Metabolism of the Phenylalanine Hydroxylation Cofactor

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SUMMARY

A simple chemical method of reducing biopterin to 7,8-dihydrobiopterin has been described. The product of this reaction appears to be identical with the phenylalanine hydroxylation cofactor isolated from rat liver extracts. With the use of the synthetic compound, the TPNH-mediated enzymatic reduction of dihydrobiopterin to the tetrahydro level has been demonstrated. This reduction is catalyzed by dihydrofolate reductase. In the presence of 7,8-dihydrobiopterin, the reductase is an essential component of the phenylalanine-hydroxylating system. The TPN-dependent oxidation of dihydrobiopterin to sepiapterin has been shown to be catalyzed by another enzyme, sepiapterin reductase.

The enzymatic conversion of phenylalanine to tyrosine requires a reduced pteridine cofactor (1). Although tetrahydrofolate can fill this role (2), a naturally occurring unconjugated pteridine is much more active. The latter compound has been isolated from rat liver extracts and shown (3) to be dihydrobiopterin (Fig. 1).

Another naturally occurring pteridine, sepiapterin (2-amino-4-hydroxy-6-lactyl-7,8-dihydropteridine) (4), had previously been shown to have high cofactor activity (5). Recently it was shown that this compound, which differs from dihydrobiopterin only in that it contains a keto rather than a hydroxyl group in position 1' of the propyl side chain, must be reduced to dihydrobiopterin before it can function as a cofactor (6). This TPNH-mediated reaction is catalyzed by sepiapterin reductase, an enzyme that has been extensively purified from rat liver extracts (6).

In addition to these naturally occurring compounds, certain synthetic tetrahydropteridines such as 2-amino-4-hydroxy-6-methyltetrahydropteridine show high cofactor activity (7). Indeed, most of the studies on the enzymatic conversion of phenylalanine to tyrosine have been carried out with these model pteridines. Since these studies showed that the tetrahydro form of the pteridine is the substrate for phenylalanine hydroxylase (8), it was anticipated that dihydrobiopterin would have to be converted to the tetrahydro derivative before it could function as a cofactor. The demonstration that phenylalanine hydroxylation in the presence of the purified rat liver cofactor is markedly stimulated by dihydrofolate reductase indicated that this enzyme catalyzed the initial reduction of the cofactor to the tetrahydro level (9). The role of dihydrofolate reductase in the hydroxylating system could not be examined directly, however, until larger amounts of dihydrobiopterin were available.

In the present paper, the enzymatic reduction of dihydrobiopterin, as well as other aspects of its metabolism, has been investigated with dihydrobiopterin prepared by a chemical procedure. The product of the chemical reduction, which appears to be identical with the pterin isolated from rat liver, is a substrate for highly purified dihydrofolate reductase. The enzyme catalyzes the TPNH-mediated conversion of dihydro- to tetrahydrobiopterin according to Equation 1.

\[ TPNH + H^+ + \text{dihydrobiopterin} \rightarrow TPN^+ + \text{tetrahydrobiopterin} \]  

In the presence of dihydrobiopterin, dihydrofolate reductase is an essential component of the phenylalanine-hydroxylating system.

The availability of larger amounts of dihydrobiopterin has also facilitated an examination of the reversibility of the sepiapterin reductase-catalyzed reaction. It has been found that the reaction is readily reversible; the enzyme catalyzes a TPN-dependent oxidation of dihydrobiopterin to sepiapterin according to Equation 2.

\[ \text{Dihydrobiopterin} + TPN^+ \rightarrow \text{sepiapterin} + TPNH + H^+ \]  

Since structural analogues of dihydrobiopterin are available, in contrast to those of sepiapterin, the reversibility of the reaction has also permitted a study of the substrate specificity of sepiapterin reductase.
Finally, the results of the present study have largely resolved the one uncertain structural feature of the phenylalanine hydroxylase cofactor: the configuration of the double bond in the pteridine ring. All of the properties of the compound strongly support the conclusion that it is the 7,8-dihydro tautomer.

EXPERIMENTAL PROCEDURE

Materials

Phenylalanine hydroxylase (rat liver enzyme) (10), glucose dehydrogenase (11), and phenylalanine hydroxylation cofactor (10), were prepared as previously described. Sepiapterin reductase was purified from rat liver extracts by Method A of a published procedure (6). Highly purified dihydrofolate reductase from chicken liver, almost free of dihydropteridine reductase, was a gift from Dr. H. Rembold. Sepiapterin was kindly supplied by Dr. M. Akino. Dihydrofolate was prepared by the method of Futterman (12). Glucose 6-phosphate was purified from Worthington. Lactic dehydrogenase was obtained from Worthington.

Methods

Preparation of Dihydropteridine Reductase Relatively Free of Dihydrofolate Reductase Dihydropteridine reductase was prepared by a published method (10). To prepare this enzyme relatively free of dihydrofolate reductase, the 0.1 M phosphate eluate from the alumina C-y step (10) was further fractionated with ammonium sulfate. The procedure was carried out at 3-5°C. To each 100 ml of gel eluate, 14 g of ammonium sulfate were added slowly with continuous mechanical stirring. The pH of the solution was adjusted to 8.0 by the dropwise addition of 1 N NH₂OH. Another 28.7 g of ammonium sulfate (for each 100 ml of starting material) were added. The precipitate was collected by centrifugation in the cold. The pH of the supernatant solution was adjusted to 6 with the addition of 4 N HCl. This solution could be stored at -20°C for months with no spectral evidence of decomposition. The yield of dihydrobipterin obtained by the zinc-alkali reduction procedure was 60 to 75%, as determined by spectral assay of the bipterin formed after boiling an aliquot of the solution in dilute HCl (3). That the pteridine is at the dihydro level of reduction was shown by iodine titration (8); 1.04 peq of iodine were consumed per µmole of dihydrobipterin. Dihydrobipterin was prepared by the same procedure.

Preparation of Tetrahydrobipterin—The tetrahydro derivative was prepared from bipterin (1.4 mg) by catalytic hydrogenation in 1 N HCl with platinum oxide (0.5 mg) as a catalyst (13). After 3 hours the hydrogenation was stopped, and the mixture was cooled to 0°C and rapidly filtered. The clear, colorless filtrate was concentrated under vacuum. Water was added to the residue, and the solution was frozen until ready for use. The yield of tetrahydrobipterin was 84%, based on titration with dichloroindophenol (8).

In 0.1 M phosphate, pH 6.8, the ultraviolet absorption spectrum, shown in Fig. 2, is essentially the same as that of other tetrahydropteridines, such as tetrahydrofolate (14) and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (8), with a peak at 298 mp. (A second peak at 220 mp with about twice the extinction value of the 298 mp peak has been omitted from the figure.) In 0.1 N HCl, the spectrum also resembles that of unconjugated tetrahydropteridines, with a single peak at 265 mp. It has been shown that in 0.1 M phosphate, pH 6.8, tetrahydropteridines are oxidized nonenzymatically to 7,8-dihydropteridines (8). The spectral changes accompanying the oxidation of tetrahydrobipterin under these conditions are shown in Fig. 2. The oxidation follows first order kinetics, with a half-life of about 16 min at 22-24°C. The compound is therefore somewhat more stable than 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (15). The spectrum of the oxidized product at this pH is the same as that of dihydrobipterin prepared by the zine-alkali reduction method (see Fig. 4). Omitted from Fig. 2 for the sake of simplicity are the absorbance changes in the low ultraviolet region of the spectrum. During oxidation, the 220 mp peak of the tetrahydro compound shifts to 250 mp; this peak has an extinction value about 1.5 times greater than the 220 mp peak of tetrahydrobipterin.

Paper Chromatography of Reduced Bipterin Derivatives—All
3936 Phenylalanine Hydroxylating system (1). The cofactor activity coincided with a purple-fluorescent spot. Determined from its absorbance at 269 \text{nm} in 0.1 \text{M} \text{K} \text{H} \text{PO} \text{4}, \text{pH} 6.8. The dashed line shows the initial spectrum. The other curves were recorded 5, 13, 23, 38, and 85 min later. The final spectrum was unchanged after another 20 min of incubation. Operations were performed in a room illuminated with a red photographic lamp. The solutions were applied to the paper with a spectroradiographic lamp. The solutions were applied to the paper with a spectroradiographic lamp. The solutions were applied to the paper with a spectroradiographic lamp. The solutions were applied to the paper with a spectroradiographic lamp. The solutions were applied to the paper with a spectroradiographic lamp.

Miscellaneous Methods—Sepiapterin was quantitatively determined from its absorbance at 269 \text{nm} in 0.1 \text{M} \text{K} \text{H} \text{PO} \text{4}, \text{pH} 6.8, calculated from the data of Nawa (10) or at 420 \text{nm} at pH 7.0 to 8.0 (\( \epsilon = 1.04 \times 10^4 \)). The compound was also determined by a dimethylphenylhydrazine procedure (17). Tyrosine was determined by the nitrosonaphthol procedure either colorimetrically (18) or fluorometrically (19).

Estimation of Molar Concentration of Phenylalanine Hydroxylating Cofactor—Even after the cofactor had been identified as dihydrobiopterin (3), it was still necessary to be able to estimate the dihydrobiopterin concentration in impure fractions of the cofactor and in highly purified fractions that had undergone decomposition on prolonged storage. Three methods have been used for determining the concentration of dihydrobiopterin in the presence of relatively small amounts of biopterin.

1. Absorption spectrum after boiling in dilute acid: When highly purified cofactor samples are boiled in dilute HCl in the dark, the major pteridine product is biopterin (3). The biopterin in such boiled solutions, corrected for the biopterin present before boiling, is a measure of the amount of reduced biopterin originally present. The biopterin can be quantitatively estimated from the optical density at 380 \text{nm} in 0.1 \text{M} \text{NaOH}. The molar extinction coefficient for biopterin at 380 \text{nm} is \( 6.10 \times 10^3 \), calculated from the data of Patterson, Milstrey, and Stokstad (21), which is about 10% lower. The molar extinction coefficient for dihydrobiopterin at 380 \text{nm} in 0.1 \text{M} \text{NaOH} is approximately \( 1.15 \times 10^4 \). The concentration of dihydrobiopterin can be calculated from the following equation, where \( B \) stands for biopterin, \( BH_2 \) for dihydrobiopterin, and O.D. for optical density at 380 \text{nm} in 0.1 \text{M} \text{NaOH}.

\[
\text{Molar concentration of } BH_2 = \frac{\text{O.D. before boiling} - \text{O.D. after boiling}}{\text{absorbance at 380 nm} \times \text{coefficient for biopterin} \times \text{volume of solution}}
\]

2. Optical density at 400 \text{nm} in dilute acid: This method is based on the fact that, in 0.1 \text{M} \text{HCl} at 400 \text{nm}, dihydrobiopterin has an absorption band whereas biopterin does not. The molar extinction coefficient for dihydrobiopterin under these conditions is about \( 4.0 \times 10^4 \).

3. Cofactor activity: The concentration of dihydrobiopterin in an impure sample can be estimated by a comparison of its cofactor activity with that of a sample of pure dihydrobiopterin, both activities determined under standard conditions (1). In practice, the activity of the unknown is compared with that of a freshly prepared solution of a secondary standard, 2-aminomethyltetrahydropteridines (7, 8).

Highly purified fractions of cofactor have about 325 cofactor units per \text{pmole} of biopterin (biopterin determined by Method 1). Assayed simultaneously, at a concentration of 10 \text{ng} per ml of reaction mixture, the dimethylpteridine has about 11 cofactor units per \text{pmole} of pteridine. From these values, the dihydrobiopterin concentration of an impure fraction of the cofactor can be determined.

Dihydrobiopterin was determined in a sample of highly purified cofactor by the three methods. The results, expressed as micro moles per ml, were: Method 1, 0.12; Method 2, 0.11; Method 3, 0.10.

RESULTS

Properties of Biopterin Reduced by Zinc and HCl—As previously shown (3), treatment of biopterin with zinc and HCl reduced the pteridine to a compound with high cofactor activity in the phenylalanine-hydroxylating system. Although this reduced product has not been fully characterized, iodine titration of a sample that had been stored frozen for several days indicated that it was a dihydro compound (3).

More recently, evidence has been obtained which indicates that the initial product formed under these conditions is the tetrahydro derivative and that the dihydro compound is formed from it by subsequent oxidation. The initial spectrum of the pteridine in 0.1 \text{M} \text{phosphate}, \text{pH} 6.8, recorded 30 sec after the zinc-HCl treatment, is shown in Fig. 3. The spectrum is the expected one for a mixture of dihydro- and tetrahydropteridines, with the latter form predominant. The spectrum is similar, for example, to that of tetrahydrobiopterin measured after a few
minutes of incubation in 0.1 M phosphate, pH 6.8, i.e. after some oxidation of the tetrahydro to the dihydro compound had occurred (see Fig. 2).

The spectral evidence indicating a mixture was confirmed by iodine and dichloroindophenol titrations carried out on fresh solutions of reduced biopterin prepared by the zinc-HCl method. These determinations indicated that the solutions contained a mixture of 80 to 85% tetrahydro- and 15 to 20% dihydropteridine. By contrast, no tetrahydro compound could be detected by dichloroindophenol titration of solutions which had been stored for several days.

The presence of a preponderance of the tetrahydro form in such solutions was further substantiated by observation of the spectral changes occurring on aerobic incubation of the reduced biopterin in 0.1 M phosphate, pH 6.8, conditions under which tetrahydropteridines are oxidized to their dihydro derivatives (8). When the reduced biopterin was incubated under these conditions (Fig. 3), its spectrum gradually changed to one which is typical of unconjugated 2-amino-4-hydroxy-7,8-dihydropteridines, with a peak at 276 to 278 m\(\mu\) and another at 325 to 330 m\(\mu\) (8, 22). These spectral changes are similar to those observed when tetrahydrobiopterin is oxidized under these conditions (see Fig. 2). As will be seen later, these changes are also similar (with the time sequence reversed) to those observed during the enzymatic reduction of dihydrobiopterin to the tetrahydro compound (see Fig. 6). It should be noted that the same two isosbestic points (at 286 to 287 m\(\mu\) and 315 to 317 m\(\mu\)) are evident in both reactions.

It seems most probable from these results that the tetrahydropteridine derivative is the initial product of the zinc-HCl reduction procedure and that subsequent storage leads to oxidation of the tetrahydro to the dihydro compound. In view of previous evidence (15), confirmed recently by Viscontini and Bobst (23), in favor of the idea that the primary oxidation product of tetrahydropteridines is a quinonoid dihydropteridine which tautomerizes to the 7,8-dihydro derivative, solutions that have been stored would probably contain both tautomeric dihydro forms as well as the tetrahydro compound.

Properties of Biopterin Reduced by Zinc in Alkali—Dihydrobiopterin (probably the 7,8-dihydro compound) uncontaminated by the tetrahydro derivative can be conveniently prepared by performing the zinc reduction in the presence of alkali, as described under “Methods.” A similar procedure has been used previously for the preparation of 7,8-dihydropteridines (8, 22, 24).

The spectrum of the biopterin reduced with zinc and alkali is shown in Fig. 4. At pH 6.8, it is similar to that of the zinc-HCl product after aerobic incubation. In contrast to the zinc-HCl product, this compound shows no change in spectrum on aerobic incubation at pH 6.8 for 20 to 30 min. At this pH, the spectrum shown in Fig. 4 is virtually identical with that of the dihydrobiopterin obtained from the sepiapterin reductase-catalyzed reduction of sepiapterin (6). It is also similar to the spectra in acid and alkali of the hydroxylation cofactor after isolation from rat liver extracts (3). The only difference is that, in acid, the spectrum of the compound isolated from liver has a slight shoulder at 275 to 280 m\(\mu\) whereas the zinc alkali product has a pronounced shoulder in this region. Occasionally, purified preparations of the rat liver hydroxylation cofactor have been obtained which have essentially identical spectra in acid, in alkali, and at neutrality with those of dihydrobiopterin prepared by the zinc-alkali method. The spectra of such a preparation are shown in Fig. 5. The slight variability in spectral characteristics of different cofactor preparations can probably be accounted for by the presence of variable amounts of the tetrahydro and quinonoid dihydro forms of the cofactor.

When chromatographed on paper in 1-propanol-1% \(\text{NH}_2\text{OH} (2:1) as described under “Methods,” the purified cofactor from
Activity of Dihydrobiopterin in Phenylalanine-hydroxylating System—It has already been reported (3) that, on a molar basis, dihydrobiopterin reduced with zinc and HCl shows the same cofactor activities, an indication that they are all the same double bond tautomers. This result also confirms the validity of the methods used to determine dihydrobiopterin concentrations, since the concentration of sepiapterin is obtained by a completely independent method.

Dihydrofolate Reductase-catalyzed Reduction of Dihydrobiopterin—The stimulation of the hydroxylation reaction by dihydrofolate reductase in the presence of the purified rat liver cofactor (9) suggested that the reduction of dihydrobiopterin could be catalyzed by this enzyme. The requirement for dihydrofolate reductase (as well as for the hydroxylase and dihydropteridine reductase) in the presence of synthetic dihydrobiopterin and the compound isolated from rat liver is shown in Table II. These results emphasize the fact that the reactions catalyzed by dihydrofolate reductase and dihydropteridine reductase are distinct: the former enzyme catalyzes the reduction of 7,8-dihydropteridines.

Table I

<table>
<thead>
<tr>
<th>Pteridine</th>
<th>Cofactor activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver cofactor</td>
<td>2.28</td>
</tr>
<tr>
<td>Dihydrobiopterin</td>
<td>2.19</td>
</tr>
<tr>
<td>Sepiapterin</td>
<td>2.10</td>
</tr>
</tbody>
</table>

a The rate of the hydroxylation reaction was measured by a determination of the rate of the phenylalanine-stimulated oxidation of TPNH. Cofactor activity is expressed as micromoles of TPNH oxidized per min, measured between 16 and 24 min after the reaction was started. Bioperterin in the rat liver cofactor and in dihydrobiopterin was determined by Method 1. Sepiapterin concentration was determined as described under "Methods."
The dihydrofolate reductase-catalyzed reduction of dihydrobiopterin can be followed spectrophotometrically, as shown in Fig. 7. Since, in this experiment, the TPNH was kept in the reduced form by the addition of a large excess of glucose and glucose dehydrogenase, the spectral changes are due only to the reduction of the pteridine. The spectrum after 36 min is typical of tetrahydropteridines, with a single peak of 265 to 266 μm. From extinction values for the dihydro and tetrahydrobiopterin derivatives, it can be estimated that the reduction was about 90% complete at this point. When the reaction mixture was acidified, the spectrum showed a sharp peak at 263 to 264 μm, with a much smaller one (about 5% of the absorbance of the 263 to 264 μm peak) at 320 to 325 μm. The latter peak is probably due to the tetrahydrobiopterin, but rather to trace amounts of biopterin formed during the incubation.

From the average decrease in absorbance at 340 μm observed in three separate experiments of the type illustrated in Fig. 7, in which the extent of reduction was estimated to be between 90 and 97%, the change in extinction coefficient for the reaction, dihydrobiopterin—tetrahydrobiopterin, was calculated to be 6.120 ± 170 M⁻¹ cm⁻¹.

When the reaction catalyzed by dihydrofolate reductase was carried out in the absence of a TPNH-generating system, i.e., without glucose dehydrogenase, the decrease in absorbance at 340 μm was about twice as great as in the presence of the generating system. Since under these conditions the decrease in absorbance at 340 μm is due to both TPNH oxidation and dihydrobiopterin reduction, the change in extinction coefficient for the reaction described by Equation 1 is equal to the sum of 6.22 × 10⁻³ (ε for TPNH at 340 μm) and 6.12 × 10⁻³ (ε for dihydrobiopterin reduction; see above), or 12.3 × 10⁻³. The finding that the decrease in absorbance at 340 μm is twice as great in the absence as in the presence of a TPNH-generating system indicates, therefore, that 1 mole of TPNH is oxidized for each mole of dihydrobiopterin reduced, in accordance with Equation 1.

The Km for dihydrobiopterin was determined from the Lineweaver-Burk double reciprocal plot shown in Fig. 8 and is equal to 2.7 × 10⁻⁴ m. This may not be significantly different from the Km for dihydrobiopterin in the phenylalanine-hydroxylating system (Km = 6.5 × 10⁻⁵ m (3)).

The Km for the reduction of dihydrofolate by the reductase was also determined under the same conditions and was found to be about 6.0 × 10⁻⁷, in reasonable agreement with the reported values of 5 × 10⁻⁷ (27) and 1.2 × 10⁻⁷ (28) for the chicken liver enzyme.

That the product of the dihydrofolate reductase-catalyzed reduction of dihydrobiopterin is tetrahydrobiopterin is strongly supported by the finding that it is active with phenylalanine hydroxylase under conditions in which no net reduction of the pteridine is possible, i.e., in the absence of TPNH and dihydropteridine reductase. As can be seen in Table III, under these conditions dihydrobiopterin is completely inactive.

The amount of tyrosine formed in this experiment was about 67% of the amount of tetrahydrobiopterin added. In other ex-
Dihydrobiopterin was enzymatically reduced with dihydrofolate reductase in a reaction mixture containing the following components, in micromoles: potassium phosphate, pH 7.4, 50; TPNH, 0.04; dihydrobiopterin, 0.09; glucose 6-phosphate, 0.6; dihydrofolate reductase, 10 μg (0.016 unit); glucose 6-phosphate dehydrogenase, 1.0 unit; and water up to 1.0 ml. The mixture was incubated at 23-24° in the dark for 2 hours and then cooled to 2°. It was then lyophilized, an aliquot of the eluate (referred to as tetrahydrobiopterin) was tested for cofactor activity in a reaction mixture containing the following components, in micromoles: potassium phosphate, pH 7.4, 50; L-phenylalanine, 2.0; phenylalanine hydroxylase, 0.07 mg; and water up to 1.0 ml. The mixture was incubated at 25°. Tyrosine was determined fluorometrically after a 4-min incubation. The pteridine was separated from substrate and pyridine nucleotides on a Dowex 1 column as described under "Methods." After lyophilization, an aliquot of the eluate (referred to as tetrahydrobiopterin in the table) was tested for cofactor activity in a reaction mixture containing the following components, in micromoles: potassium phosphate, pH 7.4, 50; L-phenylalanine, 2.0; phenylalanine hydroxylase, 0.07 mg; and water up to 1.0 ml. The reaction was stopped by the addition of trichloracetic acid after a 4-min incubation at 25°. Tyrosine was determined fluorometrically (19). The amount of tetrahydropteridine added in the hydroxylase assay was calculated from the amount of dihydrobiopterin used in the original incubation with reductase, corrected for the small loss during the Dowex 1 step.

<table>
<thead>
<tr>
<th>Reduced bioppterin added</th>
<th>Amount added</th>
<th>Tyrosine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μ mole</td>
<td>μ mole</td>
</tr>
<tr>
<td>Tetrahydro</td>
<td>0.00345</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>0.00090</td>
<td>0.0016</td>
</tr>
<tr>
<td></td>
<td>0.00138</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>0.0240</td>
<td>0.0005</td>
</tr>
<tr>
<td>Dihydro</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

It has previously been reported that the reduced form of neopterin, 2-amino-4-hydroxy-6-[(1,2,3-trihydroxypropyl)-ethyl]pteridine, a close structural analogue of bioppterin, has high cofactor activity in the phenylalanine-hydroxylating system (5). It was of interest, therefore, to see if it, too, is a substrate for dihydrofolate reductase. The dihydro derivative of neopterin was prepared by the zinc-alkali procedure. At approximately equal substrate concentrations (3 × 10⁻⁵ M) and at pH 6.90, the initial rate of reduction of dihydroneopterin was 70% that of dihydrobioppterin.

Dihydrofolate Reductase-catalyzed Reduction of Bioppterin—Recently, it has been established that folate is a substrate for chicken liver dihydrofolate reductase (27, 28, 30). This finding led to the expectation that bioppterin might also be a substrate for the enzyme. Such a reaction could be of significance for the bio-synthesis of tetrahydrobioppterin. Since folate is active as a substrate for the enzyme in the latter reaction, the possibility arises that folate might also be a substrate for the enzyme. Therefore, experiments of this type were carried out in order to test the possibility that folate is a substrate for the enzyme in the phenylalanine-hydroxylating system.

It has previously been reported that, in contrast to the cofactor activity of chemically reduced dihydrofolate, the enzymatically synthesized compound has little or no activity (29). This result indicated that only one isomer of dihydrofolate (with respect to the configuration of carbon 6 of the pteridine ring) is active in the phenylalanine-hydroxylating system and that the product of the dihydrofolate reductase reaction is the inactive isomer. From the results presented in Table III, it is clear that the enzymatically synthesized tetrahydrobioppterin is active in the hydroxylating system.

To detect reduction of bioppterin, reaction mixtures containing bioppterin, TPNH, and reductase at pH 5.6 and at pH 7.4 were incubated at room temperature for 1 hour with periodic scanning of the absorption spectrum. At both pH values, slight changes in spectrum were detected but the rates were too slow to measure quantitatively.

As a more sensitive test for bioppterin reduction, the hydroxylation cofactor activity of an aliquot of the pH 5.6-buffered reaction mixture was measured. The results (Table IV) show that preincubation of bioppterin with the reductase and TPNH led to the formation of a compound with cofactor activity. Since bioppterin, itself, is without cofactor activity, this result indicates that bioppterin was reduced during the preincubation. From the data it can be calculated that about 1% of the bioppterin had been reduced under these conditions. In three separate experiments of this type, the amount of bioppterin reduced varied between 0.8 and 1.3%. These results indicate that, at pH 5.6, bioppterin is reduced by dihydrofolate reductase at less than 1% the rate at which dihydrobioppterin is reduced.

**Enzymatic Conversion of Dihydrobioppterin to Sepiapterin**—Recently, an enzyme was purified from rat liver which catalyzes the TPNH-mediated reduction of sepiapterin to dihydrobioppterin (6). With the availability of chemically prepared dihydrobioppterin, it became feasible to examine the reversibility of this reaction. When purified sepiapterin reductase was incubated with a mixture of dihydrobioppterin and TPNH, the solution slowly became yellow. The spectral changes during the reaction are shown in Fig. 5. These changes are very similar to those reported for the reduction of dihydrofolate (6). The spectrum after 45 min is very similar to that of sepiapterin (18). The reaction was completely dependent on the enzyme and on TPNH. Additional evidence in support of the conclusion that the product is sepa-
Dihydrobiopterin was obtained by a comparison of its RF values in several solvents with those of the authentic compound. The yellow-fluorescent product and sepiapterin were not separated after paper chromatography in the following solvent systems: 1-propanol-1% NH₄OH, 2:1, RF 0.26; 1-propanol-2% ammonium acetate, 1:1, RF 0.58; 1-propanol-ethyl acetate-water, 7:1:2, RF 0.31.

If the observed conversion of dihydrobiopterin to sepiapterin is due to reversal of the sepiapterin reductase-catalyzed reaction, the other expected product should be TPNH. To detect its formation, an experiment similar to the one described in Fig. 9 was performed. After 45 min, when the absorbance at 340 μm was relatively constant, an excess of pyruvate and of lactate dehydrogenase was added. No further decrease in absorbance at 340 μm could be detected. In contrast to this result, the addition of pyruvate and lactate dehydrogenase after shorter incubations (15 to 20 min) did lead to a rapid decrease in absorbance at 340 μm. This decrease was close to the expected value, based on the amount of sepiapterin formed. These results indicate that TPNH accumulates in the reaction mixtures only during short incubations.

Independent evidence for the formation of TPNH was obtained from a study of the dismutation reaction described by Equation 3, which is the sum of Reactions 2 and 1.

\[ 2 \text{Dihydrobiopterin} + \text{tetrahydrobiopterin} + \text{sepiapterin} \rightarrow \text{TPNH} + \text{TPN} + \text{dihydropteridine} + \text{formate} \]

This decrease was close to the expected value, based on the amount of sepiapterin formed. These results indicate that TPNH accumulates in the reaction mixtures only during short incubations.

### Table V

<table>
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<th>Component omitted</th>
<th>Tyrosine formed μmole</th>
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<td>None</td>
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<tr>
<td>Sepiapterin reductase</td>
<td>0.0004</td>
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<tr>
<td>TPN</td>
<td>0.0002</td>
</tr>
<tr>
<td>Dihydrobiopterin</td>
<td>0</td>
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</table>

### Table VI

**Substrate specificity of sepiapterin reductase**

The complete reaction mixture contained the following components, in micromoles: potassium phosphate, pH 7.87, 100; TPN, 0.03; enzyme, 0.070 mg of protein; pteridines as indicated; substrate, 0.036; dihydrobiopterin, 0.029. Water was added to a final volume of 0.80 ml, and the tubes were incubated in the dark at 23-24°C. The anaerobic reactions were carried out in evacuated Thunberg tubes. After 60 min, the absorbance at 420 μm was determined and the amount of sepiapterin formed was calculated from the extinction coefficient (see "Methods"). A non-enzyme control served as the blank. In the case of dihydrobiopterin, the 360 to 420 μm range of the spectrum was scanned. The reaction was stopped by the addition of trichloracetic acid, and carbonyl groups were determined by the dinitrophenylhydrazine method (17).

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Conditions</th>
<th>Carbonyl groups</th>
<th>Spectrum</th>
<th>μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioppterin</td>
<td>Aerobic</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dihydrobiopterin</td>
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<td>0.0073</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td>Tetrahydrobiopterin</td>
<td>Aerobic</td>
<td>0.0057</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>0.0010</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The tetrahydrobiopterin probably undergoes nonenzymatic oxidation to the dihydro compound (8) and is unavailable for the hydroxylation reaction.

The results of the experiment are shown in Table V. There was a TPN- and sepiapterin reductase-dependent formation of tyrosine. In several experiments of this type, the amount of tyrosine produced was about 70% of the amount of sepiapterin formed, the latter quantity being calculated from the increase in absorbance at 420 μm.

The rate of the enzyme-catalyzed conversion of dihydrobiopterin to sepiapterin is much slower than the rate of the reaction in the opposite direction. At pH 6.8 to 6.9 it is about one-
tenth, and at pH 7.7 to 7.8, one-fourth, the rate of reduction of sepiapterin to dihydrobiopterin.

To see if the state of reduction of the pteridine ring is of importance in determining substrate activity, biopterin and tetrahydrobiopterin were tested as possible substrates for the enzyme. Since the absorption spectra of the products of these reactions are not known, the reaction was measured by a determination of appearance of carbonyl groups. The results, shown in Table VI, indicate that the enzyme is quite specific for dihydrobiopterin. Biopterin is inactive. Tetrahydrobiopterin under anaerobic conditions has little activity; the marked increase in activity in the presence of oxygen in almost certainly due to its oxidation to the active dihydro derivative. Since the tetrahydrobiopterin used in this experiment is the Dl mixture (with respect to carbon atom 6), it is still possible that either the D or the L isomer would be a good substrate for the enzyme.

It was of interest to see if dihydroneopterin is a substrate for sepiapterin reductase. When tested under the same conditions as those used in the experiment illustrated in Fig. 9, no enzyme-dependent change in spectrum was observed. The assay was sensitive enough to detect a rate of reaction 3% as fast as that with dihydrobiopterin. It is possible that isomers of neopterin other than the L-erythro compound would be active.

DISCUSSION

The product of the reduction of biopterin with zinc and alkali appears to be identical with the phenylalanine hydroxylating cofactor isolated from rat liver. They both have the same ultraviolet absorption spectra, paper chromatographic behavior, and activity (K_m and V_max) in the phenylalanine-hydroxylating systems. The evidence which shows that the compound isolated from liver is dihydrobiopterin has already been presented (3).

The results of the present study strongly support the conclusion that both the natural and synthetic compounds are the 7,8-dihydro isomers. The ultraviolet absorption spectra of these reduced pteridines at neutral, acid, and alkaline pH values are quite similar to those of authentic 7,8-dihydropteridines with simple alkyl substituents on positions 6 and 7 of the ring (8, 22). As can be seen in Fig. 4, the similarity in spectra includes one of the characteristic features of 7,8-dihydropteridines: a pronounced bathochromic shift in the long wave length absorption maximum on going from neutral to acid pH (22).

The results of the enzymatic studies provide additional support for the conclusion that the cofactor isolated from liver and the chemically prepared dihydrobiopterin are 7,8-dihydro compounds. It is known that the reduced folate isomer that is active with dihydrofolate reductase is the 7,8-dihydro derivative (26). The finding that dihydrobiopterin is a substrate for this enzyme indicates that it, too, has the same configuration.

It is also known that sepiapterin, the substrate for sepiapterin reductase, is a 7,8-dihydro compound (4). It has now been demonstrated that in the reverse direction dihydrobiopterin is also a substrate for this enzyme and that the configuration of double bonds in the pteridine ring influences the substrate activity. Since it seems highly improbable that the isomeric specificity of an enzyme would be different in that forward and reverse directions, it can be concluded that hydrogen atoms in the pyrimidine ring of dihydrobiopterin and sepiapterin have the same configuration; i.e., they are both 7,8-dihydro compounds.

The finding that dihydrofolate reductase is an essential component of the purified phenylalanine-hydroxylating system raises the question whether it is also essential for the hydroxylation reaction in vivo. It has been found that the need for dihydrofolate reductase in the hydroxylation of phenylalanine in vitro is not marked until after the cofactor has been exposed to phosphate buffers (9). This observation indicates that in crude liver extracts, and presumably in the liver cell, the cofactor exists predominantly in a form that is active in the hydroxylation system without dihydrofolate reductase, i.e., either the tetrahydro or the quinonoid dihydro form. In the course of its isolation and purification, the compound undergoes oxidation and tautomization to the 7,8-dihydro compound.

The enzyme could, however, serve an auxiliary role in the hydroxylation system in vivo. Studies with model pteridines have shown that, during the hydroxylation reaction, the pteridine cycles between the tetrahydro and quinonoid dihydro forms (15). The latter compound is unstable and can tautomerize to the 7,8-dihydro derivative, a reaction that is rapid in the absence of an efficient reducing system such as dihydropteridine reductase and TPNH. If the same cycle and side reaction operate in vivo with the biotin derivatives, the presence of dihydrofolate reductase could function to salvage any cofactor that had undergone transformation to the 7,8-dihydro derivative; otherwise it would be irreversibly lost from the hydroxylation system.

If the dihydro derivative is an intermediate in the biosynthesis of unconjugated pteridines, as it appears to be in the folate series (31, 32), dihydrofolate reductase could also be involved in the initial reduction of these derivatives to the tetrahydro level, the level which they must reach to function as hydroxylation cofactors. It seems likely, therefore, that although dihydrofolate reductase is probably not involved directly in the cyclic functioning of the cofactor in hydroxylation reactions, it may nevertheless play an important secondary role in this kind of system in vivo.

The demonstration that the sepiapterin reductase-catalyzed reaction is a reversible one indicates that sepiapterin, a pigment in amphibia, fish, and insects (33), may also be a normal constituent of mammalian tissues; up to now, however, there have been no reports of its occurrence in animals. The reversibility of the reductase reaction also suggests that this enzyme could be involved in the regulation of the activity of hydroxylating enzymes such as phenylalanine hydroxylase. Sepiapterin reductase could function in this way because it catalyzes a reaction that interconverts a coenzyme-active compound, dihydrobiopterin, and a coenzyme-inactive one, sepiapterin.

Finally, the sepiapterin reductase-catalyzed reaction may be involved in the biosynthesis of biopterin. There is evidence in favor of the idea that sepiapterin is synthesized in Drosophila by a condensation of a reduced derivative of 2-amino-4-hydroxypteridine with a 3-carbon unit (34). The next step in this biosynthetic pathway could involve the reduction of sepiapterin to dihydrobiopterin, the reaction catalyzed by sepiapterin reductase.

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