On the Mechanism of the Brain Serotonin Depletion in Experimental Phenylketonuria*

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Considerable evidence supports the view (1) that the primary metabolic lesion in phenylketonuria is a block in the conversion of phenylalanine to tyrosine due to the absence of a labile protein component of the liver hydroxylating system (2). However, the mechanism by which this metabolic defect in liver is translated into the mental retardation which clinically characterizes this disease is unclear. Several approaches to the problem have been made and the experimental model introduced by Auerbach (3) has proven particularly useful in nutritionally simulating some biochemical features of this genetic disease. More recent neurochemical and behavioral studies (4, 5) have demonstrated that animals fed diets rich in phenylalanine have diminished brain serotonin accompanied by impaired behavior in some, but not all, problem-solving tasks. A necessary connection between these two phenomena has not yet been established, although suggestive evidence has been presented from several sources.

The metabolism of serotonin from tryptophan to 5-hydroxyindole acetic acid is well established and all the intermediate steps are known to occur in brain with the exception of 5-hydroxylation of tryptophan. To date, attempts to demonstrate this reaction in nervous tissue have been unsuccessful. This failure, together with the observation that 5-hydroxytryptophan easily traverses the blood-brain barrier while serotonin does not, suggests that serotonin synthesis in the central nervous system may be intimately related to peripheral disposition of tryptophan and 5-hydroxytryptophan. Based on the known observations, several explanations have been advanced for the decreased brain serotonin accompanying phenylalanine loading in experimental phenylketonuria. These include (a) specific inhibition of 5-hydroxytryptophan decarboxylase as suggested by the findings in vitro of Davidson and Sandler (6); (b) general, nonspecific inhibition of decarboxylases and other pyridoxal phosphate-mediated enzymes as suggested by the work of Tashian (7) on glutamic acid decarboxylase; (c) inhibition of precursor transport through blood-brain barriers as indicated by the data of Schanberg and Giarman (8) and Smith (9); (d) inhibition of tryptophan hydroxylation as indicated by Freedland, Wadzinski, and Waisman (10); (e) “nutritional-stress” activation of competing corticoid-activated enzymes such as tryptophan pyrrolase and tryptophan transaminase; (f) acceleration of monoamine oxidase activity; and (g) decreased serotonin storage capacity.

The present study attempts to distinguish between some of these possibilities and to determine the sequence of the biochemical alterations.

EXPERIMENTAL PROCEDURE

Sprague-Dawley rats of mixed sex were randomly divided into two groups and housed in individual cages. Beginning at 17 days of age, animals were fed ad libitum a basic diet consisting of ground Purina laboratory chow, 18% Starlac powdered milk, and 8.7% Mazola corn oil (to obtain a moist consistency). The diet of the experimental group was supplemented with 3 or 5% by weight of l-phenylalanine (Mann). After 30 to 40 days on the diet the animals were decapitated, trunk blood collected, and tissues quickly removed and analyzed.

Livers were analyzed for tryptophan hydroxylase, EC 1.99.1.4 (10), phenylalanine hydroxylase, EC 1.99.1.2 (11), tryptophan pyrrolase, EC 1.11.1.15 (12), pyruvate-phenylalanine transaminase, and a-ketoglutarate-tyrosine and -tryptophan transaminases, EC 2.6.1.5 (13). Adrenals were analyzed for ascorbic acid (14) and corticosterone (15), serum was assayed for corticosterone (14), and brain was analyzed for serotonin (16), norepinephrine (17), and dopamine (18). Activities of brain 5-hydroxytryptophan - 3,4 - dihydroxyphenylalanine decarboxylase, EC 4.1.1.26, 4.1.1.28, and glutamic acid decarboxylase, EC 4.1.1.15, were determined by scintillation counting of 14CO2 liberated from carboxyl-14C-5-hydroxytryptophan and carboxyl-14C-glutamic acid during 15-minute incubations at 37°C in Tris (pH 8.0) or acetate buffer (pH 5.0), respectively (19). In addition, the 5-hydroxytryptophan reaction mixture was directly analyzed for serotonin formed (16). Brain cholinesterase, EC 3.1.1.7, was determined by the method of Ellman et al. (20). Protein content of supernatant fractions was determined by ultraviolet absorption (21) and the nitrogen content of whole homogenates by a Kjeldahl procedure. Specific enzymic activities were calculated on the basis of protein or nitrogen. The homogenizing medium was 0.15 M KCl for all liver assays except those for hydroxylase where 0.15 M KCl containing 0.125 M NaOH was employed. Brain enzymes were assayed on water homogenates. Assays were carried out on blocks of four experimental and four control animals to preclude differences due to chance. A t test was used to analyze the results of each individual block. Analysis of variance and Duncan's new multiple range test (22) were used to assess the significance of the combined values reported here. The fluorescent spectrum of the reaction product of the tryptophan hydroxylase assay was deter-
mined and proved to be identical with that for serotonin. This was taken as evidence for 5-hydroxylation of tryptophan.

The nature of the tryptophan hydroxylase inhibition was investigated in the following manner. Procedure A: 5.0 ml of liver supernatant fraction were dialyzed for 30 minutes against 50 ml of 0.15 M KCl containing 0.125 meq of NaOH per liter. Specific enzymic activities of the dialysates were determined as described above. Procedure B: 5.0 ml of liver supernatant fraction from control and experimental animals were heated at 100° for 2 minutes and centrifuged at 30,000 x g for 20 minutes at 4°. After centrifugation the supernatant fractions were combined to yield an experimental and a control pool. Aliquots, 1.0 ml, of the pooled supernatant fraction from control animals were added to hydroxylase reaction mixtures containing untreated liver supernatant fractions from experimental animals. Similarly, aliquots of the pooled supernatant fraction from livers of the experimental animals were added to hydroxylase reaction mixtures containing untreated liver supernatant fractions from control animals. Hydroxylase activity of these mixtures was determined as described above. Procedure C: Procedures A and B above, together with the usual hydroxylase assay, were repeated after addition to the reaction mixture of 0.001 ml of 2-mercaptoethanol, and 0.1 mg each of ascorbic acid, NADH (Boehringer) and dl-5,6,7,8-tetrahydropteroyl-L-glutamic acid (Sigma). The tetrahydrofollic acid employed was reported by the manufacturer (Sigma) to have an 

\[ \text{Em}_{27} \] of 27 in pH 7.0 buffer containing 1% mercaptoethanol. The material was used without attempting to confirm this value.

RESULTS AND DISCUSSION

The mode of dietary administration poses a methodological problem in nutritional studies. Paired feeding eliminates group differences related to body weight but may introduce those related to differential hunger, whereas feeding ad libitum may eliminate hunger at the cost of possible differences in growth rate. Because the paired feeding employed in a previous study in this series (4) could have contributed to the results, feeding ad libitum was employed in the present investigation. As may be seen from Fig. 1, animals fed a 5% phenylalanine diet gained weight at the same rate as controls while the weights of animals fed a 5% diet were somewhat lower. Final body weight did not differ statistically for the groups.

The slight decrease in the growth rate of the 5% phenylalanine-fed animals left open the possibility that the observed differences in brain serotonin reflected activation of competing "stress"-induced enzymes, such as tryptophan pyrrolase and transaminase. The results in Table I clearly show that this was not the case. None of the classical stress measures such as adrenal weight, adrenal corticoids, adrenal ascorbic acid, or serum corticoids differed significantly for the groups. More important, there were no significant differences in the activities of tryptophan pyrrolase or tryptophan transaminase. It can be concluded, therefore, that under these conditions a 5% dietary load of L-phenylalanine does not result in detectable "nutritional stress" of the kind reported in some amino acid and vitamin imbalances (23) and that the observed decrease in brain serotonin accompanying such dietary supplementation is not due to activation of these alternate metabolic pathways for tryptophan. Some rise in tyrosine transaminase activity was noted which likely reflects substrate induction accompanying increased tyrosine formation. Phenylalanine transaminase activity did not increase, however, despite the increased dietary phenylalanine.

Specific inhibition of 5-hydroxytryptophan decarboxylation is one of the more attractive mechanisms postulated for the diminished brain serotonin of the phenylalanine-fed animal. Direct estimations of brain homogenate decarboxylase activity based on either serotonin formation or ^14CO\textsubscript{2} liberated from 5-hydroxytryptophan-1^14C (Table II), however, failed to indicate such an inhibition. These results, together with those of Hsia, Nishimura, and Brenchley (24), make untenable the hypothesis of serotonin diminution by decreased aperoxhydine, or by a noncompetitive inhibitor. However, these results alone do not exclude competitive inhibition by phenylalanine metabolites. This can be seen by calculations based on assumptions which would maxi-

![Fig. 1. Growth of control and phenylalanine-fed rats.](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>5% Phenylalanine</th>
<th>5% Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean ± s.e.</td>
<td>No.</td>
</tr>
<tr>
<td>Body, initial</td>
<td>33</td>
<td>34.7 ± 0.75</td>
<td>16</td>
</tr>
<tr>
<td>Body, final</td>
<td>33</td>
<td>157.6 ± 7.35</td>
<td>16</td>
</tr>
<tr>
<td>Brain</td>
<td>26</td>
<td>1.548 ± 0.018</td>
<td>16</td>
</tr>
<tr>
<td>Liver</td>
<td>21</td>
<td>8.550 ± 0.383</td>
<td>4</td>
</tr>
</tbody>
</table>

Mean ± s.e.
and substitution of $3 \times 10^{-8}$ M. However, substrate concentration contributes little to the result.

The brain 5-hydroxytryptophan concentration is the most questionable estimate in these calculations. For example, the concentration would be only 0.008%. The brain 5-hydroxytryptophan concentration; (c) the velocity in the absence of inhibitor, $v_i$, is $0.78$ v where v is the velocity in the presence of inhibitor; (d) the steady state concentration of 5-hydroxytryptophan is $3 \times 10^{-6}$ M, or about equal to serotonin concentration; (e) $K_i$ is $1 \times 10^{-4}$ M, as calculated from the data of Davidson and Sandier (9) for the most inhibitory of the phenylalanine metabolites under the most inhibitory conditions; (d) the $K_m$ for 5-hydroxytryptophan decarboxylation is $4 \times 10^{-5}$ M (25) and that for dopa is $3 \times 10^{-3}$ M. Substitution into the expression

$$
v = \frac{K_i(S) + K_m}{K_i(S) + K_m + K_i(I)}
$$

and solving for (I) yields an inhibitor concentration of $3.4 \times 10^{-8}$ M. Estimations of decarboxylase activity in this study were carried out with a substrate concentration of 12.5 mM and involved a final 3-fold dilution of brain, so that, after dilution, (I) = $1.1 \times 10^{-3}$ M. Under these conditions only an 0.4% decrease in decarboxylase activity would occur. Analogously, Hae, Nishimura, and Brenchley (24) employed a 29-fold dilution of brain tissue and a 5-hydroxytryptophan concentration of 1 mM. The difference in calculated rate under these conditions would be only 0.008%. The brain 5-hydroxytryptophan concentration is the most questionable estimate in these calculations and is probably too large by a factor of 100. At these low levels, however, substrate concentration contributes little to the result and substitution of $3 \times 10^{-8}$ M 5-hydroxytryptophan for $3 \times 10^{-4}$ M strengthens the conclusions.

Inferences on enzymic activities based on tissue levels of product or substrate require considerable detailed information on such factors as turnover, the kinetics of competing pathways, and pool size. The metabolic interrelationship of serotonin and dopamine, however, is such that some aspects of their metabolism can be estimated from their relative concentrations. Both compounds are dependent upon 5-hydroxytryptophan-dopa decarboxylase for synthesis, the $K_m$ for 5-hydroxytryptophan being considerably smaller than that for dopa. Monoamine oxidase is the major degradative enzyme for both compounds in the central nervous system and dopamine is a better substrate than serotonin. Finally, both are released from storage sites by the same agents and dopamine binding is more sensitive to these agents than is serotonin. Because of these interrelations either competitive inhibition of the decarboxylase or acceleration of monoamine oxidase activity would be expected to lower brain dopamine more than serotonin. Although a statistically insignificant trend of decreasing dopamine with increasing dietary phenylalanine was observed (Table III), the decrease was considerably less than that observed for serotonin. Brain norepinephrine levels were unchanged under these conditions.

These results lend some indirect support to the conclusion that the decrease in brain serotonin level is not due to either decarboxylase inhibition or monoamine oxidase activation.

The results in Table III also appear to rule out generalized inhibition of pyridoxal phosphate-dependent enzymes as a mechanism for the reduction of brain serotonin. Basal levels of glutamic acid decarboxylase and tryptophan and phenylalanine transaminases failed to discriminate populations (Table III) although both classes of enzymes are pyridoxal phosphate dependent. The activities of tyrosine transaminase and glutamic acid decarboxylase are particularly sensitive to cofactor concentration and, although tyrosine transamination was increased in the 5% experimental group, pyridoxal phosphate addition only increased activity 4-fold as against an 8-fold increase in controls so that, if anything, controls suffer from a greater cofactor deficiency than experimentals (Table IV).

Tryptophan hydroxylase activity was dramatically inhibited in animals fed the phenylalanine diet (Table IV). Liver tryptophan hydroxylase is thought to be identical with phenylalanine hydroxylase (26), and conflicting reports have appeared on the influence of dietary phenylalanine on phenylalanine hydroxylase activity (27, 28). In this study mean values for phenylalanine hydroxylation were somewhat lower in experimentals than in controls, but this lowering was neither consistent within blocks nor statistically significant. As a competing substrate, high

<table>
<thead>
<tr>
<th>Table II</th>
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<tbody>
<tr>
<td>Effects of dietary phenylalanine on liver hydroxylase and brain enzymes and amines</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Serotonin, μg/g</td>
</tr>
<tr>
<td>Serotonin, μg/brain</td>
</tr>
<tr>
<td>Dopamine, μg/g</td>
</tr>
<tr>
<td>Dopamine, μg/brain</td>
</tr>
<tr>
<td>Norepinephrine, μg/g</td>
</tr>
<tr>
<td>Norepinephrine, μg/brain</td>
</tr>
<tr>
<td>Brain decarboxylase, c.p.m./hr/g protein</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Brain cholinesterase, μm/min/g nitrogen</td>
</tr>
<tr>
<td>Liver hydroxylase, μm/hr/g protein</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
</tbody>
</table>

* Control = 3% > 5% (p < 0.05).
† Control > 3% = 5% (p < 0.05).
The levels of phenylalanine could account both for inhibited tryptophan hydroxylation and uninhibited phenylalanine hydroxylation. The $K_m$ for L-tryptophan in this system has been reported as $6.8 \times 10^{-3} \text{M}$ (26) and that for L-phenylalanine as $2 \times 10^{-4} \text{M}$ (29). A phenylalanine concentration of $1.48 \text{mM}$ would be required, therefore, to reduce tryptophan hydroxylation by 75% in the presence of the $10 \text{mM}$ tryptophan used in the assay, while tryptophan inhibition would be negligible under the conditions employed for phenylalanine hydroxylase. Blood phenylalanine levels of $0.37 \text{mM}$ have been reported (30) for phenylalanine-fed animals so that a 4-fold concentration in liver could account for these results. However, the tryptophan hydroxylase activity of control samples was unaffected by addition of boiled liver supernatant fractions from experiments although a 50% reduction should have been observed if the supernatant fractions contained phenylalanine (Table V). As samples from experiments were unaffected by addition of denatured supernatant fractions from controls, it is unlikely that a heat-stable cofactor is involved in these differences. Dialysis of supernatant fractions did not alter the relative activities of control and experimental supernatant fractions, so that it is unlikely that a dialyzable competitive inhibitor is involved. Additions of mercaptoethanol, tetrahydrofolic acid, NADH, and ascorbic acid were without effect, but since they were not tested separately the possibility that one alone would have eliminated the difference cannot be excluded.

On the whole, these results suggest that the difference in the tryptophan hydroxylase activity of controls and experimental animals is due either to a noncompetitive inhibitor, excluding identity with phenylalanine hydroxylase which was not inhibited, or to a difference in functional enzyme, perhaps by cofactor saturation, in which case tryptophan and phenylalanine hydroxylation could still be related in much the same manner as is 5-hydroxytryptophan and dopa decarboxylation.

Liver tryptophan hydroxylase activity was 65% inhibited following administration of the 3% phenylalanine diet and 75% inhibited following the 5% diet. Brain serotonin levels were unaffected by the 3% diet and were decreased by 25% following the 5% diet. A relationship between these findings would indicate that liver tryptophan hydroxylation is not limiting for serotonin biosynthesis until reduced to one-third of basal activity. Because the whole relationship of liver tryptophan hydroxylation to serotonin biosynthesis is questionable (26), it is possible that the hydroxylation studied here represents a magnified reflection of the activity of an analogous system, perhaps in the central nervous system, sensitive to the same influences as the liver system but partially protected from them by interposition of blood-brain barriers.

### Table III

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>3% Phenylalanine</th>
<th>5% Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal weight, mg</td>
<td>33</td>
<td>55.3 ± 1.50</td>
<td>16</td>
</tr>
<tr>
<td>Adrenal corticosterone, µg/g</td>
<td>31</td>
<td>9.10 ± 2.30</td>
<td>15</td>
</tr>
<tr>
<td>Adrenal ascorbic acid, µg/100 g adrenal</td>
<td>10</td>
<td>397.5 ± 20.9</td>
<td>4</td>
</tr>
<tr>
<td>Serum corticoids, µg/100 ml</td>
<td>31</td>
<td>20.16 ± 5.68</td>
<td>14</td>
</tr>
<tr>
<td>Liver transaminase, µM/min/g protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>23</td>
<td>3.779 ± 0.38</td>
<td>12</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>19</td>
<td>0.608 ± 0.081</td>
<td>0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>19</td>
<td>0.0755 ± 0.0158</td>
<td>8</td>
</tr>
<tr>
<td>Liver pyrrolyase, µM/hr/g protein</td>
<td>22</td>
<td>7.87 ± 1.74</td>
<td>12</td>
</tr>
</tbody>
</table>

* Control = 3% < 5% (p < 0.05).

### Table IV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tyrosine phosphate addition</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Mean ± s.e.</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>0.604 ± 0.134</td>
</tr>
<tr>
<td>5% phenylalanine</td>
<td>4</td>
<td>1.591 ± 0.242</td>
</tr>
</tbody>
</table>

### Table V

<table>
<thead>
<tr>
<th>Treatment and additions</th>
<th>Controls</th>
<th>Experiments</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.e.</td>
<td>Mean ± s.e.</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.378 ± 0.128</td>
<td>0.309 ± 0.113</td>
</tr>
<tr>
<td>Supplement</td>
<td>1.370 ± 0.043</td>
<td>0.226 ± 0.191</td>
</tr>
<tr>
<td>Boiled supernatant</td>
<td>1.105</td>
<td></td>
</tr>
<tr>
<td>Boiled supernatant + supplement</td>
<td>1.105</td>
<td>0.148</td>
</tr>
<tr>
<td>Dialysis</td>
<td>0.546 ± 0.375</td>
<td>0.0375 ± 0.0275</td>
</tr>
<tr>
<td>Supplement</td>
<td>0.547</td>
<td>0.020</td>
</tr>
</tbody>
</table>
In the absence of evidence for central tryptophan hydroxylation, phenylalanine inhibition of 5-hydroxytryptophan transport into brain also remains a strong possibility in explaining the decrease in brain serotonin concentration. Although dopa transport is more sensitive to phenylalanine inhibition than 5-hydroxytryptophan transport (63% inhibited in brain slices (31), compared to 27% for 5-hydroxytryptophan (9)) our failure to observe a marked dopamine decrease does not weaken this hypothesis since dopa is almost certainly synthesized within the central nervous system.

The mechanisms, then, for dietary phenylalanine reduction of brain serotonin appear narrowed to decreased tryptophan hydroxylation, decreased 5-hydroxytryptophan transport, or diminished brain serotonin storage capacity. Further studies will be required for elucidation.

SUMMARY

Seven proposed mechanisms for the decreased rat brain serotonin accompanying high phenylalanine diets have been evaluated on the basis of concomitant alterations in brain, liver, adrenal, and blood constituents after administration of two dietary levels of phenylalanine. Analyses of the results suggest inhibited precursor transport as the most likely mechanism for this relationship. Inhibited tryptophan hydroxylation and decreased serotonin binding could not be excluded as mechanisms, although they appear less likely.

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On the Mechanism of the Brain Serotonin Depletion in Experimental Phenylketonuria
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