6,6-Dimethylpterins: Stable Quinoid Dihydropterin Substrate for Dihydropteridine Reductase and Tetrahydropterin Cofactor for Phenylalanine Hydroxylase†

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ABSTRACT: The tautomeric structure of the cofactor product of aromatic amino acid hydroxylases, quinoid dihydrobiopterin, is still unknown. Characterization of this molecule, which is also the substrate for dihydropteridine reductase (EC 1.6.99.7), has been hindered by the rapid rearrangement of quinoid dihydropterins to 7,8-dihydropterins. This tautomerization can be prevented by disubstitution at the 6-position. A procedure is presented for the synthesis of 6,6-disubstituted pterins from a vicinal diamine and 2-amino-6-chloro-4(3*H*)-pyrimidinone. The method is illustrated with the specific synthesis of 6,6-dimethyltetrahydropterin (6,6-Me_2PH_4). 6,6-Me_2PH_4 is a cofactor for rat liver phenylalanine hydroxylase (EC 1.14.16.1), with enzyme kinetic parameters similar to those of its positional isomer, 6,7-dimethyltetrahydropterin. The resulting quinoid 6,6-dimethyldihydropterin (q-6,6-Me_2PH_2) is stable; the half-life in 0.1 M Tris-HCl, pH 7.4, at 27 and 37 °C is 4 and 1.25 h, respectively. q-6,6-Me_2PH_2, produced either by phenylalanine hydroxylase or by chemical oxidation of 6,6-Me_2PH_4, is a substrate for dihydropteridine reductase, with a K_m of 0.4 mM and a maximum velocity double that of the natural isomer of quinoid dihydrobiopterin. In concentrations up to 0.4 mM q-6,6-Me_2PH_2 is not an inhibitor of phenylalanine hydroxylase, in contrast to 6-methyl-7,8-dihydropterin and 7,8-dihydrobiopterin which inhibit competitively, with K_i's of 0.2 mM and 0.05 mM, respectively. The stability of q-6,6-Me_2PH_2 has facilitated definitive determination of chemical and physical properties of a quinoid dihydropterin.

The exact structure of the dihydrobiopterin substrate of dihydropteridine reductase is unknown. This enzyme regenerates the tetrahydrobiopterin cofactor of phenylalanine, tyrosine, and tryptophan hydroxylases (Figure 1) (Kaufman & Fisher, 1974). A number of studies have demonstrated that the hydrogens on pteridine carbons 6 and 7 are not involved in this enzymatic oxidation/reduction cycle (Kaufman, 1964; Kwee & Lund, 1973; Scrimgeour, 1975; Ayling & Bailey, 1978; Bailey & Ayling, 1978a,b). If at physiological pH the oxidized pterin is a neutral molecule, three quinoid structures remain as possibilites (Figure 2a–c). Evidence has been presented in favor of the *p*-quinoid (Kaufman, 1964), *o*-quinoid (Kwee & Lund, 1973), and endocyclic (Scrimgeour, 1975) forms. That all three species may be in rapid tautomeric equilibrium seems not to have been considered. Furthermore, if the molecule were a monocation at physiological pH protonated on the extended guanidinium system, each of the three species represents an extreme canonical form of a single structure (Figure 2d).

The ability to observe the structure of quinoid dihydropterins directly by techniques such as ^{15}N NMR has been hampered by their propensity to rearrange rapidly to 7,8-dihydropterins. The rate-limiting step in the process has been shown to be cleavage of hydrogen from C6 (Archer & Scrimgeour, 1970). A quinoid dihydropterin, in which this rearrangement is prevented by suitable disubstitution at C6, would be stable toward this tautomerization. A tetrahydropterin disubstituted at C6 has been generated by nucleophilic attack of cyanide at C6 of 6-methyl-7,8-dihydropterin followed by reduction of the nitrile, to give the 6-methyl-6-(aminomethyl)tetrahydropterin. Upon oxidation, however, this compound did not form a stable quinoid dihydropterin but rearranged to the original 7,8-dihydropterin, with loss of the aminomethyl group as ammonia and formaldehyde (Viscontini & Cogoli-Greuter, 1971; Viscontini et al., 1971).

In this paper a method is presented for the synthesis of stable 6,6-disubstituted quinoid dihydropterins and, hence, tetrahydropterins. The synthesis, which has potential for disubstitution with a wide variety of groups, is exemplified with 6,6-dimethyldihydropterin.[1] The stability of this compound has allowed an evaluation of the chemical, physical, and enzymatic properties of the quinoid species.

The properties of 6,6-Me_2PH_4[2] and q-6,6-Me_2PH_2 as cofactor for phenylalanine hydroxylase and substrate for dihydropteridine reductase, respectively, indicate that this class of compounds may have therapeutic potential in disorders where aromatic amino acid hydroxylation is affected such as in aberrant forms of hyperphenylalaninemia and in Parkinson's disease.

Material and Methods

Reagents. 6,7-Dimethyltetrahydropterin, 6-methyltetrahydropterin, 6-methyl-7,8-dihydropterin, and phenylalanine were from Calbiochem, 7,8-dihydrobiopterin was from B. Schircks, CH8623 Wetzikon, Switzerland, peroxidase, NADH,

† From the Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688. *Received September 20, 1982.* This investigation was supported by Grant GM-30368 awarded by the National Institute of General Medical Sciences and Grant CA 31852 awarded by the National Cancer Institute. The 500-MHz NMR spectra were taken at the Southern California Regional NMR facility, supported by National Science Foundation Grant CHE79-16324. A preliminary report of part of this work has been given (Bailey & Ayling, 1982).

[1] As this paper was in preparation a report of the synthesis of 6,6-dimethyltetrahydropterin by an alternate route appeared (Armarego & Waring, 1981).

[2] Abbreviations: 6,6-Me_2PH_4, 6,6-dimethyltetrahydropterin; 6-$MePH_4$, 6-methyltetrahydropterin; 6,7-Me_2PH_4, 6,7-dimethyltetrahydropterin; q-6,6-Me_2PH_2, quinoid 6,6-dimethyldihydropterin; q-6-$MePH_2$, quinoid 6-methyldihydropterin; q-6,7-Me_2PH_2, quinoid 6,7-dimethyldihydropterin; 6-Me-7,8-PH_2, 6-methyl-7,8-dihydropterin; BH_4, tetrahydrobiopterin; pterin, 2-amino-4(3*H*)-pteridinone; HPLC, high-pressure liquid chromatography; TSP, $(CH_3)_3Si(CD_2)_2CO_2Na$; NOE, nuclear Overhauser effect; Tris, tris(hydroxymethyl)aminomethane.

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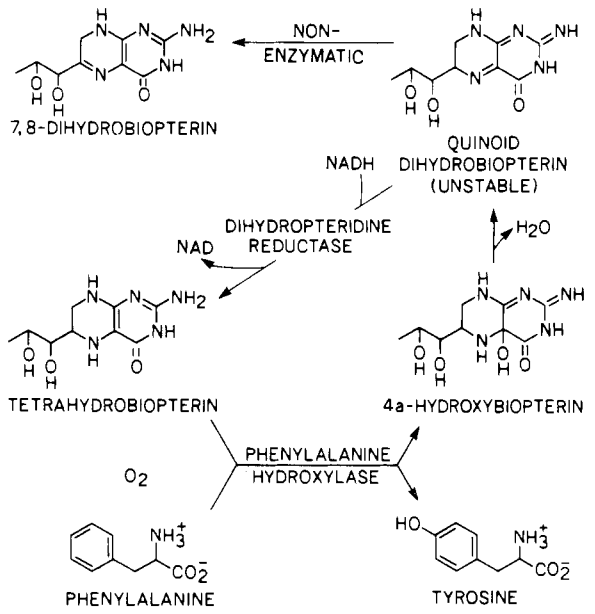

FIGURE 1: Role of dihydropteridine reductase, illustrated with phenylalanine hydroxylase. Only one of the possible structures of quinoid dihydrobiopterin is shown in this scheme (see Figure 2).

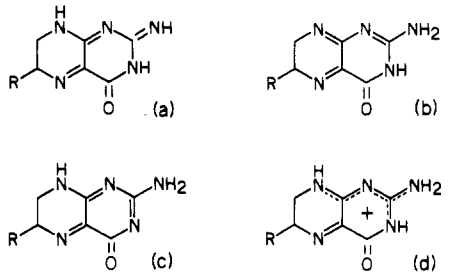

FIGURE 2: Possible structures for quinoid dihydropterin: (a) *p*-quinoid; (b) *o*-quinoid; (c) endocyclic; (d) monocation.

and NADPH were from Sigma, catalase was from Worthington, protamine sulfate was from Elanco/Eli Lilly, ascorbic acid was from Fluka, cysteine was from Biochemical Laboratories, dichlorophenolindophenol, 2-amino-6-chloro-4-(3*H*)-pyrimidinone, and 1,2-diamino-2-methylpropane (99%) were from Aldrich. The purity of the diamine was verified by the method of Lindroth & Mopper (1979). Tetrahydrobiopterin was prepared by catalytic reduction of biopterin (Regis), and the natural diastereoisomer isolated, as previously reported (Bailey & Ayling, 1978a). Partisil SCX (Whatman) was from Rainin or Alltech.

Phenylalanine hydroxylase was purified from rat liver by the method of Shiman et al. (1979). One unit of activity is defined as that amount which converts 1 μmol of phenylalanine to tyrosine in 1 min with 0.2 mM 6-MePH_4 under standard assay conditions. The standard assay is run at 27 °C in 0.1 M Tris-HCl, pH 7.4, at atmospheric oxygen, with phenylalanine at 1 mM and 2500 units of catalase/mL of reaction. Rates were determined either from the tyrosine formed (Bailey & Ayling, 1980) or by monitoring the increase in absorbance at 340 nm due to the enzyme catalyzed oxidation of cofactor (Ayling et al., 1973). The extinction coefficient at 340 nm for the conversion of 6-MePH_4 to 6-MePH_2 was measured to be 3800 M^{-1} cm^{-1}.

Dihydropteridine reductase was purified from sheep liver by the following procedure. The temperature was maintained at 0–4 °C throughout. Liver obtained fresh from slaughter was homogenized for 10 s at half-speed in a Waring blender in 0.2 M Tris-HCl, pH 7.5. The homogenate was centrifuged at 20000*g* for 30 min. To the supernatant was added dropwise with stirring 0.05 volume of 2% protamine sulfate in 0.2 M Tris-HCl, pH 7.5. Stirring was continued for 15 min, at which time the mixture was centrifuged at 20000*g* for 15 min. The supernatant was fractionated with ammonium sulfate. The fraction precipitating between 60 and 80% saturation with ammonium sulfate contained most of the activity. This fraction was dialyzed against 0.05 M, pH 8.0, Tris-HCl and the dialysate applied to a DEAE-cellulose column (Whatman-52), which had been equilibrated with 0.05 M Tris-HCl, pH 8.0. Starting with 1.2 kg of liver, a column bed volume of 500 cm^3 was used. The column was eluted with 4 L of 0.05 M Tris-HCl, pH 8.0, and 0.012 N NaCl (Lind, 1972). The dihydropteridine reductase elutes after several column volumes of buffer have passed over the column and is separated from most other proteins. The fractions containing activity were concentrated in an Amicon ultrafiltration cell. Overall yield was 80% with a purity of greater than 50%, as determined by analytical acrylamide gel electrophoresis. Beef liver enzyme was purified by a similar procedure.

Activity was determined by a modification of the procedure of Nielsen et al. (1969). Reactions of 1 mL total volume contained 100 μmol of Tris-HCl, pH 7.4, at 27 °C, 6 units of peroxidase, and, in the reaction cuvette, dihydropteridine reductase (omitted from the reference cuvette). After temperature equilibration at 27 °C for 5 min, H_2O_2, 0.4 μmol, was added to both cuvettes. Reaction was then initiated by simultaneous addition of NADH, 0.1 μmol, and 6,7-Me_2PH_4, 0.01 μmol, to both reaction and reference cuvettes, using divided mixing spoons. Since a sufficient excess of peroxide over pterin was added to maintain pterin in the quinoid form, the rate was determined from the initial increase in optical density at 340 nm by using a molar extinction coefficient of 6220 M^{-1} cm^{-1}. One unit of dihydropteridine reductase activity is defined as that amount of enzyme that can convert 1 μmol of q-6,7-Me_2PH_2 to 6,7-Me_2PH_4 in 1 min at pH 7.4 and 27 °C, when the concentration of q-6,7-Me_2PH_2 is 0.01 mM and NADH is 0.1 mM.

Equipment. UV spectra were run on a Perkin-Elmer 552 spectrophotometer attached to a Houston 100 X-Y recorder. Enzyme reactions were monitored with either a Perkin-Elmer or Aminco DW-2 spectrophotometer, both equipped with thermostated cuvette holders. HPLC analyses were performed with a Spectra Physics 8700 delivery system and monitored with a Perkin-Elmer 552 spectrophotometer modified by additional optics and microflow cells. Electrochemical detection was accomplished with a Bioanalytical TL-5a glassy carbon flow cell powered by a potentiostat constructed in this laboratory. A Keithley Model 427 current amplifier converted cell current to a recorder-compatible voltage. Mass spectra were run on a Hewlett-Packard 5982A with a 5933 data system. NMR spectra were recorded on a Varian T-60, Hitachi Perkin-Elmer R-24B, or Bruker WM-500 spectrometer. Chemical shifts are relative to Me_4Si, unless otherwise stated.

Results

Synthesis. The synthesis of 6,6-Me_2-PH_4 is illustrated in Figure 3. The commercially available 2-amino-6-chloro-4-(3*H*)-pyrimidinone (I) is nitrated by modification of published procedures (cited below). Condensation of the nitropyrimidine (II) with 1,2-diamino-2-methylpropane occurs regiospecifically, the less hindered amine displacing the halogen (Brown, 1970). The nitro group of III is then reduced either by catalytic hydrogenation or, in higher yield, by dithionite. The resulting 6-substituted derivative of 2,5,6-triamino-4(3*H*)-pyrimidinone (IV) is oxidized and the imine of quinoidal V hydrolyzed via

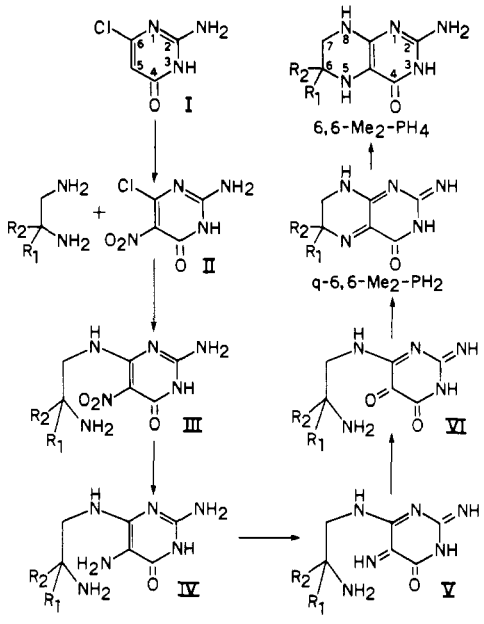

FIGURE 3: Synthetic scheme for 6,6-Me_2PH_4. $R_1 = R_2 = CH_3$.

acid catalysis to a 6-substituted derivative of quinoid divicine (VI). Upon neutralization, intramolecular Schiff's base condensation forms quinoid 6,6-dimethyldihydropterin. This can be collected or reduced to the tetrahydropterin, 6,6-Me_2PH_4.

2-Amino-6-chloro-5-nitro-4(3H)-pyrimidinone (II). The synthesis of 6,6-Me_2PH_4, essentially free of UV-absorbing impurities, in good yield, is facilitated by purification and careful nitration of the commercially available 2-amino-6-chloro-4(3*H*)-pyrimidinone (I). If the initial contaminants were not eliminated from starting material, significant losses were incurred in their removal later in the process. 2-Amino-6-chloro-4(3*H*)-pyrimidinone (I) was recrystallized once from 1 N HCl/methanol, 95/5, with minimal exposure to reflux temperature. The purity of this material was examined at various wavelengths upon elution from a Partisil SCX column (25 × 0.46 cm) with 10 mM ammonium hydroxide made to pH 3.3 with formic acid. In this buffer, pure material had the following UV-absorbing properties (λ_{max} 224 nm, 284 nm; λ_{min} 246 nm): $\epsilon_{284}/\epsilon_{246} = 10.0$ and $\epsilon_{284}/\epsilon_{320} > 300$. After 12 h in vacuo over P_2O_5, 1.64 g of I (10 mmol of monohydrate) was dissolved in 10 mL of concentrated H_2SO_4 precooled on ice to prevent hydrolysis. Nitric acid, 90% (0.8 g, 11.4 mmol), was added dropwise with stirring to the cold mixture so that its temperature remained below 6 °C. It was then allowed to warm to 25 °C and was stirred for 3 h. HPLC analysis on the above system indicated the complete disappearance of starting material. The product was dropped slowly into 100 mL of cold *tert*-butyl methyl ether with stirring on ice so that the temperature was maintained below 10 °C; the resulting suspension was kept at −15 °C overnight. The precipitate was collected by filtration and washed with ethyl ether until the filtrate contained no sulfate (approximately 150 mL) as determined with $BaCl_2$. Vacuum-dried over P_2O_5, this material weighed 1.94 g, and analysis by HPLC indicated a 3% contamination with 2-amino-4,6-dihydroxy-5-nitropyrimidine. No other significant peaks were observed by monitoring at 254, 282, or 330 nm. A broad ^1H NMR peak between 8 and 10 ppm in $(CD_3)_2SO$ integrated in comparison with an internal standard indicated the probable inclusion of 1 mol of water/mol of pyrimidine despite the primarily nonaqueous method of product recovery. Subtracting the hydrolyzed impurity, the above weight indicates a yield of 90% of monohydrate. This closely associated water appears to be responsible for the previously noted (Davoll & Evans, 1960) loss of material by hydrolysis, even when stored desiccated at −80 °C.

Four lots of commercially available 2-amino-6-chloro-4-(3*H*)-pyrimidinone hydrate were found to contain between 20% and 40% by weight of varying impurities. Although several of these contaminants are subject to nitration, most react more slowly than I which, in contrast with the literature (Lohrman & Forrest, 1964; Stuart et al., 1964; Pfleiderer & Walter, 1964), is readily nitrated by using only a 10–20% excess of reagent. The major advantage of the use of *tert*-butyl methyl ether instead of ice was found to be the differential precipitation of the desired product over most of the residual impurities in the crystallized starting material.

2-Amino-6-[(2-amino-2-methylpropyl)amino]-5-nitro-4-(3H)-pyrimidinone Monohydrochloride (III). 2-Amino-6-chloro-5-nitro-4(3*H*)-pyrimidinone, 1.94 g, was finely powdered and dissolved in approximately 200 mL of boiling absolute ethanol and filtered while hot to remove the insoluble 2-amino-4,6-dihydroxy-5-nitropyrimidine (sole contaminant), leaving a solution containing 9.0 mmol of II. This was taken again to reflux and 0.88 g (10 mmol) of 1,2-diamino-2-methylpropane added all at once with stirring. The progress of the reaction was monitored on Partisil SCX (25 × 0.46 cm) eluted with ammonium formate (pH 3.3) (0.1 M in ammonia)/methanol, 9/1, by absorbance at 254 and 330 nm. After 2-h stirring at reflux, greater than 99% of II had been consumed, and the majority of product had fallen out of solution. The reaction was kept at −15 °C overnight to complete this precipitation, filtered, washed with a few milliliters of cold absolute ethanol and 40 mL of ether, and vacuum dried over P_2O_5, giving 2.43 g of the monohydrochloride salt (97% yield): UV (0.1 N HCl) λ_{max} 331 nm, 285–290 nm sh, 233 nm sh; ^1H NMR [60 MHz, $(CD_3)_2SO$] δ 1.13 [6 H, s, $C(CH_3)_2$], 3.50 [2 H, d, $J = 5$ Hz, $-CH_2-$ (singlet if D_2O added)]. Greater than 99% of the UV_{330} absorbance and 98% of the UV_{254} absorbance in a chromatogram of this material reside in a single well-shaped peak. No evidence for the presence of any 2-amino-6-[(2-amino-1,1-dimethylethyl)amino]-5-nitro-4-(3*H*)-pyrimidinone can be seen by either HPLC or NMR.

6-[(2-Amino-2-methylpropyl)amino]-2,5-diamino-4-(3H)-pyrimidinone Dihydrochloride (IV). This compound was prepared by a modification of the procedure of Nair et al. (1975). A suspension of 2.23 g of III (8.0 mmol) in 80 mL of dimethyl formamide and 80 mL of water plus 0.16 g of NaOH (4.0 mmol) was thoroughly argonated and heated to 60 °C. Aliquots of fresh sodium dithionite, about 5 or 6 mmol each, were added via a solids addition funnel flushed constantly with argon from the top. Addition of reductant was continued until analysis on Partisil SCX (25 × 0.46 cm) eluted with ammonium formate (pH 3.3) (1 M in ammonia)/methanol/1 mM Na_2EDTA, 1/1/3, indicated only 1% of III remaining; at this time the solution was nearly clear. Approximately 5.6 g of $Na_2S_2O_4$ (32 mmol) had been used. (The required amount was found to vary between 3.5 and 6 mol/mol of III, depending on the state of the reagent and the timing of the addition.) Aside from a few minor peaks in the solvent front, due primarily to the dithionite, HPLC showed only a single impurity eluting as a rear shoulder of the main product. The majority of this byproduct, which absorbs at 262 nm in 0.1 N HCl, could be removed by centrifugation after the reaction mixture was cooled on ice. Aliquots of 1 M barium chloride were added to the resulting supernatant and precipitate removed by centrifugation. This procedure was repeated until

no further precipitate formed. A total of about 14 mL of 1 M barium chloride (approximately 40–50 mol % of the dithionite added) was needed. The final barium precipitate was resuspended in a few milliliters of cold argonated water and centrifuged. The resulting supernatant together with the initial main supernatant and 2.8 mL of concentrated HCl were rotary evaporated to dryness at 25 °C. Product was partially separated from the remaining salts by extraction into spectrophotometric grade methanol (~350 mL) until less than 1% remained in the solids. The solution was argonated, to prevent oxidation of IV. A chromatogram of this material showed that the main peak contained 90% of the UV_{269} absorbance. On the basis of this analysis and an assumed extinction coefficient of 16 000 at λ_{max} 269 nm in 0.1 N HCl, an approximate yield of 85% from III was estimated; ^1H NMR (relative to TSP) (60 MHz, D_2O/DCl) δ 1.38 [6 H, s, $C(CH_3)_2$] and 3.63 (2 H, s, $-CH_2-$).

Compound IV was also obtained by catalytic reduction of III (1 g) over an equal weight of 5% Pd on $BaSO_4$ in methanol (50 mL), stirred 14 h under 45 psi of H_2, at room temperature. A product free of UV-absorbing impurities but in somewhat reduced yield (60–65% estimated as above) was thus obtained. Care was taken to completely remove catalyst which efficiently promotes air oxidation. The methanolic solution of IV from either method may be concentrated and product precipitated with 5–10 volumes of ether to give the dihydrochloride salt of IV plus some NaCl.

Quinoid 2-Amino-6,6-dimethyldihydro-4(3H)-pteridinone (q-6,6-Me_2PH_2). A solution of IV (approximately 6.8 mmol obtained by dithionite reduction of 8.0 mmol of III) in spectrophotometric grade methanol was concentrated by rotary evaporation to a volume of about 150 mL (approximately 45 mM) and 1 mL of trifluoroacetic acid added. To this mixture, at 25 °C, was added 1.31 g of bromine (8.2 mmol) all at once with stirring. The oxidation was monitored by the same chromatographic system as used in the synthesis of IV. A further 0.32 g of bromine (2.0 mmol) was found necessary to eliminate the chromatographic peak due to IV. A requirement for greater than 40% excess of oxidant was found to be due to incomplete removal of dithionite residuals after reduction of the nitro group. Addition of the final portion of bromine was made within 5 min of the first. Within the next 5 min, all of the oxidized IV had been hydrolyzed by trace water in the methanol to the N^6-(2-amino-2-methylpropyl) derivative of quinoid divicine (VI), and the solution was then taken, over a period of 10 min, to between pH 6.6 and 7.0 (as determined by 10-fold dilution of an aliquot into water) with 1 M sodium methoxide in methanol. Upon neutralization, VI rapidly condenses forming q-6,6-Me_2PH_2 which may begin to form a bright yellow precipitate. Formation of q-6,6-Me_2PH_2 in acidic media was found to be slow in comparison to its rate of decomposition. Quinoid 6,6-dimethyldihydropterin may be collected by concentration of the neutralized solution or reduced to 6,6-dimethyltetrahydropterin.

6,6-Dimethyltetrahydropterin (6,6-Me_2PH_4). The above methanolic suspension of q-6,6-Me_2PH_2 (the result of dithionite reduction of 8.0 mmol of IV and subsequent oxidative cyclization) was rotary evaporated to dryness at ambient temperature. The yellow quinoid was reduced and thus dissolved at room temperature in a minimal volume (50 mL) of 1.0 M 2-mercaptoethanol in water. The light yellow solution was rotary evaporated together with 5 mL of concentrated HCl to an oily suspension, from which the 6,6-Me_2PH_4·2HX was extracted with several washes of methanol totalling 60 mL, leaving behind some salts.

Table I: UV-Absorbing Properties of 6,6-Dimethyltetrahydropterin

pH	buffer	λ_{max}	E_m (M^{-1} cm^{-1})
7.4	0.039 M sodium potassium phosphate (NBS)	220 303 (340)	19 700 9 700 (700)
3.3	0.01 M ammonium formate	218 266.5	27 200 12 800
1.0	0.1 N HCl	216 265	16 200 14 700
0	1.0 N HCl or 5% H_2SO_4	265 *a*	16 500

[a] No other peak above 200 nm.

This solution was concentrated to 25 mL, and 6–8 volumes of ether was added. The precipitate formed was collected by centrifugation, resuspended in 150 mL of fresh ether, and recentrifuged. The precipitate was dried under vacuum and dissolved in 25 mL of thoroughly argonated water and an equal volume of dithizone in argonated $CHCl_3$ (2 mg/100 mL) mixed in with argon bubbling. The organic layer was removed, and the aqueous solution was washed twice with 10 mL each of fresh $CHCl_3$, shell frozen, and lyophilized to dryness. The resulting light yellow powder weighed 1.98 g and contained about 29% sodium halide, primarily chloride. HPLC analysis using the same conditions as in the synthesis of IV indicated that greater than 98% of the absorbance area at either 254 or 266 nm was located in a single peak, the major impurity (0.5%) having a retention volume identical with that of the byproduct introduced by dithionite reduction of III. A UV-absorbing compound in the solvent front at this point occasionally resulted from incomplete removal of oxidized 2-mercaptoethanol during the above ether precipitation. No electrochemically active compounds (0.4 V vs. Ag/AgCl) other than 6,6-Me_2PH_4 were detected. The yield of 6,6-Me_2PH_4, determined spectrally in 0.1 N HCl by using the extinction coefficient given in Table I, was found to be 5.24 mmol (65% from III and 57% from I). Completely colorless 6,6-Me_2PH_4·2HCl of even higher purity may be obtained with good recovery by application of the method used for the crystallization of 6-methyl-5,6,7,8-tetrahydropterin (Weber et al., 1974).

Chemical Properties of 6,6-Dimethyltetrahydropterin. (*A*) *UV.* The UV-absorbing properties of 6,6-Me_2PH_4 are summarized in Table I. The concentration of a stock solution of 6,6-Me_2PH_4 was established by titration with 2,6-dichlorophenolindophenol at pH 5.5, with ascorbic acid as standard, immediately before determination of the extinction coefficient at pH 1.0 at 265 nm. This value was used to determine concentrations of all subsequent solutions including those used for the evaluation of extinction coefficients at other pHs.

(*B*) *NMR.* ^1H NMR showed the following values: [60 MHz, $(CD_3)_2SO$ plus NaOD to give the monocation] δ 1.33 [6 H, s, C6-$(CH_3)_2$], 3.30 (1 H, s, C7-H axial), and 3.47 (1 H, s, C7-H equatorial); (60 MHz, 6 N DCl) δ 1.52 [6 H, s, C6-$(CH_3)_2$] and 3.53 (2 H, s, C7-H_2). Assignment of axial and equatorial is based on the work of Weber & Visontini (1975).

^{15}N NMR showed the following values: [50.7 MHz, 0.5 M dication in $(CH_3)_2SO$/10% $(CD_3)_2SO$, relative to external formamide/10% $(CD_3)_2SO$, 25 °C] δ 133.8 (N1), 100.3 (NH_2), 78.8 (N8), and 63.9 (N5). All resonances, except that at δ 133.8, were negative. Assignments were made by comparison to spectra published for 6-methyl- and 6,7-dimethyl-5,6,7,8-tetrahydropterin (Schwotzer et al., 1978). The inability to detect N3 is most likely due to a counteractive

NOE resulting from chemical shift anisotropy induced by the higher field compared to that used for the published spectra.

(*C*) *The mass spectrum* (6,6-Me_2PH_4·2HCl by direct insertion) gave *m/z* (% relative abundance) 195 (80), 180 (100), and 165 (50).

(*D*) *Stability*. The rate of air oxidation of 6,6-Me_2PH_4 was measured in 0.1 M Tris-HCl, pH 7.4 at 27 °C, i.e., enzyme assay conditions. The half-life was 23 min with a pseudo-first-order rate constant of 0.03 min^{-1}. This is similar to the constant previously determined for 6,7-Me_2PH_4 (Ayling et al., 1973).

Chemical Properties of Quinoid 6,6-Dimethyldihydropterin. q-6,6-Me_2PH_2 is an intermediate in the synthesis of 6,6-Me_2PH_4 (see above). It can also be prepared quantitatively by bromine oxidation of 6,6-Me_2PH_4. The properties of q-6,6-Me_2PH_2 were determined on material made by the latter route. 6,6-Me_2PH_4·2HCl, 0.1 mmol, was dissolved in 3 mL of methanol, and 22 mg of Br_2 (0.14 mmol) was added. Removal of solvent by rotary evaporation left a dark orange film. The sample was repeatedly redissolved in 3 mL of fresh methanol and evaporated to dryness for a total of 6 times, until light yellow and no odor of bromine could be detected. Failure to remove the excess bromine resulted in complex absorbance changes that hindered spectral studies of q-6,6-Me_2PH_2. The quantitative yield was established by reduction back to 6,6-Me_2PH_4 with 2-mercaptoethanol, which resulted in full recovery of the original material.

(*A*) *Ionic Form of q-6,6-Me_2PH_2 at Neutral pH.* The molecular form was determined by analysis for counterion. The dihydrochloride salt of 6,6-Me_2PH_4 was converted to the dihydrobromide salt by repeated precipitation from methanol/concentrated HBr with several volumes of ether. A methanolic solution of this material was oxidized with bromine and repeatedly rotary evaporated, as above. A 40 mM solution of the resulting quinoid in methanol (2.5 mL) was titrated with 1.0 N NaOH until a sample diluted 10-fold in water reached pH 6.5. The precipitate which formed was centrifuged, briefly suspended in cold water, recentrifuged, and completely dissolved in 20 mL of methanol. HPLC analysis by a method adapted from Skelly (1982), utilizing Spherisorb-C_6, 5 μm (25 × 0.46 cm), eluted with 10 mM nonylamine plus H_3PO_4 to pH 6.2, with detection at 205 nm, showed that the precipitate contained no bromide ion, as compared to KBr standards, and was, therefore, most likely the neutral species.

(*B*) *pK_a*. The pK_a for the transition between the neutral and monocationic species of q-6,6-Me_2PH_2 was determined spectrophotometrically. A concentrated stock solution of quinoid, free of elemental bromine, in 1-propanol was diluted to 0.1 mM into a series of sodium succinate buffers at constant ionic strength (I = 0.01). Spectra, background corrected for each buffer individually, were acquired on a Perkin-Elmer 552 spectrophotometer thermostated to 25 °C. The pK_a was found to be 5.15 ± 0.05.

(*C*) *UV*. UV spectra of the neutral species of q-6,6-Me_2PH_2 (pH 7.4) and of the monocation (pH 3.0) are shown in Figure 4. The molar extinction coefficients are based on quantitative conversion of standardized 6,6-Me_2PH_4 to q-6,6-Me_2PH_2 (see above).

(*D*) *NMR*. ^1H NMR showed the following values: [60 MHz, hydrobromide salt in $(CD_3)_2SO$] δ 1.32 [6 H, s, C6-$(CH_3)_2$] and 3.45 (2 H, s, C7-H_2); (500 MHz, D_2O, pD 7.0, 25 °C) δ 1.20 [6 H, s, C6-$(CH_3)_2$] and 3.29 (2 H, s, C7-H_2).

(*E*) *Stability*. The decay of q-6,6-Me_2PH_2 in a variety of environments was monitored spectrophotometrically. In contast to quinoid dihydropterin monsubstituted at C6, and the 6-(aminomethyl)-6-methyl compound of Viscontini et al. (1971; Viscontini & Cogoli-Greuter, 1971), q-6,6-Me_2PH_2 does not appear to rearrange to a 7,8-dihydropterin. In water, a plateau of highest stability is observed near neutral pH. At pH 6.8 solutions kept on ice have a half-life greater than 100 h. In 0.1 M Tris-HCl at pH 7.4 a half-life of 4 and 1.25 h at 27 and 37 °C, respectively, is observed. Loss of quinoid is greatly accelerated in base and somewhat increased in acid (half-life = 0.75 h in 1 N HCl at 37 °C). At all pHs the disappearence of pteridine-like absorption suggests that a major ring rearrangement is occurring. This process is not affected by the presence of oxygen.

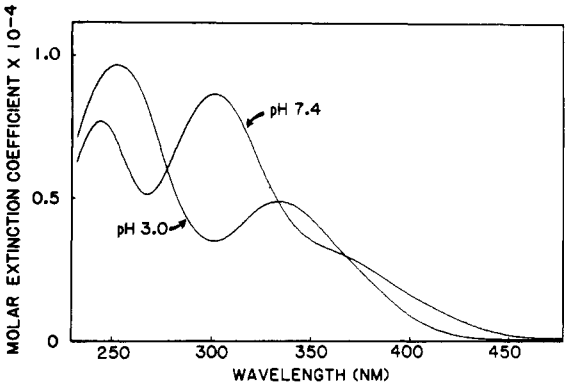

FIGURE 4: UV spectra for q-6,6-Me_2PH_2 at pH 7.4 (0.01 M Tris-HCl) and pH 3.0 (0.01 M ammonium phosphate). The molar extinction coefficients are the following: pH 7.4, 8400 (λ_{max} 303 nm), 7500 (λ_{max} 245 nm), 4900 (λ_{min} 268 nm), and 4300 (sh 340 nm); pH 3.0, 4800 (λ_{max} 335 nm), 9500 (λ_{max} 253 nm), and 3400 (λ_{min} 302 nm).

The half-life of q-6,6-Me_2PH_2 in aerated $(CH_3)_2SO$ at ambient temperature is approximately 48 h and several weeks at −80 °C. The generally increased stability in nonaqueous solvent indicates that decomposition in water may be dependent on an initial hydration.

The dry powder prepared from 6,6-Me_2PH_4, as outlined above, has been stored desiccated for several months at −80 °C with no detectable change.

(*F*) *Nonenzymatic Reduction of q-6,6-Me_2PH_2 under Physiological Conditions*. Hydroxylase cofactor analogues which are relatively stable in the quinoid dihydro form may have therapeutic potential for treatment of dihydropteridine reductase deficiency. Thus, it was of interest to determine the rate at which quinoid is reduced to the tetrahydropterin by various physiological reducing agents. Rates were measured in 0.1 M Tris-HCl, pH 7.4 at 37 °C, from the decrease in absorbance at 340 nm. A reaction first order in each component was observed. With NADH, NADPH, and ascorbic acid, the rate of reduction (k = 660 M^{-1} min^{-1}) was the same. The rate with cysteine was about 4 times slower (k = 160 M^{-1} min^{-1}).

Cofactor Properties of 6,6-Dimethyltetrahydropterin with Phenylalanine Hydroxylase. The cofactor properties of 6-methyl- and 6,7-dimethyltetrahydropterin are well-known. The kinetic constants for these cofactors are summarized in Table II, lines 1–3. It can be seen that a methyl in place of dihydroxypropyl at the 6-position has little effect on the V_{max} but decreases the affinity about 5-fold. A second methyl at the 7-position has no further effect on the binding but decreases the rate 4–5-fold.

The 6,6-dimethyl-substituted tetrahydropterin was tested for cofactor activity in a standard phenylalanine hydroxylase assay in which both cofactor consumption and tyrosine formation were monitored. As can be seen from Tables II and III, 6,6-dimethyltetrahydropterin functions as a cofactor and

Table II: Apparent Michaelis Constants and Relative Maximum Velocity for 6,6-Dimethyltetrahydropterin as Cofactor for Rat Liver Phenylalanine Hydroxylase[a]

cofactor	K_m' for cofactor (mM)	K_m' for phenylalanine (mM)	rel V_{max}
1-BH_4[c]	0.021 ± 0.003	0.17 ± 0.07[b]	1.0
6-$MePH_4$	0.1 ± 0.02	0.3 ± 0.02	0.77
6,7-Me_2PH_4[d]	0.09 ± 0.01	0.8 ± 0.1	0.17
6,6-Me_2PH_4	0.066 ± 0.01	0.65 ± 0.1	0.17

[a] Apparent K_m's for cofactor were measured at 1 mM phenylalanine, and those for phenylalanine at 0.2 mM cofactor. All reactions were at atmospheric oxygen and were run in 0.1 M Tris-HCl, pH 7.4, at 27 °C. [b] Sigmoidal K_m curve with Hill coefficient = 2; all other K_m curves were hyperbolic. [c] Data from Bailey & Ayling (1978a). [d] Data from Ayling et al. (1973).

Table III: Stoichiometry of the Reaction of 6,6-Dimethyltetrahydropterin with Phenylalanine Hydroxylase[a]

initial [6,6-Me_2PH_4] (mM)	6,6-Me_2PH_4 oxidized (nmol)	tyrosine produced (nmol)	6,6-Me_2PH_4/ tyrosine
0.01	4.4	4.27	1.03
0.03	9.1	9.77	0.93
0.06	11.76	12.57	0.94
0.1	21.18	19.25	1.10

[a] Reactions were monitored spectrophotometrically at 340 nm for 2–3 min against a reference in which phenylalanine was omitted. The reaction was terminated with trichloroacetic acid and the mixture assayed for tyrosine. Cofactor oxidized in the enzymatic reaction was calculated from the molar extinction coefficient of 3600 M^{-1} cm^{-1} for the conversion of 6,6-Me_2PH_4 to q-6,6-Me_2PH_2 at 340 nm. In determining the stoichiometry of the reaction directly, a cofactor-regenerating system was not included. Thus the above do not represent initial rates, since the reaction does not remain linear with time.

catalyzes a completely coupled reaction, in which one tyrosine is formed for each cofactor molecule consumed. An apparent Michaelis constant was determined at 1 mM phenylalanine and atmospheric oxygen with concentrations of 6,6-Me_2PH_4 ranging from 0.02 to 0.3 mM. The presence of a second methyl at the 6-position slightly increases the affinity for phenylalanine hydroxylase, since the apparent K_m was about 30% lower than that of 6-methyl- and 6,7-dimethyltetrahydropterin (Table II). The apparent K_m for phenylalanine, with 6,6-Me_2PH_4 at 0.2 mM, was 0.65 mM, which is intermediate between that observed for the 6-methyl and 6,7-dimethyl analogues under the same conditions. The maximum velocity of the reaction with 6,6-Me_2PH_4 is similar to that with the 6,7-dimethyl compound and about 6 times slower than with the natural cofactor (Table II).

Inhibition of Phenylalanine Hydroxylase by Dihydropterins. *(A) Inhibition by Quinoid Dihydropterin.* The cofactor properties of 6,6-Me_2PH_4 were utilized to investigate inhibition of phenylalanine hydroxylase by q-6,6-Me_2PH_2. When 6,6-Me_2PH_4 is used as cofactor, any electron exchange between the quinoid dihydro and tetrahydro forms[3] would not result in a change of concentration of either. Measurements were made under standard assay conditions with cofactor concentration varied between 0.05 and 0.3 mM. Stock solutions of q-6,6-Me_2PH_2 were in 0.1 M assay buffer. No inhibition of purified phenylalanine hydroxylase was observed with concentrations of freshly prepared q-6,6-Me_2PH_2 up to 0.4 mM.

(B) Inhibition by 7,8-Dihydropterins. For comparison, the inhibitory properties of 6-Me-7,8-PH_2 and 7,8-dihydrobiopterin were studied. Standard assay conditions, with 6-$MePH_4$ as cofactor over a range of 0.05–0.3 mM, were employed. Competitive inhibition was observed in both cases, with apparent K_i's of 0.2 mM and 0.05 mM, respectively.

Absorbance Spectra of Enzymatically Generated q-6,6-Me_2PH_2. Spectra were taken of the q-6,6-Me_2PH_2 synthesized from 6,6-Me_2PH_4 by phenylalanine hydroxylase or peroxidase, and a comparison was made with the chemically produced compound. The spectrophotometer was base line corrected, with sample and reference cuvettes containing all of the reaction components, except pterin in a total volume of 0.99 mL. 6,6-Me_2PH_4 (10 μL of 10 mM) was then added to the sample and H_2O (10 μL) to the reference cuvette. Scanning was begun immediately, and spectra were taken at 2-min intervals. In a phenylalanine hydroxylase reaction containing 0.1 M Tris-HCl, pH 7.4, 4 mM phenylalanine, 2500 units of catalase, and 0.3 unit of phenylalanine hydroxylase, the formation of q-6,6-Me_2PH_2 was complete within 4 min. After correction for tyrosine formed and phenylalanine consumed, the spectrum was within 3% of that produced by Br_2 oxidation of 6,6-Me_2PH_4 (Figure 4). With peroxidase, 6 units, and H_2O_2, 0.4 mM, the quinoid was completely formed in a few seconds, with a spectrum identical with that of the chemically synthesized product (Figure 4).

Substrate Properties of Quinoid 6,6-Dimethyldihydropterin for Dihydropteridine Reductase. q-6,6-Me_2PH_2 was produced by oxidation of 6,6-Me_2PH_2 by three different procedures: (i) with bromine, as outlined under Synthesis; (ii) with peroxidase (6 units/mL) and peroxide (1.2 mM); (iii) with phenylalanine hydroxylase (0.6 unit/mL), catalase (2500 units/mL), and phenylalanine (3 mM). The chemically synthesized q-6,6-Me_2PH_2 was dissolved in 0.1 Tris-HCl, pH 7.4, immediately before use to make a 10 mM stock solution, which was kept on ice. With the latter two procedures, the q-6,6-Me_2PH_2 was generated in situ and the oxidation monitored at 340 nm until complete (a few seconds with peroxidase; 1–7 min with phenylalanine hydroxylase, depending on the concentration of pterin). All reactions were in 0.1 M Tris-HCl, pH 7.4 at 27 °C. To a mixture containing q-6,6-Me_2PH_2 was added NADH (final concentration 0.1 mM), and the background rate was recorded for 1 min before addition of dihydropteridine reductase (0.01 unit/mL). Rates were calculated from the enzyme-dependent decrease in OD at 340 nm, using an extinction coefficient of 9800 M^{-1} cm^{-1} (6200 for NADH oxidation plus 3600 for quinoid dihydropterin reduction) for the chemically synthesized substrate and 6200 M^{-1} cm^{-1} for the peroxidase and phenylalanine hydroxylase containing reactions, in which the pterin is maintained in the quinoid form. Due to the high concentrations of q-6,6-Me_2PH_2 required to obtain an accurate K_m, cuvettes of 0.5-cm light path were used.

The beef liver and sheep liver enzymes showed similar activities with q-6,6-Me_2PH_2. The K_m measured in 0.1 M Tris-HCl, pH 7.4, at 27 °C, as above, was 0.4 mM for both enzymes. This K_m was obtained regardless of the method of generation of the quinoid dihydropterin. The V_{max}'s were also the same for the dihydropteridine reductase catalyzed reduction of quinoid generated by any of the three methods (Table IV). Thus, the quinoid products of each procedure appear to behave equally.

At saturating concentrations, utilization of q-6,6-Me_2PH_2 by dihydropteridine reductase is comparable to the natural quinoid dihydrobiopterin and the commonly used synthetic

[3] Redox reactions between quinoid dihydro- and tetrahydropterins generally follow a two-electron process (Kwee & Lund, 1973); thus any significant radical formation during the disproportionation is unlikely (Moorthy & Hayon, 1976).

Table IV: Michaelis Constant and Relative Maximum Velocity for q-6,6-Me_2PH_2 as Substrate for Sheep and Beef Liver Dihydropteridine Reductase[a]

quinoid substrate	method of substrate production	K_m (mM)		rel V_{max}	
		beef	sheep	beef	sheep
6,6-Me_2PH_2	bromine oxidation	0.4	0.4	1	1
6,6-Me_2PH_2	Phe hydroxylase + Phe	0.4	0.4	1	1
6,6-Me_2PH_2	peroxidase + H_2O_2	0.4		1	
6-$MePH_2$	peroxidase + H_2O_2	0.03		1.5	
6,7-Me_2PH_2	peroxidase + H_2O_2		0.03		2
dihydrobiopterin (natural isomer)	peroxidase + H_2O_2	0.004	0.004	0.5	0.5

[a] The concentration of NADH was 0.1 mM; K_m for NADH < 0.005 mM in all cases.

substrates quinoid 6-methyl- and 6,7-dimethyldihydropterin. The V_{max} of q-6,6-Me_2PH_2 is within a factor of 2 of the V_{max} of any of these substrates (Table IV). However, the affinity for enzyme is significantly affected by the second methyl group at the 6-position. The K_m is an order of magnitude higher than that for either of the two compounds with a single methyl at the 6-position and two orders of magnitude higher than that of the natural substrate (Table IV).

Discussion

A quinoid dihydropterin has been synthesized that is sufficiently stable to allow definitive characterization of its chemical and physical properties. The blocking of the normal rearrangement of 7,8-dihydropterin by disubstitution of carbon 6 with methyl groups results in a quinoid with a half-life in aqueous solution of 4 h at 27 °C and pH 7.4 and over 100 h at 0 °C and pH 6.8.

q-6,6-Me_2PH_2 is predominantly in the uncharged form (Figure 2, structure a, b, or c) at physiological pH. However, if the site of protonation for the transition with a pK_a of 5.15 is the extended guanidinium system, a mixture of tautomers, rapidly equilibrated via small concentrations of the monocation (Figure 2d), is still possible. Although the guanidinium system would seem to be favored by the gain of resonance stabilization, another candidate which should also be considered for protonation is N5, an element of a Schiff's base. However, the pK_a's of the bromo, chloro, and hydroxy C4a adducts of 5-deaza-6-methyltetrahydropterin have been determined to be 5.2, 5.3, and 6.1, respectively (Moad et al., 1979). These compounds which are comparable to q-6,6-Me_2PH_2 except for the absence of the imine 5-nitrogen and the presence of the 4a halogen or hydroxyl provide an indication of the basicity of the guanidinium system which supports the assignment of structure d, Figure 2, as the monocation of q-6,6-Me_2PH_2. If this is the case, it remains to be established whether any of the neutral tautomeric structures has sufficient stability relative to the others such that it predominates at equilibrium.

The correspondence of the UV spectra, and of the kinetic constants with dihydropteridine reductase, of q-6,6-Me_2PH_2 whether (i) generated via a phenylalanine hydroxylase reaction, (ii) produced by peroxidase and hydrogen peroxide, or (iii) chemically synthesized strongly suggests that the compound is the same in all three cases. The characterization of the tautomeric form which is substrate for dihydropteridine reductase is therefore facilitated by the availability of synthetic quinoid which can be readily prepared in quantity. The structure of q-6,6-Me_2PH_2 is currently being investigated by ^{15}N NMR.

The values reported here for the Michaelis constants of q-6,7-Me_2PH_2 and racemic q-6-$MePH_2$ agree well with those of Armarego (1979), who also resolved the isomers of the monomethyl substrate and found that the *R* and *S* isomers had almost equal affinity for sheep liver dihydropteridine reductase. It was proposed either that this could be due to the existence of sufficient room at the catalytic site to accommodate either isomer or that only the equatorial conformation of each is utilized. Our finding that the second methyl group at the 6-position decreases the affinity 13-fold, indicating restriction at the active site, is consistent with the latter proposal.

The lack of inhibition of purified phenylalanine hydroxylase by up to 0.4 mM q-6,6-Me_2PH_2 indicates that the quinoid dihydropterin under study is not the direct product of phenylalanine hydroxylase or, conversely, if quinoid is a product, its dissociation is far from rate determining. This lack of binding is in contrast to 6-Me-7,8-PH_2 and 7,8-dihydrobiopterin which are competitive inhibitors of phenylalanine hydroxylase with apparent K_i's of 0.2 mM and 0.05 mM, respectively (only a 2-fold decrease in affinity compared to the tetra reduced pterin). 7,8-Dihydropterins differ from tetrahydropterins in containing an imine conjugated via C4a with the pyrimidine ring. ^1H NMR and X-ray crystallographic data indicate that 7,8-dihydropterins are nearly planar molecules (Schircks et al., 1978; Bieri, 1977), in contrast to tetrahydropterins, in which the pyrazine ring is in a half-chair conformation (Weber & Viscontini, 1975). The inertness of q-6,6-Me_2PH_2 with phenylalanine hydroxylase is not likely attributable to the dimethyl substitution, since the K_m's of 6-$MePH_4$ and 6,6-Me_2PH_4 are nearly equal. If the discrimination against the quinoid is not a result of an overall difference in shape, the cause may lie in the difference between tetrahydro and quinoid π systems, or the absence of specific protons needed to form hydrogen bonds with enzyme.

Recent evidence strongly implicates the covalent interaction of molecular oxygen with C4a of tetrahydrobiopterin in phenylalanine (and most likely also tyrosine and tryptophan) hydroxylase (Bailey et al., 1982). Upon substrate hydroxylation, a C4a hydroxypterin (see Figure 1) is left as the initial oxidized cofactor product. The transient existence of this species in slightly alkaline phenylalanine hydroxylase reactions has been inferred by comparison of its spectrum with that of a C4a hydroxy adduct of 5-deazatetrahydropterin (Kaufman, 1975; Lazarus et al., 1981). The earlier report also indicated that the initial form of oxidized cofactor accumulating in this environment required further transformation before becoming substrate for dihydropteridine reductase. It was suggested that this transformation was the dehydration of the C4a hydroxy adduct (Kaufman, 1975). It is yet uncertain whether, physiologically, this dehydration is catalyzed by phenylalanine hydroxylase, is nonenzymatic, or as has been proposed (Kaufman, 1975) is facilitated by another protein (PHS). The stability of q-6,6-disubstituted dihydropterins has facilitated the synthesis of a series of adducts. A study of their properties, currently in progress, may clarify this issue.

Phenylalanine hydroxylase utilizes 6,6-Me_2PH_4 stoichiometrically for tyrosine formation, though at a maximum ve-

locity that is about 4.5-fold slower than homologous (*RS*)-6-$MePH_4$. Relative to the latter compound, the additional methyl group also has the effect of decreasing the K_m for cofactor by a third and increasing that for phenylalanine by 2-fold. The V_{max} of phenylalanine hydroxylase with the natural isomer of tetrahydrobiopterin is 6 times faster than with 6,6-Me_2PH_4; however, with tyrosine hydroxylase, 6,6-Me_2PH_4 is as good a cofactor as tetrahydrobiopterin.[4]

Tetrahydropterins are currently being considered for treatment of Parkinson's disease, and also for tetrahydrobiopterin deficiency states, caused by a defect either in the biosynthetic pathway or in dihydropteridine reductase (Kettler et al., 1974; Curtius et al., 1979; Danks et al., 1979; Kapatos & Kaufman, 1980; Niederwieser et al., 1982). A cofactor analogue, the quinoid form of which is stable, may overcome certain difficulties of this mode of therapy, since the success of such an approach depends on cofactor reaching the brain before conversion to the 7,8-dihydropterin. The lethality of dihydropteridine reductase deficiency indicates that, even if 7,8-dihydropterins enter the brain, the means of reduction of this form to tetrahydropterin, e.g., by dihydrofolate reductase, are inadequate. The stability of quinoid 6,6-disubstituted dihydropterins may also be sufficient to allow nonenzymatic regeneration to the tetrahydro form, by endogenous brain levels of reduced pyridine nucleotides and thiols, at an adequate rate to overcome dihydropteridine reductase deficiency. The flexibility in the choice of 6-substituents allowed by this synthesis from vicinal diamines and a readily available pyrimidine facilitates the optimization of those parameters required of an effective drug such as specificity and permeability into the brain.

A stable quinoid dihydropterin may also facilitate the assay of dihydropteridine reductase activity, both for clinical evaluation and for studies of the isolated enzyme. This would provide a considerable convenience and also allow the parameters of an enzymatic reaction (e.g., substrate concentration, extinction coefficient) to be more accurately defined, in comparison to other procedures which require in situ generation of substrate.

Finally, the study of the chemical and physical properties of 6,6-disubstituted pteridines and adducts derived therefrom will aid significantly in understanding the role of cofactor in aromatic amino acid hydroxylation and its subsequent enzymatic regeneration.

Acknowledgments

We thank Michael Braden and Shirley Dillard for excellent technical assistance and Dr. Susan Weintraub for the mass spectra.

Registry No. I, 1194-21-4; II, 1007-99-4; III·HCl, 84812-27-1; IV·2HCl, 84812-28-2; VI, 84824-59-9; q-6,6-Me_2PH_2, 84812-29-3; 6,6-Me_2PH_4, 84812-30-6; 6,6-Me_2PH_4·2HCl, 84812-31-7; 6-Me-7,8-PH_2, 17377-13-8; q-6-$MePH_2$, 70786-93-5; q-6,7-Me_2PH_2, 27644-74-2; NADH, 58-68-4; 6,7-Me_2PH_4, 611-54-1; 6-$MePH_4$, 942-41-6; BH_4, 17528-72-2; dihydrobiopterin, 6779-87-9; dihydropteridine reductase, 9074-11-7; phenylalanine hydroxylase, 9029-73-6; 1,2-diamino-2-methylpropane, 811-93-8.

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23,25-Dihydroxy-24-oxovitamin D_3: A Metabolite of Vitamin D_3 Made in the Kidney†

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ABSTRACT: Kidney homogenates of rats produced a new metabolite of 25-hydroxyvitamin D_3 which has been isolated in pure form after five column chromatographic steps. It was identified as 23,25-dihydroxy-24-oxovitamin D_3 by means of ultraviolet and infrared absorption spectrophotometry, mass spectrometry, and proton nuclear magnetic resonance spectrometry. The stereochemistry at the C-23 position is as yet unknown. 25-Hydroxy-24-oxovitamin D_3, which also has been isolated in pure form from this system, was found to be the precursor of the new metabolite in vitro. The production of the new metabolite was induced by two different methods: (a) perfusion of the kidneys with 1,25-dihydroxyvitamin D_3 contained in the perfusate and (b) injection of 1,25-dihydroxyvitamin D_3 in the intact animal. 23,25-Dihydroxy-24-oxovitamin D_3 was not biologically active in an assay for intestinal calcium transport and bone calcium mobilization in the vitamin D deficient chick at a dose level of 5.3 nmol. A metabolic pathway is proposed to describe the results; it leads from 25-hydroxyvitamin D_3 → 24(*R*),25-dihydroxyvitamin D_3 → 25-hydroxy-24-oxovitamin D_3 → 23,25-dihydroxy-24-oxovitamin D_3.

The secosteroid vitamin D_3 is known to undergo metabolic conversion before exerting its biological effects (Norman, 1979). The major circulating form of the vitamin, 25-OH-D_3,[1] is further processed by the kidney to yield 1,25$(OH)_2D_3$ or 24(*R*),25$(OH)_2D_3$. The steroid hormone-like actions of 1,25$(OH)_2D_3$ on the intestine (Tsai et al., 1972; Boyle et al., 1972), skeleton (Wong et al., 1972a,b), and a variety of other target tissues (Walters et al., 1981) are well established. In contrast, the contribution of 24(*R*),25$(OH)_2D_3$ to the spectrum of biological responses attributable to vitamin D_3 is still a controversial issue (Norman et al., 1982a,b). On the basis of experiments with a 24,24-difluorinated analogue of 25-OH-D_3, some workers have concluded that 24-hydroxylation does not play a role in the known actions of vitamin D (Miller et al., 1981; Ameenuddin et al., 1982). On the other hand, it was reported that 24(*R*),25$(OH)_2D_3$ is required for normal bone formation in the chick (Ornoy et al., 1978; Malluche et al., 1980) as well as in man (Kanis et al., 1978; Bordier et al., 1977). Also, it was shown that administration of 24(*R*),25$(OH)_2D_3$, along with 1,25$(OH)_2D_3$, was more effective in healing vitamin D deficient osteomalacia in man than 1,25$(OH)_2D_3$ alone (Bordier et al., 1978). 24(*R*),25$(OH)_2D_3$ increases intestinal absorption of calcium and phosphorus in chicks and rats (Henry et al., 1976) as well as in man (Kanis et al., 1978) and suppresses the secretion of parathyroid hormone (Canterbury et al., 1978). In addition, it was reported that 24(*R*),25$(OH)_2D_3$ is essential for normal hatchability of fertile eggs (Henry & Norman, 1978) and may stimulate sulfate incorporation into proteoglycans in isolated chondrocytes (Corvol et al., 1978).

In the chick, the further metabolic pathway of 24(*R*),25$(OH)_2D_3$ may lead to 25-OH-24-oxo-D_3 (Takasaki et al., 1981, 1982; Wichmann et al., 1981) and/or to 25,26,27-trinorvitamin D-carboxylic acid (DeLuca & Schnoes, 1979). The rate of renal synthesis of 24(*R*),25$(OH)_2D_3$ is known to be regulated by 1,25$(OH)_2D_3$ (Tanaka & DeLuca, 1974; Henry, 1979; Omdahl et al., 1980). Using isolated perfused kidneys from

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[1] Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 24(*R*),25$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 23(*S*),25$(OH)_2D_3$, 23(*S*),25-dihydroxyvitamin D_3; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25-OH-24-oxo-D_3, 25-hydroxy-24-oxovitamin D_3; 23,25$(OH)_2$-24-oxo-D_3, 23,25-dihydroxy-24-oxovitamin D_3; 25-OH-D_3-26,23-lactone, 25-hydroxyvitamin D_3 26,23-lactone; 23,25,26$(OH)_3D_3$, 23,25,26-trihydroxyvitamin D_3; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; $NaBH_4$, sodium borohydride; ICA, intestinal calcium absorption; BCM, bone calcium mobilization; $(Me_3Si)_3$, tris(trimethylsilyl) ether; $(Me_3Si)_2$, bis(trimethylsilyl) ether; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EtOH, ethanol; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/83/0422-1798$01.50/0 © 1983 American Chemical Society