TRYPTOPHAN METABOLISM

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

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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 third metabolite, xanthurenic acid, was isolated from the urine of pyridoxine-deficient rats 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 Neuro-

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spora. 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 Nye reported 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 for these metabolic pathways.1

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

EXPERIMENTAL

Isolation of Kynurenine

A young, mature rabbit was maintained for 3 weeks on a diet of polished rice, cooked in a minimum of water. On this régime, the animal lost 25 per cent of its weight. A suspension of 4.00 gm. of DL-tryptophan-$\beta$-$\text{Cl}^{14}$ in isotonic saline (30 ml.) was then 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 at room temperature to one-fifth of its volume; ethanol, sufficient to make an 80 per cent solution, was added, and the solution was allowed to stand for 2 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 were obtained. Its specific activity was determined by combustion.

The specific activity of administered tryptophan was 308 counts per minute per mg.; the specific activity of kynurenine acetate was 113 counts per minute per mg. If the entire dose of tryptophan were converted directly into kynurenine, its specific activity would have been 303 counts per minute per mg. Therefore, there was a dilution of 63 per cent by the body pool of tryptophan and kynurenine.

1 Private communication from Dr. R. Kinoshita.
Kynurenine Degradation

The kynurenine acetate thus obtained was treated with sodium hypoiodite, and iodoform of correct specific activity was obtained, indicating that only the $\beta$-carbon atom of the compound contained the label. This reaction involves the oxidation of the $\alpha$-amino acid group with the loss of carbon dioxide and ammonia to give $\alpha$-aminobenzoylacetaldehyde (15) 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 activity was determined by the direct plating technique (16) and found to be 6.8 counts per minute per mg. This sample, 35 mg., was boiled for 1 hour with $N$ sodium hydroxide, then cooled, and a solution of sodium hypoiodite was added. The iodoform, which separated immediately, was centrifuged, washed with water, and recrystallized from dilute ethanol. The specific activity, determined by direct plating, was 4.7 counts per minute per mg. (calculated, 4.8 counts per minute per mg.).

Kynurenic Acid Isolation

Two young male dogs of pure bred cocker spaniel stock were maintained on a stock diet (17), and each was given a solution of 500 mg. of DL-tryptophan-$\beta$-$\text{C}^{14}$ in 5 ml. of water containing enough hydrochloric acid to dissolve the amino acid. The urines were collected under toluene for 36 hours and then pooled and acidified with sulfuric acid. A blackish precipitate of crude kynurenic acid was obtained by centrifugation and 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—$\text{C}_{11}\text{H}_{12}\text{O}_{5}\text{N}$. Calculated, C 63.5, H 3.7; found, C 63.2, H 3.9

The specific activity of the ingested tryptophan was 460 counts per minute per mg., that of kynurenic acid, 143 counts per minute per mg. If the administered tryptophan had been converted directly into kynurenic acid, its specific activity would have been 496 counts per minute per mg. Therefore, it was diluted 71 per cent by the body pool of tryptophan and kynurenine.

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 (18). Kynurenic acid when heated to 300° under-
goes 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 car-
boxyl group.

The kynurenic acid, (I) 20 mg., was treated for 5 hours at 55° with a solu-
tion 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 acidif-
ciated, and colorless needles of o-carboxyoxanilide (II) were obtained, which
recrystallized from hot water to give 7.4 mg. of purified product, specific
activity 127 counts per minute per mg. (calculated, 130).

The tagged compound, 2.01 mg. with 30.7 mg. of carrier, was refluxed
for 3 hour with 2 ml. of 2.5 N hydrochloric acid, 100 mg. of anhydrous cal-
cium chloride in 2 ml. of water were added, and the solution was taken to
pH 8 with ammonium hydroxide. The calcium oxalate was filtered, washed
with alcohol and ether, and plated directly. Yield, 15.5 mg. (87 per cent);
specific activity, 11.6 counts per minute per 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 were de-
termined. 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 so-
dium 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 per cent corrected for the blank) and had a specific activity of
10.6 counts per minute per mg. (calculated, 10.5). The barium carbonate
derived from the carbon monoxide weighed 12.5 mg. (29 per cent) and was
completely without radioactivity. This proves that only one of the two
possible carbon atoms in the kynurenic acid was labeled. It seemed most
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 formylanthanilic
acid (III), which should liberate carbon monoxide when heated. This hy-
pothesis was tested by the use of a synthetic sample, and a 34 per cent yield
of carbon monoxide was obtained. This is in fair agreement with the 38
per cent 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.

\[
\begin{align*}
&\text{OH} &\text{COOH} \\
&\text{N} &\text{COOH}
\end{align*}
\]

\[
\begin{align*}
&\text{NHCHO} \\
&\text{COOH}
\end{align*}
\]

**TABLE I**

*Radioactivities and Fluorometric Data*

<table>
<thead>
<tr>
<th></th>
<th>N-Methylnicotinamide, total</th>
<th>Total counts per min.</th>
<th>Specific activity, counts per min. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan administered</td>
<td>γ</td>
<td>1,020,000</td>
<td>4900</td>
</tr>
<tr>
<td>Urine (before tryptophan feeding)</td>
<td>42</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot; (after &quot; &quot; )&quot;</td>
<td>450</td>
<td>7,400</td>
<td></td>
</tr>
<tr>
<td>Permutit filtrate</td>
<td>0</td>
<td>4,200</td>
<td></td>
</tr>
<tr>
<td>Permutit-KCl eluate</td>
<td>115</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>N-Methylnicotinamide picrate</td>
<td>Carrier</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**N-Methylnicotinamide Experiment**

Three young male rats (average weight, 300 gm.) of the Department of Home Economics stock colony were maintained for 1 week on a nicotinic acid-deficient diet (19) at the 12 per cent casein level, an aqueous solution of 65 mg. of DL-tryptophan-\(\beta\)-C\(^{14}\) was given each by stomach tube, 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 (20) and followed fluorometrically by the method of Huff and Perlzweig (21). 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 were
added 50 mg. of N-methylnicotinamide chloride as carrier, and the solution was concentrated to dryness in a vacuum. The residue was extracted several times with boiling 95 per cent ethanol, and the filtered extract was evaporated to dryness and reextracted 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°), which 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. If the radioactive carbon atom had been retained in this conversion, there would have been 414 counts per minute on the plate.

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.

**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 indicate 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 with that previously demonstrated in Neurospora (9). In addition the ring closure of kynurenine to kynurenic acid is clearly demonstrated for the first time, as shown in the accompanying scheme.

![Chemical diagram](http://www.jbc.org/)
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 formylkynurenine, 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 α-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 per cent 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.

SUMMARY

1. Experiments with radiocarbon prove that D,L-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 atom 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.

BIBLIOGRAPHY

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