SPECIES VARIATION IN THE METABOLISM
OF 3-HYDROXYANTHRANILATE TO
PYRIDINECARBOXYLIC ACIDS*

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The establishment of 3-hydroxyanthranilate (3-OHAA) as an interme-
diate in the conversion of tryptophan to niacin or its biological equivalent
in Neurospora (1, 2) has led to findings (3–5) which indicate that this com-
pound serves a similar role in animals. Much effort to establish the details
of the individual reactions involved has centered around studies of the
products of the oxidation of 3-OHAA in vitro. In addition to niacin (NA)
(3) and its end product, N\textsuperscript{\textbf{1}}-methylnicotinamide (4, 6), quinolinic acid
(QA) (7, 8) is formed from 3-OHAA in vivo. Studies with liver prepara-
tions have indicated that in some species 3-OHAA is oxidized to an unstable
intermediate (Compound I) (9–11) which in turn gives rise to an almost
quantitative yield of QA by a non-enzymatic process. Compound I was
reported to be converted by an enzyme ("Enzyme II") present in guinea
pig and beef liver to another product tentatively identified as picolinic
acid (PA) by $R_f$ values and ultraviolet absorption spectra (12).

Subsequent experiments (13) with use of O\textsuperscript{18} established the identity of
this product as well as the general type of reaction involved in the opening
of the aromatic ring. On the basis of studies which showed that 60 to 90
per cent of the carboxyl carbon of 3-OHAA was expired as CO\textsubscript{2} (14, 15),
and that little or no carboxyl carbon of labeled NA was expired by the rat,
Mehler and May (15) suggested that, "This is evidence that the decar-
boxylation to form picolinic acid is a major step in the metabolism of 3-
hydroxyanthranilic acid in vivo." This view failed to take into account
the possibility of alternative pathways for the metabolism of 3-OHAA or
its oxidation product (Compound I). The data which follow prove that

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PA, a test dose of which is largely excreted in the urine chiefly as the glycine conjugate (15), is not a major end product of 3-OHAA or tryptophan in the cat or rat. Information regarding the metabolism in vitro of 3-OHAA by the liver of eight other species is presented.

EXPERIMENTAL

Test Compounds

3-Hydroxyanthranilic acid randomly labeled with tritium in the benzene nucleus was prepared by exposing a mixture of lithium carbonate (110 mg.) and 2-nitro-3-methoxybenzoic acid (1 gm.) to a neutron flux of $1.8 \times 10^{12}$ neutrons per cm.$^2$ per second for 48 hours (16), followed by simultaneous demethylation and reduction (17) of the recovered 2-nitro-3-methoxybenzoic acid. The specific activity of the 3-OHAA was 12.35 μc. per mmole. A product prepared by exposure of crystalline 3-OHAA to tritium gas (18) was also used (1.5 mc. per mmole). The 3-OHAA labeled with C$^{14}$ in the carboxyl group was the same preparation previously described (6). The tryptophan used was the same sample described earlier (19).

Experiments in Vitro

The liver extracts were prepared by homogenizing fresh livers with 9 volumes of glass-distilled water and centrifuging at 14,000 X g for 30 minutes. The substrate for studies in vitro was prepared just prior to use by dissolving the 3-OHAA in dilute HCl, neutralizing to pH 7 with solid Na$_2$CO$_3$, and diluting to 2 or 4 μmole per ml. The disappearance of 3-OHAA was followed fluorometrically, and the intermediate (Compound I) was determined spectrophotometrically at 360 μM (10). The extinction coefficient used was 40,000 which appears more nearly correct than 30,000 as previously reported (10, 12). The amounts of QA formed were determined by measuring microbiologically the NA formed after decarboxylation (7). Manometric studies were carried out as described previously (10), except that the enzyme source for each flask consisted of 0.5 ml. of a 10 per cent extract of fresh liver homogenate.

The C$^{14}$O$_2$, obtained by adding 30 per cent perchloric acid to an aliquot of the contents of the center well of the Warburg flasks, was collected in an ionization chamber and counted with the vibrating reed electrometer. The C$^{14}$ in the incubation mixture was converted to CO$_2$ by wet combustion (20) and counted as a gas with the vibrating reed electrometer. Tritium analyses were made by the gas counting procedure of Wilzbach, Kaplan, and Brown (21). After adding 200 mg. of carrier PA and QA, these compounds were isolated from the deproteinized incubation mixtures by a procedure involving adsorption on and elution from Norit (7). PA was not eluted with QA from the Norit by 0.1 N NH$_4$OH, but was removed with
20 per cent phenol in 20 per cent acetic acid. After removal of the phenol by ether extraction, the PA was purified by adsorption chromatography on alumina in the same manner as QA and crystallized as the hydrochloride from ethanol.

Studies in Vivo

The 3-OHAA was suspended in isotonic saline and homogenized at a concentration of 10 mg. per ml. This suspension was injected intraperitoneally as indicated in Table IV. The labeled DL-tryptophan was dissolved in 0.9 per cent NaCl and injected intraperitoneally as described in Table V. The urines were collected during a period of 24 hours or longer. After adding 100 or 150 mg. of carrier QA and PA, these compounds were isolated from the urine after hydrolysis at 121° for 2 to 3 hours with 3 to 6 ml. of 1 N NaOH. The alkaline urine was adjusted to pH 2 with 2 N HCl, and the QA and PA were separated on a Dowex 50-H column by development with 0.1 N HCl. The QA which appeared in the first few fractions was recovered by evaporation to dryness and crystallized twice from 2 ml. of 50 per cent ethanol at pH 2.

The samples of QA isolated from the normal rat, cortisone-treated rat, and normal cats all melted with decomposition between 183-187° and left a residue (NA) which melted at 215-224° with sublimation. Authentic QA melted with decomposition at 189-191°, leaving a residue (NA) which melted at 224-230° with rapid sublimation. Addition of pure QA caused no depression of the melting points. The PA, eluted from the resin with approximately 85 ml. of 0.1 N HCl, was evaporated at pH 3 to dryness in a sublimation tube and sublimed at 100° in vacuo. It was purified by crystallization from 1 ml. of an ethanol-benzene mixture (1:1) or by resublimation. These samples began to sublime between 128-130° on the Kofler block and melted at 131-136°. Authentic PA melted at 138-139.5° and did not depress the melting point of the individual samples.

RESULTS AND DISCUSSION

It has been repeatedly observed that rat liver slices and homogenates convert 3-OHAA almost quantitatively to QA. Beef liver, which also contains a very active 3-OHAA oxidase (10), often forms much less QA than the theoretical quantity.1 This species variation and the report by Mehler (12) of a new product (PA) of this reaction prompted a more detailed study of species variation with carboxyl-labeled 3-OHAA.

The results of these experiments involving liver extracts from ten species are shown in Table I. The carboxyl carbon which was released as CO₂

1 Priest et al. (22) reported similar results in which it was noted that beef liver formed much less QA than rat or pork liver.
varied from 11.0 per cent for mouse liver to 86.0 per cent for cat liver. Essentially all of the C\textsuperscript{14} added as substrate and not released as CO\textsubscript{2} was present in the incubation mixture. Quinolinic acid accounted for essentially all of the C\textsuperscript{14} in the incubation mixture for most species studied. In the case of the mouse, however, more than 40 per cent of the C\textsuperscript{14} was present in some compound (or compounds) other than QA. Likewise with the pork, dog, and rabbit preparations, 17 per cent, 24 per cent, and 19 per cent, respectively, of the C\textsuperscript{14} were not accounted for as CO\textsubscript{2} and QA.

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Per cent of C\textsuperscript{14} in CO\textsubscript{2}</th>
<th>Remaining incubation mixture</th>
<th>Per cent of substrate converted to QA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>11.0</td>
<td>90.0</td>
<td>46.3</td>
</tr>
<tr>
<td>Pork</td>
<td>11.1</td>
<td>84.0</td>
<td>67.0</td>
</tr>
<tr>
<td>Rat</td>
<td>12.0</td>
<td>81.1</td>
<td>80.0</td>
</tr>
<tr>
<td>Hamster</td>
<td>19.0</td>
<td>76.5</td>
<td>69.5</td>
</tr>
<tr>
<td>Dog</td>
<td>26.0</td>
<td>74.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>31.0</td>
<td>49.0</td>
<td>42.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>34.0</td>
<td>63.8</td>
<td>44.5</td>
</tr>
<tr>
<td>Pigeon</td>
<td>36.8</td>
<td>52.0</td>
<td>46.5</td>
</tr>
<tr>
<td>Beef</td>
<td>49.0</td>
<td>39.4</td>
<td>41.0</td>
</tr>
<tr>
<td>Cat</td>
<td>86.0</td>
<td>12.3</td>
<td>9.0</td>
</tr>
</tbody>
</table>

* Each vessel contained 10\textsuperscript{-4} M Fe\textsubscript{2}O\textsubscript{3}, 10\textsuperscript{-3} M glutathione with 0.5 ml. of 10 per cent liver extract, and Krebs-Ringer-phosphate buffer, pH 7.4, added to 3.0 ml. The incubations were for 75 minutes at 37\textdegree with 2 \mu moles of 3-OHAA in one side arm and 0.2 ml. of 1 N HCl in the second side arm. HCl was tipped into the reaction mixture after 75 minutes to release CO\textsubscript{2}, and shaking was continued for 15 minutes.

These results, which suggested that the level of Enzyme II which catalyzes the formation of PA (12) varies markedly from one species to another, and that some livers contain enzymes which form still other products, led to a more detailed study of these reactions in vitro. The incubation mixtures, each of which contained 30 \mu moles of substrate with 7.5 ml. of a 10 per cent extract of fresh liver in a volume of 45 ml., were periodically sampled to measure the rates of disappearance of 3-OHAA, appearance of Compound I, and formation of QA. These data for the rat and cat livers are presented in Fig. 1.

For ease of comparison with earlier experiments (10), all of the results are presented on the basis of 2 \mu moles of substrate. The data for the rat and the cat were selected since they represented the extremes for C\textsuperscript{14}O\textsubscript{2} and QA formation (Table I). The time required for the disappearance of
substrate with the rat and cat livers was 5 and 10 minutes, respectively, though the initial rate of loss of fluorescence was greater for the cat. The maximal amount of Compound I formed by the rat liver was 1.80 μmoles and by the cat liver, 1.1 μmoles. This difference in the amount of Compound I formed by these extracts appears to result not from a difference in oxidase activity, but arises at least in part from differences in the rate of removal of this intermediate. The low yield of QA from the cat liver, in agreement with the data on C14 (Table I), supports this view.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Conversion of 3-OHAA to QA via Compound I with rat and cat liver extracts. The reaction mixture contained 30 μmoles of 3-OHAA, 7.5 ml. of 10 per cent enzyme extract, 3 μmoles of ferrous sulfate, and glass-distilled water to 45 ml. pH 7.4.

Similar data obtained for eight other species are not reported in detail, but a summary is presented in Table II. An 8-fold variation in oxidase activity was noted. This may not reflect the true concentrations of the enzyme, since no effort was made to insure equivalent activation by pre-incubation with reduced glutathione, a procedure which is required for maximal activity in a dialyzed extract of liver acetone powder (5). The rate of disappearance of Compound I was in general equal to or slightly greater than the rate of QA formation. The most striking exception was with the cat liver in which the Compound I disappeared more than 4 times as fast as the QA was formed. This result is in agreement with the data in Table I and presumably is due to the action of Enzyme II.

To verify the supposition that the substrate unaccounted for in the cat
liver experiments was chiefly PA, analyses for pyridinecarboxylic acids by the method of Rabinovitz et al. (23) were conducted on trichloroacetic acid filtrates from these incubation mixtures. Since QA as well as PA is measured by this method, the analysis was of little value except in the cat liver preparations for which the ratio of PA to QA concentrations was high. In this species the PA, as measured by this procedure, accounted for the remainder of the substrate (Curve X, Fig. 1).

Table II

Comparison of Various Species with Regard to Rate of Formation and Disappearance of Compound I in Presence of Soluble Liver Enzymes

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxidase activity*</th>
<th>Compound I concentration, µmoles per 3 ml</th>
<th>Compound I disappearance, µmole per 20 min. per µmole</th>
<th>QA appearance, µmole per 20 min. per µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>0.67</td>
<td>1.65 (10)†</td>
<td>0.45 (10-30)§</td>
<td>0.29</td>
</tr>
<tr>
<td>Pork</td>
<td>0.25</td>
<td>1.40 (10)</td>
<td>0.48 (10-30)</td>
<td>0.45</td>
</tr>
<tr>
<td>Rat</td>
<td>0.33</td>
<td>1.80 (5)</td>
<td>0.54 (5-25)</td>
<td>0.47</td>
</tr>
<tr>
<td>Hamster</td>
<td>2.0</td>
<td>1.90 (3 - 5)</td>
<td>0.53 (5-25)</td>
<td>0.48</td>
</tr>
<tr>
<td>Dog</td>
<td>0.67</td>
<td>1.60 (3)</td>
<td>0.52 (3-23)</td>
<td>0.40</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>2.0</td>
<td>2.25 (5)</td>
<td>0.65 (5-25)</td>
<td>0.35</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.0</td>
<td>1.5 (10)</td>
<td>0.71 (10-30)</td>
<td>0.28</td>
</tr>
<tr>
<td>Pigeon</td>
<td>1.0</td>
<td>0.95 (20)</td>
<td>0.32 (20-40)</td>
<td>0.33</td>
</tr>
<tr>
<td>Beef</td>
<td>0.5</td>
<td>1.15 (10)</td>
<td>0.39 (10-30)</td>
<td>0.48</td>
</tr>
<tr>
<td>Cat</td>
<td>1.0</td>
<td>1.10 (2)</td>
<td>0.93 (2-22)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

For the experimental conditions see Fig. 1.

* Oxidase activity = 1 ÷ time in minutes required for the disappearance of 1 pmole of substrate.
† Maximal concentration during the course of the reaction.
‡ Time at which maximal concentration was reached.
§ 20 minute time interval selected for rate calculations.

The results presented in Tables I, II, and in Fig. 1 indicate that the cat liver contains a very active enzyme which is responsible for the decarboxylation of Compound I to form PA. The rat liver apparently possesses little of this catalytic activity. To verify this hypothesis, extracts of cat and rat liver were incubated under the conditions described above with tritium-labeled 3-OHAA for 1 hour. Carrier PA and QA were added to the deproteinized incubation mixture, and, after isolating and purifying these compounds, the tritium content was determined. Approximately 80 per cent of the tritium of 3-OHAA was recovered in the two compounds. With the rat liver, approximately 90 per cent of the recovered isotope was present in the QA and with the cat liver more than 75 per cent was present in the PA (Table III). These findings confirm the pronounced differ-
ence in the manner by which 3-OHAA is metabolized \textit{in vitro} by the liver of these species.

Experiments \textit{in vivo} of a similar type were then conducted. Tritium-labeled 3-OHAA was administered intraperitoneally, carrier PA and QA were added to the urine, the conjugated acids were hydrolyzed, and the two acids were isolated and purified as already described. The report that administration of cortisone to the rat increased the concentration of the PA-forming enzyme in the liver as measured \textit{in vitro} (15) prompted a study of this effect \textit{in vivo}. The results obtained with two normal cats, a normal rat, and a cortisone-treated rat are presented in Table IV.

It is evident that PA is only a minor urinary excretion product of 3-OHAA in both the cat and the rat. The microbioassay for QA in the

<table>
<thead>
<tr>
<th>Preparation</th>
<th>3-OHAA added</th>
<th>Tritium in PA</th>
<th></th>
<th>Tritium in QA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mus.</td>
<td>Mus.</td>
<td>Per cent</td>
<td>Mus.</td>
</tr>
<tr>
<td>Rat liver extract</td>
<td>220</td>
<td>16.3</td>
<td>7.4</td>
<td>158.5</td>
</tr>
<tr>
<td>Cat &quot; &quot;</td>
<td>188</td>
<td>113.8</td>
<td>60.5</td>
<td>31</td>
</tr>
</tbody>
</table>

* The reaction mixtures are as follows: For the rat preparation, 4.78 mg. of 3 OHAA were dissolved in 7.8 ml. of water, 7.9 ml. of a 10 per cent fresh liver extract and 31.6 ml. of Krebs-Ringer-phosphate buffer, pH 7.4. For the cat preparation, 4.03 mg. of 3-OHAA were dissolved in 7.8 ml. of water, 7.9 ml. of a 10 per cent fresh liver extract, and 31.6 ml. of Krebs-Ringer-phosphate buffer, pH 7.4. Incubated for 1 hour at 37°.

The results of these experiments \textit{in vitro} strongly indicate that the differences in the manner in which the liver of various species metabolizes 3-OHAA to pyridine derivatives might result from variation in the activity of the enzyme which decarboxylates the PA precursor. This enzyme would compete with the spontaneous reaction by which this precursor is transformed into QA. It appeared that the unusually effective substitution of tryptophan for niacin in the rat might result from a lack of an enzyme (or enzymes) which decarboxylates a NA precursor. The ineffectiveness of this enzyme \textit{in vivo} in the cat, whose liver catalyzes this reaction so rapidly \textit{in vitro}, is anomalous.

That the low level of labeling of PA in these experiments \textit{in vivo} in the
rat is not the result of further metabolism of PA is indicated by the observations of Mehler (15) and by the recovery of almost all of an intraperitoneally administered test dose of 30 mg. of PA from the urine after alkaline hydrolysis. Similar experiments with the cat showed that not more than half of a 10 mg. dose of tritium-labeled PA was metabolized. The remainder was recovered from the urine by isolation after carrier addition and alkaline hydrolysis. This result, together with those recorded in Table IV, shows that no more than 10 per cent of a test dose of 3-OHAA could be converted to PA in the intact cat.

Tryptophan is probably the sole natural precursor of 3-OHAA in animals. Comparison of the extent of conversion of these two compounds to urinary pyridinecarboxylic acids might indicate the degree to which tryptophan forms 3-OHAA in vivo. This matter was studied, and the results are shown in Table V. The data indicate that PA is not a significant end product of tryptophan metabolism in the rat or cat. In the rat 3.4 per cent of the administered tryptophan was converted to QA, while the cat formed QA from only 0.65 per cent of the tryptophan-C¹⁴ given.

This inability of the cat to convert tryptophan to pyridinecarboxylic acid as compared to the rat is in agreement with the results of de Castro,
Brown, and Price (24). These workers reported that the cat is unable to metabolize tryptophan to the usual urinary end products owing to a considerably less active liver tryptophan peroxidase-oxidase system than that present in the rat. Our data support this view, since the cat converts 3-OHAA to QA and PA to approximately the same extent as the rat (Table IV) in contrast to the tryptophan results.

It appears that under the conditions of these experiments urinary PA is an end product of only a minor portion of exogenous or endogenous 3-OHAA in both of the species studied. Other urinary compounds, not yet identified, account for as much as 25 per cent of the C\textsuperscript{14} from carboxyl-

<table>
<thead>
<tr>
<th>Table V</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conversion in Vivo of Tryptophan-3a,7,7a-C\textsuperscript{14} to Quinolinate and Picolinate</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tryptophan administered, mg.</th>
<th>Rat*</th>
<th>Cat†</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot; &quot;</td>
<td>24</td>
<td>200</td>
</tr>
<tr>
<td>&quot; &quot; muc.</td>
<td>982</td>
<td>2090</td>
</tr>
</tbody>
</table>

| Amount of C\textsuperscript{14} excreted as PA, muc. | 8.35 | 2.09 |
| " " | 0.0 | 0.0 |

<table>
<thead>
<tr>
<th>Substance converted to QA, %</th>
<th>3.4</th>
<th>0.65†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate converted to QA, %</td>
<td>7.62</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* 24 mg. in one dose; urine was collected 24 hours later.
† 200 mg. in two equal doses 7 hours apart. Urine was collected 33 hours after the first injection.
‡ Subject to a large error owing to the very low specific activity of the QA isolated.
§ Based on microbioassay.

labeled 3-OHAA given to rats at levels of 3.1 and 19.1 mg. (8) and for an even larger percentage of the C\textsuperscript{14} from 1 mg. of 3-OHAA labeled in positions 1 and 2 (25). The pathway by which most of the remaining 3-OHAA is converted to CO\textsubscript{2} through metabolically active, non-aromatic compounds remains to be elucidated. It seems possible that this pathway is the one taken by the major portion of the carbon of the indole nucleus of tryptophan.

**SUMMARY**

1. Liver extracts from ten species oxidized C\textsuperscript{14}-carboxyl-labeled 3-hydroxyanthranilic acid to an unstable intermediate (Compound I). A decarboxylase, the activity of which varied with the species, catalyzed the loss of C\textsuperscript{14}O\textsubscript{2} from the labeled carboxyl carbon of Compound I to give picolinic acid in competition with the non-enzymatic reaction leading to
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quinolinic acid. The mouse, pork, and rat liver extracts, which had a much less active decarboxylase than the beef or cat liver extract, formed much quinolinic but little picolinic acid.

2. Cat liver extracts converted tritium-labeled 3-hydroxyanthranilate chiefly to picolinate, while extracts of rat liver formed chiefly quinolinate.

3. Both the cat and the rat converted approximately 10 per cent of the tritium-labeled 3-hydroxyanthranilate to urinary quinolinate and approximately 4 per cent to picolinate in vivo. Tryptophan-C\textsuperscript{14} led to labeled urinary quinolinic acid in both species, especially in the rat, but not to labeled picolinic acid.

4. In both species most of an exogenous dose of picolinate was recovered from the urine.

BIBLIOGRAPHY

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