STUDIES WITH CARBOXYL-LABELED 3-HYDROXY-ANTHRANILIC AND PICOLINIC ACIDS IN VIVO AND IN VITRO

By ALAN H. MEHLER and EVERETTE L. MAY

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland)

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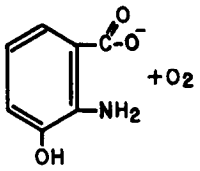
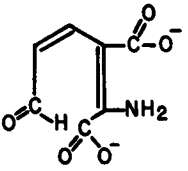
The formation of picolinic acid by the action of liver enzymes on 3-hydroxyanthranilic acid has been described previously (1). The reaction was postulated at that time to involve the intermediate formation of an acyclic compound that could undergo either a spontaneous cis-trans isomerization and cyclization to form quinolinic acid or through enzyme action lose a carboxyl group and cyclize to become picolinic acid. In the present work evidence is presented that the carboxyl group of 3-hydroxyanthranilic acid

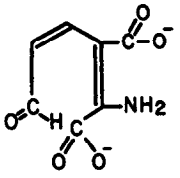
1. 

3-Hydroxyanthranilate

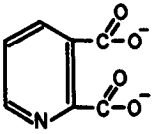
3-Hydroxyanthranilic Oxidase

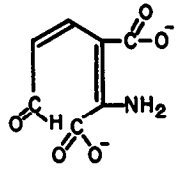


2-Acroleyl-3-aminofumarate

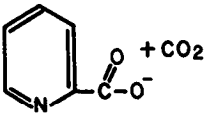
2a. 

Spontaneous


Quinolinate

2b. 

Picolinic Carboxylase



Picolinate

is the group that is cleaved through the action of the second enzyme of the sequence, now called picolinic carboxylase. The accompanying reactions are therefore proposed to represent the metabolism of 3-hydroxyanthranilic acid by liver.

3-Hydroxyanthranilic acid is an important intermediate in tryptophan metabolism and is known to be a precursor of nicotinic acid (2). The labeled carboxyl group of 3-hydroxyanthranilic acid persists in nicotinic acid derivatives (3). In the present work it was found that most of the isotope of a small dose of carboxyl-labeled 3-hydroxyanthranilic acid given to rats appears as CO_2, whereas carboxyl-labeled nicotinic acid does not yield any labeled CO_2. This is evidence that the decarboxylation to form picolinic acid is a major step in the metabolism of 3-hydroxyanthranilic acid *in vivo*.

These studies involved the syntheses of carboxyl-labeled 3-hydroxyanthranilic acid and carboxyl-labeled picolinic acid. The former was prepared according to the combined sequences of D'Angeli *et al.* (4) for the preparation of 2-nitro-3-methoxybenzoic acid from *m*-methoxybenzoic acid, Ciereszko and Hankes (5) for the conversion of this product to carboxyl-labeled 2-nitro-3-methoxybenzoic acid, and Nyc and Mitchell (6) for the reduction and hydrolysis to 3-hydroxyanthranilic acid. However, it was found necessary to alter the described procedures in certain steps, probably because of the reduced scale.

Methods

Preparation of Carboxyl-C^{14}-Labeled 3-Hydroxyanthranilic Acid from 3-Methoxy-2-nitroiodobenzene—The synthesis of carboxyl-labeled 3-hydroxyanthranilic acid devised by Ciereszko and Hankes (5) involves substitution of labeled cyanide for the halogen of 3-methoxy-2-nitroiodobenzene. The nitrile is then hydrolyzed to the corresponding carboxylic acid, which is reduced and hydrolyzed to 3-hydroxyanthranilic acid. In the exchange of cyanide for the iodine of 3-methoxy-2-nitroiodobenzene (5) very poor yields were obtained when the dry reagents were heated together, apparently because of sublimation of the iodo compound. This reaction was found to proceed smoothly with ethylene glycol as a solvent. A magnetically stirred mixture of 0.35 gm. of 3-methoxy-2-nitroiodobenzene (5), 0.11 gm. of $Cu_2(C^{14}N)_2$, and 3 ml. of ethylene glycol was brought quickly to boiling and refluxed briskly for 4 to 5 minutes. The cooled mixture was diluted with water, cooled to 0°, and filtered. The precipitate was extracted with three to four portions of boiling methanol, each extract being filtered from cuprous iodide. Dilution of the filtrate with an equal volume (about 10 ml.) of water, cooling to 0°, and filtering gave 0.19 gm. (90 per cent) of nitrile-C^{14}-labeled 3-methoxy-2-nitrobenzonitrile, m.p. 121.5–122°. This product, 0.3 gm. of sodium hydroxide, 2 ml. of water, and 1.5 ml. of alcohol were refluxed gently for 30 minutes with nitrogen bubbling through the solution. The solution was cooled, acidified with concentrated hydrochloric acid, cooled to 0°, and filtered to give 0.14 gm. of crude acids which were sublimed at an air bath temperature of 125–150° (0.5 mm.). Collected as the

more volatile sublimate were 25 mg. of *m*-methoxybenzoic acid, m.p. 104–105° (identified by elemental analysis and comparison with an authentic specimen) and then 95 mg. of 3-methoxy-2-nitrobenzoic acid, m.p. 253–256° (decomposition). Reduction of this nitro acid by the procedure of Nyc and Mitchell (6) yielded 70 mg. of 2-amino-3-methoxybenzoic acid, m.p. 167–170°. The latter, 15 mg. of red phosphorus, and 1.4 ml. of 55 per cent hydriodic acid, in a sealed tube, were kept in a steam bath for 24 hours. The contents of the tube were diluted with water and the solution was filtered. The filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 3 ml. of boiling concentrated hydrochloric acid and cooled gradually (finally to 0°) to give 58 mg. (73 per cent based on 2-amino-3-methoxybenzoic acid, 25 per cent based on radioactive cuprous cyanide) of carboxyl-C^{14}-labeled 3-hydroxyanthranilic acid hydrochloride, m.p. 229° (decomposition). Additional product was obtained from the mother liquor by the addition of carrier 3-hydroxyanthranilic acid.

$C_7H_8ClNO_3$(189.6). Calculated, C 44.34, N 4.25; found, C 44.42, H 4.39

Picolinic acid labeled in the carboxyl group was prepared essentially as described by Murray *et al.* (7) by conversion of 2-bromopyridine to 2-pyridyl lithium in ether under helium and carboxylation of the lithium compound with $C^{14}O_2$ liberated from $BaC^{14}O_3$.

After adding 3 N HNO_3 to the reaction mixture, the aqueous phase was put on a column of Dowex 1 acetate, 1 cm. × 20 cm., which was then washed with water. The picolinic acid was eluted by a gradient method in which approximately 5 N acetic acid was added to a mixing flask containing 100 ml. of water. The picolinic acid, detected by its radioactivity, appeared in a well defined fraction shortly after the eluate became acidic. The solution of picolinic acid was taken to dryness under reduced pressure and the residue was dissolved in water. This solution was chromatographed on a small column of Dowex 50 (1 cm. × 12 cm.) with 4 N HCl in the reservoir and 50 ml. of water in the mixing flask. The fractions containing picolinic acid were dried *in vacuo*. The residue was dissolved in water and again chromatographed on Dowex 1 acetate as before. This procedure yielded fractions that exhibited the spectral characteristic of picolinic acid, and these were used without further isolation when very small quantities of highly radioactive material were desired. Other preparations were crystallized from HCl-saturated ethanol, after the addition of carrier picolinic acid, and recrystallized from ethanol-ether.

An aqueous extract of rat liver acetone powder was used as a source of 3-hydroxyanthranilic oxidase free from picolinic carboxylase (1). Picolinic carboxylase purified from guinea pig liver was used in some experiments, but it was found more convenient to use crude extracts of livers from rats

treated with cortisone, which increase the level of picolinic carboxylase many times.[1] Carboxyl-labeled nicotinic acid was obtained commercially.

C^{14} was determined in a windowless gas flow counter with sample sizes sufficiently small to cause negligible self-absorption, except in the case of the measurement of expired CO_2. In this case the CO_2 was trapped in lithium hydroxide, which was diluted to about 0.001 M before plating. The self-absorption of 0.05 ml. aliquots of this solution was of the order of 10 to 20 per cent.

Results

Enzymatic Formation of CO_2 from 3-Hydroxyanthranilic Acid—CO_2 liberated from carboxyl-labeled 3-hydroxyanthranilic acid was determined in systems containing both 3-hydroxyanthranilic oxidase and picolinic carboxylase and in controls with only the oxidase present. The incubation was carried out in Warburg vessels with two side arms and a center well. 0.2 ml. of 1 N LiOH was placed in the center well. The main chamber contained 100 μmoles of pyrophosphate buffer, pH 9.4, enzyme, and water to give a total volume of 4.0 ml. One side arm contained 25 γ of carboxyl-labeled 3-hydroxyanthranilic acid hydrochloride in 0.1 ml.; this solution contained 15,800 c.p.m. In one series the other side arm contained 0.1 ml. of 0.5 M malic acid, sufficient to bring the pH of the vessel contents to 4 to facilitate liberation of CO_2. The enzyme was either oxidase alone, 0.2 ml. of rat liver acetone powder extract (1:10), or oxidase plus carboxylase, 0.5 ml. of an extract of cortisone-treated rat liver. The acetone powder extract contained sufficient oxidase to consume all of the substrate within 2 minutes at 25°. The fresh liver extract contained somewhat more of the oxidase and sufficient carboxylase to remove all of the intermediate within 3 minutes at 25°. The vessels were sealed with greased glass stoppers and the substrate was tipped in immediately. The vessels were placed in an incubator at 37° and shaken for 3 hours. The malic acid was then tipped in, and the incubation was continued for about 10 minutes. The lithium hydroxide was then removed with a pipette and the center well was rinsed with water. The combined alkali and rinses from each vessel were diluted to 5.0 ml. and 0.05 ml. aliquots were counted. Aliquots of the incubation mixtures were also counted directly.

The results shown in Table I demonstrate the release of labeled CO_2 when the picolinic-forming enzyme is present in addition to 3-hydroxyanthranilic oxidase. It may be noted that CO_2 was readily distilled into alkali from an incubation mixture near pH 9, since no difference was found in experiments in which the pH remained near 9 and in those in which acid was added.

[1] The role of cortisone and other hormones in controlling the level of this enzyme will be discussed in a separate publication.

Experiments in Vivo; Picolinic Acid Metabolism—82 γ of picolinic acid containing 520,000 c.p.m. were injected intraperitoneally into a rat. The urine was collected under toluene. Over half of the isotope was excreted into the urine within 6 hours, and almost all could be accounted for in 24 hours. No label was detected in the CO_2. Paper chromatography in "formix" (1) and paper electrophoresis at pH 4.5 showed all of the radioactivity to move as a single component, different from picolinic acid. In a separate experiment in which unlabeled picolinic acid was given to twenty-eight rats and the labeled urinary compound served as a tracer, the excreted product was isolated and identified as the glycine conjugate, picolinuric acid, which had been described previously as a urinary excretion product of rabbits, dogs, and frogs given picolinic acid (8). The product was

TABLE I

Distribution of C^{14} after Enzymatic Degradation of Labeled 3-Hydroxyanthranilate

	Experiment 1				Experiment 2	
	Oxidase		Oxidase + carboxylase		Oxidase	Oxidase + carboxylase
	Acidified	Neutral	Acidified	Neutral		
CO_2	0	0	12,000	12,500	0	11,700
Residue	11,700	15,300	1,860	1,800	12,500	1,680

purified by chromatography on Dowex 1 acetate, by using the same general method described above for the purification of picolinic acid. The radioactive fractions were evaporated to dryness and crystallized from 95 per cent ethanol. The spectrum showed an absorption maximum at 266 mμ, compared with the peak at 264 mμ characteristic of picolinic acid (1). Elementary analysis for C, H, and N gave results consistent with picolinuric

Calculated, C 53.88, H 4.42, N 15.54; found, C 53.33, H 4.81, N 15.49

acid. On hydrolysis with 6 N HCl for 16 hours the spectrum of picolinic acid appeared and an equivalent amount of glycine was produced.[2]

3-Hydroxyanthranilic Acid Metabolism—300 γ of 3-hydroxyanthranilic hydrochloride containing 682,000 c.p.m. were injected intraperitoneally into rats placed in glass metabolism cages. Air was drawn through the cage with a slight vacuum and CO_2 was trapped in wash bottles containing 200 ml. of 1 M LiOH. Parallel experiments were carried out with carboxyl-labeled nicotinic acid; 28 γ containing 420,000 c.p.m. were used.

No isotope was detected in the CO_2 obtained from animals given labeled

[2] We are indebted to Dr. J. Rabinowitz of this Institute for the glycine determination.

nicotinic acid. In contrast, in 24 hours approximately 90 per cent of the label of 3-hydroxyanthranilic acid was found in expired CO_2.

The 24 hour urine from rats given 3-hydroxyanthranilic acid contained 43,500 c.p.m. or 6.4 per cent of the administered dose. Preliminary paper chromatograms show only one radioactive compound, but minor components could easily have escaped detection. The administered radioactive nicotinic acid was largely retained by the rat, as only 27,100 c.p.m., or 6.5 per cent, was found in the urine.

DISCUSSION

If the quantities of 3-hydroxyanthranilic acid administered to rats were absorbed within a few hours, the amount reaching the liver would not greatly exceed the amount presumably formed from tryptophan during that period. This comparison is based on the excretion of picolinic acid by the normal rat (unpublished experiments, A. H. M.), in which several hundred γ per day appeared in the urine. The metabolism of the labeled compound, therefore, appears to be an indication of the normal processes in the intact organism. The conversion of the bulk of the labeled compound to CO_2 is evidence that most of the 3-hydroxyanthranilic acid formed *in vivo* is similarly oxidized and decarboxylated.[3] The decarboxylation probably does not involve the other known products of 3-hydroxyanthranilic acid metabolism, quinolinic acid (10) and nicotinic acid. The latter has been found not to give rise to CO_2 when administered in the quantities used in these experiments and only small amounts of CO_2 were found by others when larger doses were given (11). Rats have been found to excrete the bulk of administered quinolinic acid unchanged (12), and quinolinic is not decarboxylated by liver enzymes that form picolinic acid from 3-hydroxyanthranilic acid (1). The previously reported formation of quinolinic acid from 3-hydroxyanthranilic acid *in vivo* (10) is a reflection of the spontaneous cyclization of the intermediate in the presence of large amounts of substrate.

The reaction mechanism previously proposed for the formation of picolinic acid is supported by the release of labeled CO_2 by the combined action of the oxidase and the carboxylase. The structure of the intermediate oxidation product, of course, cannot be established conclusively on the basis of available information, but, since it is at the oxidation level of quinolinic acid (1), the action of picolinic carboxylase can only be a simple non-oxidative decarboxylation. The physiological importance of this en-

[3] Hankes and Henderson (9) have recently reported experiments in which carboxyl-labeled 3-hydroxyanthranilic acid was administered to rats. In these experiments larger doses than those used in the experiments reported in this paper were also largely converted to CO_2.

zyme remains obscure, since picolinic acid is retained to a very limited extent, if at all, by the intact animal.

SUMMARY

Picolinic acid is formed by a decarboxylation of the oxidation product of 3-hydroxyanthranilic acid in which the original carboxyl group of the substrate is removed. This reaction appears to proceed at a sufficient rate *in vivo* to cause most of parenterally administered carboxyl-labeled 3-hydroxyanthranilic acid to yield labeled CO_2. Picolinic acid labeled with C^{14} has been found to be excreted essentially quantitatively as its glycine conjugate by rats.

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