Metabolism of 3-Hydroxyanthranilic Acid-Carboxyl-C^{14} in the Human.* (29122)

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The tryptophan metabolite 3-hydroxyanthranilic acid has been established as an intermediate in the conversion of tryptophan to niacin(1) and some of the intermediate steps have been demonstrated(2). The recent observations of the elevated levels of urinary hydroxyanthranilic acid in patients with bladder cancer(3,4) and the confirmed carcinogenicity of hydroxyanthranilic acid for the mouse bladder(5,6) have focused attention on this and other tryptophan metabolites. A disorder of tryptophan metabolism was shown(7) in which abnormal levels of aromatic amines derived from tryptophan were found in the urines of spontaneous bladder cancer patients but not in patients with bladder cancer caused by exposure to industrial aromatic amines. Abnormal urinary levels have also been detected in various diseases (8,9,10,11).

In view of these observations it was of interest to determine how the human would metabolize labeled hydroxyanthranilic acid as compared to other animals(12).

Experimental. A small amount of carboxyl-labeled 3-hydroxyanthranilic acid was administered to a female patient with achondroplasia to measure blood, respiratory and urinary products. This patient had previously been found to have normal tryptophan metabolism as determined by quantitative urinary analysis for a number of tryptophan metabolites(13,14,15,16,17,18) before loading with tryptophan (Table I). After loading with 2.0 g of L-tryptophan, anthranilic acid, acetylkynurenine and kynurenine fractions were slightly higher than controls but these elevations were small compared with gross abnormalities sometimes observed(11). She was given orally, in a gelatin capsule, 14.088 mg of carboxyl-labeled 3-hydroxyanthranilic acid containing 51.055 μc of C^{14}. Respiratory CO_2 was collected over a 24-hour period in 2 N NaOH. Urine was collected for three 24-hour periods under toluene in a refrigerated dark bottle. Blood samples were drawn at 0, 0.5, 2, 4, 8, 12 and 24 hours from time of isotope administration and the sera saved for analysis. The patient was 51 years old, weighed 41.53 kg and was 135 cm tall. Before and during the experiment, she was maintained on a standardized hospital diet providing approximately 1 g of tryptophan per day but which avoided niacin rich foods such as liver and nuts.

Methods. The $C^{14}O_2$ solutions, urines and serums were analyzed for C^{14} by the method of Van Slyke, Steele, and Plazin(19). The urines were assayed for nicotinic acid(20), quinolinic acid(21), N^1-methylnicotinamide (17) and N-methyl-3-pyrodine-5-carboxamide (15) and these metabolites were isolated by carrier techniques.

Isolation of quinolinic acid and nicotinic acid from urine. A 100 ml sample of urine was evaporated to dryness in a rotary evaporator and the dry residue was extracted with two 50 ml portions of normal hexane to remove the anti-foaming agent used. The dried residue was autoclaved with 24 ml of 1 N NaOH for one hour at 10 lb pressure, and the hydrolysate was adjusted to pH 7 with 5 N HCl. To this neutralized solution quinolinic acid and nicotinic acid were added as carriers. The solution was then taken down to dryness and the dry residue was extracted with two 75 ml portions of absolute methanol. This methanol extract was taken to dryness and the residue was extracted with small portions of water, which were filtered through a small sintered glass funnel. The final volume of filtrate was approximately 10 ml.

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TABLE I. Urinary Excretion of Tryptophan Metabolites by the Patient with Achondroplasia Compared with Normal, Healthy Control Women.

		Metabolite*										
		KA	XA	ISA	AAG	o-AHA	AcK+AA	KYN	HKYN	N'-MN	MPCA	4-PA	CR
Achondroplastic (1)	Pre-tryptophan load	12	5	621	6	17	8	9	34	15	57	4.00	.81
	Post-tryptophan load	52	26	556	16†	32	31†	66†	56	23	86	2.36	.69
Control women (18)	Pre-tryptophan load	13 ± 4	8 ± 2	371 ± 145	4 ± 1	21 ± 4	10 ± 2	10 ± 3	21 ± 14		89 ± 31	3.87 ± 1.40	1.17 ± .20
	Post-tryptophan load	60 ± 19	30 ± 8	500 ± 270	7 ± 2	40 ± 11	14 ± 3	28 ± 13	43 ± 14	N'-MN	131 ± 36	3.34 ± 1.16	1.15 + .22

* The abbreviations used are KA, kynurenic acid; XA, xanthurenic acid; ISA, a crude fraction predominantly indoxylsulfate (indican); AAG, anthranilic acid glycuronide; o-AHA, o-aminohippuric acid; AcK+AA, a fraction predominantly N-acetylkynurenine with traces of anthranilic acid; KYN, kynurenine; HKYN, hydroxykynurenine; N'-MN, N'-methylnicotinamide; MPCA, N-methyl-2-pyridone-5-carboxamide; 4-PA, 4-pyridoxic acid; CR, creatinine. Values are given as micromoles per 24 hr except creatinine which is given as g per 24 hr. The loading dose of L-tryptophan was 2.0 g, given orally. Control values are the mean values ± standard deviations.

† Indicates values different from controls by more than 2 standard deviations.

A column (22 × 40 mm) of Dowex 50W-8 (200-400 mesh, H^+ form) was washed with distilled water. The sample was put on the column and the effluent from the column passed through a continuous flow ultraviolet analysis apparatus set at a wavelength of 272 mμ and collected in a fraction collector. Quinolinic acid came off after about 120 ml of water had passed through the column and was all removed in about 210 ml of eluate. After washing the column with an additional 100 ml of water, nicotinic acid was eluted with 3 N HCl, the nicotinic acid coming off in about 200 ml of eluate. The solutions were taken to dryness in a rotary evaporator. The quinolinic acid was purified by several recrystallizations from 50% ethanol. The nicotinic acid was placed in a vacuum sublimer and sublimed at 6 mm pressure and 105°C. The sublimed nicotinic acid was recrystallized several times from water.

Isolation of carrier N^1-methylnicotinamide and N-methyl-2-pyridone-5-carboxamide (pyridone). Ten 1% aliquots of a 24-hour urine were passed through 10 columns of Dowex-1 acetate (200 to 400 mesh) each 0.9 cm wide and 7.0 cm high. The filtrates were then passed through columns of Dowex-50W (H^+ form, 200 to 400 mesh), each column being 0.9 cm × 3.5 cm. Each Dowex-1 column was washed with 70 ml of water which was also then passed through the corresponding Dowex-50W columns. The filtrates and washes were combined and saved for pyridone isolation.

The N^1-methylnicotinamide which was retained on the Dowex-50W columns was eluted from each column with 100 ml of 1.75 N HCl after first washing the columns with 50 ml of 1.25 N HCl. This column procedure was shown to give essentially quantitative recovery of the desired compounds from urine (15,17). Carrier N^1-methylnicotinamide chloride (80.0 mg) was added to the combined 1.75 N HCl fractions from these columns and the solution was promptly concentrated to dryness using a rotary vacuum evaporatory. The residue was then dissolved in 50 ml of water, the pH adjusted to 7.0 and the solution passed through a column of IRC-

50 resin, 1.6 cm × 11.0 cm, which had been pre-washed with 200 ml of 20% acetic acid and water until all acid was removed (about 200 ml). N^1-methylnicotinamide ion was retained on the column while other components of this fraction such as trigonelline and creatinine were not. After washing the column with 250 ml of water, N^1-methylnicotinamide was eluted with 300 ml of 5% acetic acid. This fraction was concentrated to dryness, dissolved in a minimum volume of hot 90% ethanol (about 15 ml) and 4 ml of saturated picric acid in ethanol was added. The crystals of N^1-methylnicotinamide picrate which formed were isolated and recrystallized to constant specific activity from 95% ethanol.

The pyridone fractions from the Dowex-50W columns were combined, 80.0 mg of pyridone carrier was added, and this solution was concentrated to dryness on a rotary evaporator. The residue was hydrolyzed to N-methyl-2-pyridone-5-carboxylic acid by heating at 100°C with 10 ml of 2.0 N NaOH for 2 hours. The hydrolyzed solution was diluted to 100 ml with water and passed through a column of Dowex-50W (H^+), 1.6 cm × 6.5 cm. The column was washed with 40 ml of water and the combined filtrate and wash was passed through a column of Dowex-1 (OH^-), 1.6 cm × 4.0 cm which retained the pyridone acid. After washing the column with 40 ml of water, the desired compound was eluted with 400 ml of 1 N HCl which was then evaporated to dryness. The residue was dissolved in 25 ml of 0.1 N HCl and passed through a charcoal column (Nuchar C), prepared as described previously(15). After washing with 200 ml of water, and 300 ml of 5% ammonium hydroxide, the pyridone acid was eluted with 400 ml of 2% pyridine solution. The pyridone acid was eluted in the fraction between 250 and 400 ml of pyridine solution. This fraction was concentrated to dryness, dissolved in about 2 ml of water, treated with a small amount of charcoal, filtered, and precipitated by addition of a few drops of 5 N HCl. The crystals which formed were long white needles. These were recovered and recrystallized to constant specific activity from small volumes of hot water.

Preparation of serum samples for analysis. A 50 microliter sample of each serum was dried in a Van Slyke combustion tube and analyzed for C^{14} by the gas analysis method (19). The protein in the serum was analyzed for C^{14} in the following way to determine the per cent of bound activity in the serum. To 100 μl of serum in a Van Slyke centrifuge combustion tube 0.9 ml of tungstic acid (3 ml of 10% sodium tungstate mixed with 24 ml of 1/12 N H_2SO_4) was added slowly with shaking. The precipitated serum protein was centrifuged into the tip of the tube with a force of 1100 × *g*. The precipitated protein was resuspended and spun down twice more and then dried for C^{14} analysis. All of the supernatants were saved and concentrated to dryness in combustion tubes for analysis.

Results. After ingestion of the labeled hydroxyanthranilic acid, 34.82% of the activity appeared in the urine within the first 24 hours, 0.98% in the second 24 hours and 0.51% in the third 24-hour period for a total urinary excretion of 36.31%. Most of this urinary activity was in quinolinic acid (Table II) with much lesser amounts in nicotinic acid, N^1-methylnicotinamide and N-methyl-2-pyridone-5-carboxamide. The specific activity of excreted quinolinic acid in the first day was relatively high compared with that of the other niacin metabolites on the first day. However, on the second and third day the specific activity of all the pyridine compounds was more nearly comparable.

Measurements of serum radioactivity (Table III) showed appreciable activity within 0.5 hours of ingestion of the tracer dose. The total activity remained high for 2 hours, then decreased steadily. The per cent of serum activity precipitated with serum proteins increased indicating that much of the activity remaining in the serum at the later times was firmly attached to the serum proteins.

Fig. 1 shows the cumulative excretion of $C^{14}O_2$ from the patient compared with previous data obtained in rats given the same compound(22). As in the rat studies, there was a rapid appearance of C^{14} in the breath. More than 40% of the dose was expired within the first 6 hours with only 1.6% expired

TABLE II. 72-Hour Excretion of Quinolinic Acid, Nicotinic Acid, N-Methylpyridone Carboxamide and N¹-Methylnicotinamide by a Patient with Achondroplasia Given 51.055 μc of 3-Hydroxyanthranilic Acid-Carboxyl-C¹⁴ with a Specific Activity of 554.472 μc/mmole.

Day	Quinolinic acid			Nicotinic acid			N¹-methylnicotinamide			N-methyl-2-pyridone-5-carboxamide		
	1	2	3	1	2	3	1	2	3	1	2	3
mmoles excreted	.049	.027	.026	.0036	.0034	.0034	.0353	.0258	.0240	.064	.050	.044
Sp. act. excreted μc/mmole	315.88	6.83	2.09	3.10	2.51	1.93	1.056	1.75	1.61	1.44	2.104	1.31
% injected C¹⁴	30.02	.37	.110	.022	.0170	.0130	.073	.088	.076	.180	.205	.160
Rel. spec. act.	.5697	.012	.004	.006	.005	.004	.0019	.0032	.0029	.0030	.0040	.0024

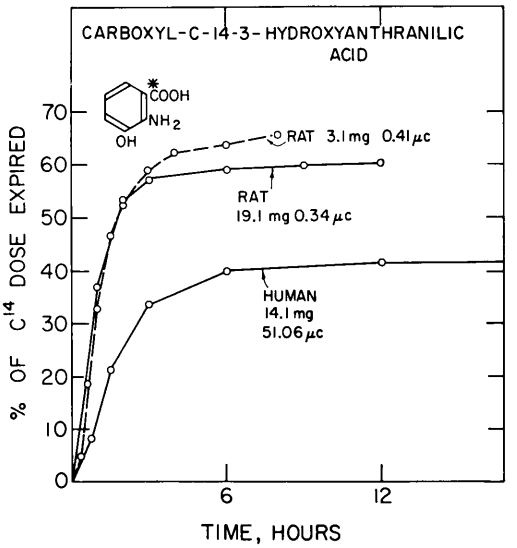

FIG. 1. Rate of expiration of $C^{14}O_2$ from 3-hydroxyanthranilate labeled in the carboxyl group with C^{14}. The upper curves obtained with rats were plotted from previously published data(22).

during the following 18 hours. The specific activity of the expired CO_2 reached a maximum during the first 90 minutes. In the rat studies approximately 60 to 65% of the 3 mg and 19 mg doses appeared in the breath in a similar period, but with a much sharper rise in $C^{14}O_2$ specific activity.

Discussion. The expiration of $C^{14}O_2$ by the patient was similar to that observed in rats; however, rats expired a higher percentage of the dose as $C^{14}O_2$. This difference may be due to the fact that the rats received a dose equivalent to a 0.7-4.4 g dose in a human. Studies with DL-tryptophan-2-C^{14} in humans have shown that the $C^{14}O_2$ levels in the expired air will change from 5% to about 25% if the dose is increased from 40 mg to 2 g(23). The 14 mg dose of 3-hydroxyanthranilic acid-C^{14} given the patient here was apparently in the physiological range.

About 34.8% of the dose was excreted in the urine in the first 24 hours of which 86% was accounted for as quinolinic acid. In comparison, a rat receiving 19 mg of hydroxyanthranilic acid excreted 28.7% in the urine of which only 11% was accounted for as quinolinic acid. If the 19 mg dose in the rat was a large overdose, a large percentage of

TABLE III. Carbon-14 Levels in Human Serum After C^{14}-Carboxyl-Labeled 3-Hydroxyanthranilic Acid Administration.

	Time (hr)						
	0	.5	2	4	8	12	24
Whole serum, DPM/ml	4	3674	3509	1407	599	414	239
Precipitated protein, DPM/ml	0	349	206	225	210	171	139
% of whole serum		9.5	5.9	16	35	41.3	58.2

the urinary C^{14} would have been expected to appear as quinolinic acid along with the high percentage of $C^{14}O_2$ produced. The difference in per cent urinary C^{14}-quinolinic acid in man as compared to the rat suggests some quantitative difference in the manner in which hydroxyanthranilic acid is metabolized in the two species. Of the activity in the patients' urine it was possible to account for 16.00 μc of the 18.54 μc excreted over a 72-hour period as quinolinic acid, N^1-methylnicotinamide, N-methyl-2-pyridone-5-carboxylamide and nicotinic acid. The remaining 2.54 μc probably was present at least in part as unmetabolized hydroxyanthranilic acid. Paper chromatography of a sample of the first 24-hour urine and subsequent autoradiographic analysis showed radioactivity in areas with the Rf of hydroxyanthranilic acid as well as quinolinic acid, N-methyl-2-pyridone-5-carboxamide, N^1-methylnicotinamide, and nicotinic acid. The order of magnitude of per cent of activity present in each isolated component (Table II) suggested that renal clearance of compounds as well as dosage administered plays a role in the overall results obtained with labeled compounds. Considering that the normal pathway of hydroxyanthranilic acid metabolism was assumed to be *via* quinolinic acid, nicotinic acid, N^1-methylnicotinamide, and then pyridone, it is noteworthy that the decreasing order of components excreted was quinolinic acid, pyridone, N^1-methylnicotinamide and nicotinic acid. The excretion of less nicotinic acid than pyridone or N^1-methylnicotinamide may be due to the fact that the latter two products are not reabsorbed in the renal tubules and therefore gave higher renal clearances than nicotinic acid, which is partially reabsorbed(24, 25).

The total serum radioactivity seemed to be made up of at least two components, a major portion which does not precipitate with the serum proteins and a minor portion which is precipitated. At the early times the non-precipitable portion constitutes most of the serum activity but at 24 hours the activity attached to proteins was approximately half of the total activity. This bound activity decreased with time at a much slower rate than did the soluble activity. The chemical nature of the protein-bound radioactivity has not been determined. It may result from incorporation of breakdown products of hydroxyanthranilic acid or from binding of o-quinone or quinone-imine metabolites as reported for other o-aminophenols(26,27). Hydroxyanthranilic acid has also been observed to bind to ascites tumor cells(28). It is possible that this kind of tissue binding of hydroxyanthranilic acid or its metabolites may play a role in carcinogenesis.

Summary. 1. Orally administered 3-hydroxyanthranilic acid labeled with C^{14} in the carboxyl group was metabolized by a human subject with achondroplasia in such a manner that approximately 40% of the isotope appeared as $C^{14}O_2$ within 6 hours. 2. Within the first 24 hours 35% of the label appeared in the urine. Of this urinary activity, 80% was in quinolinic acid with smaller amounts present in nicotinic acid, N^1-methylnicotinamide, and N-methyl-2-pyridone-5-carboxamide. 3. The per cent of C^{14} appearing in the urinary metabolites corresponded more to the renal clearance pattern of the compounds rather than to their known precedence in the metabolic pathway from 3-hydroxyanthranilic acid to N-methyl-2-pyridone-5-carboxamide. 4. Appreciable radioactivity was found in blood serum during the first day, with an increasing percentage of the serum activity in a form precipitable with serum proteins.

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Organ and Intracellular Location of the Methionine Methyl Group Synthesizing System of the Rat.* (29123)

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Many publications have dealt with the *de novo* biosynthesis of the methionine methyl group by animal systems *in vitro*(1-5), but no work has been reported on the organ and intracellular location of this biosynthetic system. This is the purpose of this communication.

Procedures. To determine which organs were the most active in synthesizing methionine methyl groups, 2 rats were used in each of 3 experiments. The brain, pectoralis muscles, kidneys, and liver were removed immediately upon killing. The tissues were rinsed, patted dry, weighed, and homogenized in a Servall "Omni-mixer" at full speed at 0°C for 2 minutes with 4 ml of 0.05 M phosphate buffer (pH 7.2) per g of tissue. The homogenates were then centrifuged at ca. 25,000 $\times$ *g* for 3 hours at 0°C and the supernatants used in the assays.

In the investigations on the intracellular location, the liver was used because of results obtained in the first part of this study, a single rat being used per experiment. After

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