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TRYPTOPHAN METABOLISM. I.

CONCERNING THE MECHANISM OF THE MAMMALIAN CONVERSION OF TRYPTOPHAN INTO KYNURENINE, KYNURENIC ACID AND NICOTINIC ACID

Charles Heidelberger, Mary E. Gullberg, Agnes Fay Morgan and Samuel Lepkovsky

March 15, 1949

Berkeley, California

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ABSTRACT

March 15, 1949

1. Experiments with radiocarbon prove that DL-tryptophan is converted into kynurenine in rabbits and kynurenic acid in dogs.

2. The β-carbon atom of the tryptophan becomes the β-carbon atom of kynurenine and the 3-carbon of kynurenic acid. Thus, the conversion takes place by only one mechanism.

3. The administered dose of tryptophan is considerably diluted by the body pool of this substance and other intermediates.

4. The side-chain of tryptophan is lost during its conversion into nicotinic acid.

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* The work described in this paper was sponsored by the Atomic Energy Commission.

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One of the first metabolic products of tryptophan to be reported was kynurenic acid, which was isolated by Ellinger in 1904 (1) from the urine of dogs that had been fed the amino acid. He characterized the compound correctly except for the assignment of the carboxyl group to the 3-position in the quinoline ring, but its correct location at the 2-position was determined soon afterward (2). Another apparent metabolic product, kynurenine, was discovered in 1931 by Kotake and Iwao (3), who assigned an incorrect structure to this molecule. In 1943, however, Butenandt et. al (4) determined its structure and confirmed it by synthesis. A

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** Present address: McArdle Memorial Laboratory, The Medical School, University of Wisconsin, Madison, Wisconsin.

(1) A. Ellinger, Z. Physiol. Chem., 43, 325 (1904).
(2) A. Homer, J. Biol. Chem., 17, 509 (1914).
(3) Y, Kotake and J. Iwao, Z. Physiol. Chem., 195, 139 (1931).
(4) A. Butenandt, W. Weidel, and von Derjugin, W., Z. Physiol. Chem., 279, 27 (1943).

third metabolite, xanthurenic acid, was isolated from the urine of pyridoxin deficient dogs that had previously been fed tryptophan (5).

Krehl et. al. (6) in 1945 showed that rats would grow rapidly on low nicotinic acid diets containing corn provided that additional tryptophan was added, and called attention to the possibility of a metabolic interrelation of this amino acid and nicotinic acid. They suggested that these results might be due at least in part to the intestinal flora. Shortly afterwards Rosen, Huff and Perlzweig (7) demonstrated an increased excretion of nicotinic acid and N-methylnicotinamide in the urine of rats following the administration of tryptophan and suggested that tryptophan is converted into nicotinic acid. Since that time this conversion has been found to take place in humans (8) and several other mammals. A first understanding of the mechanism of this transformation resulted from the work of Beadle, Mitchell and Nyc (9), who demonstrated that kynurenine is an intermediate in the conversion of tryptophan into nicotinic acid in mutant strains of Neurospora. It seemed desirable to ascertain whether this same mechanism might apply to mammals as well, and to gain further definite evidence as to the exact nature of the conversion of tryptophan into kynurenine and kynurenic acid. Accordingly, the experiments with isotopic tryptophan (10) were undertaken, and a preliminary summary of our results has already been reported (11). During the course of this work, Mitchell and Nyc reported

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(5) S. Lepkovsky, E. Roboz and A. J. Haagen-Smit, J. Biol. Chem., 149, 195 (1943).
(6) W. A. Krehl, L. J. Teply, P. S. Sarma and C. A. Elvehjem, Science, 101, 489 (1945).
(7) F. Rosen, J. W. Huff and W. A. Perlzweig, J. Biol. Chem., 163, 343 (1947).
(8) H. R. Sarett and G. A. Goldsmith, J. Biol. Chem., 167, 293 (1947).
(9) G. W. Beadle, H. K. Mitchell and J. F. Nyc, Proc. Nat. Acad. Sci., 33, 155 (1948).

that 3-hydroxyanthranilic acid is also an intermediate in the conversion of tryptophan into nicotinic acid in Neurospora (12), and they obtained evidence from dietary experiments (13) that the compound also participates in the sequence of reactions in the rat. Further confirmation was afforded by the experiments of Albert, Scheer and Deuel (14), who demonstrated increased excretion of N-methylnicotinamide in the urine of rats that had been given the hydroxyanthranilic acid. Kotake apparently has obtained independent evidence of these metabolic pathways (15).

EXPERIMENTAL

Isolation of Kynurenine: A young, mature rabbit was maintained for three weeks on a diet of polished rice, cooked in a minimum of water. On this regime, the animal lost 25% of its weight, and then a suspension of 4.00 g. of DL-tryptophan-β-C^{14} in isotonic saline (30 ml.) was injected subcutaneously. The urine was collected under toluene, and the L-kynurenine sulfate was isolated by slight modifications of the method of Butenandt et. al. (4) The 48-hour urine which gave a strong qualitative test for kynurenine was acidified with sulfuric acid and filtered. The filtrate was concentrated

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(10) C. Heidelberger, J. Biol. Chem.
(11) C. Heidelberger, M. E. Gullberg, A. F. Morgan and S. Lepkovsky, J. Biol. Chem., 175, 471 (1948).
(12) H. K. Mitchell and J. F. Nyc, Proc. Nat. Acad. Sci., 34, 1 (1948).
(13) H. K. Mitchell, J. F. Nyc and R. D. Owen, J. Biol. Chem., 175, 433 (1948).
(14) P. W. Albert, B. T. Scheer and H. J. Deuel, J. Biol. Chem., 175, 479 (1948).
(15) Private communication from Dr. R. Kinoshita.

at room temperature to one-fifth of its volume; ethanol, sufficient to make an 80% solution was added, and the solution was allowed to stand for two days in the refrigerator. At the end of this time, the crystalline kynurenine was filtered and recrystallized from dilute ethanol. The kynurenine sulfate which still contained considerable inorganic impurities was converted into the acetate salt by crystallization from acetic acid-benzene, and 350 mg. of an analytically pure sample was obtained. Its specific activity was determined by combustion.

Specific activity of administered tryptophan, 308 cts./min/mg.

Specific activity of kynurenine acetate, 113 cts./min./mg. If the entire dose of tryptophan were converted directly into kynurenine, its specific activity would have been 303 cts./min./mg. Therefore, there was a dilution of 63% by the body pool of tryptophan and kynurenine.

Kynurenine Degradation: The kynurenine acetate thus obtained was treated with sodium hypoiodite and iodoform of correct specific activity was obtained, indicating that only the β-carbon atom of the compound contained the label. This reaction involves the oxidation of the α-amino acid group with the loss of carbon dioxide and ammonia to give o-aminobenzoylacetaldehyde (16) which is then cleaved in alkali in either of the two possible ways, each of which would give iodoform derived from the same carbon atom on reaction with another molecule of hypoiodite.

The labeled kynurenine acetate, 3.2 mg., was diluted with 50.4 mg. of carrier kynurenine acetate, and after one recrystallization its specific

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(16) viz. H. T. Clarke in Gilman's "Organic Chemistry," John Wiley and Sons, Inc., (1943) Vol. II, p. 1101.

activity was determined by the direct plating technique (17), and was found to be 6.8 cts./min./mg. This sample, 35 mg., was boiled for one hour with N sodium hydroxide, was then cooled and a solution of sodium hypoiodite was added. The iodoform separated immediately, and was centrifuged, washed with water, and recrystallized from dilute ethanol. The specific activity was determined by direct plating and was 4.7 cts./min./mg. (Calculated, 4.8 cts./min./mg.)

Kynurenic Acid Isolation: Two young male dogs of pure-bred cocker spaniel stock were maintained on a stock diet (18), and each was given a solution of 500 mg. of DL-tryptophan-β-C^{14} in 5 ml. of water containing enough hydrochloric acid to dissolve the amino acid. The urines were collected under toluene for 36 hours and were then pooled and acidified with sulfuric acid. A blackish precipitate of crude kynurenic acid was obtained by centrifugation, and was clarified three times with charcoal in an alkaline solution, followed by acidification. The light yellow crystalline solid was then recrystallized twice from dilute acetic acid, and 150 mg. of pure kynurenic acid was obtained.

Analysis: Calcd for $C_{10}H_7O_3N$: %C, 63.5; %H, 3.7. Found: C, 63.2; H, 3.9. Specific activity of the ingested tryptophan, 460 cts./min./mg. Specific activity of kynurenic acid, 143 cts./min./mg. If the administered tryptophan had been converted directly into kynurenic acid, its specific activity would have been 496 cts./min./mg. Therefore, it was diluted 71% by the body pool of tryptophan and kynurenine.

(17) C. Heidelberger, M. R. Kirk and M. S. Perkins, Cancer, 1, No. 2, 263 (1948).
(18) A. F. Morgan, M. Groody and H. E. Axelrod, Am. J. Physiol., 146, 723 (1946).

Kynurenic Acid Degradation: The position of the labeled carbon atom in the radioactive kynurenic acid was established with considerable certainty by use of a series of reactions described in the literature (19). Kynurenic acid when heated to 300° undergoes smooth decarboxylation, and 4-hydroxyquinoline is obtained. When this reaction was carried out on the labeled compound, the evolved carbon dioxide was devoid of radioactivity, proving that the label is not in the carboxyl group.

The kynurenic acid, (I) 20 mg., was treated for five hours at 55° with a solution containing 10 mg. of potassium hydroxide and 64 mg. of potassium permanganate in 3 ml. of water. The manganese dioxide was removed by centrifugation, and the excess permanganate was decolorized by the addition of a few drops of sodium sulfite. The colorless solution was acidified, and colorless needles of o-carboxyoxanilide (II) were obtained, and recrystallized from hot water to give 7.5 mg. of purified product, specific activity 127 cts./min./mg. (Calculated, 130)

The tagged compound, 2.01 mg. with 30.7 mg. of carrier, was refluxed for one-half hour with 2 ml. of 2.5 N hydrochloric acid, 100 mg. of anhydrous calcium chloride in 2 ml. of water was added, and taken to pH 8 with ammonium hydroxide. The calcium oxalate was filtered, washed with alcohol and ether, and was plated directly. Yield, 15.5 mg. (87%); specific activity, 11.6 cts./min./mg. (Calculated, 12.2) This proves that the label is in the side-chain of the o-carboxyoxanilide and must have been derived from positions 2 or 3 of the kynurenic acid.

When heated above its melting point, o-carboxyoxanilide evolves carbon dioxide and carbon monoxide and the radioactivities of these gases

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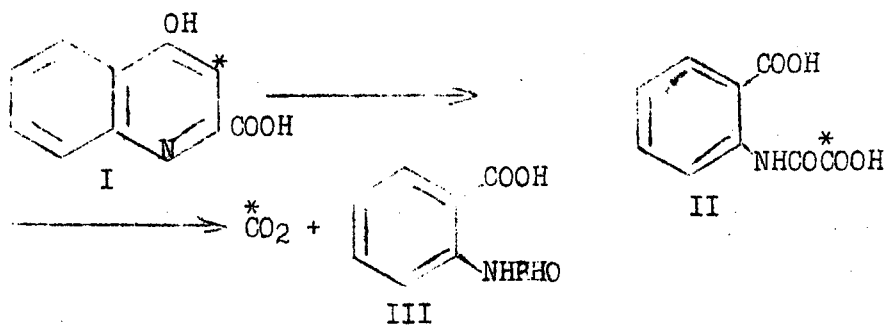
(19) F. Kretschy, Monats., 5, 16 (1884).

were determined. A mixture of 3.79 mg. of active and 42.4 mg. of carrier oxanilide was placed in a small flask and heated to 215°. The effluent gases were swept with nitrogen through a sodium hydroxide bubbler, then through a tube filled with copper oxide at 600° and finally through a second sodium hydroxide bubbler. The carbon dioxide was absorbed first, and the carbon monoxide was oxidized to carbon dioxide in the combustion tube, trapped in the second bubbler and then precipitated as barium carbonate. The barium carbonate derived from the evolved carbon dioxide weighed 34.5 mg. (80% corrected for the blank), and had a specific activity of 10.6 cts./min./mg. (Calculated, 10.5). The barium carbonate derived from the carbon monoxide weighed 12.5 mg. (29%) and was completely without radioactivity. This proves that only one of the two possible carbon atoms in the kynurenic acid was labeled. It seemed more likely that the carbon dioxide obtained during the decomposition of the o-carboxyoxanilide (II) arose from the carboxyl group and the carbon monoxide from the carbonyl group. If this were true, the intermediate formed after the initial loss of carbon dioxide would be formylanthranilic acid (III), which should liberate carbon monoxide when heated. This hypothesis was tested by the use of a synthetic sample, and a 34% yield of carbon monoxide was obtained. This is in fair agreement with the 38% that would be predicted on the basis of the results obtained from the decomposition of the o-carboxyoxanilide. Thus, it is extremely probable that only the 3-position in kynurenic acid was labeled.



N-Methylnicotinamide Experiment: Three young male rats (average weight, 300 g.) of the Home Economics Department stock colony were maintained for one week on a nicotinic acid-deficient diet (20) at the 12% casein level, were each given by stomach tube an aqueous solution of 65 mg. of DL-tryptophan-β-B^{14}, and their urines were collected under toluene for 36 hours and pooled. The N-methylnicotinamide was isolated by the procedure of Hochberg, Melnick and Oser (21) and was followed fluorometrically by the method of Huff and Perlzweig (22). The urine was diluted with acetate buffer at pH 4.5 and the mixture was then passed over a Permutit column. The N-methylnicotinamide was then eluted with hot potassium chloride solution. Both filtrate and eluate were collected. Each step was followed by fluorometric analysis for N-methylnicotinamide and by radioactivity measurements. The results are shown in Table I. To the potassium chloride eluate was added 50 mg. of N-methylnicotinamide chloride as carrier and the solution was concentrated to dryness in vacuum. The residue was extracted several times with boiling 95% ethanol, and the filtered extract was evaporated to dryness and re-extracted with ethanol. The solution was concentrated to 3 ml., and several drops of a saturated solution of picric acid in ethanol were added. The picrate salt of N-methylnicotinamide was obtained (35 mg., m.p. 178-183°) and was recrystallized from ethanol. The melting point was 187-188° and was not depressed on admixture with an authentic sample. A direct plate was made from 12.3 mg. of this purified picrate, and no radioactivity could be detected even when it was placed in the Nucleometer.

(20) J. M. Hundley, Nutrition, 34, 253 (1947).
(21) M. Hochberg, D. Melnick and B. L. Oser, J. Biol. Chem., 158, 265(1945).
(22) J. W. Huff and W. A. Perlzweig, J. Biol. Chem., 167, 157 (1947).

If the radioactive carbon atom had been retained in this conversion, there would have been 414 cts./min./mg. on the plate.

Table I

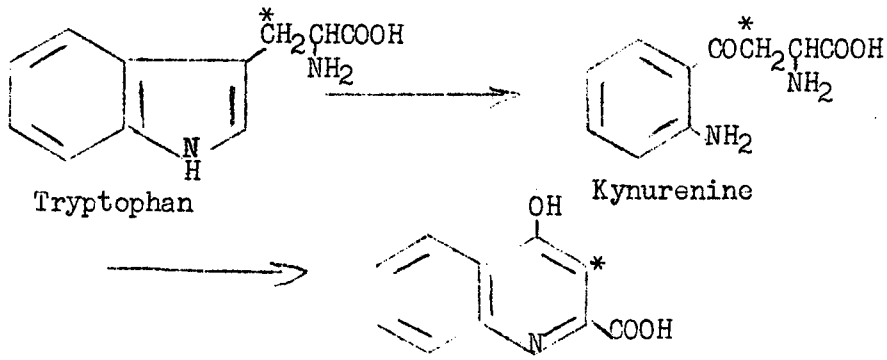
Radioactivities and Fluorometric Data

	γ of N-methyl nicotinamide (total)	Total cts./min.	Specific Activity cts./min./mg.
Tryptophan administered		1,020,000	4900
Urine (before tryptophan feeding)	42	0	
Urine (after tryptophan feeding)	450	7400	
Permutit filtrate	0	4200	
Permutit KCl eluate	115	210	
N-Methylnicotinamide picrate	carrier	0	0

A similar experiment was carried out in which the labeled kynurenine, obtained from rabbit urine, was administered to nicotinic acid-deficient rats. Although the N-methylnicotinamide excretion was increased from 145 to 730 γ, there was no detectable radioactivity in the picrate isolated as described above.

DISCUSSION

The fact that kynurenine and kynurenic acid, isolated from the urines of animals that had been given labeled tryptophan, were radioactive proves that the conversion of the amino acid into these products had actually taken place. The lowered specific activities of the products indicates considerable dilution of the administered dose by the body pools of tryptophan and the other intermediates. The demonstration that the β-carbon atom of tryptophan becomes the β-carbon of kynurenine proves that the change takes place by only one possible mechanism, which is indicated in the formulas, and which is identical to that previously demonstrated in Neurospora (9). In addition, the ring-closure of kynurenine to kynurenic acid is clearly demonstrated for the first time:



It is evident that the indole ring becomes oxidized, possibly through the intermediate of 2-hydroxytryptophan. The next product of the oxidation might then be formyl kynurenine, which would immediately be hydrolyzed in the body to kynurenine. The conversion of kynurenine to kynurenic acid might be formulated by the oxidative deamination of the α-amino group in kynurenine (another example of this well-known biochemical transformation) followed by an intramolecular condensation between the o-amino group and the α-keto

group to give the quinoline derivative, kynurenic acid. It had seemed possible that kynurenic acid might further be oxidized to give nicotinic acid, an attractive hypothesis because of the pyridine ring already present in the molecule. If this were the case, the nicotinic acid isolated would have been radioactive. The lack of radioactivity of the N-methylnicotinamide picrate shows that under the conditions of our experiments less than 0.005% of the observed conversion could have taken place by such a mechanism. Therefore, the side-chain of tryptophan must be lost during the transformation. We have no direct evidence in these experiments for the participation of 3-hydroxyanthranilic acid as an intermediate, but our results are certainly compatible with such a mechanism.

ACKNOWLEDGMENTS

We are grateful to Mrs. Martha Kirk and Mrs. Olga Nave for technical assistance.

SUMMARY

1. Experiments with radiocarbon prove that DL-tryptophan is converted into kynurenine in rabbits and kynurenic acid in dogs.

2. The β-carbon atom of the tryptophan becomes the β-carbon of kynurenine and the 3-carbon of kynurenic acid. Thus, the conversion takes place by only one mechanism.

3. The administered dose of tryptophan is considerably diluted by the body pool of this substance and other intermediates.

4. The side-chain of tryptophan is lost during its conversion into nicotinic acid.