A method was developed to study the unsupplemented phenylalanine hydroxylase system in rat liver slices. All of the components of the system—tetrahydrobiopterin, dihydropteridine reductase, and the hydroxylase itself—are present under conditions which should be representative of the actual physiological state of the animal. The properties of the system in liver slices have been compared to those of the purified enzyme in vitro. The three pterins, tetrahydrobiopterin, 6,7-dimethyltetrahydropterin, and 6-methyltetrahydropterin, all stimulate the hydroxylation of phenylalanine when added to the liver slice medium in the presence of a chemical reducing agent. The relative velocities found at 1 mM phenylalanine and saturating pterin concentrations are: tetrahydrobiopterin, 1; 6,7-dimethyltetrahydropterin, 2.5; 6-methyltetrahydropterin, 13. This ratio of activities is similar to that found for the purified, native phenylalanine hydroxylase and indicates that the enzyme in vivo is predominantly in the native form. Rats pretreated with 6-methyltetrahydropterin showed enhanced phenylalanine hydroxylase activity in liver slices demonstrating for the first time that an exogenous tetrahydropterin can interact with the phenylalanine hydroxylase system in vivo. This finding opens up the possibility of treating phenylketonurics who still possess some residual phenylalanine hydroxylase activity with a tetrahydropterin like 6-methyltetrahydropterin which can give a large increase in rate over that seen with the natural cofactor, tetrahydrobiopterin.

The phenylalanine hydroxylase system in mammalian liver carries out the following reactions (1):

\[
\text{Phenylalanine} + \text{O}_2 + \text{tetrahydropterin} \rightarrow \text{tyrosine} + \text{H}_2\text{O} + \text{quinonoid dihydropterin} \quad (1)
\]

\[
\text{Quinonoid dihydropterin} + \text{reduced pyridine nucleotide} \rightarrow \text{tetrahydropterin} + \text{oxidized pyridine nucleotide} \quad (2)
\]

The first reaction is catalyzed by phenylalanine hydroxylase and the second by dihydropteridine reductase. Various reducing agents, such as mercaptans and ascorbate, also can regenerate the tetrahydropterin from the quinonoid dihydro compound (1).

Normally, in measuring phenylalanine hydroxylase activity in a particular tissue, an extract is made and sufficient amounts of cofactors, tetrahydropterin-regenerating system and H2O2-destroying systems are added so that the formation of tyrosine is proportional to the amount of phenylalanine hydroxylase present (2). The other components of the system must be measured in separate assays.

To study the physiological regulation of the hydroxylase system, it would be useful to be able to determine the rate of the conversion of phenylalanine to tyrosine in a relatively intact tissue preparation in which all of the components of the system might be present at physiological levels. With such an assay, the effects on the hydroxylating system of various pharmacological and physiological interventions in whole animals could be assessed.

In the present paper, we describe the development of a simple method for the measurement of the activity of the phenylalanine hydroxylase system in liver slices. Some of the properties of the system in slices are compared with those in extracts prepared from the same livers.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sprague-Dawley rats weighing 100 to 150 g were used for all experiments. Uniformly 14C-labeled L-phenylalanine was from New England Nuclear. 6,7-Dimethyltetrahydropterin (3), 6-methyltetrahydropterin (4), and tetrahydrobiopterin (5) were synthesized in this laboratory. Solutions of the tetrahydropterins were prepared and their concentration determined as previously described (6). All reagents were prepared in deionized, glass-distilled water.

DL-m-[2-14C]Tyrosine was synthesized by condensation of 3-methoxybenzyl chloride with [2-14C]ethylenimine-dicyclohexylacetate (New England Nuclear) as described for the preparation of o-[2-14C]Tyrosine (7). After treatment with 48% HBr, the compound was purified by chromatography on a Beckman amino acid column (8).

**Methods**

**Preparation of Liver Slices**—Animals were killed by decapitation.
and the livers were quickly removed and placed in ice-cold 0.25 M sucrose or 0.15 M KCl. Slices were made with a Stadie-Riggs tissue slicer (A. H. Thomas Co.) and placed in cold 0.15 M KCl. They then were blotted carefully, weighed, and added to the reaction medium.

**Measurement of Tyrosine Formation in Liver Slices**—Liver slices were incubated in 25-ml Erlenmeyer flasks in a total volume of 2 ml containing 0.1 M potassium phosphate buffer, pH 6.8, and 1 μCi of L-[14C]phenylalanine plus added cold L-phenylalanine as desired. Optimum conditions for uptake and hydroxylation of the phenylalanine were determined as described below. After incubation, the reactions were stopped by the addition of 0.3 ml of 3 M HClO4. The medium and the slices were homogenized in a glass homogenizer; the mixture then was centrifuged to remove the precipitated proteins. A 10- to 20-μl aliquot of the supernatant fraction was applied to a cellulose MN-300-coated glass plate (2 x 10 cm, Analtech) together with carrier phenylalanine and tyrosine. The plates were developed by ascending chromatography with a solvent system composed of CHCl3-methanol-NH4OH-H2O (5:8.3:2:0.8:0.2, v/v). In this system, the solvent runs to the top of the plate in 10 min. The plate was removed, air dried, and sprayed with ninhydrin reagent (Sigma). The plate then was warmed until the spots just began to appear. The Rf values for phenylalanine and tyrosine are 0.6 and 0.2, respectively. The areas corresponding to the two amino acids were scraped off the plate with a razor blade into a scintillation vial and moistened with 0.5 ml of H2O. After addition of 10 ml of Aquasol (New England Nuclear), radioactivity was measured in a liquid scintillation counter (Beckman). The amount of liver slice-dependent tyrosine formation was calculated from the per cent of the radioactivity in the tyrosine area compared to the sum of that in the tyrosine and phenylalanine areas (correcting for the small amounts of radioactivity in the tyrosine area from a boiled tissue sample). There were no significant counts in any other areas of the chromatograms.

**Phenylalanine Hydroxylase Assay in Liver Extracts**—Liver extracts were prepared and assayed essentially as described by Kaufman (2).

**RESULTS**

**Uptake and Hydroxylation of Phenylalanine.** The uptake of phenylalanine by liver slices was the same in Krebs-Ringer buffer as in phosphate buffer and was not affected by the presence of dinitrophenol or by the absence of oxygen. These results indicate that facilitated transport of phenylalanine into liver slices does not occur. Therefore, a simplified incubation medium consisting only of 0.1 M potassium phosphate buffer, pH 6.8, was used in all subsequent studies reported here.

In preliminary experiments, it was found that at 25°, uptake of phenylalanine (up to 1 mM) is relatively slow, taking about 30 min to reach a distribution ratio of about 1. During this time, some phenylalanine is converted to tyrosine. Since the rate of hydroxylation of phenylalanine under these conditions is limited by the rate of uptake of phenylalanine, the rate of tyrosine formation is not a reliable measure of phenylalanine hydroxylase activity. To circumvent this problem, we took advantage of the fact that the hydroxylase-catalyzed conversion of phenylalanine to tyrosine has a high temperature coefficient (Q10 approximately 4 to 5). Exploiting this characteristic of the system, we found that it was possible to incubate liver slices at 15° until equilibration with the medium phenylalanine was reached (30 min) under conditions where very little tyrosine is formed (Fig. 1). The incubation flasks then were transferred to a 25° water bath to allow the hydroxylation to take place. This protocol was followed in all subsequent experiments.

**Characteristics of Phenylalanine Hydroxylation System in Liver Slices**—Hydroxylation of phenylalanine by liver slices is linear with respect to time. The results of a typical experiment are shown in Fig. 1. In several other experiments (not shown here) linearity with time was reproducibly observed. The hydroylation is also linear with respect to tissue concentration. In Fig. 2 are shown the effects of increasing amounts of liver slices on the rates of tyrosine formation in livers from both female and male rats. It had previously been shown (9) that extracts from male rat livers have approximately 25% higher activity than those from female rats. With liver slices, the phenylalanine hydroxylation system from male rats had 50% more activity than that from females. With liver slices from both sexes, linearity was found in the range of 20 to 300 mg of tissue weight.

When the initial concentration of phenylalanine in the medium was varied, the formation of tyrosine reached a plateau at about 1 mM and showed moderate substrate inhibition at higher concentration (Fig. 3), under conditions where uptake of phenylalanine was not limiting. The apparent Km for phenylalanine for this system is 0.2 to 0.3 mM. The Km for phenylalanine with purified phenylalanine hydroxylase is 0.2 mM (10) in the presence of tetrahydrobiopterin.

The determination of phenylalanine hydroxylase activity in liver slices by the measurement of the conversion of [14C]phenylalanine to [14C]tyrosine is not complicated by further metabolism of the [14C]tyrosine. This was demonstrated by incubating [14C]tyrosine with liver slices under the

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same conditions described above for phenylalanine, at a concentration equal to that which would have been produced from phenylalanine alone. More than 90% of the [14C]tyrosine was recovered unaltered. This result indicates that further metabolism of tyrosine by liver slices, under the conditions used for the measurement of phenylalanine hydroxylation activity, does not occur to a significant extent. This could be due to the rapid equilibration of the tyrosine in the liver slice with the tyrosine in the surrounding medium since the concentration of tyrosine was found to be the same in both the slice and the medium during the experiment shown in Fig. 1.

Stimulation of Phenylalanine Hydroxylation by Exogenous Pterins—Tetrahydropterins added to the medium together with either a chemical or an enzymatic tetrahydropterin-regenerating system can markedly stimulate the rate of conversion of phenylalanine to tyrosine. In Fig. 4 are shown the results of addition of 6MPH₄ and dithiothreitol to the incubation medium. The maximum stimulation occurs at 0.2 mM 6MPH₄ and is dependent on the concentration of the chemical-regenerating system. Dithiothreitol alone stimulates only slightly under these conditions. Tetrahydrobiopterin gives a 1.6-fold increase in activity when added to the medium at a concentration of 0.045 mM (Table I), indicating that either the enzyme system is already almost saturated with the natural cofactor or that tetrahydrobiopterin is not transported well into the slice.

Various reducing agents were tested for their ability to stimulate phenylalanine hydroxylation in the presence of different tetrahydropterins (Table I). Ascorbate appears to be somewhat better than the mercaptans.

Effect of Pretreatment of Rats with Agents that Modify Phenylalanine Hydroxylase Activity in Vitro—Several inhibitors of the phenylalanine hydroxylase system are known which indirectly by inhibiting dihydropteridine reductase (13). This inhibition prevents the regeneration of the active tetrahydropterin. When liver slices were assayed for phenylalanine hydroxylase activity in the presence of methotrexate in the medium there was potent inhibition, reaching 50% at injected intraperitoneally with a dose of 300 mg/kg of DL-p-chlorophenylalanine methyl ester. Forty-eight hours after the injection, the conversion of phenylalanine to tyrosine in liver slices and extracts from the treated animals was 64 and 62% inhibited, respectively, as compared to that from control animals. After 96 hours, there was 41% inhibition in an extract and 43% inhibition in the slice. In addition, when the phenylalanine hydroxylase activity was assayed in the whole animal by a new in vivo assay based on the incorporation of deuterium into water from ring-deuterated phenylalanine (12), there was 41% inhibition in the group of rats that had been treated with the inhibitor 96 hours previously.

Methotrexate inhibits the phenylalanine hydroxylase system indirectly by inhibiting dihydropteridine reductase (13). This inhibition prevents the regeneration of the active tetrahydropterin. When liver slices were assayed for phenylalanine hydroxylase activity in the presence of methotrexate in the medium there was potent inhibition, reaching 50% at

The abbreviations used are: 6MPH₄, 6-methyltetrahydropterin; DMPH₄, 6,7-dimethyltetrahydropterin; DOPA, 3,4-dihydroxyphenylalanine.

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** The effect of varying phenylalanine concentration on the formation of tyrosine by liver slices. The tyrosine formed was measured after a preincubation for 30 min at 15°, followed by a 30-min incubation at 25°.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Conversion of phenylalanine to tyrosine by liver slices in the presence of added 6-methyltetrahydropterin and dithiothreitol. Slices were preincubated with 1 mM phenylalanine for 30 min at 15°, followed by a 30-min incubation at 25°. In the absence of added 6-methyltetrahydropterin or dithiothreitol, the rate of tyrosine formation was 0.6 μmol/g/30 min.

**Table I**

<table>
<thead>
<tr>
<th>Tetrahydropterin</th>
<th>Reducing agent</th>
<th>Tyrosine μmol/10 min/g</th>
</tr>
</thead>
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<tr>
<td>None</td>
<td>None</td>
<td>0.14</td>
</tr>
<tr>
<td>6-Methyl</td>
<td>6-Methyl</td>
<td>0.95</td>
</tr>
<tr>
<td>6-Methyl</td>
<td>2-Mercaptoethanol</td>
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<td>6-Methyl</td>
<td>Glutathione</td>
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<tr>
<td>6-Methyl</td>
<td>Ascorbate</td>
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<tr>
<td>6-Methyl</td>
<td>Dithiothreitol</td>
<td>2.87</td>
</tr>
<tr>
<td>6,7-Dimethyl</td>
<td>Dithiothreitol</td>
<td>0.54</td>
</tr>
<tr>
<td>Biopterin</td>
<td>Dithiothreitol</td>
<td>0.22</td>
</tr>
</tbody>
</table>

The concentration of tetrahydrobiopterin was 0.045 mM, 6-methyltetrahydropterin was 0.095 mM, 6,7-dimethyltetrahydropterin was 0.5 mM, phenylalanine was 1 mM, and the reducing agents were each 20 mM. In each case, the addition of the reducing agent alone had no significant effect.
<0.018 mM. When rats were given an intraperitoneal injection of methotrexate and their livers were assayed for phenylalanine hydroxylase activities in slices, there was a progressive decrease in activity with time, reaching 90% inhibition within 4 hours after a dose of 10 mg/kg (Fig. 5).

Treatment of Rats with 6-Methyltetrahydropterin—A group of rats were injected with a solution of 25 mg of 6MPP, in 1 ml of 0.9% NaCl containing 100 mg of sodium ascorbate. At 30, 60, and 120 min, animals were killed and their phenylalanine hydroxylase activities were measured in liver slices in the presence of 20 mM dithiothreitol. The results are shown in Fig. 6. Within 30 min after the injection of 20 mg of 6MPP, there was a 6-fold increase in phenylalanine hydroxylase activity when assayed in the presence of 20 mM dithiothreitol, and a 3-fold increase in the absence of added dithiothreitol. This is the first demonstration that an exogenous tetrahydropterin can be transported into the liver and interact with the phenylalanine hydroxylase system in vivo.

DISCUSSION

With the recent demonstration that the liver of a patient with classical phenylketonuria has 0.27% of the normal level of phenylalanine hydroxylase (8), the possibility has been raised that stimulation of that enzyme activity could be used as an alternate means of therapy for phenylketonuria. It is known that there are at least two forms of phenylketonuria: classical phenylketonuria, characterized by very low levels of phenylalanine hydroxylase leading to mental retardation if untreated by dietary restriction of phenylalanine; and atypical phenylketonuria or hyperphenylalaninemia, where there is about 5% of the normal activity (15) and apparently normal I.Q. (16). It seems likely, therefore, that a 20-fold stimulation of the low hydroxylase activity in patients with classical phenylketonuria could protect the developing brain from the damage that is somehow caused by the lack of phenylalanine hydroxylase.

To explore ways in which the level of hepatic phenylalanine hydroxylase might be elevated in vivo, we have attempted to develop assays for the hydroxylase in whole animals (12), as well as in an intact tissue preparation such as liver slices. At both of these levels of organization, the multicomponent hydroxylase system can be studied with a minimum perturbation of the relationship between the individual components. The results obtained to date with both techniques are in excellent agreement.

As can be seen in Figs. 1 and 3, phenylalanine hydroxylase activity in liver slices is approximately linear with respect to both time and the amount of tissue. In addition, the slice system seems to possess many of the properties which had been demonstrated previously for the isolated purified enzyme when it utilizes the natural cofactor, tetrahydrobiopterin. Just as with the purified enzyme, the hydroxylase reaction in slices has a high temperature coefficient, as shown in Fig. 1. The relationship between the rate of formation of tyrosine and phenylalanine concentration is hyperbolic with half-maximal velocity occurring at phenylalanine concentrations similar to those found with the isolated enzyme in the presence of tetrahydrobiopterin (about 0.2 to 0.3 mM). It should be noted that the $K_m$ for phenylalanine in the presence of either 6MPP, or DMPH, is about 4 to 6 times higher than it is in the presence of tetrahydrobiopterin (17). The low $K_m$ for phenylalanine in liver slices thus provides independent support for the conclusion that the functioning hydroxylase cofactor in liver is tetrahydrobiopterin. In addition, there is moderate substrate inhibition at high phenylalanine concentrations, which is similar to that seen when phenylalanine hydroxylase activity is measured with tetrahydrobiopterin in the presence of phenylalanine hydroxylase-stimulating protein found in liver (17).

One of the most characteristic properties of native phenylalanine hydroxylase is the relative $V_{max}$ values for the three pterins, tetrahydrobiopterin, 6MPP, and DMPH. The relative velocities for liver slices (at 1 mM phenylalanine, which is a saturating concentration with tetrahydrobiopterin) are: tetrahydrobiopterin 1; DMPH, 2.5; 6MPP, 13 (Table I). The relative rates in vitro with purified rat liver phenylalanine hydroxylase using similar concentrations of substrates are: tetrahydrobiopterin, 1; DMPH, 2.9; 6MPP, 14.9. When the hydroxylase is activated by either limited proteolysis or by exposure to lysolecithin, the relative activities of the hydroxylase increase significantly.

4 S. Milstien and S. Kaufman, unpublished observations.
ase with the different pterins is markedly altered; e.g., after activation, the maximum velocity with tetrahydrobiopterin is 3 to 4 times greater than with DMPH$_4$ (10). The relative activities that were found in slices (Table I) indicate, if there is no difference in uptake of the different pterins by the liver cell, that hepatic phenylalanine hydroxylase is predominantly in the nonactivated state in vivo.

Another property that is characteristic of the activated phenylalanine hydroxylase is the ability to catalyze the conversion of m-tyrosine to DOPA when tetrahydrobiopterin is the cofactor. The native enzyme is essentially devoid of activity toward m-tyrosine (18). Liver slices were incubated with m-[14C]tyrosine and [14C]DOPA formation was determined by chromatography on a Beckman amino acid resin column (8). There was no detectable [14C]DOPA formed over that from a boiled tissue incubation. This result also indicates that there is no difference in uptake of the different pterins by the liver cell, this phenomenon would lead to marked variation of the apparent $K_m$ with hydroxylase concentration (21); for example, the apparent $K_m$ of the purified hydroxylase in the liver for tetrahydrobiopterin is about 0.025 mM, a value that is about 10 times higher than that of the purified enzyme (1). A reasonable explanation for this difference may be found in our previous observation that at pH 8, the apparent $K_m$ of the purified hydroxylase for tetrahydrobiopterin varies markedly with hydroxylase concentration (21); for example, the apparent $K_m$ is 0.035 mM at about 10 μM of hydroxylase/ml and is 0.140 mM at about 40 μM of hydroxylase/ml. Even if a much less marked variation of the apparent $K_m$ with hydroxylase concentration occurs at pH values below 8 (such as might be expected in the cytosol of the liver cell), this phenomenon would lead to the expectation that the $K_m$ of the hydroxylase for tetrahydrobiopterin in vivo would be higher than that of the isolated enzyme.

This expectation is further supported by the estimated concentration of the hydroxylase in liver. From the specific activity of the essentially pure hydroxylase and the units of hydroxylase activity in liver extracts (22) it can be estimated that the concentration of the hydroxylase in rat liver is about 500 μg/g, a concentration that is in the range where variation of the apparent $K_m$ for tetrahydrobiopterin with hydroxylase concentration is highly likely.

Finally, known inhibitors of the phenylalanine hydroxylase system have been shown to cause parallel and expected inhibitions of activity in slices and extracts, an indication that the hydroxylase is behaving similarly at these two different levels of tissue organization in its susceptibility to these drugs. p-Chlorophenylalanine, which irreversibly inhibits the hydroxylase (11), inhibited the hydroxylase to the same extent in slices and in extracts. Methotrexate, on the other hand, which only inhibits the regeneration of reduced cofactor, caused marked inhibition of activity in the slice, but as expected, does not inhibit the hydroxylase activity in extracts that are supplemented with a nonenzymatic tetrahydropterin regeneration system.

The observed maximum increase in the rate of phenylalanine hydroxylation in liver slices by addition of 6-methyltetrahydropterin (Table I) is approximately the extent of stimulation that would be required to raise the activity of hydroxylase from the low level characteristic of classical phenylketonuria (i.e., 0.27% of normal) to the relatively high level that is characteristic of hyperphenylalaninemia (i.e., 5% of normal). It may also be possible to design and synthesize tetrahydropteridines that have even more suitable properties than 6MPPH for the in vivo stimulation of hepatic phenylalanine hydroxylation. These properties would include: ease of transport into the liver, high affinity for the hydroxylase and reductase, and ability to support a high maximum velocity of phenylalanine hydroxylation. We are currently engaged in the synthesis of analogues of biopterin for this purpose.

REFERENCES

Studies on the phenylalanine hydroxylase system in liver slices.
S Milstien and S Kaufman


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