The Isolation and Characterization of Dihydropteridine Reductase from Sheep Liver

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SUMMARY

Dihydropteridine reductase has been obtained from sheep liver in essentially homogeneous form. The enzyme exists as a dimer of molecular weight 41,000 to 42,800. The subunit molecular weight has been determined to be 21,300. In the presence of the quinonoid tautomer of either dihydrobiopterin or dihydro-6,7-dimethylpterin, DPNH is a better substrate than TPNH. Evidence indicates that the activity of this enzyme is far greater than that of the known pterin-dependent hydroxylases in all tissues examined.

More recently, it was reported that with 7,8-dihydrobiopterin, (the form of the cofactor that had been isolated from rat liver), a third enzyme is an essential component of the hydroxylation system (7). This enzyme is dihydrofolate reductase, which catalyzes the TPNH-mediated reduction of 7,8-dihydrobiopterin to the tetrahydro form according to Reaction d, Fig. 1 (7). Starting with 7,8-dihydrobiopterin, this reaction initiates the hydroxylation cycle (i.e. Reactions a and e), by converting the cofactor to the active, tetrahydro form (7). This reaction may also serve to salvage any 7,8-dihydrobiopterin that might be formed from the unstable quinonoid-dihydro isomer by nonenzymatic tautomerization (Fig. 1, Reaction c) (8). It should be noted that the sum of Reactions a and e or a, c, and d in Fig. 1 equals Equation 1.

In 1969, Nielsen et al. (9) determined the nucleotide specificity of crude sheep liver dihydropteridine reductase for the first time. They found that the enzyme can utilize either TPNH or DPNH, but that it is far more active with DPNH.

In the present paper, we report the purification to homogeneity and the chemical and physical characterization of sheep liver dihydropteridine reductase. Our kinetic studies confirm the nucleotide specificity reported by Nielsen et al. (9) and Scrimgeour and Cheema (10).

EXPERIMENTAL PROCEDURE

Materials

Phenylalanine hydroxylase (11), tyrosine hydroxylase (12), and glucose dehydrogenase (13) were prepared as previously described. Crystalline catalase, horseradish peroxidase, 3-hydroxybutyrate dehydrogenase, and TPNH were obtained from Boehringer-Mannheim Corp. DPNH and iodoacetamide were obtained from Sigma Chemical Co. 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride (DMPH, 2HCl) was obtained from Aldrich. Tetrahydrobiopterin was the kind gift of Dr. R. F. Long of Hoffmann-La Roche, Inc. Methotrexate was the gift of Dr. D. Kaufman of the National Institutes of Health. L-[3,5,3H]tyrosine was obtained from New England Nuclear and was purified as described (14) using the modification of Shiman et al. (12). 3,3'-Dithiobis-[6-nitrobenzoic acid] was obtained from Eastman Organic Chemicals, p-dimethylaminobenzaldehyde was obtained from Fisher Scientific Co.; folate acid and ammonium sulfate (enzyme grade) were obtained from Mann Research Laboratories. Dihydrofolate was prepared by the method of Futterman (15). DEAE-cellulose (DE 52 micro-
granular anion exchanger) was obtained from Whatman. Calcium phosphate gel was prepared by the method of Keilin and Hartree (16).

**Methods**

**Assay of Dihydropteridine Reductase**

*Method 1—* Dihydropteridine reductase was assayed spectrophotometrically by following the disappearance of reduced pyridine nucleotide (DPNH or TPNH) at 340 nm at 25°. In this assay, quinonoid dihydropterin was generated with peroxidase (Equation 2).

\[
\text{Tetrahydropterin} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{quinonoid dihydropterin} + 2H_2O \tag{2}
\]

Equation 2 thus replaces Reaction a shown in Fig. 1. The amount of quinonoid dihydropterin formed under these assay conditions (reduced pyridine nucleotide omitted) was determined by measurement of the absorption spectrum of the pterin product. The results demonstrated that there is a quantitative conversion of the tetrahydropterin to the quinonoid dihydropterin, i.e. the concentration of the quinonoid dihydro compound in the reaction mixture is equal to the concentration of the tetrahydro compound present initially.

The standard assay contained the following components in micromoles: Tris-HCl buffer, pH 7.2, 50; hydrogen peroxide, 0.9; reduced pyridine nucleotide, 0.1; DMPH4, 0.01; peroxidase, 8 µg; and dihydropteridine reductase. The final volume of the reaction mixture was 1.0 ml. Blank rates (i.e. the nonenzymatic conversion of quinonoid dihydropterin to tetrahydropterins to quinonoid dihydropterins (17), is a modification of the method of Nielsen et al. (18). In this case, tyrosine hydroxylase and tyrosine replace phenylalanine hydroxylase and phenylalanine, respectively, in Fig. 1, Reaction a. Tritium release from L-[3,5-3H]tyrosine during the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine was measured by the method of Nagatsu et al. (19). Quinonoid-dihydromethylpterin (quinonoid-DMPH4) was prepared by the method of Kaufman (8). Each reaction mixture contained the following components in micromoles: Tris-maleate buffer, pH 6.8, 50; L-tyrosine (containing 200,000 cpm of L-[3,5-3H]tyrosine), 0.05; α-glucose, 62.5; quinonoid-dihydromethylpterin, 0.01; reduced pyridine nucleotide, 0.005; catalase, 200 µg; excess tyrosine hydroxylase and glucose dehydrogenase; and bovine adrenal medulla dihydropteridine reductase. Incubation was carried out for 30 min at 37° with constant shaking.

Dihydrofolate reductase was assayed by the method of Osborn and Huennekens (20), except that 0.05 M potassium phosphate buffer, pH 6.8, was used. Optical density changes were converted to enzyme units using 5050 M⁻¹ cm⁻¹ as the molar extinction coefficient for the reduction of dihydrofolate to tetrahydrofolate at 340 nm (21).

Phenylalanine hydroxylase was assayed by measurement of the increase in optical density at 340 nm due to the oxidation of tetrahydropterins (22).

In all cases, 1 unit of enzyme activity is defined as that amount of enzyme which will catalyze the oxidation of 1 µmole of reduced pyridine nucleotide at 25° in 1 min.

Protein was determined by the method of Warburg and Christian (23).

**Extraction for Tissue Survey**

Various tissues of the rat were examined for dihydropteridine reductase activity. The tissues were prepared in the following manner. Animals were killed by decapitation; the various tissues were rapidly removed, weighed, and homogenized in 3 volumes of cold 0.03 M potassium phosphate buffer, pH 7.2, using a glass homogenizer equipped with a Teflon pestle. Adrenal it is necessary to add relatively large amounts of the rat liver enzyme to this assay. In the presence of low amounts of pterin, a portion of the pterin is bound to the hydroxylase making it unavailable for the reductase reaction. This results in lower reductase activity than is seen when the reaction is coupled to pterin hydroxylase. However, when small amounts of reductase are measured at low pterin concentration and the amount of hydroxylase can therefore be lowered, the activity of the hydroxylase- and peroxidase-coupled reactions is equal.
medulai were dissected from the capsule and cortex prior to homogenization. The homogenates were centrifuged for 1 hour at 45,000 x g. The supernatant material was assayed for the presence of dihydropteridine reductase. In addition, when the brain was examined, cerebrum, cerebellum, midbrain, and hindbrain were homogenized separately and subfractionated by the method of Gray and Whittaker (24).

Amino Acid Analysis

Alkylation or alkylation plus reduction was performed by the method of Adelstein and Kuehl (25). Excess reagents were removed by exhaustive dialysis. Samples were dried in a vacuum desiccator and taken up in 1.0 ml of 6 N HCl. The hydrolysates were flushed three times with nitrogen and were sealed. Hydrolysis was carried out for 24 or 72 hours at 105°. The samples were dried over NaOH pellets. In order to remove excess ammonia, the samples were adjusted to pH 11 to 12 with NaOH and, once again, were dried in a vacuum desiccator. Finally, the samples were dissolved in water and adjusted to pH 2.2.

Amino acid analyses were performed by the method of Spackman et al. (26) using a Spinco model 120 amino acid analyzer. Tryptophan and tyrosine content of sheep liver dihydropteridine reductase was measured by the method of Beaven and Holiday (27). Tryptophan was also measured spectrophotometrically by the method of Spies and Chambers (28). Cysteine was measured by the method of Ellman (29) and by amino acid analysis of the carboxymethylated residue.

Disc Gel Electrophoresis

Polyacylamide gel electrophoresis was carried out according to Ornstein (30) and Davis (31). Gels of differing acrylamide content were formed by varying the amount of Reagent C (20 g of acrylamide monomer and 0.8 g of N,N'-methylene-bisacrylamide diluted to 100 ml with distilled water) added to the gel polymerization mixture. Gels were stained overnight with 0.05% Coomassie brilliant blue and were destained in 7.5% acetic acid.

Electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate was carried out by the method of Weber and Osborn (32).

Determination of Physical Properties

Sucrose density gradients were prepared with a Beckman gradient former in 5-ml polycarbonate tubes. A solution containing 20% sucrose (w/v) buffered with 0.01 M Tris-HCl, pH 8.0, was used in the dense solution syringe. The light solution was 0.01 M Tris-HCl, pH 8.0. Samples were centrifuged at 178,880 x g for 21 hours in a Beckman L2-65-B centrifuge. The gradients were monitored for protein by passage through a Gilford spectrophotometer equipped with a flow cell. Ten-drop fractions of approximately 0.2 ml were collected.

Stokes radii were determined as described by Ackers (33) on a Sephadex G-100 column measuring 2.5 x 35 cm.

RESULTS

Purification of Sheep Liver Dihydropteridine Reductase—All of the steps in the purification of this enzyme as well as both the rat and human liver dihydropteridine reductases were carried out in the cold at 2–4°, unless specified otherwise. Mechanical stirring was used during all additions. Ammonium sulfate precipitates were collected by centrifugation for 20 min at 18,000 x g, and ethanol precipitates were generally obtained by centrifugation for 15 min at 4,000 x g. Water which had been deionized and then glass-distilled was used throughout these procedures. Enzyme fractions were assayed with DPNH using the peroxidase-coupled system (Method 1). The procedure carried out for the sheep liver enzyme through the calcium phosphate gel step is essentially the same as the one we have previously described (3).

Extraction—Sheep livers, removed immediately after death, were frozen at -20° until ready for use. When the extraction was to be performed, the liver was allowed to thaw partially at room temperature. It was then cut into small pieces in the cold room and homogenized with 1.5 volumes of cold 0.03 M acetic acid for 1 min at about three-fourths full speed in a Waring Blender (model 1041). Another 1.5 volumes of 0.03 M acetic acid were added and the blending was continued for another minute at about one-third full speed. The mixture was centrifuged for 35 min at 4000 x g.

First Ammonium Sulfate Fractionation—Ammonium sulfate, (29.3 g) was added to each 100 ml of extract. The mixture was centrifuged and the residue was discarded. An additional 17.5 g of ammonium sulfate were added and the centrifugation was repeated. The precipitate was dissolved in approximately one-seventh of the original volume of 0.025 M Tris-HCl buffer, pH 7.4, and the solution was dialyzed overnight against a large excess of 0.01 M Tris-HCl buffer, pH 7.4, in a rocking dialyzer.

Zinc-Ethanol Fractionation—The dialyzed solution from the previous step was diluted with 0.01 M Tris-HCl buffer, pH 7.4, to about 12.5 mg of protein per ml and treated with 0.135 volume of 0.2 M zinc acetate.

The cloudy solution was fractionated with ethanol between 0 and 6.5% (v/v) ethanol at -1° and between 6.5 and 25% (v/v) ethanol at -6°. The precipitate collected from the higher alcohol concentration was dissolved in a volume of 0.033 M potassium phosphate buffer, pH 6.8, equal to one-eighth of the volume after dilution with Tris-HCl buffer. Any undissolved material was removed by centrifugation and the supernatant fluid was dialyzed overnight against a large excess of the same buffer.

Alkaline Ammonium Sulfate Fractionation—The dialyzed solution was diluted with 0.033 M potassium phosphate buffer, pH 6.8, to a protein concentration of about 10 mg per ml. To each 100 ml of diluted solution, 14 g of ammonium sulfate were added. The pH of the solution was then adjusted to 8.2 by the dropwise addition of 1 N ammonium hydroxide. Another 23.8 g of ammonium sulfate were added to each 100 ml of solution (based on original diluted volume plus the volume of ammonium hydroxide which was added). After centrifugation the precipitate was discarded. An additional 18.3 g of ammonium sulfate were added to each 100 ml of solution, as before. The precipitate was dissolved in 0.01 M Tris-HCl buffer, pH 7.4, and dialyzed against the same buffer overnight.

Adsorption and Elution from Calcium Phosphate Gel—The solution from the alkaline ammonium sulfate step was diluted with 0.01 M Tris-HCl buffer, pH 7.4, to a protein concentration of about 10 mg per ml. Approximately 0.3 volume of calcium phosphate gel (dry weight = 20 to 22 mg per ml) was added (the amount of gel necessary to adsorb 85% of the activity was determined on a trial run). After centrifugation, the supernatant fluid was discarded. The gel was eluted successively with 0.005 M and 0.01 M potassium phosphate buffer, pH 6.8, in each case with a volume of eluting solution equal to the starting volume.
Dialysis against two changes of 0.01 M Tris-HCl buffer, pH 7.8.

**DEAE-cellulose Chromatography**—The dialyzed enzyme was applied to a DEAE-cellulose column (2.5 x 35 cm) which had been previously equilibrated with the dialyzing buffer. The flow rate was 1 ml per min. Following application of the enzyme, the column was washed with equilibrating buffer until no further protein was eluted (approximately 2 bed volumes). A 2-liter linear gradient from 0.0 to 0.5 M KCl in 0.01 M Tris-HCl buffer, pH 7.8, was used to elute the enzyme. Five-milliliter fractions were collected and these were monitored for enzyme activity and protein concentration. The most active fractions were pooled.

**Sephadex Gel Filtration**—The pooled fractions from the previous step were concentrated to 20 mg of protein per ml with the use of an Amicon ultrafiltration device. No more than 2 ml per run were applied to a Sephadex G-100 column (2.5 x 35 cm) which had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.4. The flow rate was 6 ml per hour. Fractions (1.25 ml) were collected and monitored for enzyme activity and protein concentration. The fractions with the highest specific activities were pooled. Most of the activity eluted between 70 and 95 ml. A summary of the purification of sheep liver dihydropteridine reductase is presented in Table I.

**Purification of Human Liver Dihydropteridine Reductase**—Adult male rats were killed by decapitation and the livers were quickly removed. The livers were cut into small pieces and homogenized as described in the extraction procedure for the sheep liver enzyme. After centrifugation, the supernatant fraction was adjusted to pH 7.4 by the dropwise addition of 1 N KOH. The precipitate that formed was removed by centrifugation for 10 min at 10,000 X g.

**Ammonium Sulfate Fractionation**—To each 100 ml of extract, 35 g of solid ammonium sulfate were added over a period of 30 min. Following the addition, the mixture was stirred for an additional 30 min and then centrifuged. An additional 10 g of solid ammonium sulfate were added to each 100 ml (based on extract volume) of the supernatant over a period of 15 min. The mixture was stirred for an additional 30 min and was centrifuged as above. The precipitate was dissolved in 0.01 M Tris-HCl buffer, pH 7.8, and adjusted to a final volume equal to 20% of the extract volume.

**DEAE-cellulose Chromatography**—The ammonium sulfate fraction was dialyzed against two changes of 0.01 M Tris-HCl buffer, pH 7.8 for 3 hours each. The enzyme was applied to a DEAE-cellulose column as described in the purification of the sheep liver enzyme and was eluted in the same manner. A summary of the purification of the rat liver dihydropteridine reductase is presented in Table II.

**Catalytic Properties of Sheep Liver Dihydropteridine Reductase**

The enzyme used for the following studies was shown to contain less than 10% impurities as measured by disc gel electrophoresis.

**Assay Requirements**—Fig. 2 shows the proportionality between enzyme activity and protein concentration for both the DPNH-
TABLE III
Requirements of dihydropteridine reductase assay

The complete reaction mixture contained 0.12 mM DPNH and 7.2 μM DMPH₄. The concentrations of the other components are given under "Methods."

<table>
<thead>
<tr>
<th>Components</th>
<th>nmoles/min</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete reaction</td>
<td>41.4</td>
<td>100</td>
</tr>
<tr>
<td>- DMPH₄</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>- DPNH</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>- Peroxidase</td>
<td>2.1</td>
<td>5.1</td>
</tr>
<tr>
<td>- H₂O₂</td>
<td>8.3</td>
<td>20.0</td>
</tr>
</tbody>
</table>

and TPNH-dependent reduction of the quinonoid-pterin. As shown in Table III, enzymic activity is dependent on all components of the reaction mixture being present. The activity observed in the absence of added peroxide could be completely eliminated by the addition of an excess of catalase, an indication that some peroxide was being generated from the nonenzymatic oxidation of the tetrahydropterin.

Effect of Substrate Concentration on Sheep Liver Dihydropteridine Reductase—Limiting Michaelis constants for quinonoid-dihydrobiopterin and DPNH were determined by the method of Florini and Vestling (34). Fig. 3 shows the effect of DPNH concentration on enzymic activity at varying concentrations of quinonoid-dihydrobiopterin and Fig. 4 shows the effect of quinonoid-dihydrobiopterin concentration on enzymic activity at varying DPNH concentrations. A plot of the reciprocal of the apparent Vₘₐₓ (1/Vₘₐₓ vs. DPNH) versus reciprocal of molar concentration of DPNH. The following concentrations of DPNH were used: 16.5 μM (O—O); 11.0 μM (●—●); 6.6 μM (△—△); 4.4 μM (▲—▲); and 2.2 μM (□—□). Under experimental conditions, quinonoid-dihydrobiopterin concentration is equal to the concentration of added tetrahydrobiopterin (biopterin-H₄). The concentrations of the other components are described under assay Method 1 under "Methods."

It was of some interest to determine the relative rates of oxidation of DPNH and TPNH by the sheep liver reductase in an

TABLE IV
Summary of kinetic data for sheep liver dihydropteridine reductase

<table>
<thead>
<tr>
<th>Pterin used</th>
<th>Pyridine nucleotide used</th>
<th>Kₘ (μM)</th>
<th>Vₘₐₓ (μM)</th>
<th>Vₘₐₓ nmoles/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopterin-H₄</td>
<td>DPNH</td>
<td>1.0</td>
<td>4.0</td>
<td>42.8</td>
</tr>
<tr>
<td>Biopterin-H₄</td>
<td>TPNH</td>
<td>0.9</td>
<td>70.9</td>
<td>12.4</td>
</tr>
<tr>
<td>DMPH₄</td>
<td>DPNH</td>
<td>15.2</td>
<td>5.7</td>
<td>132.3</td>
</tr>
<tr>
<td>DMPH₄</td>
<td>TPNH</td>
<td>6.3</td>
<td>80.6</td>
<td>18.0</td>
</tr>
</tbody>
</table>

lower Kₘ and a higher Vₘₐₓ), thus confirming the observation of Nielsen et al. (9).

It was of some interest to determine the relative rates of oxidation of DPNH and TPNH by the sheep liver reductase in an
The pyridine nucleotide specificity of sheep liver dihydropteridine reductase in the presence of a mixture of TPNH and DPNH. The reaction mixtures contained the following components in micromoles: potassium phosphate, pH 8.2, 30; TPNH or DPNH, 0.15; phenylalanine, 1.2; tetrahydrobiopterin, 0.024; catalase, 200 µg; phenylalanine hydroxylase, 80 µg; and dihydropteridine reductase, 100 µg. The volume was 1.0 ml and the temperature was 25°C. At Arrow 1, 30 µmoles of n-3-hydroxybutyrate and 15 µg of 3-hydroxybutyrate dehydrogenase were added. At Arrow 2, 0.1 µmole of TPNH was added to the cuvette that originally contained DPNH and the pen position was readjusted so that it was still on the chart.

An experiment was designed to present the enzyme with an equal mixture of DPNH and TPNH in such a manner that the oxidation of only TPNH would be measured. This was accomplished by the addition of a regenerating system for DPNH (a DPN-specific dehydrogenase and its substrate). The results of this experiment are shown in Fig. 6. For this experiment, the coupled hydroxylase-reductase assay was used in which the phenylalanine-dependent oxidation of the reduced pyridine nucleotides is followed at 340 nm (1). It should be noted that the reductase was in excess of the hydroxylase so that the reaction rate is independent of the nature of the pyridine nucleotide used. Two reaction mixtures, one with DPNH (Curve a) and the other with TPNH (Curve b) were assayed. Initially, as expected, the rate of oxidation of both pyridine nucleotides were identical. At Arrow 1, an excess of DPN-specific 3-hydroxybutyrate dehydrogenase and 3-hydroxybutyrate was added to both cuvettes; in the DPNH mixture, the DPN that had been formed during the prior period was reduced. At Arrow 2, TPNH was added to the DPNH mixture. In the presence of both pyridine nucleotides, there was no detectable decrease in absorbance (i.e. no detectable TPNH oxidation), an indication that DPNH was being oxidized exclusively. This result demonstrates that in the presence of both DPNH and TPNH, only DPNH is utilized by the sheep liver dihydropteridine reductase.

Effect of Methotrexate on Sheep Liver Dihydropteridine Reductase—It has been reported previously that folic acid antagonists such as aminopterin could inhibit phenylalanine hydroxylation in the range of 10^{-5} to 10^{-4} M (35). The results of these experiments, however, were obtained with the use of a coupled system (both rat liver phenylalanine hydroxylase and sheep liver dihydropteridine reductase were present), and although we suggested that dihydropteridine reductase was the sensitive component, we did not prove it. Fig. 7 shows that at 69 µM methotrexate, dihydropteridine reductase is inhibited and that this inhibition is competitive with respect to bioperin. The K_i for methotrexate was calculated to be 38 µM. Methotrexate was also found to inhibit rat liver phenylalanine hydroxylase activity (measured as described under “Methods”); however, this inhibition was only observed at concentrations of methotrexate ten times higher than those required to produce inhibition of the reductase. In addition, it was found that the inhibition of phenylalanine hydroxylase was noncompetitive with respect to tetrahydrobiopterin.

Specificity of Other Dihydropteridine Reductases—Partially purified preparations of rat liver and human liver dihydropteridine reductases were tested for their nucleotide specificity in the presence of quinonoid-dihydrobiopterin. The data presented in Table V indicate that for both enzymes DPNH is relatively more effective than TPNH based on both V_{max} and K_m values.
Table VI

Distribution of dihydropteridine reductase in various tissues of rat

<table>
<thead>
<tr>
<th>Tissue</th>
<th>nmoles of DPNH oxidized/min/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>10.4</td>
</tr>
<tr>
<td>Liver</td>
<td>8.2</td>
</tr>
<tr>
<td>Brain</td>
<td>2.0</td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>1.6</td>
</tr>
<tr>
<td>Heart</td>
<td>0.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.5</td>
</tr>
<tr>
<td>Lung</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table VI shows the distribution of dihydropteridine reductase in various tissues of the rat. Tissues were prepared as described under “Methods.” Each region of the brain examined contained approximately the same amount of reductase and the majority of the activity was found in the supernatant fraction. The synaptosomes in all regions were found to contain approximately 5% of the total activity.

Mussacchio has reported the presence of a TPNH-dependent dihydropteridine reductase in bovine adrenal medulla (36). Bovine adrenal medullary dihydropteridine reductase was prepared in the same manner as described for the rat-tissue survey and was assayed with both DPNH and TPNH according to assay Methods 1 and 3. In both assays, the enzyme activity was 15 times higher with DPNH than with TPNH (1.5 units per g of tissue with DPNH and 0.1 unit per g of tissue with TPNH). We were also unable to confirm the report that the adrenal enzyme is insensitive to inhibition by methotrexate. We found that at 35 μM methotrexate (and 10 μM DMPH), the adrenal enzyme was inhibited 60%.

Measurement of Dihydrofolate Reductases in Rat and Sheep Liver—Because of its role in the hydroxylation reaction when 7,8-dihydrobiopterin is used as the cofactor, it was of interest to determine the nucleotide specificity of rat liver and sheep liver dihydrofolate reductase. As a source of the sheep liver enzyme, fractions from the DEAE-cellulose step of dihydropteridine reductase preparation containing dihydrofolate reductase activity were used (the peak fractions from the Sephadex step are completely devoid of dihydrofolate reductase activity). A rat liver extract was used as the source of the rat liver dihydrofolate reductase. The activity of the enzyme from rat liver was 15 times higher and that from sheep liver was six times higher with TPNH than with DPNH, thus confirming the previous findings (37) with the chicken liver enzyme that dihydrofolate reductase (at neutral pH) is relatively specific for TPNH. In addition, the activity of the sheep liver enzyme was six times higher with TPNH than with DPNH when 7,8-dihydrobiopterin replaced dihydrofolate as substrate.

Physical Properties of Sheep Liver Dihydropteridine Reductase

Disc Gel Electrophoresis—Electrophoresis of the purified protein was performed as described under “Methods.” The peak fractions produced a single band when stained with Coomassie brilliant blue, a result consistent with the presence of a single protein (Fig. 8). A single band was also observed in gels subjected to electrophoresis in 0.14 M acetate buffer, pH 4.3.

Amino Acid Composition—The results of an amino acid analysis carried out on pure sheep liver dihydropteridine reductase are shown in Table VII. The results have been calculated on the basis of a subunit molecular weight of 21,000. The cysteine value was the same for both reduced and nonreduced carboxymethylated protein. The partial specific volume was calculated from the amino acid analysis and found to be 0.73. It was found that the cysteine residue could not be measured in the absence of sodium dodecyl sulfate using the procedure of Ellman (29). The addition of 200 μg of sodium dodecyl sulfate to the reaction mixture gave a value of 1.08 residue per subunit (mol wt = 21,000). This result indicates that the cysteine residue is reduced but not exposed in the native protein. In addition, the enzyme was inhibited only 12% by 1 × 10⁻⁴ M p-chloromercuribenzoate, an indication that it is unlikely that the sulfhydryl group is involved in the enzymatic reaction.

Molecular Weight Determination Using Different Per Cent Polyacrylamide Gels—The molecular weight of the reductase was determined by the method of Hedrick and Smith (38). Fig. 9 shows a plot of the slope of the relative migration of the reductase and of protein standards versus gel concentration as a function of molecular weight. The results indicate a molecular weight of 41,000 for sheep liver dihydropteridine reductase.

Molecular weights of polypeptides may also be determined with polyacrylamide gels containing sodium dodecyl sulfate (32). Fig. 10 shows a plot of the log of the molecular weight versus the relative migration for five standard proteins and for sheep liver dihydropteridine reductase. A molecular weight of 21,300 was calculated for the reductase. The results shown in Figs. 9 and 10...
### TABLE VII

**Amino acid analysis**

<table>
<thead>
<tr>
<th>Residue</th>
<th>24 hrs</th>
<th>72 hrs</th>
<th>Residues per subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles/mg enzyme</td>
<td>24 hrs</td>
<td>72 hrs</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.63</td>
<td>0.67</td>
<td>12.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.20</td>
<td>0.19</td>
<td>4.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.04</td>
<td>0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>S-carboxymethyleysteine</td>
<td>0.01</td>
<td>0.01</td>
<td>1.0</td>
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<tr>
<td>Aspartic acid</td>
<td>0.75</td>
<td>0.74</td>
<td>15.1</td>
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<td>Threonine</td>
<td>0.65</td>
<td>0.63</td>
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<td>Serine</td>
<td>0.91</td>
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</tr>
<tr>
<td>Glutamic acid</td>
<td>1.18</td>
<td>1.14</td>
<td>21.9</td>
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<td>Proline</td>
<td>0.41</td>
<td>0.37</td>
<td>8.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.08</td>
<td>1.00</td>
<td>21.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.28</td>
<td>1.25</td>
<td>25.4</td>
</tr>
<tr>
<td>Valine</td>
<td>0.61</td>
<td>0.70</td>
<td>12.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.16</td>
<td>0.15</td>
<td>3.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.04</td>
<td>0.05</td>
<td>4.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.86</td>
<td>0.85</td>
<td>17.1</td>
</tr>
<tr>
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<td>0.01</td>
<td>0.01</td>
<td>2.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>0.03</td>
<td>5.6</td>
</tr>
<tr>
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<td>2</td>
<td></td>
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<tr>
<td>Tryptophan*</td>
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<tr>
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</tr>
<tr>
<td>Cysteine*</td>
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<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by the method of Beaven and Holiday (27).

+ Determined by the method of Spies and Chambers (28).

+ Determined by the method of Ellman (29).

---

**Fig. 9.** Determination of molecular weight of sheep liver dihydropteridine reductase by varying gel concentration in polyacrylamide electrophoresis. BSA1 and BSA2, bovine serum albumin monomer and dimer, respectively; OVA, ovalbumin. Indicate the presence of 2 subunits in the native form of dihydropteridine reductase.

The Stokes radius of the enzyme was measured with the use of a Sephadex G-100 column. Gel pore size was calculated from the data presented in Table VIII. Based on a gel pore size of 11.63 nm, a Stokes radius of 2.68 nm was calculated for sheep liver dihydropteridine reductase. From the Stokes radius, a diffusion constant of $8.01 \times 10^{-7} \text{ cm}^2 \text{s}^{-1}$ was also calculated.

The sedimentation constant for the reductase was measured by the method of Martin and Ames (39). The data, summarized in Table IX, indicate a value of 3.80 S for the sedimentation constant for this enzyme.

The molecular weight of dihydropteridine reductase calculated from the diffusion constant, sedimentation constant, and partial specific volume was found to be 42,800. This value is in excellent agreement with the value determined from the data in Fig. 9.

**DISCUSSION**

With the use of the peroxidase-coupled assay to follow the activity, we have purified to homogeneity dihydropteridine re-
ductase from sheep liver. Its molecular weight, determined by two independent methods, is between 41,000 and 42,800. The enzyme appears to be composed of 2 subunits, each with a molecular weight of approximately 21,000. Our values are somewhat different from those reported recently, in preliminary form, by Scrimgeour and Cheema (10). From sedimentation equilibrium data, these workers calculated a molecular weight for the sheep liver reductase of 52,000, and from polyacrylamide gel electrophoresis in sodium dodecyl sulfate, they obtained a value of 27,000 for the molecular weight of the subunits.

With our homogeneous enzyme preparation, we have confirmed the finding (9), made with a relatively crude enzyme preparation, that the reductase is more active with DPNH than with TPNH. With tetrahydrobiopterin as the source of the quinonoid dihydropterin substrate, the \( K_m \) for DPNH is about one-fifteenth that of TPNH, and the \( V_{max} \) with DPNH is more than three times as great as it is with TPNH. Similar differences in these kinetic constants were observed when the cofactor analog, dimethyltetrahydropterin, served as the source of the pterin substrate. For reasons that are not clear, our values for the \( K_m \) values for DPNH and TPNH (in the presence of dimethylpterin) are only one-tenth to one-sixth as great as those reported by Scrimgeour and Cheema (10).

The finding that dihydropteridine reductase from all tissues examined is more active with DPNH than with TPNH, whereas dihydrofolate reductase is relatively specific for TPNH, indicates that the nucleotide specificity of the phenylalanine hydroxylating system in any tissue will be determined by two variables: (a) the state of oxidation of biopterin and (b) the relative activities of dihydropteridine and dihydrofolate reductases.

As can be seen in Fig. 1, if, under any condition, 7,8-dihydrobiopterin is the predominant form of the cofactor, the nucleotide specificity of the hydroxylation reaction will be determined by the reductase reaction that is rate-limiting (i.e. either reaction \( d \) or \( e \)). If the dihydropteridine reductase reaction (Reaction \( e \)) is limiting, the initial rate of the hydroxylation reaction will be faster with DPNH than with TPNH; if the dihydrofolate reductase-catalyzed reaction (Reaction \( d \)) is limiting, the hydroxylation reaction will be faster with TPNH than with DPNH.

If, on the other hand, tetrahydrobiopterin is the predominant form of the cofactor, Reaction \( d \) will play only a minor role in the conversion of phenylalanine to tyrosine and the nucleotide specificity of the system will be determined by the specificity of dihydropteridine reductase (Reaction \( e \)), i.e. the hydroxylation reaction will be relatively specific for DPNH.

The above considerations provide a plausible explanation for the earlier observation that the hydroxylation reaction studied with relatively impure enzyme preparations is more active with TPNH than DPNH (1). The protein fractions used in the earlier study were still unresolved with respect to the endogenous pterin cofactor, which was subsequently identified as 7,8-dihydrobiopterin (5). Under these conditions, the first step leading to tyrosine formation is Reaction \( d \) (Fig. 1), catalyzed by the TPNH-specific dihydrofolate reductase. That this reduction was the rate-limiting step in the hydroxylation reaction is supported by the observation that under these conditions, the hydroxylation reaction is characterized by a pronounced lag period that can be overcome by preliminary incubation of 7,8-dihydrobiopterin with TPNH and a sheep liver fraction containing dihydrofolate reductase (4, 40). With tetrahydrobiopterin, there is no lag period (7). Thus, TPNH was more effective than DPNH because the two conditions leading to this specificity were fulfilled: the cofactor was present in the 7,8-dihydro form, and dihydrofolate reductase was limiting.

Dihydropteridine reductase catalyzes a reaction that is similar to the one catalyzed by dihydrofolate reductase. The substrates for both enzymes are dihydropteridines, the quinonoid dihydro isomer in the former case (6), and the 7,8-dihydro isomer in the latter case (41, 42). Although the two enzymes differ in their pyridine nucleotide specificity and in their pterin structure requirements (e.g. 6,7-dimethylpterin is a substrate for the dihydropteridine reductase (43), whereas it is inactive with dihydrofolate reductase (44)), their similarities prompted us to examine the effect of methotrexate, a noncompetitive inhibitor of dihydrofolate reductase (45), on dihydropteridine reductase. We were also interested in testing this inhibitor because of our previous finding that it inhibits a component of the phenylalanine hydroxylation system (35). We found that the drug inhibits dihydropteridine reductase with a \( K_i \) of \( 4 \times 10^{-5} \) m, and that the inhibition is competitive with the pterin cofactor. This \( K_i \) value is close to that reported for aminopterin with sheep liver dihydropteridine reductase (10), although in that case, noncompetitive inhibition was observed. Thus, although both dihydropteridine and dihydrofolate reductases are sensitive to inhibition by antifolate compounds, dihydrofolate reductase is much more sensitive (\( K_i \) for aminopterin is about \( 10^{-7} \) m) (45).

Musacchio et al. (18) have postulated that under certain conditions, bovine adrenal-medullary dihydropteridine reductase may be rate-limiting in the conversion of tyrosine to dihydroxyphenylalanine. Under optimal conditions, the rate of tyrosine hydroxylation was found to be 2 nmoles per min per g of adrenal medulla (18). The present data on the activity of dihydropteridine reductase in adrenal medulla indicate that the activity of the reductase is 750 times greater than that of the hydroxylase. In view of this great excess of reductase over hydroxylase, it seems improbable that the reductase-catalyzed step could limit the rate of tyrosine hydroxylation in this tissue.

The same is true with regard to the role of the reductase in limiting the rate of either tyrosine or tryptophan hydroxylation in brain. Tyrosine hydroxylation in rat brain has been estimated to be 25 nmoles per hour per g of tissue (46), whereas that of tryptophan hydroxylation is about 1 n mole per hour per g of tissue (47). Our value for the activity of rat brain reductase (Table VI) is 5,000 and 120,000 times the rates of tyrosine and tryptophan hydroxylation, respectively. This large excess of dihydropteridine reductase over the known pterin-dependent hydroxylases in brain and the uniform distribution of the reductase in the various regions of this organ suggest the possibility that other pterin-dependent oxygenases, as yet undiscovered, may be present in this tissue.

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