Riboflavin and Hepatic Kynurenine Hydroxylase*

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After the ingestion of L-tryptophan, riboflavin-deficient rats excrete abnormally large amounts of kynurenic acid (1). This observation led to the suggestion (1) that the conversion of L-kynurenine to xanthurenic acid is decreased during a riboflavin deficiency. Consistent with this hypothesis is the observed increased excretion of anthranilic acid and its derivatives in response to tryptophan in deficient rats (2, 3). It was observed that riboflavin-deficient animals excrete more niacin metabolites (4) and quinolinic acid (5, 6) in response to L-tryptophan. L-Kynurenine (6) conversion to these metabolites was also impaired, but little change in response to 3-hydroxy-L-kynurenine or 3-hydroxyxanthranilic acid was noted as a result of riboflavin deficiency (5, 6). Urinary xanthurenic acid levels of riboflavin-deficient rats are as much as 14-fold higher than those of normal control animals (2, 6) when L-tryptophan is given. It has been found (6) that, in riboflavin-deficient animals, about the same amounts of xanthurenic and quinolinic acids are excreted whether L-tryptophan or L-kynurenine is given and the excretion pattern in both cases approximates that of control animals given L-kynurenine. These results could be interpreted as indicating an accumulation of L-kynurenine when L-tryptophan is given to riboflavin-deficient rats.

With the discovery (7, 8) of a mitochondrial enzyme system capable of hydroxyating L-kynurenine to 3-hydroxy-L-kynurenine it was possible to extend the studies in vivo cited above to riboflavin-deficient rats. An examination of the role of riboflavin in the hepatic mitochondrial hydroxylation system is also reported here.

Material for Enzyme Assay—L-kynurenine sulfate was prepared enzymatically (9). 3-Hydroxy-L-kynurenine was a gift of Dr. R. R. Brown. 3-Hydroxy-DL-kynurenine was prepared by Dr. R. E. Koski (8). Digitonin, nicotinamide, and glucose 6-phosphate were obtained from the California Foundation for Biochemical Research. Practical grade Tris was obtained from the Matheson Company, Inc.

TPNH, 95 to 100 per cent pure, FAD, 90 per cent pure, and riboflavin phosphate, free from contamination, were obtained from the Sigma Chemical Company. Glucose 6-phosphate dehydrogenase was purified 4-fold from dried brewers' yeast, a gift of the Anheuser-Busch Brewing Company, according to the method of Kornberg and Horcoker (10) through the first ammonium sulfate step. This preparation was used to generate TPNH.

Preparation and Disruption of Mitochondria—Livers, removed immediately from decapitated rats and chilled in ice-cold 0.25 M sucrose, were used for the isolation of mitochondria according to the method of Schneider (11) modified to the extent that the mitochondrial pellet was obtained by centrifugation at higher speed (18,000 × g). The pellet was washed twice by suspending in 0.25 M sucrose. The washed mitochondria were suspended in a volume of 0.25 M sucrose equivalent to one-half the original weight of fresh liver.

To solubilize the kynurenine hydroxylase, equal volumes of 2 per cent sodium cholate or 1 per cent digitonin and the mitochondrial suspension were blended thoroughly. The mixture was stirred frequently at 0° for 90 minutes. After the addition of another volume of 0.25 M sucrose, the mixture was centrifuged 15 to 20 minutes at 29,000 × g at −5°. The supernatant fraction or extract was subjected to centrifugal forces of 105,000 × g for 90 minutes or 144,000 × g for 60 minutes.3

Assay of Enzymatic Activity—Components of the standard assay system were: 1 µmole of L-kynurenine sulfate, 0.2 to 0.5 µmole of TPNH, 80 µmoles of nicotinamide, 30 µmoles of potassium cyanide, 30 µmoles of potassium chloride, 5 µmoles of cysteine, 150 µmoles of Tris, an aliquot of an enzyme preparation, and water to a volume of 3.0 ml at pH 8.3. After the addition of TPNH the mixture was incubated at room temperature at

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TABLE I
Solubilization of hepatic mitochondrial kynurenine hydroxylase with 1 per cent digitonin or 2 per cent sodium cholate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction*</th>
<th>Recovery†</th>
<th>Relative activity²</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Mitochondrial suspension</td>
<td>100</td>
<td>0.051</td>
</tr>
<tr>
<td>2 per cent sodium cholate</td>
<td>29,000 \times g extract</td>
<td>235</td>
<td>0.305</td>
</tr>
<tr>
<td></td>
<td>29,000 \times g residue</td>
<td>21</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>144,000 \times g supernatant</td>
<td>224</td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>29,000-144,000 \times g residue</td>
<td>26</td>
<td>0.070</td>
</tr>
<tr>
<td>1 per cent digitonin</td>
<td>29,000 \times g extract</td>
<td>272</td>
<td>0.634</td>
</tr>
<tr>
<td></td>
<td>29,000 \times g residue</td>
<td>109</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td>144,000 \times g supernatant</td>
<td>149</td>
<td>0.579</td>
</tr>
<tr>
<td></td>
<td>29,000-144,000 \times g residue</td>
<td>130</td>
<td>0.700</td>
</tr>
</tbody>
</table>

* Fractions were stored at -10⁰ until assayed.
† Per cent recovery in fractions is based on the activity in an equivalent volume of original mitochondrial suspension.
² Mole of 3-hydroxykynurenine formed per hour per mg. of protein in the standard assay system containing 0.4 μmole TPNH and 0.2 ml. of a fraction. Duplicate incubations were performed at 36⁰. 3-Hydroxykynurenine was determined by the nitrous acid colorimetric method.

RESULTS

Comparison of Solubilizing Methods—Saito et al. (8) have reported solubilization of kynurenine hydroxylase by sonication of rat liver mitochondria. These workers also stated that digitonin or sodium cholate was effective in extracting the enzyme from 21-24⁰, or at 36⁰ in a Dubnoff metabolic shaker. When a large number of samples were to be assayed, it was found convenient to determine 3-hydroxykynurenine spectrophotometrically with the nitrous acid method (12). The rate of 3-hydroxykynurenine formation at room temperature or 36⁰ was constant over the initial 30 minutes of incubation. Employing two-dimensional chromatography with butanol, acetic acid, and water and then with the use of 20 per cent potassium chloride as solvent systems it was possible to detect (13) L-kynurenine and 3-hydroxykynurenine but not 3-hydroxyanthranilic acid, anthranilic acid, xanthurenic acid, or kynurenic acid.

The course of the reaction was also determined in some experiments by measuring the decrease in optical density at 340 mp or the increase at 400 mp in a 15-minute period. Making the corrections described by Saito et al. (8), it was possible to calculate the rates of TPNH oxidation and 3-hydroxykynurenine formation from these data. In all cases TPNH oxidation rates corresponded closely with 3-hydroxykynurenine formation rates.

Protein concentrations were determined spectrophotometrically according to the method of Warburg and Christian (14) after dialysis of samples.

![Fig. 1. Dependence of hydroxylation rate upon enzyme concentration.](http://www.jbc.org/)

The standard assay system containing 0.4 μmole of TPNH, was incubated 10 (○) or 20 (●) minutes at 21⁰. 3-Hydroxykynurenine was measured directly by observing the increase in optical density at 400 mp.
mitochondria, but gave no details of their method or the extent of solubilization accomplished. Consequently, it was necessary to examine these treatments before adopting one or the other for routine use. Fractions obtained by differential centrifugation of digitonin- or sodium cholate-treated mitochondrial suspensions were assayed for enzymatic activity and protein content to obtain the results summarized in Table I.

With digitonin and sodium cholate treatments the total recovery of enzyme in the 29,000 × g residue and 29,000 × g extract was 381 and 256 per cent, respectively. This 2- to 3-fold increase in activity was observed consistently upon treatment of mitochondrial suspensions with digitonin or sodium cholate. A 130 per cent recovery, with an apparent 14-fold increase in specific activity, was observed in a particulate submitochondrial fraction, the 29,000 to 144,000 × g mitochondrial suspensions with digitonin or sodium cholate. Consequently, the sodium cholate method was adopted for routine use.

Rate Studies—For these experiments a 29,000 × g sodium cholate extract of mitochondria was prepared. After storage at −10° for 12 hours this extract was centrifuged 90 minutes at 105,000 × g and the supernatant dialyzed for 16 hours against two changes of distilled water to obtain a "cholate-solubilized enzyme.

To obtain the results presented in Fig. 1, initial reaction rates were determined with five levels of enzyme and incubation periods of 10 minutes and 20 minutes. Initial TPNH oxidation rates are not shown since they corresponded closely with 3-hydroxykynurenine formation rates at all five levels of enzyme. Rates were a linear function of enzyme concentration up to 0.5 ml. of cholate-solubilized enzyme in 3.0 ml.

In other studies it was observed that the initial reaction rate is limited by TPNH concentrations below 0.2 μmole in 3.0 ml. Omission of potassium cyanide or potassium chloride lowered initial reaction rates. The omission of both of these components resulted from differences in mitochondrial permeability. Therefore the cholate method without centrifugation was used to disrupt the mitochondria before measurement of hydroxylase activity. In Table II, the results from Groups 33 and 34 constitute a comparison between riboflavin-deficient and inanition control rats. The specific activity of L-kynurenine hydroxylase was 3-fold lower in disrupted liver mitochondria from the riboflavin-deficient rats than in those from inanition control rats. Preincubation with riboflavin phosphate, FAD, or a boiled liver extract did not increase enzymatic activity in the preparations from riboflavin-deficient rats.

**TABLE II

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver weight</th>
<th>Mitochondrial protein</th>
<th>Hydroxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>mg.</td>
<td>μg.</td>
</tr>
<tr>
<td>33—Inanition control, 10 rats</td>
<td>4.12</td>
<td>50.5</td>
<td>80</td>
</tr>
<tr>
<td>34—Riboflavin-deficient, 11 rats</td>
<td>3.83</td>
<td>62.2</td>
<td>80</td>
</tr>
</tbody>
</table>

*Additions to 2.0 ml. of 2 per cent cholate disrupted mitochondria during 60 minutes preincubation at 0°.
†μMoles of 3-hydroxykynurenine formed per hour per mg. of protein in the standard assay system containing 0.5 μmole of TPNH and 0.6 ml. of preincubated disrupted mitochondria from individual livers. Incubations were at 21° for the time intervals specified.
‡Average body weights in Groups 33 and 34 were 144.8 ± 3.6 and 73.5 ± 4.0 gm., respectively after 8 weeks.
§S. e. m. = \sqrt{\frac{\text{dev.}^2}{n(n - 1)}}
|| Addition of a boiled liver extract gave no significant restoration of activity in 4 disrupted mitochondrial samples from riboflavin-deficient rats.

**Discussion**

Since mitochondria appear to be the only site of 3-hydroxykynurenine formation in liver cells (7), a depression in the hydroxylase system is bound rather tightly to the mitochondrial structure and that its release involves more drastic structural alterations than simply rupture of a membrane. A recovery of enzyme in the 29,000 × g residue and 29,000 × g extract of digitonin- or sodium cholate-treated mitochondrial suspensions resulted from differences in mitochondrial permeability. Therefore the cholate method without centrifugation was used to disrupt the mitochondria before measurement of hydroxylase activity. In Table II, the results from Groups 33 and 34 constitute a comparison between riboflavin-deficient and inanition control rats. The specific activity of L-kynurenine hydroxylase was 3-fold lower in disrupted liver mitochondria from the riboflavin-deficient rats than in those from inanition control animals. Preincubation with riboflavin phosphate, FAD, or a boiled liver extract did not increase enzymatic activity in the preparations from riboflavin-deficient rats.
Flavin coenzyme to liver homogenates from riboflavin-deficient rats is consistent with the postulation (2) that the conversion of L-kynurenine to 3-hydroxy-L-kynurenine is impaired by a riboflavin deficiency. Failure to observe significant restoration of activity by the addition of riboflavin phosphate or FAD to disrupted mitochondria from deficient animals places a coenzyme role for riboflavin in doubt. However, Burch et al. (15) have observed similar results in their studies of tissue levels of six flavoprotein enzymes during riboflavin deficiencies in the rat. Only limited restoration of ω-amino acid oxidase or glycolic acid oxidase activity could be obtained by adding the appropriate flavin coenzyme to liver homogenates from riboflavin-deficient rats. Little or no restoration of activity could be observed for the other four enzymes tested. A depression of apoenzyme levels during riboflavin deficiency was offered as an explanation for these results.

Another possible explanation for failure to obtain restoration of hydroxylase activity with the addition of riboflavin phosphate or FAD to disrupted mitochondria is indicated by the report of Singer et al. (16). The linkage(s) binding FAD to the protein in succinic dehydrogenase preparations from beef heart mitochondria are not broken completely by mild acidic hydrolysis conditions (16). Partial enzymatic hydrolysis of the enzyme preparations gives rise to a series of compounds containing amino acids firmly attached to the coenzyme, but no free coenzyme. On the basis of these findings it is conceivable that reconstitution of these enzymes and of the kynurenine hydroxylase system would not be possible by simply adding FAD to preparations from riboflavin-deficient rats even if normal amounts of the apoenzyme were present in these preparations.

Further studies of the possible function of riboflavin as a coenzyme for kynurenine hydroxylase will take into consideration the above possibilities. It is interesting to note that phenylalanine hydroxylase, a similar enzyme system, requires an unidentified organic cofactor for maximal activity (17). Such a cofactor, tissue levels of which may be reduced by a riboflavin deficiency, may also be functioning in the kynurenine hydroxylase system. The requirement for such a cofactor could not be shown by dialysis.

SUMMARY

1. A 2- to 3-fold increase in kynurenine hydroxylase activity resulted from digitonin or sodium cholate treatment of hepatic mitochondria. Of this enzymatic activity 90 and 34 per cent were present in the supernatant from 144,000 × g centrifugation of cholate and digitonin treated mitochondria, respectively. After digitonin treatment 30 per cent of the activity was found in submitochondrial particles.

2. Potassium cyanide and potassium chloride, each at 10⁻² M, doubled the activity of a dialyzed, soluble preparation. In the presence of optimal concentrations of reduced triphosphopyridine nucleotide and L-kynurenine, there was a linear dependence of initial reaction rate on enzyme concentration.

3. The specific activity of L-kynurenine hydroxylase in cholate disrupted hepatic mitochondria from riboflavin-deficient rats was 30 to 50 per cent that of similar preparations from inanition control animals. Addition of riboflavin phosphate, flavin adenine dinucleotide, or a boiled liver extract had no statistically significant effect on hydroxylase activity of preparations from deficient or control animals.

REFERENCES

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