THE EFFECT OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS UPON TYROSINE METABOLISM IN THE SCORBUTIC GUINEA PIG*

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The ingestion of large amounts of aromatic amino acids included in diets practically devoid of ascorbic acid results in the urinary excretion of several intermediary metabolites of these substances in both guinea pigs (1, 2) and premature infants (3). The abnormal constituents of the urine following tyrosine ingestion have been identified as p-hydroxyphenylpyruvic acid, p-hydroxyphenyllactic acid, and small amounts of tyrosine (1, 3). Homogentisic acid has also been recovered from guinea pig urine (1). The degree of hydroxyphenyluria observed seems to depend upon the duration of the scorbutigenic regime as well as the amount of tyrosine ingested (2). This apparent defect in tyrosine metabolism disappears following the administration of small amounts of L-ascorbic acid (1, 2, 4).

The failure of d-isoascorbic acid to produce the same effect, except in doses 20 times that of the naturally occurring isomer (1), suggests that the vitamin C activity of this compound is the essential property involved. This hypothesis is confirmed by the observation of transitory and minimal responses following the administration of several dicarboxylic acids of the Krebs cycle (5) and liver extract (4, 6) as well as the failure of the longer recognized components of the vitamin B complex to influence the metabolic aberration.

The reports that pteroylglutamic acid (PGA) increased the oxidation of tyrosine by suspensions of liver from sulfonamide-treated rats (7) and that the high excretion of phenolic compounds by patients having pernicious anemia in relapse was reduced by liver therapy (8) suggested to us that PGA might have an effect upon the tyrosine metabolism of the scorbutic guinea pig. Results have been presented demonstrating that PGA abolishes the hydroxyphenyluria of tyrosine-fed guinea pigs on a scorbutigenic diet (9). This report provides additional details of the methods employed in these studies and presents further studies showing the pre-

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vention of this defect by continuous administration of PGA. In addition the influence of some related substances has been studied.

Methods

Albino guinea pigs of both sexes weighing approximately 300 gm. were housed in wire bottom cages and fed a scorbutigenic diet of the following composition: skimmed milk powder heated for 2 hours at 100°, 30; rolled oats (fortified), 39; wheat bran, 20; sodium chloride, 1; cod liver oil, 2; and cottonseed oil, 8 per cent. A sample of this diet was found to contain approximately 0.8 γ per gm. of total pteroylglutamates as determined by microbiological assay.

This diet plus ascorbic acid or cabbage in suitable amounts permitted growth and maintained the animals in good condition for periods up to several times the length of those represented by the present study. During the experimental periods 5 per cent L-tyrosine (General Biochemicals) was incorporated in the diet and the animals were kept in metabolism cages of the usual type. Collections of urine were made for serial 24 hour periods in bottles containing 2.5 ml. of 2 N HCl and a small amount of mineral oil. The urines were diluted to a standard volume of 100 ml., cleared with 1.5 gm. of Lloyd’s reagent, and the filtrates stored in the cold until analyzed. Control runs demonstrated the stability of the several aromatic fractions measured under these conditions.

Determinations of total hydroxyphenyl compounds were made by a photoelectric adaptation of the method of Folin and Ciocalteu (10) and expressed as tyrosine. Keto acids were estimated by an adaptation of the procedure of Friedemann and Haugen (11), and the standardization made against phenylpyruvic acid. Homogentisic acid was measured by the method of Neuberger (12). The results are expressed as per cent of the added tyrosine intake for the two preceding 24 hour periods. Ascorbic acid, when administered, was fed daily by pipette directly into the mouths of the animals. PGA and related compounds were injected subcutaneously. The liver extract was given intramuscularly.

EXPERIMENTAL

Four pairs of animals were fed the basal diet plus 5 per cent L-tyrosine over a 20 day period. Group I served as controls and received no supplements. Group II was given a supplement of 5 mg. of PGA daily.

1 This analysis was kindly performed for us by Dr. Paul L. Day of the Department of Biochemistry, University of Arkansas School of Medicine, Little Rock.

2 Administered as a solution of folvite (Lederle). We are grateful to Dr. Stanton M. Hardy and Dr. Thomas H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, for generous supplies of the pteroylglutamates and crude methylfolic acid used in these studies.
III received 25 mg. of ascorbic acid daily. Group IV received both vitamins in the dosages indicated above. The average 24 hour excretions of hydroxyphenyl (as tyrosine) for the 20 day period were 37.3 ± 16.5, 6.6 ± 4.1, 5.2 ± 1.0, and 4.6 ± 2.2 per cent of added dietary tyrosine respectively for Groups I through IV. Homogentisic acid excretion was minimal for all groups. The data for the individual animals and groups were consistent. Individual charts depicting the data on four of the animals are found in Fig. 1. Additional studies of serum ascorbic acid concentrations as influenced by the pteroylglutamates will be considered in a subsequent report.\(^3\) In a second experiment the amount of tyrosine ingested was equalized by administering it by pipette in aqueous suspension, as was done by Painter and Zilva (2). The animals did not tolerate this procedure well and consumed but little of the basal diet. Owing to the poor condition of all of the animals the experiment was terminated on the 9th day of tyrosine supplementation. However, the results presented in Table I are comparable to the first experiment. Homogentisic acid excretion by these animals was not determined. These data confirm the pre-

\(^3\) Stockell, A. K., Woodruff, C. W., and Darby, W. J., to be published.
liminary findings previously reported and show the similar effects of both vitamins.

Excretions of keto acid and homogentisic acid of less than 1 per cent of the added tyrosine are probably not significant. The average excretion of hydroxyphenyl compounds for all of the treated animals was $5.4 \pm 2.58$ and probably represents the "normal" excretion of these substances under conditions of tyrosine feeding. Quite similar results were obtained by Painter and Zilva (2), although they administered glycyltyrosine parenterally in various dosages. Whenever significant hydroxyphenyluria occurs, an essentially constant ratio of keto acids to total hydroxyphenyl compounds has been found (2). Our studies confirm this finding. Since the $p$-hydroxyphenylpyruvic acid is also measured quantitatively as hydroxyphenyl, the more time-consuming keto acid determinations have been

<table>
<thead>
<tr>
<th>Vitamin supplement</th>
<th>No. of animals</th>
<th>No. of determinations</th>
<th>Average dietary intake</th>
<th>Average excretion per 24 hrs. ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>gm. per 24 hrs.</td>
<td>Hydroxyphenyl compounds</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>25</td>
<td>4</td>
<td>24.0 ± 10.7</td>
</tr>
<tr>
<td>Ascorbic acid, 25 mg. daily by mouth</td>
<td>2</td>
<td>18</td>
<td>13</td>
<td>9.2 ± 6.2</td>
</tr>
<tr>
<td>PGA, 5 mg. daily subcutaneously</td>
<td>3</td>
<td>24</td>
<td>5</td>
<td>6.5 ± 1.9</td>
</tr>
</tbody>
</table>

omitted in subsequent experiments except for occasional estimations in each new situation.

Five guinea pigs were placed on the scorbutigenic diet containing 5 per cent L-tyrosine. After 4 to 7 days on the diet the excretion of hydroxyphenyl compounds was measured for 2 to 3 days. For the next 4 days each animal was given 5 U. S. P. units of concentrated antipernicious anemia liver extract daily. The excretions were measured during the liver extract treatment and for 2 or 3 days after the completion of the injections. The results are reported in Table II. No significant decrease in hydroxyphenyluria was noted during the treatment period. Homogentisic acid determinations were not made. This batch of liver extract had been assayed clinically. Obviously 5 units of active antipernicious anemia liver extract did not alter the hydroxyphenyluria.

"We wish to thank Dr. Thomas H. Jukes of the Lederle Laboratories Division for making this information available to us."
The action of two synthetic conjugates of PGA in preventing the defect has been studied. Pteroyldiglutamic acid and pteroyltriglutamic acid in doses equivalent to 5 mg. of PGA were given daily to two pairs of guinea pigs receiving the basal diet containing 5 per cent L-tyrosine. Another pair served as controls. These animals were not albinos. The excretion of homogentisic acid and hydroxyphenyl compounds between the 11th and 16th days on the diet is reported in Table III. These days coincided with the greatest excretion of metabolites by the control animals. The

**TABLE II**

Effect of Daily Intramuscular Injection of 5 Units of Purified Liver Extract upon Hydroxyphenyluria of Five Tyrosine-Fed Guinea Pigs

<table>
<thead>
<tr>
<th>Period</th>
<th>No. of determinations</th>
<th>Average tyrosine intake (mg. per 24 hrs.)</th>
<th>Average 24 hr. excretion of hydroxyphenyl compounds ± S.D. (per cent added tyrosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment, 2 or 3 days</td>
<td>12</td>
<td>486</td>
<td>20.9 ± 14.0</td>
</tr>
<tr>
<td>During &quot; 4 days</td>
<td>20</td>
<td>507</td>
<td>24.9 ± 14.4</td>
</tr>
<tr>
<td>After &quot; 2 or 3 days</td>
<td>13</td>
<td>411</td>
<td>25.9 ± 14.6</td>
</tr>
</tbody>
</table>

**TABLE III**

Effects of PGA Conjugates on Hydroxyphenyluria of Tyrosine-Fed Guinea Pigs during 6 Day Period

The results represent twelve determinations on two animals.

<table>
<thead>
<tr>
<th>Vitamin supplement*</th>
<th>Average tyrosine intake (mg. per 24 hrs.)</th>
<th>Average excretion per 24 hrs. ± S.D.</th>
<th>Hydroxyphenyl compounds (per cent added tyrosine)</th>
<th>Homogentisic acid (per cent added tyrosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>504</td>
<td>17.3 ± 12.0</td>
<td>0.6 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Pteroyldiglutamic acid subcutaneously</td>
<td>571</td>
<td>29.9 ± 15.7</td>
<td>0.5 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Pteroyltriglutamic</td>
<td>604</td>
<td>3.7 ± 1.0</td>
<td>3.8 ± 1.98</td>
<td></td>
</tr>
</tbody>
</table>

* Administered in doses equivalent to 5 mg. of PGA daily.

effect of the triglutamate was comparable to that of PGA, while the diglutamate had no apparent effect upon tyrosine metabolism under these conditions.

Although Sealock and Silberstein (1) identified homogentisic acid in the urine of guinea pigs excreting hydroxyphenyl compounds under conditions similar to those employed in the present study, Painter and Zilva (2) failed to confirm this observation. The inconstant presence of alkaptonuria in

* Administered as diopterin and teroceptor (Lederle), respectively.
the experiments here reported and its occurrence only in the absence of significant hydroxyphenyluria suggested that the excretion of homogentisic acid by tyrosine-fed guinea pigs may not be related to this apparent defect in tyrosine metabolism. We have attempted to produce a deficiency of PGA in several animals by including a metabolic antagonist, crude methylfolic acid (13), in the basal diet at a level of 1 and later 2 per cent, supplementing this diet with 25 mg. of ascorbic acid daily and 5 per cent L-tyrosine as in the previous experiments. The one surviving animal at 65 days was still gaining weight and had normal hematological findings. There was no hydroxyphenyluria. Homogentisic acid excretion reaching a level of 10 per cent of the added tyrosine appeared at about the 3rd week. Treatment with 5 mg. of PGA daily for 1 week failed to alter the homogentisic acid excretion. The appearance of alkaptonuria during ascorbic acid ingestion and its failure to disappear following the administration of large amounts of PGA would also suggest that some other mechanism is involved in its production. Further investigations of this subject are now in progress.

DISCUSSION

These experiments show that PGA will both prevent and abolish the hydroxyphenyluria seen in guinea pigs fed diets containing large amounts of tyrosine and devoid of vitamin C. Similarly, pteroyltriglutamic acid will prevent its development. However, in the two animals studied, the diglutamic derivative was without effect. In curative experiments employing a liver extract of known antipernicious anemia potency, no significant effect was found. Sealock and Lepow (6), who employed slightly different dietary conditions and measured the keto acid rather than the total hydroxyphenyl excretion, used larger amounts of liver extract. However, the moderate reduction in keto acid excretion which they have reported is considerably less than that which we find after 5 mg. of PGA. This difference in results may be a manifestation of the qualitative difference between PGA and the antipernicious anemia substances present in liver extract as well as the difference in dosage. Studies on the effect of vitamin B₁₂ on hydroxyphenyluria are in progress.

Several examples suggesting an interrelationship between ascorbic acid deficiency and megaloblastic anemias may be found in the literature. This subject has recently been discussed by R. W. Vilter (14) who cites two cases of pernicious anemia from the British literature which appeared to be unresponsive to liver therapy until ascorbic acid was administered. The experience of Dyke et al. (15) is especially interesting because a review

We are indebted to Dr. H. B. Lewis for the sample of homogentisic acid used in the standardization of our method.
of all their cases of pernicious anemia maintained on liver therapy showed a slight, temporary reduction in red cell counts simultaneously with an increase in clinical scurvy in the British Isles (16) during the spring of 1942. The anemia seen in patients with clinical scurvy is occasionally macrocytic in type and often improves on hospital diets low in vitamin C without specific treatment (17). In six of twelve patients with nutritional macrocytic anemia or pernicious anemia in relapse, showing biochemical evidence of vitamin C deficiency, the daily administration of 500 to 1000 mg. of ascorbic acid was followed by reticulocyte responses (18). Recent investigations of megaloblastic anemias in infants have revealed a remarkably frequent occurrence of diets practically devoid of vitamin C among the anemic children. Eleven of the twenty-five patients reported by Zuelzer and Ogden (19) and all of the fourteen patients receiving a simulated breast milk reported by May (20) had partaken of diets quite low in ascorbic acid. The observations on tyrosine metabolism in pernicious anemia which suggested the present study constitute another example from investigations on the human which strongly indicate a possible metabolic interrelationship between ascorbic acid and the hemapoietic vitamins.

The effect of PGA upon this apparent defect in tyrosine metabolism in premature infants has recently been studied (21). In four of ten infants developing hydroxyphenyluria while receiving high protein diets devoid of vitamin C, the administration of 5 to 30 mg. of PGA daily resulted in a diminution in the excretion of these compounds. Subcutaneous injection of this vitamin was found to be more effective than oral administration, a finding in keeping with our experience in guinea pigs (9).

The observations of Johnson and Dana (22) that scorbutic symptoms appeared in rats maintained on a PGA-deficient diet and that a response followed ascorbic acid administration seemed to provide further experimental evidence of an interrelationship between these two vitamins. Johnson has indicated, however, that this effect may not be a specific one, but that it is more probably related to the quantity of food consumed by the animals.

SUMMARY

1. The hydroxyphenyluria produced in guinea pigs fed scorbutigenic diets containing 5 per cent L-tyrosine is prevented by both PGA and its triglutamic homologue as well as by ascorbic acid. These pteroylglutamates do not protect the guinea pig against scurvy.

2. Neither pteroyldiglutamic acid nor liver extract in the amounts used had a demonstrable effect upon the hydroxyphenyluria.

7 Johnson, B. C., personal communication.
3. Observations upon the excretion of homogentisic acid under these conditions are reported.
4. Possible interrelationships between the metabolic rôles of PGA and ascorbic acid are discussed.

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