Biochemical Pharmacology, Vol. 27, pp. 1939–1945

0006-2952/78/0801-1939$02.00/0

QUINONOID DIHYDROPTERIN REDUCTASE—I

PURIFICATION AND CHARACTERIZATION OF THE BOVINE BRAIN ENZYME*

HARRY SNADY and JOSÉ M. MUSACCHIO
Department of Pharmacology, New York University Medical Center, New York, NY 10016, U.S.A.

(*Received* 21 *July* 1977; *accepted* 26 *January* 1978)

Abstract—Quinonoid dihydropterin reductase (DHPR, EC 1.6.99.7) from bovine brain was purified 340-fold over the initial extract to an activity of 76 units/mg of protein with an overall recovery of 10 per cent. Analytical polyacrylamide gel electrophoresis, combined with protein staining, indicated that the single band of purified enzyme could have contained no more than 7% contaminants. Furthermore, using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, only one protein band was observed for the SDS-treated enzyme. Using a sucrose density gradient, the sedimentation coefficient and the molecular weight of the native enzyme were determined to be 3.70 and 47,000 ± 1,400 respectively. Molecular weight using 10% SDS–gel electrophoresis was found to be 22,400 ± 230, indicating that the brain reductase is composed of two equivalent subunits. Other characteristics of DHPR which were determined were the isoelectric point (pH 5.7), the K_m for quinonoid 6,7-dimethyl-dihydropterin (18 μM) and the K_m for NADH (26 μM). Purified brain DHPR was used as an antigen to produce specific antibodies to the enzyme. The specificity of the antibody was established according to the following criteria: (1) the antibody was found to be a potent inhibitor of partially purified and homogeneous DHPR; (2) a single precipitin arc formed upon running either partially purified or homogeneous DHPR against the antibody on a double immunodiffusion plate, or by immunoelectrophoresis; (3) the antibody inhibited DHPR activity from rat brain, rat kidney and rat liver; and (4) neither inhibition of activity, nor formation of precipitin arcs was exhibited by the antibody when reacted with other catecholamine-synthesizing enzymes partially purified from different rat tissues.

Quinonoid dihydropterin reductase (DHPR, EC 1.6.99.7) catalyzes the conversion of paraquinonoid dihydropterins (p-H_2P) to tetrahydropterins (H_4P). This enzyme was first found in sheep liver [1] where it functions in the enzymatic conversion of phenylalanine to tyrosine. The enzyme has since been shown to be present in other organs, including brain [2, 3].

A few studies have indicated that DHPR is responsible for the maintenance of the H_4P cofactor for pterin-requiring oxygenases [2–4]. Several of the oxygenases include enzymes involved in the biosynthesis of neurotransmitters, specifically norepinephrine and serotonin. During the hydroxylase-catalyzed reactions, the formation of the hydroxylated compound is coupled to the oxidation of the H_4P to the q-H_2P [5–9]. The reduction of q-H_2P back to the tetrahydro level, catalyzed by DHPR, completes the oxygenase cycle in which the unconjugated pteridine functions as a coenzyme. Since q-H_2P is unstable, it can isomerize to an inactive 7,8-dihydropterin [5, 9–11]. However, the 7,8-isomer is a substrate for folic acid reductase (EC 1.5.1.3) which can reduce it back to H_4P. Thus, to function catalytically, pterin-requiring hydroxylation systems involve at least two or perhaps three enzymes.

In order to further characterize brain DHPR, we purified the enzyme from bovine brain and investigated some of its properties. Specific antibodies to DHPR were produced and the immunochemical properties of the enzyme were studied. The regional and subcellular distribution of the rat brain enzyme is described in the companion paper [12].

MATERIALS AND METHODS

Materials. The following materials were purchased from the indicated companies: fresh bovine brains from Max Insel Cohen, Newark, NY; Diaflo ultrafiltration membranes-type PM 10 from Amicon Corp., Lexington, MA; acrylamide (electrophoresis purity grade), hydroxyapatite Bio-Gel-HT (modified calcium phosphate) *N,N'* - methylene - bis - acrylamide (electrophoresis purity grade), and *N,N,N',N'*-tetramethylethylenediamine from Bio-Rad Laboratories, Richmond, CA; 6,7 - dimethyl - 5,6,7,8 - tetrahydropterin·HCl·1.5 H_2O from Cal-Biochem, San Diego, CA; Freund's adjuvant (complete and incomplete) and Special agar-noble from Difco Laboratories, Detroit, MI; Folin-Ciocalteau phenol reagent from Fisher Scientific Co., Fair Lawn, NJ; fluorescamine from Hoffmann-La Roche, Nutley, NJ; ampholine and electrofocusing equipment from LKB Instruments, Inc., Washington, D.C.; cytochrome *c* (horse heart-lyophilized powder, salt free), Coomassie Brilliant Blue R-250 and ovalbumin (2x crystallized) from Mann Research Labs, New York, NY; albumin from bovine serum (crystallized and lyophilized), nicotinamide adenine dinucleotide reduced from dihydrodiphosphopyridine nucleotide, disodium salt from yeast (grade III), catalase from beef liver (2x

*This work was presented by H. S. in partial fulfillment of the requirements for the degree of Ph.D. in the Department of Pharmacology at New York University.

crystallized), dihydronicotinamide adenine dinucleotide phosphate (triphosphopyridine nucleotide, reduced), peroxidase from horse-radish (salt free powder-type II) and Trizma base [Tris-(hydroxymethyl) aminomethane] reagent grade from Sigma Chemical Co., St. Louis, MI.

Measurement of DHPR activity. DHPR activity was measured according to the method of Nielsen *et al.*[13]. The following reaction mixture, to a final volume of 1 ml, was prepared in a cuvette: 100 μmoles Tris–Cl, pH 6.8; 250–350 μg peroxidase; 1–150 μl DHPR; and 100 μmoles $DMPH_4$ followed within 5 sec by 10 μmoles H_2O_2. The reaction was initiated exactly 25 sec later with the addition of 100 μmoles NADH dissolved in 5 mM Tris–Cl, pH 9.5. A reaction blank, containing no DHPR, was prepared simultaneously. The difference in extinction at 340 nm between the two systems was followed for 3 min in a Beckman model 25 double beam spectrophotometer. The amount of peroxidase added to each cuvette was adjusted to insure that the conversion of $DMPH_4$ to quinonoid 6,7-dimethyltetrahydropterin (q-$DMPH_2$) would not be rate-limiting. The linear part of the curve was used as a measure of the initial velocity. The activity of DHPR is expressed in units defined as μmoles NADH oxidized/min. The non-specific oxidation of NADH was investigated as above but in the absence of $DMPH_4$ and it was found to be negligible.

Protein determination. Protein concentration was determined by the method of Lowry *et al.*[14] in the tissue extracts, using the ammonium sulfate and hydroxyapatite steps. The absorbance at 280 nm was used to measure the concentration of protein in samples from the electrofocusing and preparative electrophoresis steps.

Determination of molecular weight. The approximate molecular weight of DHPR was determined by sucrose density gradient centrifugation[15] and by SDS–polyacrylamide gel electrophoresis[16]. The DHPR used for this purpose was the purified enzyme eluted from the preparative electrophoresis gel. The proteins used as markers in the density gradient centrifugation were assayed as follows: hemoglobin content was determined at 414 nm and cytochrome *c* content at 400 nm; catalase activity was determined by the method of Beers and Sizer[17].

Determination of Michaelis constants. The enzyme preparation used to determine Michaelis constants was obtained from electrophoresis peak fractions. The data is shown as Lineweaver–Burk double-reciprocal plots; Michaelis constants obtained from these plots were calculated by regressing each line according to the method of least squares.

Analytical gel electrophoresis. DHPR eluted from a peak 2 mm cross section of the preparative electrophoretic gel was concentrated by ultrafiltration in a colloid bag to approximately 0.5 mg/ml (50 units/ml). Analytical gel electrophoresis was carried out at 10° according to the method of Maizel[18] using Tris–glycine buffer, pH 8.3, on 10% (w/v) polyacrylamide gels. The system was run at 2 mA/gel tube for a period of 12 hr. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out at 25° according to the method of Weber and Osborn[16] except that the protein sample was initially incubated in 1% SDS–1% 2-mercaptoethanol for 10 min at 90° instead of 37° for 2 hr. Gels were stained for protein with a 0.015% Coomassie Brilliant Blue solution for 3 hr and destained overnight with a 5% acetic acid–0.5% Dowex 1 × 8 resin suspension.

RESULTS

Enzyme purification. Table 1 outlines the various stages of purification of DHPR. All procedures were carried out at 0–4°, unless otherwise specified. Glass distilled water was used to prepare all solutions. Since enzyme activity was noted to decrease when bovine brains were stored frozen, fresh bovine brains (usually two to six) were washed quickly in saline, minced and homogenized in a Waring blender for 1 min in 3 vol. of 0.03 M acetic acid. The crude homogenate was centrifuged at 14,000 *g* for 20 min. The supernatant fraction was considered the initial extract typically having a specific activity of 0.22 units/mg.

Ammonium sulfate precipitation. Solid ammonium sulfate was added to the extract under

Table 1. Purification of quinonoid dihydropterin reductase from bovine brain*

Step	Vol. (ml)	Total units	Total protein (mg)	Specific activity (units/mg)	Yield
Extract	5630	2082	9336	0.22	100
Ammonium sulfate	392	1290	2867	0.45	62
Hydroxyapatite gel eluate	72	826	333	2.48	40
Electrofocusing peak fractions	39	470	14.7	32	23
Electrophoresis peak fractions	45	212	2.8	76	10

*Starting material was 1.87 kg brain. DHPR activity and protein concentration were measured for each step as described in Materials and Methods. Units of enzyme activity were defined as μmoles NADH oxidized/min. The recovery of activity for each stage in the purification compared to the previous stage was as follows: hydroxyapatite gel fractionation, 64 per cent; electrofocusing fractionation, 57 per cent; and preparative electrophoresis fractionation, 45 per cent.

constant stirring to give a saturation of 55%. After 30 min, the mixture was centrifuged at 14,600 *g* for 20 min and the pellet was discarded. Additional solid ammonium sulfate was added to reach 77% saturation; after 30 min, centrifugation was repeated as before. The supernatant fraction was discarded and the 55–77% ammonium sulfate precipitate was dissolved in 0.01 M Tris–Cl buffer, pH 7.4, in a volume equivalent to 7% of the initial extract volume. The resuspended precipitate was dialyzed overnight against 100 vol. of the same Tris buffer. This step provided a 2-fold increase in specific activity.

Hydroxyapatite gel fractionation. The dialyzed 55–77% ammonium sulfate fraction was diluted to a protein concentration of 10 mg/ml. By adding 1 *g* (dry weight) of hydroxyapatite gel/135 units of DHPR, about 90 per cent of the DHPR in the ammonium sulfate fraction was adsorbed. The suspension was stirred for 1 hr and then centrifuged at 14,600 *g* for 10 min. The supernatant fraction was discarded; the gel pellet was successively eluted with 0.0125 M and 0.11 M potassium phosphate buffer, pH 6.8, using 100 ml of each eluting buffer/225 units of adsorbed DHPR. The second eluate, which contained most of the DHPR activity, was adjusted to 1 mM dithiothreitol and concentrated to one-tenth the initial volume using an Amicon cell with PM 10 membranes. This step provided a 5-fold increase in the specific activity.

Isoelectric focusing fractionation. The procedure was carried out as outlined in the LKB 8100 Ampholine Instruction Manual[19]. Carrier ampholine solutions of 40% (w/v) were used to generate the two pH gradients employed (3.5 to 7 and 3.5 to 8). To 15–20 ml (maximum total protein of 130 mg) of the previously concentrated DHPR solution eluted from the hydroxyapatite, sucrose and ampholine were added to make the final concentrations 10 to 15% and 0.5 to 1.0% respectively. This solution was then layered over the sucrose gradient in a three-fourths filled column. After filling the rest of the column, electrofocusing was carried out at a constant voltage of 550–600 V with an initial current of 17–18 mA. The run was considered complete when the current had dropped to a steady 3.5 mA. During the run, the column was cooled with circulating water at 5°. This step resulted in a 15-fold increase in specific activity. Peak activity corresponded to a pH of 5.7 ± 0.08.

Preparative gel electrophoresis. Preparative gel electrophoresis was carried out at 5° according to the method of Maizel[18] using Tris–glycine buffer, pH 8.3, on 7.5% (w/v) polyacrylamide gel. The peak electrofocusing fractions were concentrated to 50–60 units DHPR/ml (1.8 mg protein/ml) by Amicon ultrafiltration (PM 10 membranes) and then layered onto the preparative gel. The system was run at a constant current of 6.5 mA until the DHPR activity was localized in a band around 6 cm from the top of the gel. To recover the DHPR from the preparative gel, the gel was cut into 2 mm cross sections. These sections were crushed in a test tube and mixed with saline (when DHPR was to be used for preparation of antibodies) or eluted with 0.01 M phosphate buffer, pH 7.4, containing 0.1 mM 2-mercaptoethanol (when DHPR was used for any other study). The tubes were centrifuged after 5 hr and the clear supernatant fraction was removed. If a second elution was required, additional eluting buffer was added to the gel pellet and centrifugation was carried out after 8 hr. This step provided another 2.5-fold increase in the specific activity.

The overall purification of DHPR was 345-fold to a specific activity of 76 units/mg of protein with an overall yield of 10 per cent (Table 1). DHPR purified through electrofocusing or electrophoresis was stable at 5° for approximately 2 weeks.

Purity of enzyme. SDS–polyacrylamide gel electrophoresis of the purified enzyme (Fig. 1) revealed the presence of only one protein band. Homogeneity was also shown by immunological

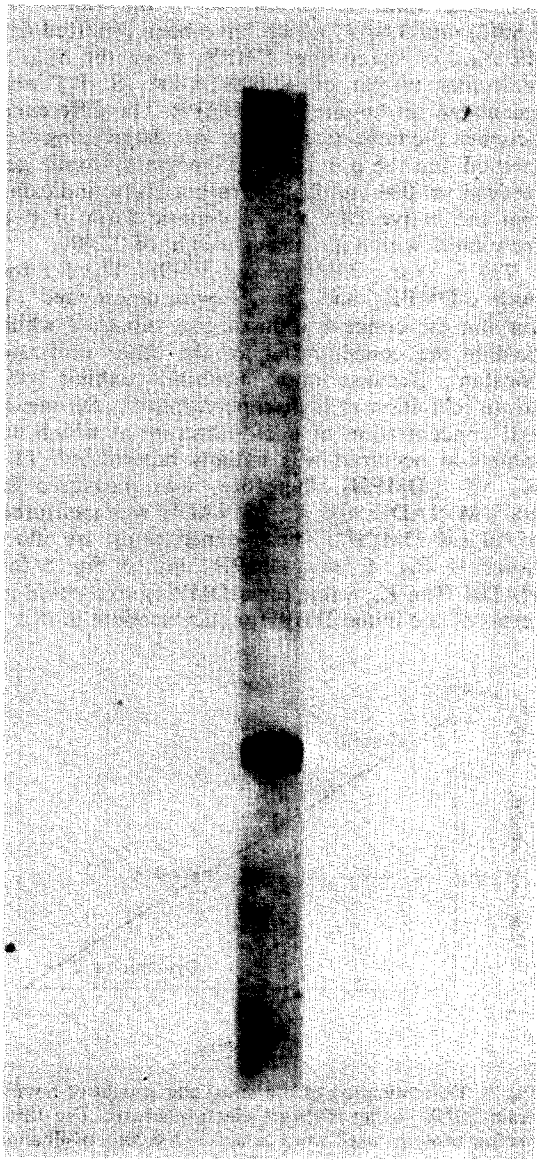

Fig. 1. SDS–polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining of SDS-treated DHPR. Using a 10% SDS–polyacrylamide gel, 10 μg of purified DHPR was subjected to electrophoresis at room temperature for 5 hr at 8 mA/gel.

criteria using double immunodiffusion. Solutions of purified DHPR obtained from preparative electrophoresis gels showed only one precipitin line when reacted against antiserum containing antibodies to DHPR.

Molecular weight. The molecular weight was determined by the method of Weber and Osborn[16]. The mobility of DHPR compared to that of various protein standards in SDS–polyacrylamide gels was used to estimate the molecular weight of the enzyme (Fig. 2). The molecular weight of DHPR was estimated to be 22,400 ± 230 (S. E.).

Using sucrose density gradient centrifugation, the sedimentation coefficient of DHPR was measured by comparing the mobility of the enzyme to the mobility of protein standards (Fig. 3). The $S_{20,w}$ of DHPR was estimated at 3.70 ± 0.07 (S. E.). This value is very close to the values of 3.80[2] and 3.60[4] which have been reported for the $S_{20,w}$ of sheep liver DHPR. From the $S_{20,w}$ a molecular weight of 47,000 ± 1,400 (S. E.) was calculated for bovine brain DHPR. The difference between the molecular weight calculated using this method and the previous SDS–polyacrylamide gel method (47,000 and 22,400, respectively) indicates that the native DHPR is a dimeric form of two monomers with a molecular weight of 22,400.

The K_m for q-DMPH$_2$ and NADH. The K_m for both q-DMPH$_2$ and NADH was determined by varying the concentration of one substrate while holding the concentration of the other substrate constant. Because both substrates exhibit substrate inhibition at high concentrations, the maximal concentration of each substrate at which no inhibition occurred was initially determined. The K_m of q-DMPH$_2$, therefore, was measured at 100 μM NADH; the K_m of NADH was measured at 50 μM DMPH$_4$. These experiments are illustrated in Fig. 4 for q-DMPH$_2$ and in Fig. 5 for NADH. The K_m value for q-DMPH$_2$, expressed in terms of the initial DMPH$_4$ concentration, is 18 ± 3

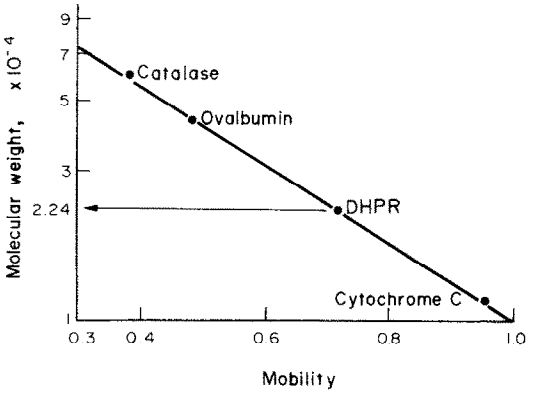

Fig. 2. Determination of the molecular weight of bovine brain DHPR using SDS–gel electrophoresis. The three marker proteins used were catalase (250,000), ovalbumin (45,000) and cytochrome *c* (12,400). The marker proteins and DHPR were all pretreated with SDS and each point on the graph represents the mean of three separate determinations. The arrow indicates the molecular weight (22,400) corresponding to the mobility measured for SDS-treated DHPR, 0.717 ± 0.002 (S. E.).

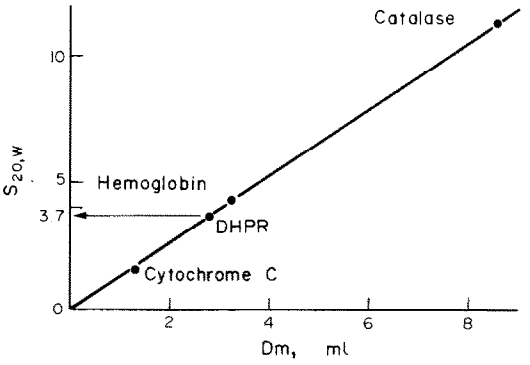

Fig. 3. Determination of the sedimentation coefficient ($S_{20,w}$) of bovine brain DHPR. DHPR (28 units), purified through the hydroxyapatite step, and the markers indicated were layered onto 5–20% (w/v) linear sucrose density gradients prepared in cellulose nitrate tubes. The gradients were centrifuged in an SW 41 rotor for 18 hr at 41,000 rev/min. The abscissa represents the distance migrated (Dm) (measured in ml from the top of the gradient). Each point represents the mean of five separate determinations. The distance migrated by DHPR was 2.82 ± 0.05 ml (S. E.). This value was used to determine the sedimentation coefficient of the enzyme, which is indicated by the arrow.

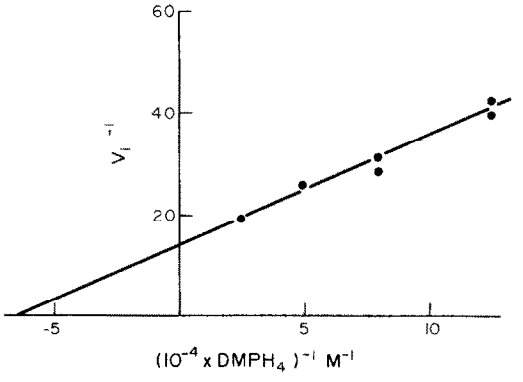

Fig. 4. Double reciprocal plot of q-DMPH$_2$ concentration against velocity. The enzyme activity was measured in μmoles NADH oxidized/min as described in Materials and Methods. The concentration of NADH was 100 μM; the concentration of q-DMPH$_2$ is expressed in terms of the initial DMPH$_4$ concentration.

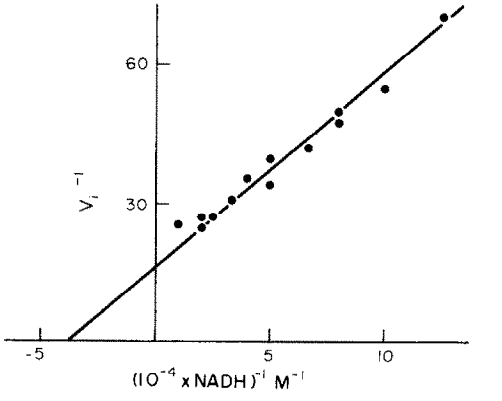

Fig. 5. Double reciprocal plot of NADH concentration against velocity. The enzyme activity was measured in μmoles NADH oxidized/min as described in Materials and Methods. The concentration of DMPH$_4$ was 50 μM.

(S. E.) μM; the K_m for NADH is 26 ± 3 (S. E.) μM.

Immunochemistry. Gel sections from several preparative electrophoresis runs were ground up in saline as described (0.5 ml saline/0.5 mg of purified enzyme). This suspension was mixed with an equal volume of Freund's complete adjuvant. A volume of 4–6 ml was injected subcutaneously into white New Zealand rabbits along the vertebral column. Two boosters were given at 3-week intervals and the antibody was harvested 5–6 days after the second booster.

The antibody produced from the purified DHPR eluted from the preparative electrophoresis gel was checked for specificity to the DHPR-antigen by three methods.

The first method employed immunodiffusion[20]; the antibody was reacted against DHPR in all stages of purification. Figure 6 shows two immunodiffusion patterns for the various reactants. The formation of only one precipitin line between all outer wells and the center well demonstrates the presence of identical antigenic determinants in the compared reactants. In additional immunodiffusion tests for specificity, the antiserum did not form precipitin lines with a preparation of purified tyrosine hydroxylase, phenylethanolamine - *N* - methyltransferase, tryptophan hydroxylase, dopamine - β - hydroxylase or aromatic-L-amino acid decarboxylase; nor did antibodies to tyrosine hydroxylase, dopamine - β - hydroxylase or tryptophan hydroxylase form precipitin lines with pure DHPR.

A second method, immunoelectrophoresis at pH 8.6[21], also demonstrated specificity of the antibody. When the antiserum was reacted against either partially purified or purified DHPR samples, only one precipitin line formed between the reactants[22].

The third method for verifying the specificity of the antibody to DHPR was to titrate a known quantity of DHPR with different amounts of the antibody and then measure the enzymatic activity. Immunotitration of DHPR activity was performed as follows; immunoglobulin IgG was precipitated from the serum at 50% saturation with ammonium sulfate and redissolved in saline. To a constant amount of DHPR solution, decreasing aliquots of preimmune, control IgG were added to a series of tubes followed by the appropriate increasing aliquots of specific anti-DHPR IgG so that the final volume and serum protein content were always constant. The samples were incubated for 1 hr at 25°, centrifuged, and the DHPR activity remaining in the supernatant fraction was measured; all determinations were made in duplicate. Figure 7 shows two examples of immunotitration. The sources of antigen used were: (1) bovine brain DHPR purified through the ammonium sulfate step; and (2) bovine brain DHPR purified through the last step according to Table 1. Increased inhibition of DHPR activity is observed as increasing amounts of DHPR antibody (IgG) solution are added to either of the two initial DHPR samples. This experiment indicates that the antibody binds specifically to the enzyme. It should be noted that there is a significant difference ($P \leq 0.002$) between the titration end points for the two sources of enzyme that will be discussed below. The lack of effect of the antibody on the activities of rat brain tyrosine hydroxylase, dopamine - β - hydroxylase and tryptophan hydroxylase is additional evidence of the antibody specificity.

To determine the cross-reactivity of the antibody, DHPR solutions, prepared from bovine brain, rat liver, rat brain and rat kidney, were purified through the ammonium sulfate step and were used for immunotitration studies. The results

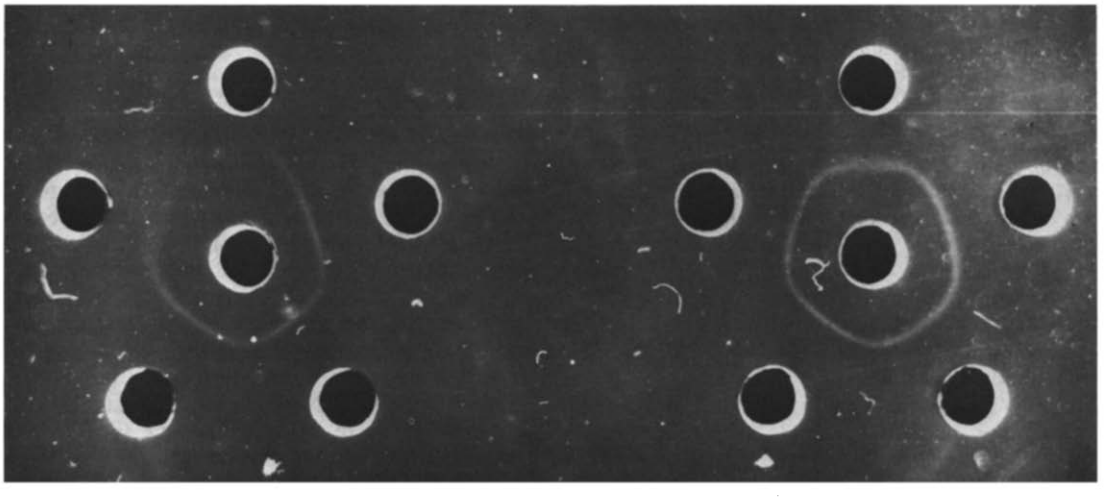

Fig. 6. Immunodiffusion demonstration of the specificity of antibodies to DHPR. The two precipitin arc patterns were observed when antiserum in the center wells was reacted against DHPR of varying stages of purity in the outer wells. The outer well pattern on the left side was set up as follows: 12 o'clock (o.c.) well, extract stage; 2 o.c. well, ammonium sulfate stage; 5 o.c. well, hydroxyapatite gel stage; 7 o.c. well, electrofocusing stage; and 10 o.c. well, pure DHPR. The outer well pattern of the right side of the figure was: 12 o.c. well, pure DHPR; 2 o.c. well, hydroxyapatite gel stage; 5 o.c. well, pure electro focusing stage; 7 o.c. well, hydroxyapatite gel stage; and 10 o.c. well, electrofocusing stage. In all cases, no more than one precipitin line formed between the center well and any other well.

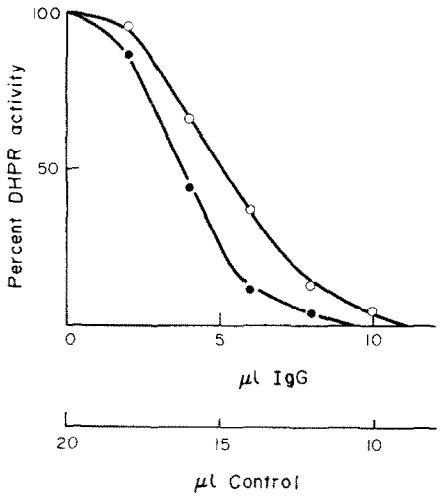

Fig. 7. Immunotitration of DHPR enzyme activity. Starting with an equal number of activity units of both DHPR purified through the ammonium sulfate step (●) and pure (○), increasing amounts of the specific antibody (IgG) were added to equivalent aliquots of the DHPR-antigen solutions. The appropriate amount of control serum was also added to each aliquot to keep the total serum added at a constant 20 μl. After incubation for 1 hr at 25°, each sample was centrifuged and the enzyme activity of the supernatant fractions was measured as described in Materials and Methods. All determinations were made in duplicate.

Table 2. Specificity of inhibition of DHPR activity by antiserum*

Serum added	Source of DHPR: Bovine brain	Rat brain	Rat liver	Rat kidney
Control	1.00	1.10	1.13	1.03
Antiserum	0	0.09	0.09	0.08

*With the same amount of control serum (control was added to equivalent aliquots of DHPR activity from the four tissue sources), only a dilutional effect on the initial DHPR activity was observed, whereas equivalent amounts of the specific DHPR antiserum (Antiserum) produced nearly complete or complete inhibition of the DHPR activity. The activity of the control bovine brain was arbitrarily set at 1.00. Each result is the average of two experiments.

shown in Table 2 demonstrate that the DHPR antibody cross-reacts with DHPR obtained from sources other than bovine brain, such as rat brain, rat kidney and rat liver.

DISCUSSION

Throughout all the purification procedures, DHPR activity was always found in a homogeneous peak, indicating that, in brain, only one protein catalyzes the conversion of quinonoid dihydropterins to tetrahydropterins using NADH as cofactor. The advantages of the present method for DHPR purification are: (1) it is simple, (2) the yield is high (10 per cent), and (3) the specific activity of the purified enzyme (76 units/μg of protein) is comparable to that reported for other methods [4].

The enzyme prepared in this study is 93–100% homogeneous. If there are minor impurities in the purified DHPR preparation, they cannot represent more than 7 per cent of the total protein or they would have been detected by gel staining or precipitin arc formation. Furthermore, the impurities must be non-immunogenic at these low levels because the antiserum contained antibodies directed only against the DHPR-antigen.

During purification, DHPR seems to be partially inactivated. The inactive enzyme cannot be separated from the active form by standard procedures. This is indicated by the significant difference between the curves of the immunotitration study (Fig. 7). The sample of pure DHPR is inactivated to a lesser extent than the ammonium sulfate fraction, indicating that the pure fraction contains some denatured enzyme that is immunologically active but enzymatically inactive. Similar observations have been made during the purification of dopamine - β - hydroxylase [23].

The results obtained with sucrose density centrifugation and SDS–polyacrylamide gel electrophoresis indicate that DHPR is a dimer consisting of two equivalent monomers. This is in support of the results of Cheema *et al.* [4] and Craine *et al.* [2]. With the sucrose density gradient method of Martin and Ames [15], the molecular weight estimated for bovine brain DHPR was 47,000. This agrees exactly with the molecular weight estimated for sheep brain DHPR by Cheema *et al.* [4] using chromatography on Sephadex G-200. The agreement between the molecular weights for brain DHPR estimated by either the sedimentation coefficient or gel filtration indicates that the molecular weight determined by either method is probably accurate. Therefore, DHPR from several tissues and species have molecular weights in the range of 41,000–52,000 and all are composed of two equivalent subunits with a molecular weight in the range of 21,000–27,000 [2, 4].

Using purified DHPR from bovine brain, we found a value of 18 μM for the K_m of q-$DMPH_2$ which is close to the values of 10 μM for the sheep brain enzyme and 12 μM for the sheep liver enzyme reported by Cheema *et al.* [4]. We found a K_m value for NADH of 26 μM which is quite similar to the values of 20 μM for the sheep brain enzyme and 30 μM for the sheep liver enzyme as reported by Cheema *et al.* [4].

In studies on sheep liver DHPR, several investigators found NADH to be a better substrate than NADPH in the presence of the quinonoid form of either dihydrobiopterin or 6,7-dimethyldihydropterin [2, 13, 24]. With tetrahydrobiopterin as the source of substrate, the K_m for NADH is about one-fifteenth that of NADPH. These investigators observed similar differences with the 6,7-dimethyl cofactor analog. Also, the K_m for the putative natural cofactor, tetrahydrobiopterin, is 15-fold smaller than the K_m for $DMPH_4$ with NADH as the other substrate. Thus, the kinetic constants are largely determined by the

structure of the substrate with which the enzyme is functioning. The K_m values observed in this study, therefore, can be used to compare the relative kinetic characteristics of DHPR from bovine brain to DHPR from other sources. Any estimation of the rate of formation of reduced pterins *in vivo* could only be performed by using *in vitro* the putative natural substrate, quinonoid dihydrobiopterin. Moreover, any further extrapolations to the *in vivo* situation would have to take into account many other factors such as the concentration of the pterin substrate present in the tissue under study and the availability of the pterin substrate to the enzyme.

The antibody produced in the present study is not only directed against DHPR from bovine brain but also against DHPR from rat brain, rat kidney and rat liver. This finding supports the existence of some structural similarity of DHPR from different species and tissues. Additional evidence in support of this conclusion includes the following. A thiol group appears to be essential for the activity of DHPR; Cheema *et al.*[4] found that both parachloromercuribenzoate and $HgCl_2$ inhibit the activity of the enzyme from different tissues. These investigators also showed that the amino acid content of DHPR from sheep brain, sheep liver and beef adrenal gland was quite similar; only a few minor differences were observed and these were related to the valine, lysine and arginine content. Another indication of similarity is that the reductase from sheep brain has the same pH optimum as the reductase from sheep liver, between pH 6.8 and 7.0[4].

The presence of only one protein capable of catalyzing the reduction of quinonoid dihydropterins using NADH as cofactor supports the hypothesis that DHPR plays an important role in the maintenance of the tetrahydropterin coenzyme(s), not only for pterin-requiring hydroxylase systems, which are involved in the biosynthesis of various neurotransmitters, but also for all other pterin-requiring systems.

Acknowledgements—The authors wish to express their deepest appreciation to Dr. Tong H. Joh and Dr. Donald J. Reis from the Department of Neurology of Cornell University Medical Center, New York, NY 10021, for their valuable help and suggestions regarding all the immunological aspects of this work. This work was supported in part by PHS Grant AM 13128. José M. Musacchio is the recipient of a PHS Research Scientist Award, MH 17785.

REFERENCES

1. S. Kaufman, *J. biol. Chem.* **226**, 511 (1956).
2. J. E. Craine, S. E. Hall and S. Kaufman, *J. biol. Chem.* **247**, 6082 (1972).
3. J. M. Musacchio, G. L. Craviso and R. Wurzburger, *Life Sci.* **11**, 267 (1972).
4. S. Cheema, S. J. Soldin, A. Knapp, K. T. Hofmann and K. G. Scrimgeour, *Can. J. Biochem.* **51**, 1229 (1973).
5. M. C. Archer and K. G. Scrimgeour, *Can. J. Biochem.* **48**, 278 (1969).
6. A. Bobst, *Helv. chim. Acta* **50**, 1480 (1967).
7. P. Hemmerich, in *Pteridine Chemistry* (Eds. W. Pfleiderer and E. C. Taylor), pp. 143–67. Pergamon Press, Oxford (1964).
8. S. Kaufman, *J. biol. Chem.* **236**, 804 (1961).
9. S. Kaufman, *J. biol. Chem.* **239**, 332 (1964).
10. S. Kaufman, in *Pteridine Chemistry* (Eds. W. Pfleiderer and E. C. Taylor), pp. 307–22. Pergamon Press, Oxford (1964).
11. S. Kaufman, *J. biol. Chem.* **242**, 3934 (1967).
12. H. Snady and J. M. Musacchio, *Biochem. Pharmac.* **27**, 1947 (1978).
13. K. H. Nielsen, V. Simonsen and K. E. Lind, *Eur. J. Biochem.* **9**, 497 (1969).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. R. G. Martin and B. N. Ames, *J. biol. Chem.* **236**, 1372 (1960).
16. K. Weber and M. Osborn, *J. biol. Chem.* **244**, 4406 (1969).
17. R. F. Beers and I. W. Sizer, *J. biol. Chem.* **195**, 133 (1951).
18. J. V. Maizel, Jr., in *Methods in Virology* (Eds. K. Maramorosch and H. Koprowski), Vol. V, pp. 179–246. Academic Press, New York (1971).
19. *LKB* 8100 *Ampholine Electrofocusing Equipment Instruction Manual.* LKB-Produkter AB, Sweden (1970).
20. O. Ouchterlony, in *Progress in Allergy* (Ed. P. Kallos), Vol. V, pp. 1–78. Karger, Basel (1958).
21. J. J. Scheidegger, *Int. Archs Allergy appl. Immun.* **7**, 103 (1955).
22. H. Snady, *Ph. D. Thesis*, New York University, New York (1975).
23. R. A. Rush, S. H. Kindler and S. Udenfriend, *Biochem. biophys. Res. Commun.* **61**, 38 (1974).
24. K. G. Scrimgeour and S. Cheema, *Ann. N.Y. Acad. Sci.* **186**, 115 (1971).