 1000 ml. of ammonium formate buffer with a linear gradient increase in molarity from 0 to 0.25*M*, pH 3.5. Fractions of 10 ml. were collected in a fraction collector. The effluents were scanned by counting every other fraction to locate those which were radioactive. These were identified by comparisons of their elution positions with known compounds, by ultraviolet absorption spectral analysis, and by paper chromatography.

Aliquots of the pooled fractions were concentrated to near dryness and nonlabeled carriers NAA and naphthaceturic acid routinely were added before the aliquots were applied as a line to the paper strips. The chromatograms were developed by the ascending technique on Whatman 3 mm. paper. The three solvent systems used were (1) 1-butanol saturated with 1*N* hydrochloric acid, (2) 1-butanol saturated with water, and (3) 80% ethanol in water. After the chromatograms were dried, they were scanned in a 4-π windowless radiochromatogram scanner (Baird-Atomic, Inc., Valley Stream, N. Y.). The carriers were made visible by being photochemically converted to hydroxyl derivatives (*7*) which in turn were located by spraying with diazotized sulfanilic acid. NAA and its metabolites gave a pink spot by this method. Naphthaceturic acid became yellow when sprayed with 4% *p*-dimethylaminobenzaldehyde in acetic anhydride and heated a few seconds over a hot plate. At larger doses (100 and 250 mg.) urinary glucuronides were detected as blue spots on strips without the addition of a carrier when sprayed with 2% naphthoresorcinol in 33% trichloroacetic acid and heated.

Urinary glucuronides were cleaved with β-glucuronidase (Ketodase, Warner-Chilcott, Morris Plains, N. J.). The ketodase was added to aliquots of the column eluates previously adjusted to pH 5 until the concentration was 1000 Fishman units of β-glucuronidase per ml. of solution. An equal volume of 2*M* acetate buffer, pH 5, was added, and the solutions were incubated at 37° C. for 24 hours.

In series 2, the metabolites in the bile and urine were not quantitated by column chromatography.

Results

Urine and Feces Collection. The excretion of C^{14} following single oral doses of NAA-C^{14} is given in Table I. From 71 to 90% of the administered radioactivity was excreted in the urine, the majority within 24 hours with the exception of the highest dose. With the 250-mg. dose, a greater percentage was excreted on the second day. From 3 to 10% of the radioactivity was present in the feces with the smaller doses. This proportion was increased to 20% at the largest dose level. No measurable amount of radioactivity was detected in the excreta after the third day.

Figure 1 shows the elution pattern of the urinary radioactivity for each of the representative dose levels after chromatography on DEAE columns. Scintillation counting of the effluents indicated five radioactive peaks representing two major and three minor metabolites. Peak A was the only one which could be eluted with water, which indicated that it was a neutral or nonacidic material. After peak A was eluted with the water wash, gradient elution was started, and a small peak B was eluted which had a position corresponding to that of authentic NAA. This peak was followed by the elution of two major peaks, C and D, and a smaller peak E.

The fractions in each of the radioactive peaks were combined, and aliquots of each were counted for quantitative measurements. The results are given in Table II. The two major metabolites C and D accounted for 70 to 93% of the urinary metabolites, and the three minor peaks accounted for 6 to 29% of the total radioactivity in the urine. Peak C was the predominant metabolite after the smaller doses of 0.1 and 1.0 mg., representing approximately 58% of the total urinary activity. Peak D was the major metabolite after larger doses of 100 and 250 mg. were given.

A series of qualitative tests was performed on the pooled fractions from the column. After alkali hydrolysis, peak C gave a positive test for glycine (*1*). Peak D gave a positive test for glucuronides when treated with naphthoresorcinol (*9*). Peak E gave a slight turbidity with barium chloride when tested for ethereal sulfates (*13*).

After the metabolites C and D were

Table I. Excretion of Radioactivity by Rats Following Single Oral Doses of NAA-C^{14}

Rat Number	Rat Wt., Grams	Dose, Mg.	% of Dose: Urine 1st day	Urine 2nd day	Urine 3rd day	Feces 1st & 2nd day	Feces 3rd day	Total Recovery of Radioactivity
4	255	0.097	83.5	0.9	0.03	8.0	0	92.5
8	270	0.116	80.0	2.4	0.9	9.7	0	93.0
1	265	1.001	89.0	0.8	0.3	Not analyzed		
5	251	0.992	77.8	5.7	0.6	3.2	0	87.3
3	255	93.6	60.3	13.6	0.2	14.3	0.2	88.4
7	280	100.6	55.0	16.1	0	21.2	0	92.3
6	260	247.7	29.1	39.8	7.2	14.1	3.1	93.3
9	280	243.1	24.5	42.4	10.2	18.4	2.0	97.5

each chromatographed on paper and the isolated radioactive areas were eluted with water, the ultraviolet spectra indicated both to be similar to NAA, with peaks at 270, 279, and 290 mμ. However, neither was NAA since they had slower mobilities than NAA in the several solvent systems used.

When metabolite C was chromatographed with naphthaceturic acid added as a carrier, the radioactive peak coincided with that of the naphthaceturic acid carrier. When metabolite D was hydrolyzed with β-glucuronidase, the ether extract of the hydrolyzate was chromatographed on paper to give a peak coinciding with that of carrier NAA. Peak B gave a paper chromatographic pattern identical with that of unchanged NAA.

The minor peaks A and E have not been positively identified. The elution position of peak A suggests a carbonate. The elution position of peak E indicates, possibly, a stronger acid than the other peaks. Since a slight turbidity occurred when tested for ethereal sulfates, a small percentage of NAA may be hydroxylated and then conjugated with sulfuric acid. No attempt was made to define the chemical nature of the radioactivity in the feces.

Urine and Bile Withdrawal. Results of the excretion by rats with cannulated bile ducts are given in Table III. After the dose of 0.1 mg., the amount excreted in the urine was approximately 4 times that excreted into the bile. When the duration of the experiment was extended to 6 hours, there was only a slight increase in the percentage of C^{14} found in both urine and bile.

The excretory pattern was reversed after the 100-mg. dose, with more radioactivity appearing in the bile than in the urine. There was, also, a significant increase when the collection period was extended to 6 hours, with the percentages in both urine and bile being doubled. The bile and urine were chromatographed on paper after both dose levels, and representative results are shown in Figure 2.

Following the 0.1-mg. dose, naphthaceturic acid was the major metabolite in the urine. A considerably smaller peak, corresponding to the glucuronide conjugate of NAA, was also present, but no NAA could be detected. The glucuronide conjugate was the major metabolite in the bile. Smaller peaks corresponding to naphthaceturic acid, unchanged NAA, and an unidentified peak were also present in the bile.

Paper chromatography following the 100-mg. dose showed the glucuronide to be the predominant peak in both urine and bile, with much smaller peaks, corresponding to the glycine conjugate, appearing in both cases. Unchanged NAA appeared in the bile, but none was detected in the urine.

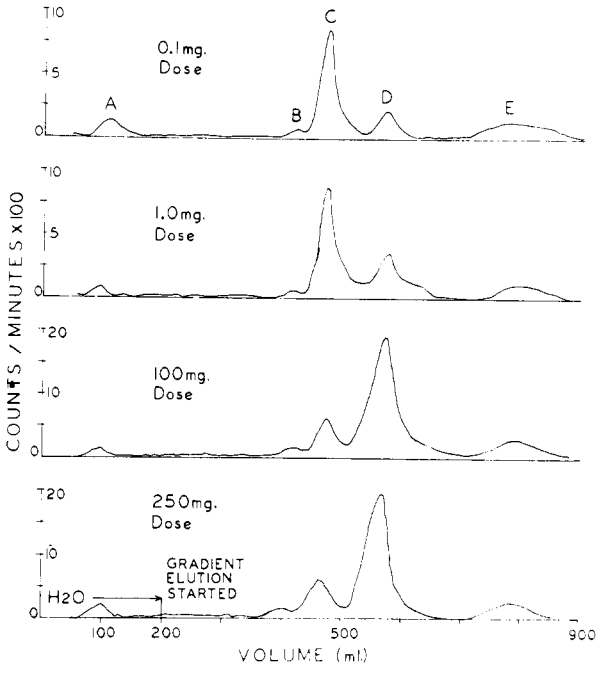

Figure 1. Representative diagrams of DEAE-cellulose chromatography of the first day's urine from rats which received NAA–C^{14} orally at levels from 0.1 mg. to 250 mg.

Peak A: unidentified. B: unchanged NAA. C: naphthaceturic acid. D: naphthacetylglucosiduronic acid. E: unidentified

Table II. Metabolites of NAA-C^{14} in Rat Urine 24 Hours after a Single Oral Dose

Rat Number	Dose, Mg.	Per Cent of Total Urinary Radioactivity				
		A unidentified	B unchanged NAA	C naphthaceturic acid	D acetylglucosiduronic acid	E unidentified
4	0.097	7.7	2.0	67.1	15.0	19.1
8	0.116	8.4	1.7	54.5	17.9	8.8
1	1.001	3.5	1.5	51.8	23.0	2.1
5	0.992	3.7	2.9	59.5	25.1	2.7
3	93.6	0.9	5.8	22.0	63.6	4.9
7	100.6	1.7	7.7	12.7	56.7	6.7
6	247.7	1.6	0.9	19.6	56.2	3.4
9	243.1	1.3	0	20.2	73.1	8.6

Table III. Excretion of Radioactivity by Rats with Cannulated Bile Ducts Following Single Oral Doses of NAA-C^{14}

Rat Number	Rat Wt., Grams	Dose, Mg.	Duration of Experiment, Hours	% of Dose	
				Urine	Bile
10	400	0.083	2	20.4	5.8
12	435	0.104	2	53.4	12.5
14	430	0.104	2	37.7	9.1
16	380	0.092	2	34.5	8.3
23	355	0.112	4	35.5	10.2
21	340	0.112	6	38.6	9.7
22	360	0.116	6	42.7	8.0
24	345	0.118	6	36.1	9.6
11	360	106.1	2	2.2	8.0
13	410	106.8	2	1.8	7.6
15	380	94.5	2	1.1	3.8
16	365	94.3	2	6.1	7.7
20	350	95.4	3.5	3.6	11.5
18	370	95.0	6	3.3	16.7
19	360	95.4	6	7.4	21.3

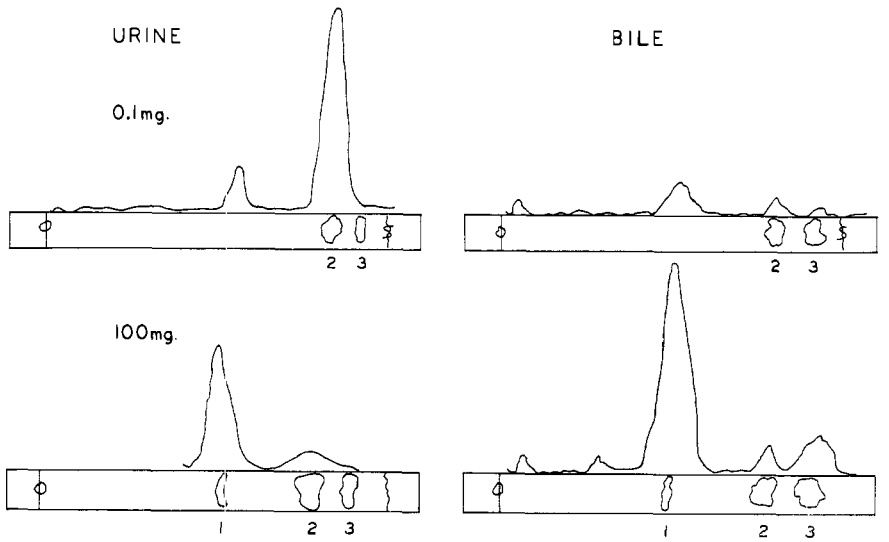

Figure 2. Paper chromatographic pattern of radioactivity of urine and bile in series 2 developed with butanol saturated with 1.0N HCl

On the corresponding paper strips the position of the glucuronide conjugate (1) was made visible by spraying with the naphthoresorcinol reagent, and the carriers naphthaceturic acid (2) and unchanged NAA (3) were made visible by spraying with diazotized sulfanilic acid after photochemically converting them to hydroxyl derivatives

Discussion

Naphthaleneacetic acid-C^{14} is readily absorbed from the gastrointestinal tract of rats following oral administration of the compound. The rates and routes of elimination varied markedly with the different dosage levels. Following small dosages of 0.1 and 1.0 mg., the majority of the C^{14} was excreted in the urine within 24 hours. With larger dosages a decreasing percentage of C^{14} was eliminated in the urine, and the percentage excreted in the feces increased. At all levels elimination was over 90% complete at the end of 3 days.

The results of this study show that when NAA-C^{14} is administered orally to the rat, it is quickly conjugated with glycine and glucuronic acid. Bernhard and Caflisch-Weill (*3*) found the glycine conjugate in the urine of a dog which had been fed NAA, but they did not detect this metabolite in the urine of a rat and a rabbit.

The conjugation with glycine is the major metabolic route with small doses, whereas the glucuronide conjugation becomes the major route with larger doses. The rate of synthesis with glucuronic acid appears to depend on the dose level, whereas the rate of synthesis with glycine is independent of the dose level. The rate of synthesis with glycine appears to depend on the amount of glycine which is immediately available for the synthesis. This is in agreement with the work of Arnstein and Neuberger (*2*), who reported that the formation of hippuric acid from benzoic acid depends upon the amount of free glycine which is immediately available for conjugation and on the rate at which bound glycine can be mobilized for the synthesis. Simkin and White (*12*) reported that the concentration of free glycine in the liver and blood of rats on a glycine-poor diet was diminished after administration of benzoic acid, but no change was detected in liver-protein glycine or other tissue glycine.

The proportion of the C^{14} found in the bile of rats in series 2 (ratio of bile to urine) was greater than that found in the feces of rats in series 1 (ratio of feces to urine). This suggests that the metabolites excreted into the bile are then reabsorbed from the intestines and excreted by way of the kidneys. Possibly the glucuronide conjugate excreted into the bile is hydrolyzed by β-glucuronidase known to be present in the gut content. The free NAA so liberated could be reabsorbed from the intestines. This cycle could be repeated until all of the material is eliminated from the animal.

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Received for review March 24, 1966. Accepted July 5, 1966.

Correction

Protein Composition and Functional Properties of Meat

In this article by T. H. Donnelly, E. H. Rongey, and V. J. Barsuko [J. AGR. FOOD CHEM. **14,** 196 (1966)], on page 198, the left and right halves of Figure 2 are reversed.