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Received December 2, 1966. P.S.E.B.M., 1967, v124.

Effects of Loading with Tryptophan Metabolites on Metabolism of DL-Tryptophan-7a-C^{14} in Rats.* (31968)

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Previous work with human subjects has demonstrated a relationship between the amount of carrier added to a tracer dose of DL-tryptophan-2-C^{14} and the percent of the C^{14} expired as CO_2. As the level of carrier added to a dose of labeled tryptophan was increased from zero to 2 g, the percent of C^{14} expired as CO_2 in 24 hours increased from 4.5 to 25%(1). The data suggested that given quantities of tryptophan were converted into proteins and enzymes, but that the excess was metabolized to CO_2 probably *via* the glutarate pathway(2,3). In view of this and several reports of *in vitro* and *in vivo* effects of tryptophan metabolites on enzymes related to this pathway, as well as other systems(4,5,6), and because of the increasing evidence that some tryptophan metabolites are carcinogenic (7,8), it was considered of interest to investigate the *in vivo* metabolism of DL-tryptophan-7a-C^{14} as affected by loading doses of L-tryptophan, DL-kynurenine, 3-hydroxyanthranilic acid and quinolinic acid. The loading doses used were equivalent, on a molar basis, to a 2 g load of tryptophan in a 70 kg man, a load which has been used extensively(9), and which has been recommended as a standard test dose for studies of tryptophan metabolism in humans.

* This research was supported by U. S. Atomic Energy Commission and in part by Grant E-202 from Am. Cancer Soc., and Nat. Cancer Inst., Nat. Inst. Health. Preliminary report given at Federated Society Meetings, Chicago, Illinois, 1964, v23, 421.

Experimental. An aqueous solution of DL-tryptophan-7a-C^{14} (5.76 μc/mg) containing 1.064 mg per ml was injected intraperitoneally into pairs of rats (400-500 g) at a level of 0.67 ml per kg of body weight. The unlabeled tryptophan, DL-kynurenine sulfate, 3-hydroxyanthranilic acid and quinolinic acid were also injected intraperitoneally at a dosage level of 0.14 mMole per kg of body weight, which would represent on an equimolar basis a 2.0 g dose of L-tryptophan in a 70 kg man. The respiratory CO_2 was collected for 12 hours and the urine for two 12-hour periods. The urine samples were assayed for quinolinic acid (QA)(10) and nicotinic acid(11). By use of carrier techniques these labeled metabolites were isolated from only the first 12-hour urines(12), since the major portion of the excreted radioactivity and metabolite were in this fraction of urine. The urines were not assayed for kynurenine, o-aminohippuric acid, xanthurenic acid, N^1-methylnicotinamide, or N-methyl-2-pyridone-5-carboxamide because of limited amounts of sample, but the carbon-14 labeled components of these metabolites were isolated by carrier techniques.

For the isolation of o-aminohippuric acid, N^1-methylnicotinamide, kynurenine, xanthurenic acid, and N-methyl-2-pyridone-5-carboxamide, the column separation described by McCoy and Chung(13) was used. To 30 percent of the rat urine was added 1.0 mg of the above compounds to allow spectrophotometric

analysis of the fractions eluted from the columns. The kynurenic acid-xanthurenic acid fraction was further resolved into these two components by chromatography on a column of Dowex-1-formate as previously described (14). To the xanthurenic acid fraction in dilute NH_4OH was added 100 mg of xanthurenic acid as additional carrier. This was recrystallized to constant specific activity by acidification with acetic acid.

N^1-Methylnicotinamide was eluted from the Dowex-50 column in the 1 N HCl fraction. This fraction was concentrated to dryness *in vacuo* after adding 100 mg of N^1-methylnicotinamide chloride as carrier. Upon dissolving the white residue in a minimum of 95% ethanol the N^1-methylnicotinamide was isolated as the picrate by adding a saturated solution of picric acid in ethanol. The product was recrystallized from 90% ethanol until the specific activity was constant(12).

Kynurenine, which appears in the same fraction with hydroxykynurenine, was further purified by passage through a column of Dowex-2 in the borate form (Brown, unpublished) and re-isolated on a column of Dowex-50W (H^+). The kynurenine was then eluted with 5 N HCl and after addition of 100 mg of kynurenine sulfate, the solution was concentrated to dryness and the product recrystallized to constant specific activity from dilute ethanol in the presence of slight excess of sulfuric acid. The recovery of these compounds from the column procedures was checked by spectrophotometric analysis.

All CO_2 and compounds isolated from urine were analyzed for C^{14} by the techniques of Van Slyke, Steele and Plazin(15).

Samples (0.5 ml) of each urine were reduced to a small volume with a stream of nitrogen prior to spotting on Whatman No. 4 chromatographic paper and the sheets placed in a solvent system of methanol, butanol, benzene, and water, (2:1:1:1), to which 1% glacial acetic acid was added(16). After running the chromatograms in an ascending fashion overnight, the dried chromatogram was observed under ultraviolet light and the fluorescent spots marked. The chromatogram was then affixed to sheets of industrial type KK x-ray film and autoradiographed.

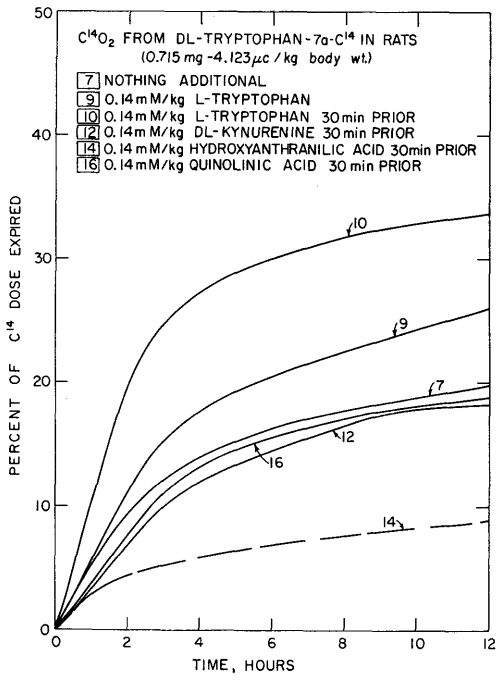

FIG. 1. Expiration of $C^{14}O_2$ by rats injected intraperitoneally with DL-tryptophan-7a-C^{14} alone and combined with other tryptophan metabolites.

Results and discussion. When a tracer dose of DL-tryptophan-7a-C^{14} was administered to rats, 19.8% of the C^{14} was expired as $C^{14}O_2$ (Fig. 1) in the first 12 hours. Rats receiving a load of L-tryptophan at the same time as labeled tryptophan expired 26.0% of the dose as $C^{14}O_2$. If the L-tryptophan was given 30 minutes prior to the labeled tryptophan, the $C^{14}O_2$ excretion was increased to 33.6%. This increased excretion of $C^{14}O_2$ may be the response to an increased tryptophan pyrrolase enzyme activity by adaptation(17,18) with the formylkynurenine formed subsequently converted to $C^{14}O_2$ *via* the kynurenine-hydroxyanthranilic acid-acetate pathway (glutarate pathway)(2). Although tryptophan loading resulted in a 3- to 4-fold increase in the percent of dose in urinary QA, the absolute increase of C^{14} in QA was only a small fraction of that in CO_2.

Injection of a load of DL-kynurenine or quinolinic acid 30 minutes prior to injection of DL-tryptophan-C^{14} had little or no effect on $C^{14}O_2$ production (Fig. 1) or urinary levels of C^{14} (Table I). However, when a load of

3-hydroxyanthranilic acid was administered 30 minutes prior to the tryptophan-C^{14}, $C^{14}O_2$ excretion was depressed to a level of 8.8%. This low level of $C^{14}O_2$ may be accounted for by several possible mechanisms: (a) the flooding of the hydroxyanthranilic acid-acetate-CO_2 pathway by the excess hydroxyanthranilic acid; (b) an inhibition of the hydroxyanthranilic acid oxidase enzyme system by the binding of active sites of the

TABLE I. DL-Tryptophan-7a-C^{14} Metabolites in Rat Urine (1st 12-Hour Urine).

Group	7	9	10	12	14	16
Supplement, 0.14 mM		Tryptophan	Tryptophan	Kynurenine	Hydroxyanthranilic acid	Quinolinic acid
C^{14} inj, μc	3.681	3.8651	3.4884	3.8344	3.6810	4.0489
Urinary C^{14}, μc	.282	.387	.318	.277	.87	.288
% of dose	7.67	10.01	9.12	7.24	23.62	7.12
Quinolinic acid						
Exc, mM	.0012	.0023	.002	.0013	.0023	.0718
S.A., μc/mM	5.764	13.67	13.12	5.438	1.535	.09
$\frac{\text{S.A. QA}}{\text{S.A. Tryp}} =$	.0049	.012	.0111	.0046	.0015	.0001
% of dose	.19	.82	.76	.18	.10	.81
% Urinary activity	2.53	8.20	8.32	2.46	.41	2.35
Nicotinic acid						
Exc, mM	.0002	.001	.0009	.0008	.0007	
S.A., μc/mM	5.643	4.414	1.203	1.822	.497	
$\frac{\text{S.A. NA}}{\text{S.A. Tryp}} =$	.0048	.0038	.001	.0016	.0004	
% of dose	.03	.12	.03	.039	.009	
% Urinary activity	.34	1.18	.35	.54	.038	
Picolinic acid						
% of dose			.187		.084	
% Urinary activity			2.047		.354	

S.A. Injected DL-tryptophan-7a-C^{14}—1176 μc/mM.

TABLE II. Additional Urinary Metabolites from DL-Tryptophan-7a-C^{14}. (1st 12-hour urine)

Group	7	9	10	12	14	16
Supplement, 0.14 M		Tryptophan	Tryptophan	Kynurenine	Hydroxyanthranilic acid	Quinolinic acid
Kynurenine						
% of dose	.0084	.0102	.0076	.0214	.0066	.0059
% urinary activity	.1	.103	.083	.296	.028	.083
o-aminohippuric acid						
% of dose	.027	—	.042	.026	.092	.028
% urinary activity	.35	—	.46	.36	.39	.40
Xanthurenic acid						
% of dose	.051	.24	.104	.094	.048	.079
% urinary activity	.662	2.4	1.14	1.3	.204	1.109
N^1-methyl-nicotinamide						
% of dose	.1	.169	.248	.097	.016	.096
% urinary activity	1.3	1.69	2.715	1.34	.069	1.35
N-methyl-2-pyridone-5-carboxamide						
% of dose	.0097	—	.028	.015	.015	—
% urinary activity	.126	—	.303	.211	.062	—

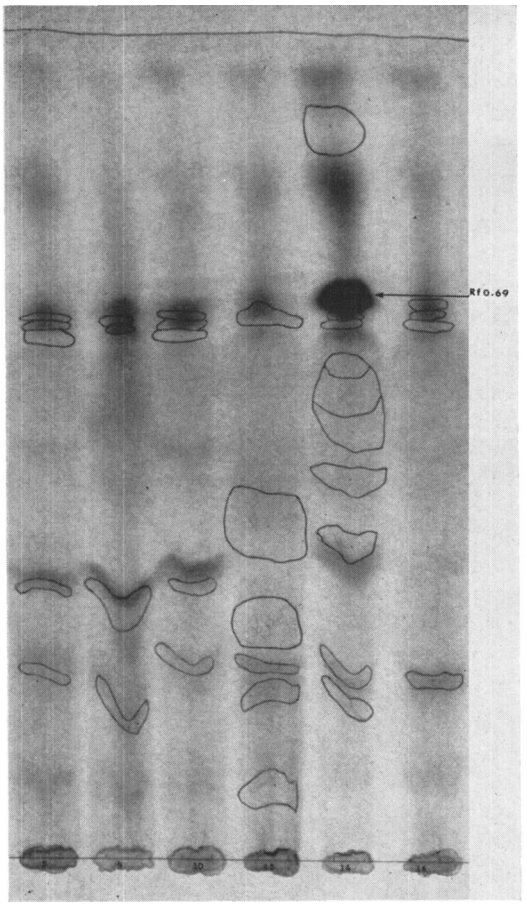

FIG. 2. Autoradiograph of paper chromatogram of urines from rats injected intraperitoneally with DL-tryptophan-7a-C^{14} alone and combined with other tryptophan metabolites. Spot at Rf 0.69 is unknown labeled component.

hydroxyanthranilic acid oxidase by excess substrate; (c) inhibition of tryptophan pyrrolase(5). Evidence has been presented that 3-hydroxyanthranilic acid or its metabolites can bind to serum proteins in humans(12); therefore, the binding of the excess hydroxyanthranilic acid on the enzyme proteins would be a possibility. Further evidence suggesting that either the tryptophan pyrrolase or the hydroxyanthranilic acid oxidase was inhibited was shown in the slightly lower lever of quinolinic acid-C^{14} isolated from the urine of the animals receiving the hydroxyanthranilic acid load. This is different from the expected result from a loading dose of hydroxyanthranilic acid. The data (Table II) showing that the hydroxyanthranilic acid load did not alter the percent C^{14} appearing in kynurenine or xanthurenic acid suggests that tryptophan pyrrolase was not inhibited by the load of hydroxyanthranilic acid.

The autoradiograph of the urine chromatograms (Fig. 2) shows that with the various metabolites given prior to the labeled tryptophan, the quantities of various fluorescent and labeled metabolites coming out in the urine will vary. In Fig. 2, the circled areas indicate fluorescent components seen under ultraviolet light. The radioactive spot on the autoradiograph of most interest was the intense spot in urine No. 14 at Rf 0.69. In the system used for the chromatogram, xanthurenic acid and the 2-pyridone metabolite of niacin have Rf values of 0.67 and 0.72 respectively. For this reason, the xanthurenic acid and pyridone were isolated by carrier technique from the urines. If the administered hydroxyanthranilic acid inhibited the hydroxyanthranilic acid oxidase, a buildup of metabolically produced hydroxyanthranilic acid as well as hydroxykynurenine could be expected with a significant increase in urinary xanthurenic acid produced from the hydroxykynurenine. However, as shown in Table II the total percent of urinary C^{14} present in the xanthurenic acid fraction would not account for the high concentration of activity in the radioactive spot with Rf 0.69. After the initial isolations of urinary components a small quantity of urines No. 10 and 14 remained. Since the isolated components could not account for the major share of urinary carbon-14, we decided to isolate 2 additional metabolites, picolinic acid and glutaric acid, which were likely to contain carbon-14. As shown in Table I, the picolinic acid isolated from the urine of rats receiving the load of tryptophan accounted for 2% of the urinary activity, while the picolinic acid in the urine from the animals receiving the 3-hydroxyanthranilic acid load accounted for only 0.35% of the activity. This additional data may not have accounted for the intense activity at Rf 0.69, but additional evidence of a metabolic inhibition by the 3-hydroxyanthranilic acid was presented. The glutaric acid isolated from urine No. 14 accounted for 0.72% of the urinary carbon-14 and 0.17% of the dose.

However, these quantities of activity cannot account for the intense area of activity in urine from rat No. 14 at Rf 0.69. Hydroxyanthranilic acid has the ability to inhibit several enzyme systems(5), and this could be a reflection of its ability to bind to proteins (10). The depressed $C^{14}O_2$ could have been interpreted as a depression of the tryptophan pyrrolase system except that there was no depression of C^{14} in kynurenine or xanthurenic acid. Even though the pyrrolase system was blocked this would not show as an intense quantity of radioactivity in the tryptophan area at Rf 0.53 to 0.59, since tryptophan is so well reabsorbed by the kidney and, therefore, poorly excreted.

The observation that none of the components isolated (Table II) accounted for the increase in activity of the urine from the animals treated with hydroxyanthranilic acid suggests that all pathways to CO_2 formation from tryptophan were inhibited with the excretion of the major share of the activity in an unidentified component in the urine.

These experiments with the prior administration of L-tryptophan or 3-hydroxyanthranilic acid were repeated and the accelerated output of CO_2 by tryptophan and the depression of $C^{14}O_2$ by hydroxyanthranilic acid was obtained again. The effects of hydroxyanthranilic acid might be explained if hydroxyanthranilic acid inhibited further metabolism of α-aminomuconic-semialdehyde to CO_2(3) allowing the non-enzymic formation of picolinic acid to proceed to a greater extent. However, there was no large amount of activity found in the picolinic acid from rat No. 14. The hydroxyanthranilic acid did not decrease the percent of C^{14} appearing in kynurenine as it should have if tryptophan pyrrolase were inhibited.

If the effect of the tryptophan load is that of flooding the system with kynurenine and subsequent metabolites, and to increase the pyrrolase activity, there should have been some effect of tryptophan load seen on the percent of C^{14} found in kynurenine. The anticipated effect was seen in xanthurenic acid levels, and in quinolinic acid and to a lesser extent in N^1-methylnicotinamide and pyridone.

The hydroxyanthranilic acid had some apparent stimulatory effect on percent of tryptophan carbon-14 which appeared in orthoaminohippuric acid but no effect on kynurenine or xanthurenic acid carbon-14 content, a result which seems discordant with that expected.

The increased urinary carbon-14 from tryptophan after hydroxyanthranilic acid is probably not due to vit. B_6 deficiency produced by hydroxyanthranilic acid, since in vit. B_6 deficiency there is an enhanced urinary excretion of xanthurenic acid, kynurenine and hydroxykynurenine(19), a result not found in these experiments in which kynurenine and xanthurenic acid were unaltered in the hydroxyanthranilic acid treated rats. Perhaps most of these results can be explained if there is good renal tubular reabsorption of tryptophan but only poor absorption or even secretion of kynurenine.

Summary. DL-tryptophan-7a-C^{14} was administered intraperitoneally to adult rats at a level of 0.715 mg/kg of body weight. In some experiments loads of L-tryptophan, kynurenine, hydroxyanthranilic acid or quinolinic acid (equivalent on a molar basis to 2 g of L-tryptophan in a 70 kg man) were given 30 minutes prior to the labeled tryptophan. Rats given a tracer dose of DL-tryptophan-7a-C^{14} excreted 18-20% of the C^{14} as $C^{14}O_2$ and 7.2-7.6% in the urine in the first 12 hours. The injection of a load of L-tryptophan 30 minutes prior to the DL-tryptophan-7a-C^{14} caused an increase in $C^{14}O_2$ to 33.6% of the dose and the urinary C^{14} level increased to 9.1%. The injection of 0.14 mM/kg of 3-hydroxyanthranilic acid 30 minutes prior to administration of the labeled tryptophan caused a depression of the $C^{14}O_2$ excretion to 8.8% and increased the urinary C^{14} level to 23.6%. Autoradiographs of chromatograms of the urines and C^{14} analyses of more than 8 of the known tryptophan metabolites isolated from the urines showed that the major share of activity present in the urine from rats receiving the 3-hydroxyanthranilic acid load was present in an unidentified component.

We wish to acknowledge the technical assistance of Margaret Kowitz.

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Received December 5, 1966. P.S.E.B.M., 1967, v124.

Chronic Administration of Hydroxylamine and Derivatives in Mice. (31969)

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The recent speculation on the possible carcinogenicity of the mutagen hydroxylamine (1) has prompted us to describe the results of several studies we have performed with hydroxylamine or its derivatives. Within recent years the mutagenic and teratogenic effects of hydroxylamine derivatives on micro-organisms and on *in vitro* systems have been demonstrated(2-4). However, relatively little has been reported of the chronic effects of such materials in animals. For this reason hydroxylamine, hydroxyurea, useful in treatment of some leukemias(5-8), and methylhydroxylamine (methoxyamine) reputedly an even more effective mutagen than hydroxylamine(9), were administered to mice.

Materials and methods. Compounds. Hydroxyurea was kindly provided by the Drug Development Branch, CCNSC, or through the courtesy of Dr. L. J. Lerner and Miss Barbara Stearns of the Squibb Institute for Medical Research. Hydroxylamine sulfate, O-methoxyamine hydrochloride and urethan (ethyl carbamate) were Eastman White Label chemicals.

Animals. Male random-bred Swiss-Webster or female C3H/HeN mice, 4 weeks old, from the NIH Animal Production Section were kept in plastic cages, usually 6-8 animals per cage. Purina Laboratory Chow was available *ad lib.* The mice were weighed weekly.

Toxicology. Tolerated dosage was determined on 6 mice at each level. The compounds were given in the drinking water at several concentrations for 2 weeks until signs of toxicity (death, loss of weight) appeared. From these results a suitable dose for chronic administration was selected.

Chronic administration of compounds. The compounds were given in the drinking water. With hydroxyurea enough solution was prepared for a 2-day supply to each cage to avoid the gradual hydrolysis of the material. Solutions of the other compounds were prepared