Tyrosyluria Resulting from Inhibition of p-Hydroxyphenylpyruvic Acid Oxidase in Vitamin C-deficient Guinea Pigs

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Tyrosyluria, the excretion of p-hydroxyphenyl metabolites of tyrosine, such as p-hydroxyphenylpyruvic acid and p-hydroxyphenyllactic acid, occurs after the administration of tyrosine to scorbutic guinea pigs (1, 2), scorbutic man (3, 4), and to premature infants (5). A persistent tyrosyluria consisting mainly of p-hydroxyphenylpyruvic acid was also present in the unique case of tyrosinosis reported by Medes (6).

Earlier studies in vitro have shown that p-hydroxyphenylpyruvic acid oxidase has an unusual susceptibility to inhibition by high concentrations of its substrate (7-9). Recently it has been demonstrated that p-hydroxyphenylpyruvic acid oxidase injected intraperitoneally inhibits p-hydroxyphenylpyruvic acid oxidase in scorbutic guinea pigs. In contrast, animals which received adequate amounts of vitamin C were completely protected from this inhibition (10).

In view of these findings, it was of interest to determine whether the tyrosyluria subsequent to the feeding of large amounts of tyrosine to vitamin C-deficient animals was due to an inhibition of this oxidase through the formation of the corresponding $\alpha$-keto acid.

EXPERIMENTAL PROCEDURE

Materials—p-Hydroxyphenylpyruvic acid was obtained from the H. M. Chemical Company, Ltd. 2,6-Dichlorophenindole was purchased from Eastman Organic Chemicals Department, Eastman Kodak Company. Glutathione, l-tyrosine, $\alpha$-ketoglutaric acid, pyridoxal-5-phosphate, and $l$-glucoscorbic acid were commercial preparations from the Nutritional Biochemicals Corporation. $d$-Isoscorbic acid was purchased from Hoffman-La Roche, Inc. $l$-Ascorbic acid was obtained from Merek and Company, Inc. Homogenatase acid was purchased from the Cyleo Chemical Corporation. $n, n'$-Hydroxypyrollactic acid was generously supplied by Dr. H. J. Cahnmann.

Animals—Male, albino guinea pigs, weighing approximately 200 g, were placed on a vitamin C-free diet prepared as described by Woodruff et al. (11). Control groups were given the same diet supplemented orally with 25 mg per day of $l$-ascorbic acid. After 1 week, the animals on the vitamin C-free diet were still gaining weight and showed no signs of scurvy, and the concentration of ascorbic acid in the liver was approximately 3.0 mg/100 g wet weight of liver. Animals on the vitamin C-free diet for 2 weeks developed the typical signs of scurvy: weight loss, swollen joints, and hemorrhages in the knee joints. At this time the concentration of ascorbic acid in the liver had fallen to less than 1.0 mg/100 g wet weight of liver.

Preparation of Liver Homogenate—At the time of death, the livers were removed and a 33% homogenate was prepared. Liver (5 g) was homogenized with 10 ml of 0.2 M sodium phosphate buffer, pH 6.5, in a Potter-Elvehjem type glass homogenizer at 5°. The homogenate was centrifuged at 10,000 x g for 10 minutes and the resulting supernatant fraction was used in the experiments described below. Homogenates of kidney were prepared in a similar way.

Liver Ascorbic Acid Determination—Liver (1 g) was homogenized with 12.5 ml of 4% trichloroacetic acid at 5°. After centrifugation, suitable aliquots of the deproteinized supernatant fraction were taken for the determination of ascorbic acid according to the method of Roe et al. (12).

Determination of Tyrosine and p-Hydroxyphenylpyruvic Acid in Plasma and Urine—Plasma from heparinized blood samples was deproteinized by the addition of an equal volume of 7% perchloric acid. The protein-free, supernatant fraction was neutralized with 14% KOH and the resulting perchlorate removed by centrifugation. The supernatant fraction was then analyzed for tyrosine by the method of La Du and Michael (13) and for p-hydroxyphenylpyruvic acid by the same method with the snake venom L-amino acid oxidase omitted. Dilute neutralized urine was analyzed for tyrosine and p-hydroxyphenylpyruvic acid directly.

Determination of p-Hydroxypyrollactic Acid in Urine—Acidified urine was extracted twice with 10 times its volume of acidi"fied, washed, peroxide-free ether. The pooled ether extracts were evaporated at room temperature with a stream of air in the presence of 1.0 ml of 0.2 M sodium phosphate buffer, pH 6.5. After the ether had been completely removed, the residual material was made up to either 2 or 5 ml with the phosphate buffer. The content of p-hydroxypyroline acid in the extracts was determined manometrically in the presence of yeast lactic acid dehydrogenase (14) with methylene blue as the hydrogen acceptor. The main compartment of the Warburg vessels contained 1.0 ml of yeast lactic acid dehydrogenase, 5 $\mu$moles of methylene blue, and 0.2 mol sodium phosphate buffer, pH 6.5, to make a total volume of 2.0 ml. The side arm contained aliquots

1 Obtained from Nutritional Biochemicals Corporation.
Effect of feeding tyrosine on enzymes in normal and scorbutic guinea pigs

Assay conditions. Tyrosine transaminase—The reaction was measured spectrophotometrically at 25° by following the appearance of the enol-borate complex of p-hydroxyphenylpyruvate at 310 mμ (16). p-Hydroxyphenylpyruvate and homogentisic acid oxidase—The spectrophotometric assay for p-hydroxyphenylpyruvate oxidase activity was carried out at 25° and the manometric assay of homogentisic acid oxidase activity at 37°, as previously described (10).

<table>
<thead>
<tr>
<th>Enzyme assay</th>
<th>Normal animals</th>
<th>Scorbutic animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (6)c</td>
<td>Treated (6)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine transaminase</td>
<td>17.9 ± 6.9</td>
<td>23.0 ± 11.4</td>
</tr>
<tr>
<td>p-Hydroxyphenylpyruvic acid oxidase</td>
<td>30.4 ± 6.1</td>
<td>24.8 ± 5.1</td>
</tr>
<tr>
<td>Homogentisic acid oxidase</td>
<td>127 ± 22.4</td>
<td>152 ± 20.5</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Hydroxyphenylpyruvic acid oxidase</td>
<td>0.6 ± 0.9</td>
<td>5.3 ± 1.7</td>
</tr>
</tbody>
</table>

* Enzyme activities are based on initial rate of oxidation. Transaminase activity was calculated after the initial lag period.

RESULTS

Effect of Feeding L-Tyrosine on Tyrosine Oxidation Enzymes in Scorbutic Guinea Pigs—Guinea pigs on the vitamin C-free diet for 2 weeks with typical signs of scurvy, and animals on the same diet supplemented with vitamin C, were divided into control and experimental groups of 6 to 10 animals. The experimental animals were given a total of 400 mg of tyrosine suspended in 4 ml of water (sweetened with sucrose) by feeding 100-mg portions at hourly intervals for 4 hours. One hour after the last dose of tyrosine, the animals were killed and the liver and kidneys rapidly removed and chilled. Homogenates were prepared and used for enzymatic assay as described under the section on “Enzyme Assay Methods.” The activities of tyrosine transaminase, p-hydroxyphenylpyruvic acid oxidase, homogentisic acid oxidase in liver, and p-hydroxyphenylpyruvic acid oxidase in kidney are given in Table I. It was found that feeding tyrosine to normal guinea pigs had no effect on these enzymes. However, there was a marked decrease, over 80%, in the activity of p-hydroxyphenylpyruvic acid oxidase in the liver and kidney of scorbutic animals fed tyrosine. The latter group also showed a significant increase in the activity of tyrosine transaminase. p-Hydroxyphenylpyruvic acid oxidase was also lower in the scorbutic animals not given tyrosine than in the corresponding control group.

The amount of p-hydroxyphenylpyruvic acid and p-hydroxyphenylactic acid excreted during the 5-hour experimental period was determined, and the degree of tyrosyluria was proportional to the level of tyrosine in the plasma (Table II). It is of interest that at the time of death there was no detectable p-hydroxyphenylpyruvic acid in the blood, even though the major tyrosyl metabolite in the urine was p-hydroxyphenylpyruvic acid. The urine also contained small amounts of p-hydroxyphenylactic acid, but no detectable tyrosine. This elevation of the amino acid in the blood and the excretion of the corresponding α-keto acid resembles the biochemical findings in phenylketonuria, in which phenylalanine is elevated in blood and phenylpyruvic acid is excreted in the urine.

<table>
<thead>
<tr>
<th>Table II</th>
</tr>
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<tbody>
<tr>
<td>Tyrosyluria after feeding tyrosine to normal and scorbutic guinea pigs</td>
</tr>
</tbody>
</table>

The estimations of plasma tyrosine, urine p-hydroxyphenylpyruvic acid, and urine p-hydroxyphenylactic acid were performed as described under the experimental section. Average values are given.

<table>
<thead>
<tr>
<th>Plasma Tyrosine</th>
<th>Untreated (6)</th>
<th>Treated (6)</th>
<th>Untreated (10)</th>
<th>Treated (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg per 100 ml of plasma</td>
<td>0.5</td>
<td>12</td>
<td>1.0</td>
<td>73</td>
</tr>
</tbody>
</table>

Urine

<table>
<thead>
<tr>
<th>p-Hydroxyphenylpyruvate</th>
<th>&lt;0.1</th>
<th>0.6</th>
<th>&lt;0.1</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxyphenylactic</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Numbers in parentheses = number of animals.
Effect of Repeated Feeding of Tyrosine to Vitamin C-deficient Guinea Pigs—Further experiments were undertaken to determine whether guinea pigs less deficient in vitamin C, i.e. not frankly scorbutic, would also be susceptible to inhibition of p-hydroxyphenylpyruvic acid oxidase by feeding tyrosine. It was also desirable to determine whether inhibition of p-hydroxyphenylpyruvic acid oxidase, once induced, might be maintained by repeated small oral supplements of tyrosine. Several groups of guinea pigs were placed on the vitamin C-free diet for 1 week. At this time they were fed 400 mg of tyrosine in 100-mg portions at hourly intervals. Some of the animals were killed 1 hour after the fourth dose of tyrosine, and in all the liver p-hydroxyphenylpyruvic acid oxidase activity was found to be inhibited over 90% (Table III). The remaining animals were continued on the vitamin C-free diet and were given 100 mg of tyrosine orally every 12 hours for the next 3 days. Groups of animals were then killed 1 hour, 4 hours, and 12 hours after the last dose of tyrosine, and the activities of tyrosine transaminase and p-hydroxyphenylpyruvic acid oxidase were determined (Table III). Although p-hydroxyphenylpyruvic acid oxidase was depressed to about one half of its normal value for several hours, it had returned to its previous normal value by 12 hours. The recovery of p-hydroxyphenylpyruvic acid oxidase may represent newly synthesized enzyme or the reactivation of previously inhibited enzyme. It is of interest that under these experimental conditions, the level of tyrosine transaminase remained elevated to about 5 times its value in control animals.

Ability of Various Compounds to Prevent Inhibition of p-Hydroxyphenylpyruvic Acid Oxidase In Vivo—Earlier studies on the inhibition of p-hydroxyphenylpyruvic acid oxidase by excess substrate in vitro have shown that ascorbic acid is not specifically modified by their physiological distribution and retention within the liver. From these results one would predict that other compounds which have been found to be effective in preventing inhibition of p-hydroxyphenylpyruvic acid oxidase by excess substrate in vitro would be able to prevent inhibition of this enzyme in vivo. The concentration of tyrosine in the plasma was elevated in the groups with inhibited p-hydroxyphenylpyruvic acid oxidase activity of the liver was determined. Plasma levels of tyrosine, p-hydroxyphenylpyruvic acid, and liver ascorbic acid were determined as described in the "Experimental Procedure" section. Average values are given.

Table III

Effect of prolonged feeding of L-tyrosine on liver p-hydroxyphenylpyruvic acid oxidase and tyrosine transaminase in vitamin C-deficient guinea pigs

<table>
<thead>
<tr>
<th>Treatment with tyrosine*</th>
<th>Time of death, hrs after last dose of tyrosine*</th>
<th>Liver enzyme activities†</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg each hr for 4 hrs</td>
<td>Followed by 100 mg twice daily for 3 days</td>
<td>μmoles of substrate oxidized/hr/g fresh liver</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>p-Hydroxyphenylpyruvic acid oxidase</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>Tyrosine transaminase</td>
</tr>
<tr>
<td>+</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* The regimen followed in administering tyrosine orally is described in the text.
† Assays for tyrosine transaminase and p-hydroxyphenylpyruvic acid oxidase were as described under Table I. The values given represent the average of 4 animals in each group.

Table IV

Protection of p-hydroxyphenylpyruvic acid oxidase in vivo by various compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Liver p-hydroxyphenylpyruvic acid oxidase</th>
<th>Plasma tyrosine</th>
<th>Plasma p-hydroxyphenylpyruvic acid</th>
<th>Liver &quot;ascorbic acid&quot;††</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (6)+</td>
<td>1.6</td>
<td>50.3</td>
<td>5.6</td>
<td>3.1</td>
</tr>
<tr>
<td>L-Ascorbic acid (4)</td>
<td>32.2</td>
<td>12.5</td>
<td>&lt;0.4</td>
<td>28.0</td>
</tr>
<tr>
<td>D-Isosorbic acid (4)</td>
<td>31.0</td>
<td>13.2</td>
<td>&lt;0.1</td>
<td>13.3</td>
</tr>
<tr>
<td>L-Glucoascorbic acid (4)</td>
<td>9.5</td>
<td>24.5</td>
<td>0.8</td>
<td>4.5</td>
</tr>
<tr>
<td>2,6-Dichlorophenolinodophenol (oxidized) (5)</td>
<td>29.0</td>
<td>10.2</td>
<td>&lt;0.1</td>
<td>2.6</td>
</tr>
<tr>
<td>2,6-Dichlorophenolinodophenol (reduced) (4)†</td>
<td>29.5</td>
<td>5.9</td>
<td>&lt;0.1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* D-Isosorbic acid and D-glucoascobic react in this method like ascorbic acid and have a molar extinction coefficient in the same order as the latter.
† Numbers in parentheses = number of animals.
†† Dye reduced with glutathione before injection.

and then given 400 mg of tyrosine in 100-mg portions at hourly intervals. The test compounds were given intraperitonelally, 10 mg one half hour before the first tyrosine feeding, and 10 mg more 2 hours later. Animals were killed 1 hour after the last tyrosine dose and the p-hydroxyphenylpyruvic acid oxidase activity of the liver was determined. Plasma levels of tyrosine, p-hydroxyphenylpyruvic acid, and liver ascorbic acid were measured at the time of death (Table IV). The results indicated that 2,6-dichlorophenolinodophenol and D-isosorbic acid were able to completely prevent any inhibition of p-hydroxyphenylpyruvic acid oxidase, and glucoascobic acid gave partial protection of the enzyme. The concentration of tyrosine in the plasma was elevated in the groups with inhibited p-hydroxyphenylpyruvic acid oxidase and was proportional to the degree of inhibition observed. The concentration of ascorbic acid in the liver indicated that there was a greater retention of D-isosorbic acid than glucoascobic acid in this tissue and this finding was in agreement with their relative ability to protect p-hydroxyphenylpyruvic acid oxidase.

From these results one would predict that other compounds which have been found to be effective in preventing inhibition of p-hydroxyphenylpyruvic acid oxidase by excess substrate in vitro would be able to prevent inhibition of this enzyme in vivo. However, the effectiveness actually observed in vivo will also be modified by their physiological distribution and retention within the liver.

The ability of 2,6-dichlorophenolinodophenol to protect the
Discussion

The tyrosyluria resulting from feeding tyrosine to vitamin C-deficient guinea pigs can now be explained in terms of an inhibition of p-hydroxyphenylpyruvic acid oxidase in the liver and kidney. Since it has been shown previously that p-hydroxyphenylpyruvic acid oxidase is inhibited in vivo by the injection of p-hydroxyphenylpyruvic acid (10), resulting in the accumulation and excretion of p-hydroxyphenylpyruvic acid, it appears likely that tyrosine also exerts its inhibitory effect via this α-keto acid. The ability of vitamin C and several related compounds to protect p-hydroxyphenylpyruvic acid oxidase from substrate inhibition, both in vivo and in vitro, is further evidence in accord with this mechanism.

It is of interest that guinea pigs need not be frankly scorbutic in order to exhibit inhibition of their p-hydroxyphenylpyruvic acid oxidase by tyrosine feeding. However, the susceptibility to inhibition increases as the vitamin C deficiency becomes more severe. The degree of enzyme inhibition depends upon two factors: the tissue concentration of vitamin C and the load of tyrosine given. These observations recall the findings of Painter and Zilva (20) in studies on the tyrosyluria of vitamin C-deficient guinea pigs. These authors found that the amount of p-hydroxyphenylpyruvic acid excreted was dependent upon the relative intake of tyrosine and of vitamin C.

The 4- to 5-fold increase in tyrosine transaminase activity induced by tyrosine feeding in the vitamin C-deficient animals would also favor the accumulation of p-hydroxyphenylpyruvic acid and help to perpetuate the metabolic defect. This increase in tyrosine transaminase might be an adaptive change secondary to the intake of substrate, but other possibilities must be considered, such as a protective (21) or activating effect (22, 23) of the amino acid. It should be noted that no increase in tyrosine transaminase activity occurred in the normal guinea pigs fed large amounts of tyrosine.

In view of these results, it is of interest to reconsider the origin of the tyrosyluria associated with feeding tyrosine or phenylalanine to premature infants. This tyrosyluria can be corrected by the administration of ascorbic acid (3), and it is reasonable to assume that inhibition of p-hydroxyphenylpyruvic acid oxidase by p-hydroxyphenylpyruvic acid accounts for the tyrosyluria found in these individuals.

Tyrosyluria has also been induced in guinea pigs by the administration of diethylthiocarbamate (24). This agent, a potent inhibitor of p-hydroxyphenylpyruvic acid oxidase in vitro (7), also inhibits this enzyme in vivo, thus leading to the excretion of p-hydroxyphenylpyruvic acid in the urine when tyrosine or phenylalanine are fed. Inhibition of the oxidase by diethylthiocarbamate takes place even in the presence of high tissue concentrations of ascorbic acid, and this compound appears to inhibit the oxidase directly.

The tyrosyluria reported in man in the unique case of tyrosinosis described by Medes (6) should also be mentioned. This condition has been assumed to be a metabolic disease in which the ability to further metabolize p-hydroxyphenylpyruvic acid is blocked, perhaps because of the lack of p-hydroxyphenylpyruvic acid oxidase. However, the exact nature of the metabolic defect has not been established (25).

The ability to block tyrosine metabolism at specific steps may be of value in further studies of the alternative pathways available for the metabolism of this amino acid. Induced metabolic blocks may also be useful in the treatment of metabolic disorders. For example, if a block could be induced at an earlier step than homogentisic acid oxidase, the pigmentation, ochronosis, and arthritis associated with alcaptonuria might be alleviated.

Summary

1. The inhibition in vivo of p-hydroxyphenylpyruvic acid oxidase has been demonstrated in vitamin C-deficient guinea pigs, as well as severely scorbutic animals when fed extra tyrosine.

2. The excretion of p-hydroxyphenylpyruvic acid and other tyrosyl compounds in these animals can be attributed to the inhibition in vivo of p-hydroxyphenylpyruvic acid oxidase.

3. Inhibition of p-hydroxyphenylpyruvic acid oxidase in vitamin C-deficient animals can be prevented by the administration of ascorbic acid and some other compounds such as pyridoxine, α-tocopherol, and n-glucosidase. These compounds also protect the enzyme from inhibition by excess substrate in vitro.

4. The tyrosine transaminase activity is increased 4- to 5-fold in scorbutic animals fed tyrosine and this may help to maintain the metabolic defect.

References

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