The question of actual head-group orientation for phospholipids in bilayers is one that needs more experimental investigation. Our results show that the line shape will be highly dependent on this orientation and that unambiguous interpretation of ^{31}P NMR results for these systems is possible only if the head-group orientation can be determined. The orientation has been determined for phosphatidylcholine in bilayers where the molecule has been shown to assume a bent conformation (Griffin et al., 1978). Presumably, electrostatic interactions between neighboring molecules are at least partially responsible for this preferred orientation. It seems reasonable that changes in pH, temperature, or ionic strength could affect the electrostatic interactions and head-group orientations. In a similar fashion, electrostatic binding of proteins to the membrane surface or intercalation of molecules within the bilayer might also allow head-group reorientation. All these factors have been postulated, on the basis of NMR evidence alone, to induce bilayer to hexagonal phase changes in some systems. It is our feeling that simple head-group reorientation is an equally plausible explanation of the observed spectra in most instances and cannot be ruled out without further experimental investigation.

It is obvious that ^{31}P NMR spectroscopy is a sensitive probe of phospholipid orientation and mobility. It is a powerful technique when used in conjunction with other methods such as electron microscopy and X-ray crystallography and is also useful when the phospholipid head-group conformation is constrained, as it is in the cardiolipin molecule. However, the results of this theoretical study show that caution is in order when interpreting ^{31}P NMR results alone.

References

Campbell, R. F., Melrovitch, E., & Freed, J. H. (1979) *J. Phys. Chem. 83*, 525–533.

Cullis, P. R., & DeKruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Griffin, R. G., Powers, L., & Pershan, P. S. (1978) *Biochemistry 17*, 2718–2722.

Herzfeld, J., Griffin, R. G., & Haberkorn, R. A. (1978) *Biochemistry 17*, 2711–2718.

Hitchcock, P. B., Mason, R., Thomas, M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036–3040.

Hui, S. W., Stewart, T. P., Yeagle, P. L., & Albert, A. D. (1981) *Arch. Biochem. Biophys.* (in press).

Kohler, S. J., & Klein, M. P. (1977a) *Biochemistry 16*, 519–526.

Kohler, S. J., & Klein, M. P. (1977b) *J. Am. Chem. Soc. 99*, 8290–8293.

Mehring, M. (1976) in *High Resolution NMR Spectroscopy in Solids*, Springer-Verlag, New York.

Mehring, M., Griffin, R. G., & Waugh, J. S. (1971) *J. Chem. Phys. 55*, 746–755.

Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.

Seelig, J., & Gally, H.-U. (1976) *Biochemistry 15*, 5199–5204.

Siderer, Y., & Luz, Z. (1980) *J. Magn. Reson. 37*, 449–463.

Singer, S. J., & Nicholson, G. L. (1972) *Science (Washington, D.C.) 175*, 720–731.

Van, S. P., Birrell, B., & Griffith, O. H. (1974) *J. Magn. Reson. 15*, 444–459.

On the Mechanism of Action of Phenylalanine Hydroxylase†

Robert A. Lazarus, Robert F. Dietrich,‡ David E. Wallick, and Stephen J. Benkovic*

ABSTRACT: The oxidation of 6-methyltetrahydropterin and tetrahydrobiopterin coupled to the formation of tyrosine by phenylalanine hydroxylase generates a precursor species to the quinonoid product that is tenatively identified as a 4a-hydroxy adduct based on its spectral similarity to the 4a-hydroxy-6-methyl-5-deazatetrahydropterin. The rate of appearance of this intermediate and that of tyrosine are equal and hydroxylase catalyzed in accord with the completion of the hydroxylation event. This observation, which confirms and extends an earlier one by Kaufman [Kaufman, S. (1975) in *Chemistry and Biology of Pteridines* (Pfleiderer, W., Ed.) p 291, Walter de Gruyter, Berlin], serves to link the reaction courses followed by pterin and pyrimidine cofactor analogues and supports the hypothesis that the 4a position is a site of O_2 attachment. Thus, as expected, no prereduction of the enzyme was observed in anaerobic experiments utilizing stoichiometric amounts of enzyme and tetrahydropterin in the presence or absence of 1 mM phenylalanine. Activation of the hydroxylase by 1 mM lysolecithin leads to oxidation of the tetrahydropterin in the absence of phenylalanine. A ring-opened pyrimidine analogue of the tetrahydropterin, 2,5-diamino-4-[(*meso*-1-methyl-2-aminopropyl)amino]-6-hydroxypyrimidine, was studied to examine the possibility of tetrahydropterin ring opening in the enzymatic reaction prior to 4a-hydroxy adduct formation. However, no hydroxylase-catalyzed ring closure was observed.

L-Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1), an essential enzyme of mammalian metabolism, catalyzes the formation of L-tyrosine from L-phenylalanine and molecular oxygen by utilizing tetrahydrobiopterin as the natural cofactor (Kaufman & Fisher, 1974). In the course of the reaction the tetrahydropterin cofactor is oxidized to the unstable quinonoid dihydropterin which rearranges in a buffer-catalyzed reaction to 7,8-dihydropterin or can be recycled back to the tetrahydro species by using either dithiothreitol (Bublitz, 1977) or NADH and dihydropteridine reductase (Craine et al., 1972; Kaufman, 1957) to regenerate the cofactor.

† From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received January 19, 1981; revised manuscript received July 13, 1981.* This investigation was supported by Grant PCM-7803847 from the National Science Foundation.

‡ Recipient of a National Institutes of Health Postdoctoral Fellowship.

0006-2960/81/0420-6834$01.25/0

In an attempt to provide information about the exact role of the tetrahydropterin cofactor in the hydroxylase-catalyzed reaction, we have examined a variety of synthetic cofactor analogues including tetrahydropterins and substituted pyrimidines. In this paper, we report the results of experiments designed to look for potential intermediates along the hydroxylase-catalyzed oxidative pathway, as well as several UV binding experiments utilizing stoichiometric amounts of enzyme and substrates under a variety of conditions. Results are also given for a non-phenylalanine-dependent oxidation of tetrahydropterins catalyzed by phenylalanine hydroxylase in the presence of lysolecithin.

Experimental Procedures

Materials

Doubly distilled deionized water was used throughout. All reagents were the highest grade of commercially available material. Phenylalanine hydroxylase (PAH)[1] was purified from the livers of Wistar rats at least through step IIB or IIC of the method of Shiman et al. (1979) to an apparent purity of ca. 95% as judged by $NaDodSO_4$ gel electrophoresis. We could not detect by $NaDodSO_4$ slab gel electrophoresis the presence of any PAH stimulator protein (M_r 12 500/subunit) in our preparation (Huang et al., 1973). Catalase, dihydropteridine reductase (DHPR), L-phenylalanine, NADH, 2,6-dichloroindophenol, dithiothreitol (DTT), 2,4,5-triamino-6-hydroxypyrimidine sulfate (TAP), and lysolecithin were purchased from Sigma Chemical Co. L-Tyrosine (Nutritional Biochemicals Corp.) was recrystallized from water.

Tetrahydrobiopterin (BH_4) was prepared by catalytic reduction over palladium according to the method of Bailey & Ayling (1978a) from biopterin (Calbiochem). 6,7 Dimethyltetrahydropterin ($DMPH_4$) was prepared by the method of Mager et al. (1967). 6-Methyltetrahydropterin ($6MPH_4$) and 7-methyltetrahydropterin were prepared by catalytic hydrogenation over 10% Pd/C of the corresponding pterins (Storm et al., 1971). 6-Methyl-5-deazatetrahydropterin (**1**) and the 4a-bromo- (**2a**), 4a-chloro- (**2b**), or 4a-hydroxy-6-methyl-5-deazatetrahydropterins (**2c**) were prepared as previously described (Moad et al., 1979). 6-Methyl-8-deazatetrahydropterin (**3**) was prepared from 2,4-dihydroxy-6-methyl-1,3,5-triazanaphthalene (Irwin & Wibberly, 1967) as modified by D. Gottschall (unpublished results). 5,6,7-Trimethyl-5,6,7,8-tetrahydropterin dihydrochloride monohydrate (Viscontini & Bieri, 1972) was synthesized by D. Gottschall by the method of Whitely et al. (1969). Guanine, 2,4-diamino-6-hydroxy-5-nitropyrimidine (**4a**), and 2,4-diamino-6-hydroxy-5-thiocyanatopyrimidine (**4d**) were gifts from Smith Kline & French. 2,4-Diamino-6-hydroxy-5-hydroxymethylpyrimidine (**4e**) was synthesized according to the method of Rembold & Schramm (1963). 2,4-Diamino-5,6-dihydroxypyrimidine (**4f**) (divicine) and 2,4-diamino-6-hydroxy-5-nitrosopyrimidine (**4h**) were synthesized by the method of Chesterfield et al. (1964). 2,4-Diamino-6-hydroxy-5-phenylazopyrimidine (**4g**) was synthesized as described by Benson et al. (1950).

2,4-Diamino-6-hydroxy-5-bromopyrimidine (**4b**). To a warm solution of 2.0 g (0.014 mol) of 2,4-diamino-6-hydroxypyrimidine monohydrate (Aldrich) in 120 mL of 0.1 M $NaHCO_3$ (pH 9.4) was added 2.5 g (0.015 mol) of Br_2 over 5 min followed by an additional 0.5 g of Br_2 to ensure an excess. The pale yellow needles that formed upon cooling (2.5 g, 82%) had mp 243–244 °C dec [lit. mp 243 °C dec (Bendich & Clements, 1953)].

2,4-Diamino-6-hydroxy-5-iodopyrimidine (**4c**) was prepared similarly except that the reaction was run in 0.5 N NaOH at 50 °C. After addition of I_2, the solution was neutralized with HOAc, cooled, and filtered to give a 71% yield of pale yellow crystals: mp 233 °C dec [lit. mp 233–236 °C dec (Bendich & Clements, 1953)].

2,4-Diamino-6-hydroxy-5-(thiophenoxy)pyrimidine Monohydrochloride (**4i**). To a filtered solution of sodium ethoxide (0.52 mol) and dry guanidine carbonate (4.4 g, 24.4 mmol) in 50 mL of dry DMF was added 2.0 g (9.05 mmol) of ethyl phenylthiocyanoacetate (Bryson et al., 1976). The precipitated pyrimidine was filtered after 44.5 h at reflux and washed with H_2O to give 1.47 g of a white solid. The free base was converted to the monohydrochloride by crystallization from 6 N HCl–acetone (Norit A) to give a total yield of 1.62 g (66%): mp 290–298 °C dec. Anal. Calcd for $C_{10}H_{11}N_4SOCl$: C, 44.36; H, 4.10; N, 20.69; Cl, 13.10. Found: C, 44.06; H, 3.77; N, 20.67; Cl, 12.87.

5-(Benzylamino)-2,4-diamino-6-hydroxypyrimidine (BTAP). To a solution of 11.4 g (8.4 mmol) of $NaOAc{\cdot}3H_2O$ and 5.4 g (21 mmol) of $TAP{\cdot}H_2SO_4{\cdot}H_2O$ in 80 mL of H_2O was added 3.0 g (29 mmol) of benzaldehyde in 60 mL of 50% EtOH–H_2O. After the mixture was stirred several hours under N_2, the yellow crystals were collected and triturated with hot EtOH and filtered to give 4.44 g (92%) of pale yellow crystals of 5-(benzylidineamino)-2,4-diamino-6-hydroxypyrimidine: UV (95% EtOH) λ_{max} 362 nm, 287, 240 sh. GC–MS (3% OV-17) 175–250 °C at 10 °C/min of the trimethylsilylated derivative gave *m/e* 373 and 445 (EI) for the bis(trimethylsilyl) and tris(trimethylsilyl) derivatives, respectively. The Schiff base was reduced by adding 0.9 g (3.9 mmol) in aliquots to 0.25 g (3.9 mmol) of $NaCNBH_3$ (Aldrich) in 100 mL of MeOH at room temperature, followed by addition of 2 N HCl to maintain pH <7. The solution was stirred for 30 min, the pH was adjusted to 3, and then the solution was rotary evaporated. The resulting pale yellow solid was triturated with acetone and filtered to give 1.1 g (105%) of crude product. Recrystallization from 1 N HCl, acetone, and Et_2O afforded pale yellow crystals as the dihydrochloride monohydrate: UV (0.1 N Tris, pH 7.4), λ_{max} 279 nm (ϵ_{279} 11 200). Anal. Calcd for $C_{11}H_{17}N_5O_2Cl_2$: C, 41.00; H, 5.31; N, 21.74; Cl, 22.00. Found: C, 40.63; H, 5.43; N, 21.89; Cl, 21.88.

2-Amino-5-(benzylamino)-6-hydroxy-4-(methylamino)pyrimidine (**5**). 2,5-Diamino-6-hydroxy-4-(methylamino)pyrimidine was prepared by the method of Fidler & Wood (1957). This was then treated as above to give the desired product.

2-Amino-4-[(meso-1-methyl-2-aminopropyl)amino]-6-hydroxy-5-nitropyrimidine (**6**). *meso*-2,3-Diaminobutane monohydrochloride (10 g, 80 mmol) (Gullotti et al., 1972) was dissolved in 40 mL of 2 N NaOH and heated to 45 °C. To this stirred solution 2.5 g (131 mmol) of 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine (Pfleiderer & Walter, 1964) was added in small portions over a period of 1.5 h, and the temperature was maintained at 40–50 °C. After addition was complete, the stirred suspension was heated on a steam bath an additional 1 h, and the reaction mixture was cooled overnight at 4 °C. The light yellow solid product was filtered and washed with water, ethanol, and finally ether. After the product was air-dried, the yield of the adduct was 2.7 g (97 mmol): UV λ_{max} 333 nm in 0.1 M Tris-HCl, pH 7.4. The

[1] Abbreviations used: PAH, phenylalanine hydroxylase; $NaDodSO_4$, sodium dodecyl sulfate; DHPR, dihydropteridine reductase; DTT, dithiothreitol; TAP, 2,4,5-triamino-6-hydroxypyrimidine; BH_4, tetrahydrobiopterin; $DMPH_4$, 6,7-dimethyltetrahydropterin; $6MPH_4$, 6-methyltetrahydropterin; $7MPH_4$, 7-methyltetrahydropterin; BTAP, 5-(benzylamino)-2,4-diamino-6-hydroxypyrimidine.

crude product was further purified by suspension of the slightly yellow powder in 0.1 N HCl (ca. 1 g in 10 mL), filtration, and concentration of the filtrate to a white solid in vacuo. The solid was dissolved in the minimum amount of hot water, brought by careful addition of acetone to the cloud point, and allowed to stand at room temperature until crystallization was complete. After filtration and drying, the crystals analyzed as the monohydrochloride salt. Anal. Calcd $C_8H_{14}N_6O_3$·HCl: C, 34.48; H, 5.43; N, 30.16; Cl, 12.72. Found: C, 34.27; H, 5.56; N, 29.98; Cl, 12.46. The 200-MHz ^{1}H NMR spectrum (0.5 N DCl) showed peaks at δ 1.23 (3 H, doublet), 1.26 (3 H, doublet), 3.58 (1 H, multiplet), and 4.67 (1 H, multiplet) relative to DSS. The UV spectrum in 0.1 N Tris-HCl, pH 7.4, showed λ_{max} 333 nm (ϵ 15 400) with a shoulder at 290 nm (ϵ 5100).

2,5-diamino-4-[(meso-1-methyl-2-aminopropyl)amino]-6-hydroxypyrimidine (**7**). A solution of **6** (35.1 mg, 12.6 mmol) in 12.0 mL of 0.05 N HCl was treated with 5 mg of 10% Pd/C and hydrogenated on a Parr apparatus under 45 psi of H_2 (g) with shaking for 4 h. The product was immediately filtered and concentrated to dryness in vacuo. The 200-MHz ^{1}H NMR spectrum (0.5 N DCl) (taken immediately after concentration to dryness) showed peaks at δ 1.18 (3 H, doublet), 1.20 (3 H, doublet), 3.48 (1 H, multiplet), and 4.42 (1 H, multiplet) relative to DSS. A sample derivatized with trifluoroacetic anhydride gave a peak in the chemical ionization mass spectrum corresponding to the trifluoroacetylated product. There were no peaks corresponding to derivatized $DMPH_4$. The UV spectrum showed λ_{max} 269 nm in 0.1 N HCl (ϵ 15 700) and λ_{max} 291 nm (ϵ 11 600) in 0.1 N Tris-HCl at pH 7.4. All assays and experiments utilizing **7** were carried out on freshly reduced material.

Methods

UV spectra were recorded on a Cary 118 spectrophotometer. Kinetic assays were carried out on a Gilford 240 or 252 instrument. ^{1}H NMR were recorded on a Bruker WH-200 spectrometer. Fluorescence spectra were measured with a Perkin-Elmer MPF 44A instrument. MS and GC–MS were carried out on a Finnigan 3200, 9500, 6000 system. HPLC were performed on an Altex 100 solvent metering system equipped with a UV detector at 254 nm. pH measurements were made with a Radiometer 22 instrument equipped with a Model PHA 630 Pa scale expander and a Radiometer GK-2302C electrode. Melting points are uncorrected. Microanalyses were performed by MHW Laboratories, Phoenix, AZ. $NaDodSO_4$–polyacrylamide gel electrophoresis, 10% and 12.5% acrylamide, was performed according to Laemmli (1970). Analysis for *m*-tyrosine was carried out by derivatization (Kaiser et al., 1974) to the *N*-(trifluoroacetyl)-*n*-butyl ester followed by GC (Varian 3700) on a 3% OV-17 on a Chromosorb WHP 80/100 column (2000 × 2 mm) at 160 °C. Retention time for phenylalanine, *m*-tyrosine, and *p*-tyrosine are 5.8, 6.6, and 7.6 min, respectively.

Enzyme Assays. PAH was assayed at 25 °C unless stated otherwise by either direct measurement of the rate of formation of oxidized cofactor (Ayling et al., 1973a) (assay 1), measurement of the NADH oxidation which is coupled to quinonoid formation through dihydropteridine reductase (Kaufman, 1979) (assay 2), measurement of the rate of tyrosine formation by following the change in absorbance at 275 nm (Shiman et al., 1979) (assay 3), or measurement of tyrosine formation from the fluorescence of the nitrosonaphthol derivative (Waalkes & Udenfriend, 1957) (assay 4). Protein was measured by the modified Lowry procedure (Peterson, 1977).

Kinetics. These experiments were performed in 1 mL of 0.02 M Tris, pH 7.8, containing 1 mM L-phenylalanine, 65 μg of catalase, and 5–25 μg of PAH (sp act. ~ 6 units/mg; assay 3), followed by preincubation at 25 °C for 3 min and addition of 16–72 nmol of tetrahydropterin. Aliquots were taken at various times and analyzed for tyrosine (assay 4) and the remaining tetrahydropterin by quenching into 90 μM 2,6-dichloroindophenol ($\epsilon_{600} \simeq 16\,100$ for the oxidized form of the dye) in 0.04 M potassium phosphate, pH 7.0, and measuring the amount of dye reduction at 600 nm. Plots of ln (tyrosine$_\infty$ − tyrosine$_t$) vs. time and ln [(6MPH$_4$)$_t$ − (6MPH$_4$)$_\infty$] vs. time were linear over at least two half-lives. The formation and decomposition of the intermediate were measured spectrophotometrically at 244 nm; the decomposition rate (k_2) was pseudo first order. Rapid turnover experiments were performed by monitoring the change in the UV spectrum of the tetrahydropterin or pyrimidine cofactor. Experiments were done in 1 mL of 0.1 M Tris, pH 7.4, or 0.1 M potassium phosphate, pH 8.0, containing either 1 mM or 2 mM L-phenylalanine, 20–50 μg of catalase, and 5–10 μM PAH (based on 50 000 daltons/subunit). After preincubation for 3 min at 25 °C, an aliquot of tetrahydropterin or pyrimidine in 0.05 N HCl was added to the sample cuvette to give a concentration of 10–20 μM cofactor. The initial spectrum was recorded within 15 s. At the end of the reaction, the contents were analyzed for tyrosine. The amount of 7,8-dihydropterin found was estimated from the extinction coefficient at ca. 324 nm determined from Br_2 oxidation of the tetrahydropterin at pH 7.4: 7,8-BH_2 (ϵ_{324} 4615), 7,8-$DMPH_2$ (ϵ_{324} 5800), 7,8-7MPH_2 (ϵ_{321} 4935), and 7,8-6MPH_2 (ϵ_{324} 6300).

Anaerobic Experiments. All stock solutions were made up in degassed water. PAH (7 mg/mL) was dialyzed against degassed elution buffer under N_2 for 4 h at 4 °C. All operations were carried out in a glove box under inert atmosphere. To a cuvette containing 12.7 μM PAH, 1 mM phenylalanine, 100 mM glucose, 50 units of glucose oxidase (Sigma), and 300 μg of catalase in 1 mL of 0.1 M Tris, pH 7.4, at 25 °C (incubated 30 min) was added 13 μM BH_4 or $DMPH_4$. The cuvettes were stoppered and the UV spectra of the pterin were recorded with time. An aliquot was quenched anaerobically and analyzed for tyrosine. The cuvettes were then exposed to air for 100 min, and their UV spectrum was monitored and analyzed for tyrosine again. The blank was treated the same.

Cyclization of **7**. The relative amounts of cyclization of **7** at various pH values (7.4–11.0) were determined following the oxidation of an aliquot in 0.2 M Tris, pH 8.0, with excess Br_2 (5-fold excess) by comparison with a standard curve of the fluorescence of solutions of known concentration of 6,7-dimethylpterin, λ_{exc} 350 nm and λ_{em} 438 nm. The product of the PAH-catalyzed oxidation of **7** was treated with excess Br_2 (3-fold) and analyzed in the same manner. The fluorescence spectra of the Br_2 oxidation product, the enzyme oxidation product followed by Br_2 treatment, and 6,7-dimethylpterin were all identical. HPLC analysis (Whatman Partisil PXS 10/25 SCX; 4.6 mm × 25 cm; 1.5 mL/min; 0.1 N sodium acetate, pH 3.5) after oxidation of **7** with 1.0 equiv of Br_2 showed peaks with retention times identical with those of 7,8-$DMPH_2$ and 6,7-dimethylpterin. No other peaks were observed under these conditions.

Cofactor Analogues. The various compounds tested were assayed for substrate activity by monitoring any changes in their UV spectrum caused by PAH (assay 1 conditions) and/or their ability to produce tyrosine (assay 4). Inhibition experiments were carried out vs. $DMPH_4$ under conditions and inhibitor concentrations as follows: **1** (assay 1, 0.0–0.4 mM);

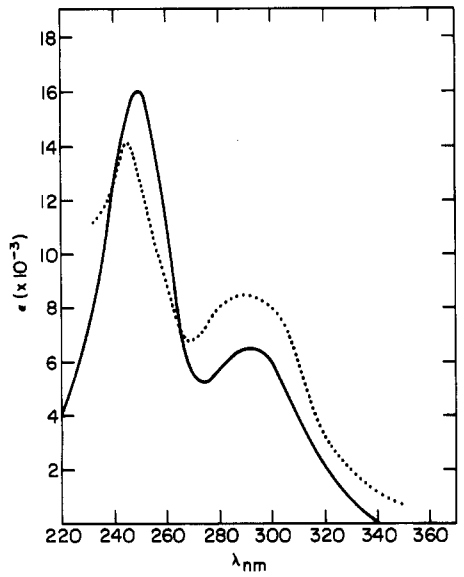


FIGURE 1: UV spectra of 4a-hydroxy-6-methyl-5-deazatetrahydropterin (Moad et al., 1979) in 0.01 M Tris, pH 8.06 (—), and the intermediate generated from the addition of 16 nmol of $6MPH_4$ to a 1-mL solution of 1 mM phenylalanine and 18 μg of PAH in 0.02 M Tris, pH 8.0 (···). The reference cuvette contained everything except $6MPH_4$. The UV spectrum was recorded after 45 s (OD_{244} had maximized) and is corrected for the amount of quinonoid–$6MPH_2$ generated.

2a–c (Moad et al., 1979), **3** and **4e** (assay 2, **3** and **4e** = 0.25 mM and $DMPH_4$ = 0.047 mM); 5,6,7-trimethyltetrahydropterin (assay 1, 0.0–0.4 mM); guanine (assay 1, 0.0–1.5 mM); **4d** (assay 1, **4d** = 0.1–2.0 mM and $DMPH_4$ = 0.075–0.25); **4f** (assay 3, **4f** = 0.12 mM and $DMPH_4$ = 0.01 mM); **4g** (assay 1, 0.0–0.2 mM); **4h** (assays 1, 3, and 4, 0.0–0.2 mM); **4i** (assay 3, 0.0–0.038 mM). The apparent K_M for **7** was determined by using assay 2 conditions.

5 was tested for cofactor activity in 0.1 M Tris, pH 7.4, containing 2 mM phenylalanine, 50 μg of catalase, 19 μg of PAH, and varying amounts of **5** (0.08–0.9 mM) in a 1-mL volume, followed by quenching with 0.1 mL of 30% trichloroacetic acid after 20 min and assaying for tyrosine (assay 4). Tyrosine formation was linear with time over 20 min.

Results

Initial Turnover Products Derived from Tetrahydropterin and Pyrimidine Cofactors. We have now extended the observation of a tetrahydropterin-derived intermediate during PAH turnover to $6MPH_4$. In 0.2 M Tris-HCl, pH 7.8, the initial tetrahydropterin UV spectrum was rapidly converted to one that resembles the 4a-hydroxy-5-deazatetrahydropterin adduct (Figure 1). This intermediate spectrum changed to one consistent with quinonoid–$6MPH_2$ which then rearranged nonenzymically ($t_{1/2}$ = 56 min) in a buffer-catalyzed reaction (Archer & Scrimgeour, 1970) to give 7,8-$6MPH_2$ as the final observable product of the reaction.

The pseudo-first-order rate of disappearance of $6MPH_4$ (k_1) was equal to the pseudo-first-order rate of formation of tyrosine (k_{Tyr}); both were strictly enzyme-catalyzed reactions (Figure 2). The formation of quinonoid–$6MPH_2$ (k_2) had both a slight pH-independent enzyme-catalyzed and a pH-dependent spontaneous rate (Figure 2). The spontaneous rates at pH 7.4, 7.8, and 8.25 were 0.75, 0.40, and 0.30 min^{-1}, respectively. Both k_1 and k_{Tyr} were inhibited by 6-methyl-5-deazatetrahydropterin (Moad et al., 1979), however, k_2 was not affected. No intermediate was observed when either 1 mM *p*-fluorophenylalanine or 1 mM lysolecithin was used instead of 1 mM phenylalanine. Attempts to trap a potential peroxide intermediate using thioxane, I^-, or glutathione and glutathione peroxidases (both Se and non-Se enzymes) proved to be unsuccessful.[2]

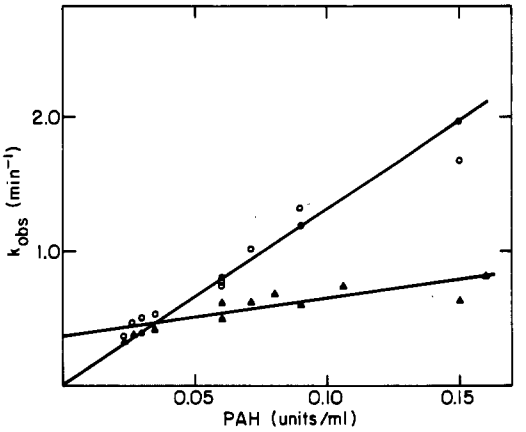


FIGURE 2: Dependence of the pseudo-first-order rate constants for disappearence of $6MPH_4$ [k_1 (●)], appearance of tyrosine [k_{Tyr} (○)], and dehydration of the carbinolamine intermediate [k_2 (▲)] on enzyme concentration. Reactions were run in 0.02 M Tris-HCl, pH 7.8, containing 65 μg/mL catalase, 1 mM L-phenylalanine, and either 36 or 72 μM $6MPH_4$.

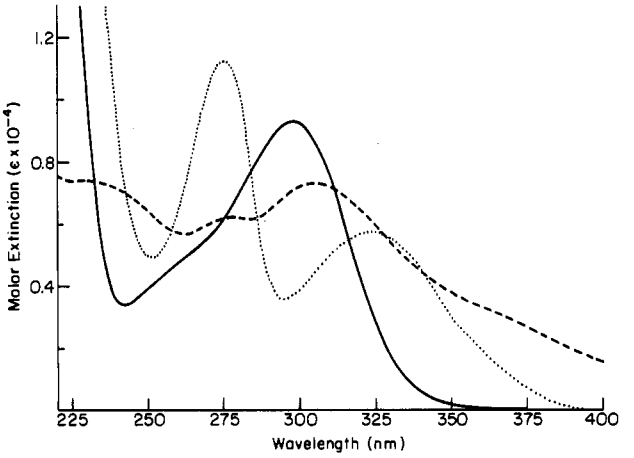


FIGURE 3: UV spectra of $DMPH_4$ (—), quinonoid–$DMPH_2$ (---), generated from $DMPH_4$ by treatment with 1 molar equiv of Br_2, and 7,8-$DMPH_2$ (···) in 0.1 M Tris-HCl, pH 7.4.

An intermediate with very similar properties to the one generated from $6MPH_4$ was also observed with BH_4. The primary kinetic difference was that quinonoid–BH_2 rearranged nonenzymically ca. 10 times faster than quinonoid–$6MPH_2$ so that its spectrum was not observable. When 6,7-$DMPH_4$ was used as the cofactor, no intermediate is observed. Analysis for *p*-tyrosine showed that the reactions are all tightly coupled (tyrosine formed/tetrahydropterin oxidized > 95%) for BH_4, $6MPH_4$, and 6,7-$DMPH_4$. No *m*-tyrosine was observed.

In the presence of 0.3–1.0 active site equiv of PAH based on 50 000/subunit molecular weight, phenylalanine, and O_2, the only pterin-derived products by examination of the UV spectra were the respective quinonoid dihydropterin and its rearrangement product, the 7,8-dihydropterin, as well as tyrosine at both pH 7.4 and pH 8.0. Essentially all of the oxdized cofactor could be accounted for in the UV spectrum either as the quinonoid species or as 7,8-dihydropterin in the

[2] These were performed under the conditions of assay 1 (pH 7.8) and monitored for tyrosine formation (assay 4). There was no effect upon addition of either 0.29 mM thioxane, 0.1 M NaI, or 30 mM glutathione and the glutathione peroxidases which were supplied by Dr. Channareddy.

cases of the fully coupled tetrahydropterins BH_4 (94%), $DMPH_4$ (95%), and $6MPH_4$ (93%) as well as the partially uncoupled cofactor, $7MPH_4$ (58%). The dihydropterin products formed in each case were identical with those formed upon addition of 1.0 molar equiv of Br_2 under the same conditions (pH and buffer) to the appropriate tetrahydropterin (Figure 3).

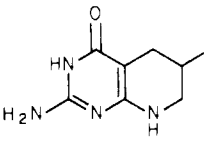
The UV spectra of the PAH-catalyzed initial oxidation products of two pyrimidine analogues were examined and shown to be different from the nonenzymatic oxidation products, in agreement with the results of Bailey & Ayling (1980). When 23.8 μM BTAP in pH 7.4 Tris-HCl containing 2 mM phenylalanine was treated with PAH to give an active site concentration of 16.7 μM, the spectrum of the oxidation product was nearly identical with the spectrum of oxidized divicine. The spectrum showed two peaks, one at 240 nm (OD = 0.395) and another at 275 nm (OD = 0.145). The spectrum of authentic divicine, allowed to air oxidize at the same pH and concentration, showed absorption maxima at 239 nm (OD = 0.340) and 275 nm (OD = 0.143). The BTAP oxidation product spectrum was determined after the absorbance at 240 nm had maximized. The reaction solution was then treated with DTT (final concentration 7 mM) to give a product λ_{max} 281 nm (OD = 0.21). Divicine at the same concentration has λ_{max} 281 nm (OD = 0.24). Assay for tyrosine showed 60–65% of the BTAP oxidized led to tyrosine formation. Bromine oxidation, on the other hand, leads to uncharacterized product(s) with an initial large increase in absorbance at 320 nm, whereas there is essentially no increase in absorbance at 320 nm in the enzyme-catalyzed oxidation. Similarly, when TAP was utilized as a cofactor in the PAH-catalyzed reaction, an initial oxidation product was formed whose spectrum was consistent with oxidized divicine and tyrosine was formed equal to 40–45% of the TAP turned over. Bromine oxidation of TAP led to a quinonoid species which when reduced with DTT is converted back to TAP (Dietrich & Benkovic, 1979). The UV spectrum of quinonoid TAP (0.15 mM) generated by Br_2 oxidation in 0.1 M Tris, pH 7.4, was unaffected by the presence of 1.3 μM PAH and/or 1 mM phenylalanine.

UV Binding Experiments. Evidence for the oxidation of the tetrahydropterin by PAH in the absence of phenylalanine but in the presence or absence of O_2 was sought by determining the UV difference spectrum of the bound cofactor under various conditions. In pH 7.4 Tris-HCl with $[BH_4]$ = 19.2 μM, [PAH] = 42.8 μM, and $[O_2] \simeq 0.25$ mM, where 61% of the tetrahydropterin is bound to the enzyme,[3] there was essentially no change in the pterin spectrum which was followed over a 15-min period.

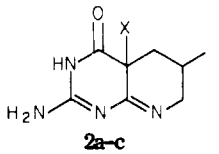
UV binding experiments in which stoichiometric amounts of the tetrahydropterin (BH_4 or $DMPH_4$) and PAH (ca. 13 μM) were incubated anaerobically in the presence of 1 mM phenylalanine showed no change over 1 h in the UV spectrum from the tetrahydropterin in the absence of enzyme and O_2. Analysis for tyrosine after 1 h as a control for residual oxygen indicated that the tyrosine/tetrahydropterin ratio was 0.075 for BH_4 and 0.0 for $DMPH_4$. Upon addition of oxygen, tyrosine was produced with the concomitant change in the UV spectrum indicative of oxidation to the 7,8-dihydropterin. After 100 min the tyrosine/tetrahydropterin ratio was 0.36 and 0.40 for $DMPH_4$ and BH_4, respectively.

Lysolecithin Activation. The UV spectrum of the tetrahydropterin in the presence of both PAH and 1 mM lysolecithin (aerobic) shows a rapid oxidation to the quinonoid dihydropterin, followed by rearrangement to the 7,8-dihydropterin. An apparent $K_M \cong 0.12$ mM was obtained for $6MPH_4$ by using assay 1 in the presence of either 1 mM phenylalanine or 1 mM lysolecithin; however, the relative V_{max} for tetrahydropterin oxidation by using 1 mM lysolecithin was 42% that of the rate in the presence of phenylalanine. L-Tyrosine had no effect on the oxidation of the tetrahydropterin under conditions that have been reported previously (Fisher & Kaufman, 1973). Addition of 1 mM phenylalanine to a solution of 6.25 μM $6MPH_4$, 0.3 mg of PAH, 50 μg of catalase in 0.1 Tris-HCl, pH 7.4, after oxidation was complete (90 s), followed by analysis for tyrosine (assay 4) showed no tyrosine present.

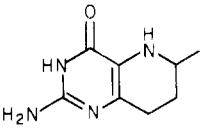
Cofactor Analogues. In order to further define the structural features required for cofactor activity and to limit the possible structures for intermediates derived from PAH oxidation of the cofactors, we examined a variety of tetrahydropterin and pyrimidine structures. It has been known for some time that a variety of tetrahydropterins can serve as efficient cofactors (Ayling et al., 1973b). Recently we have reported that 5-deaza-6-methyltetrahydropterin (**1**) and its



1



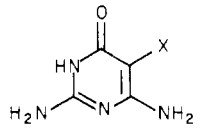
2a–c
X = Br, Cl, OH



3

4a-Br (**2a**), -Cl (**2b**), and -OH (**2c**) adducts will not serve as cofactors (Moad et al., 1979). Whereas **1** is a good competitive inhibitor (K_I = 50 μM) vs. $DMPH_4$ ($K_M \cong 100$ μM), none of the 4a adducts bind to PAH to inhibit the reaction. Surprisingly, 8-deaza-6-methyl-tetrahydropterin **3** is neither a substrate *nor* an inhibitor. However, 5,6,7-trimethyltetrahydropterin and guanine are competitive inhibitors with K_I = 0.33 mM and 0.90 mM, respectively, vs. $DMPH_4$.

As noted above, both TAP and BTAP function as cofactors and promote tyrosine formation. The structural requirements for pyrimidine supported hydroxylation, however, are quite restrictive. 2,4-Diamino-6-hydroxy-5-substituted pyrimidines **4** where X = NO_2, NO,[4] Br,[5] I,[5] SCN, CH_2OH, and OH act



4

neither as substrates nor as inhibitors. Only when X = N=

[3] The fraction of BH_4 bound was calculated from the apparent K_M = 20 μM in the presence of 1 mM phenylalanine (Bailey & Ayling, 1978b) presuming $K_M \simeq K_d$. The K_d in the absence of phenylalanine has not been measured and may be quite different, thus altering the percent of cofactor bound; however, phenylalanine does not seem to affect the K_M of $DMPH_4$ (Kaufman & Fisher, 1974).

[4] It was originally thought that PAH deaminates **4h** at the 2 position. This has since been shown to be due to a contaminating amount of guanase activity (R. A. Lazarus and S. J. Benkovic, unpublished results) which is no longer present in our current preparations of PAH.

[5] Inhibition studies with **4b** and **4c** were complicated by the fact that thiols, NADH, or tetrahydropterins are oxidized by the pyrimidine. With DTT and β-mercaptoethanol, the pyrimidines are dehalogenated (R. A. Lazarus and S. J. Benkovic, unpublished results).

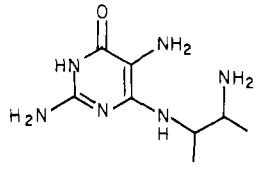
Table I: Results of Oxidative Cyclization of the Ring-Opened Analogue 7

pH	% cyclization from Br_2 oxidation	% cyclization from PAH oxidation	% tyrosine formation	rel V_{max}/K_M
7.4	14.1 ± 2	12 ± 3	10 ± 2	0.07 ± 0.04
8.0	24.0 ± 2	22 ± 2	7 ± 1	0.07 ± 0.04
9.0	36.0 ± 2	34 ± 2	5 ± 1	0.12 ± 0.02
10.0	29.3 ± 2	n.d.[a]		
11.0	18.7 ± 2	n.d.		

[a] n.d. = not determined.

N—C_6H_5 or S—C_6H_5, which resemble BTAP, was competitive inhibition (K_I = 21 μM or 4.7 μM, respectively) but no cofactor activity observed. Addition of a methyl group to the 4-amino group of BTAP gives **5**, which is isosteric with 6-phenyltetrahydropterin. This serves as a cofactor (K_m = 0.89 mM; V_{rel} = 2.9% that of $DMPH_4$); however, the reaction is largely uncoupled since tyrosine formation/pyrimidine oxidized ≃ 0.2–0.4.

For reference, the oxidative cyclization of **7**— a model



7

reaction for the hypothesized cyclization by PAH of a ring-cleaved pterin stemming from a precursor 4a-dioxytetrahydropterin adduct—was carried out in the presence of excess bromine at pH values between 7.4 and 11.0 by determining the amount of 6,7-dimethylpterin that formed from its fluorescence. The amount of cyclization, summarized in Table I, was shown to be pH dependent with a maximum at approximately pH 9 with 36% of **7** converted to 6,7-dimethylpterin.

When examined as a cofactor in the PAH-catalyzed reaction by using assay 2, **7** acted as a substrate with an apparent K_M of 2 ± 1 mM in the presence of 2 mM phenylalanine and showed no significant pH dependence. The amount of tyrosine produced in rapid turnover experiments at pH values between 7.4 and 9.0 showed tyrosine production decreasing with increasing pH whereas the fraction of **7** cyclizing increased (Table I). The fraction cyclized at various pH values was identical with that of the nonenzymatic oxidation. No direct relationship was observed between cyclization and tyrosine formation.

Discussion

The findings that (1) 2,4,5-triamino-6-hydroxypyrimidines can function as cofactors in the PAH-catalyzed reaction (Bailey & Ayling, 1980; Kaufman, 1979) leading to the formation of tyrosine and divicine (replacement of the 5-amino by a 5-hydroxy substituent) and (2) tetrahydrobiopterin at pH 8.0 reportedly forms during PAH turnover an uncharacterized intermediate hyothesized to be a 4a-hydroxy adduct (Kaufman, 1975) has furnished support to the argument that the cofactor is the site for the oxygen activation required for aromatic hydroxylation. The results of our experiments will be discussed in this context.

The observation of a PAH-catalyzed tetrahydropterin intermediate derived from both 6-MPH_4 and BH_4 that is a precursor of the quinonoid dihydropterin is in agreement with the proposed 4a-hydroxy adduct based on the following facts. (1) The UV spectrum of the PAH-generated intermediate closely resembles that of the 4a-hydroxy-5-deazatetrahydropterin adduct. The initial UV spectrum of an 8a adduct derived from 6-methyl-8-deazatetrahydropterin shows only a single absorption at 235 nm, $\epsilon \sim 16\,000$ (Gottschall, 1981). (2) Since the rate of formation of tyrosine is equal to the rate of disappearance of the tetrahydropterin, indicating oxygen transfer has already occurred, the most straightforward explanation is that the initial products off the enzyme are tyrosine and the 4a-hydroxy adduct. (3) The rate of dehydration of the carbinolamine intermediate to give quinonoid dihydropterin increases with decreasing pH as expected[6] (Jencks, 1969). (4) A 4a-hydroperoxide adduct, which could not be detected, is disfavored based on its presumed greater reactivity contrasted with that of the hydroxy adduct in the elimination reaction that would lead to an uncoupled reaction, which was not observed. A 4a-peroxyphenylalanine adduct is similarly disfavored. The fact that no intermediate is observed with $DMPH_4$ may simply reflect its differing K_M and V_{max} values such that $k_2 > k_1$ (k_{Tyr}). It should be noted that the dependence of k_2 on PAH concentration is sufficient to prevent the detection of the adduct by conventional methods at PAH levels equivalant to that of the pterin.[7] (5) The formation of the adduct from both 6-MPH_4 and BH_4 eliminates a unique tricyclic species formed from intramolecular condensation of the hydroxy side chain in the quinonoid BH_4.

Although the above experiments suggest that the pterin is the initial site of oxygen attachment, the requirement of Fe^{3+} for catalytic activity (Fisher et al., 1972; Gottschall, 1981) raises the possibility that the pterin may act to reduce the metal ion preparing it for O_2 binding. In the course of turnover, PAH-catalyzed hydration might then generate the 4a-hydroxy species. However, incubation of PAH and BH_4 anaerobically or aerobically in the absence of phenylalanine at pH 7.4 showed no UV difference spectral change attributable to the generation of the quinonoid dihydropterin. Similarly stoichiometric amounts of BH_4 or $DMPH_4$ incubated anaerobically with PAH in the presence of 1 mM phenylalanine which activates the enzyme for turnover (Shiman & Gray, 1980) did not result in 7,8-dihydropterin formation. Thus, this particular partial reaction is eliminated from consideration, in agreement with previous results (Kaufman & Fisher, 1974). It is noteworthy that in the presence of O_2 and lysolecithin, which can substitute for phenylalanine as an activator, the tetrahydropterins are oxidized by PAH.[8] The possibility of generating an active hydroxylating species of >1-min lifetime through lysolecithin activation was probed by quenching (after 90 s) the reaction with phenylalanine and analyzing for tyrosine; however, none was observed.

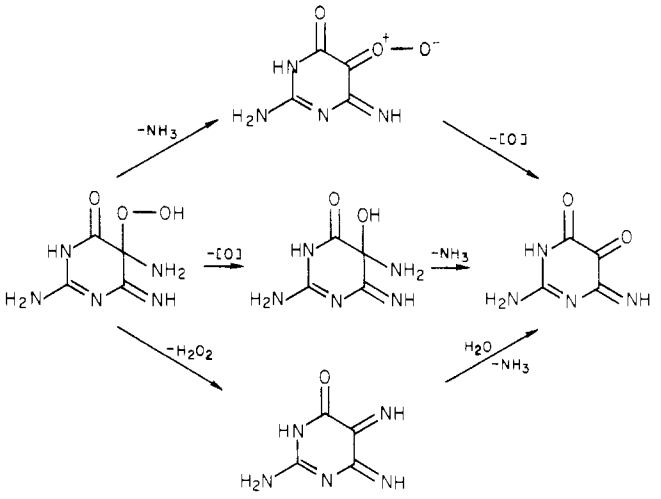
In short, oxidation of the tetrahydropterin minimally requires activated PAH and O_2 but not phenylalanine. Other reducing agents such as DTT (6 mM) or L-ascorbate (6 mM) in the presence of 1 mM phenylalanine and/or lysolecithin and/or **1** were found to be incapable of catalyzing tyrosine formation (assay 4). Therefore, tyrosine formation requires either a tetrahydropterin or pyrimidine (vide infra) as a reducing agent.

[6] The rate of dehydration (k_2) is not first order in H^+, suggesting that protonation at N-5 might be kinetically important.

[7] The apparent catalysis of the 4a adduct dehydration by PAH might be due to the presence of a small, not readily detected amount of "stimulator protein" (Kaufman, 1975).

[8] Neither lysolecithin nor PAH alone was capable of oxidizing the tetrahydropterin, but in contrast to the results of Fisher & Kaufman (1973), the oxidation did not require L-tyrosine. Tyrosine (0.3 mM) does not inhibit PAH (assay 3).

Scheme I



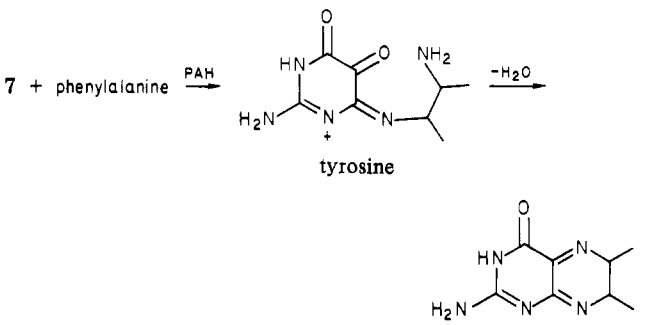
In view of the above observations with pterins, the reaction course followed with the pyrimidine cofactor analogues is in close agreement. Our results on the PAH-catalyzed oxidation of the pyrimidines, TAP and BTAP, agree with the earlier observations of Bailey & Ayling (1980) that (1) hyroxylation of phenylalanine can be achieved with a pyrimidine cofactor analogue rather than a tetrahydropterin with the degree of coupling dependent on the nature of the substituent at N-5, (2) the oxidation of the pyrimidine cofactor analogue leads quantitatively to the formation of oxidized divicine regardless of the extent of coupling to hydroxylation, and (3) the divicine product, after reduction, does not support hydroxylation since the amount of tyrosine produced in the presence or absence of the recycling system is the same. Our coupling factors of 40% for TAP (i.e., 0.4 equiv of tyrosine formed/1.0 equiv of TAP oxidized) and 60% for BTAP approach those reported by Bailey & Ayling (1980) and not Kaufman (1979) since the latter neglected the continued oxidation of NADH by reduced divicine in the presence of O_2.

One interpretation of the irreversible loss of the 5-amino substituent is through the formation of a 5-peroxy adduct (see Scheme I) which (1) may generate a carbonyl oxide, a hypothesized hydroxylating agent, (2) may decompose through deamination of the carbinol amine formed after oxygen atom transfer, or (3) may generate quinonoid TAP which then undergoes hydrolysis. The third is disfavored on the basis that the UV spectrum of quinonoid TAP is unaffected by the presence of PAH and phenylalanine.

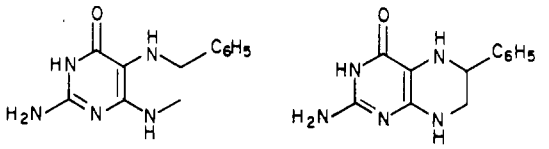
According to part 1 the difference between the pyrimidine and pterin behavior stems from the latter's potential for recyclization to the quinonoid form. Thus one might expect PAH to catalyze recyclization of **7**, and yet the direct interpretation of the data in Table I argues that **7** is responsible for tyrosine formation by simply acting as a pyrimidine analogue that remains acyclic throughout PAH turnover. There is no correlation between cyclization of **7** to dihydropterin and tyrosine formation; however, there is a direct relationship between the Br_2-promoted cyclization of **7** and that catalyzed by PAH over the pH range 7.4–11.0. Consequently, the PAH conversion of **7** to dihydropterin follows Scheme II, where the cyclization of oxidized **7** occurs nonenzymatically.

The failure of the ring-opened analogue **7** to exhibit any evidence of PAH-catalyzed ring closure, while it does not eliminate mechanisms featuring ring opening, fosters a degree of skepticism toward them. The high K_M of **7** relative to that of $DMPH_4$ (2 mM to 0.1 mM) indicates less than optimum binding of **7** to the active site of PAH. The aminoalkyl side

Scheme II



chain of **7** might be bound in a conformation which would retard the enzyme-catalyzed ring closure. In this case PAH-mediated ring formation might occur only if product release were rate limiting, provided sufficient conformational flexibility of the side chain existed to permit the adoption of the proper conformation. This increase in K_M for 4-aminoalkyl substituents is apparent upon comparison of the K_M for BTAP or 6-phenyltetrahydropterin of 3 μM (Bailey & Ayling, 1978b) to that of **5** (900 μM) which is isosteric with 6-phenyltetra-



hydropterin (**8**). The dichotomy between the products formed from the pyrimidines and tetrahydropterins during the PAH turnover as well as the greatly reduced V_{max} for the former [<5% (Bailey and Ayling, 1978b)] may indicate an incorrect positioning of the pyrimidine, resulting in the decomposition of its oxidized form. Given the presence of iron in PAH (Fisher et al., 1972; Gottschall, 1981), the question arises as to whether it also contributes to oxygen activation by interacting with the cofactor which might alter pyrimidine vs. pterin behavior.

The examination of other 5-substituted 2,4-diamino-6-hydroxypyrimidines demonstrates that amino substitution is probably required for cofactor activity since 5-Br, -I, -SCN, $-CH_2OH$, -OH, $-N{=}N-C_6H_5$, and $-S-C_6H_5$ do not support hydroxylation. The phenyldiazo (**4g**) and thiophenoxy (**4i**) compounds are potent competitive inhibitors of PAH with K_I = 21 μM and 4.7 μM, respectively. The requirement for amino substitution extends to the tetrahydropterins since neither the 5-deazatetrahydropterin, **1**, nor the 8-deazatetrahydropterin, **3**, supports hydroxylation. Surprisingly, **3** does not bind to PAH.

In conclusion, an intermediate—tentatively identified as the 4a-hydroxypterin adduct—is observed in hydroxylase-catalyzed reactions utilizing $6MPH_4$ and BH_4. It is probable that it is generated in all cases but is not detected owing to $k_2 > k_1$. The striking difference in the dependence of k_1 and k_{Tyr} vs. k_2 on PAH concentration suggests that the dehydration of the quinonoid is probably not an intrinsic property of PAH and supports the suggestion that a stimulating protein is required for the efficient regeneration of the tetrahydropterin cofactor by the reductase (Kaufman, 1975).

Acknowledgments

We thank Dr. Robert Henrie II for the synthesis of **4i**, Dave Gottschall for the synthesis of **3** and 5,6,7-trimethyl-5,6,7,8-tetrahydropteridin dihydrochloride monohydrate, Paul Domanico for technical assistance, and Dr. Ross Shiman for many

helpful discussions.

References

Archer, M. C., & Scrimgeour, K. G. (1970) *Can. J. Biochem. 48*, 526.
Ayling, J. E., Pirson, R., Pirson, W., & Boehm, G. (1973a) *Anal. Biochem. 51*, 80.
Ayling, J. E., Boehm, G. R., Textor, S. C., & Pirson, R. A. (1973b) *Biochemistry 12*, 2045.
Bailey, S. W., & Ayling, J. E. (1978a) *J. Biol. Chem. 253*, 1598.
Bailey, S. W., & Ayling, J. E. (1978b) *Biochem. Biophys. Res. Commun. 85*, 1614.
Bailey, S. W., & Ayling, J. E. (1980) *J. Biol. Chem. 255*, 7774.
Bendich, A., & Clements, G. C. (1953) *Biochim. Biophys. Acta 12*, 462.
Benson, F. R., Hartzel, L. W., & Savell, W. L. (1950) *J. Am. Chem. Soc. 72*, 1816.
Bryson, T. A., Donelson, D. M., Dunlap, R. B., Fisher, R. R., & Ellis, P. D. (1976) *J. Org. Chem. 41*, 2066.
Bublitz, C. (1977) *Biochem. Med. 17*, 13.
Chesterfield, J. H., Hurst, D. T., McOmie, J. F. W., & Tute, M. S. (1964) *J. Chem. Soc.*, 1001.
Craine, J. E., Hall, E. S., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 6082.
Dietrich, R., & Benkovic, S. J. (1979) *J. Am. Chem. Soc. 101*, 6144.
Fidler, W. E., & Wood, H. C. S. (1957) *J. Chem. Soc.*, 4157.
Fisher, D. B., & Kaufman, S. (1973) *J. Biol. Chem. 248*, 4300.
Fisher, D. B., Kirkwood, R., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 5161.
Gottschall, D. (1981) Ph.D. Thesis, The Pennsylvania State University.
Gullotti, M., Pasini, A., Fantucci, P., Ugo, R., & Gillard, R. D. (1972) *Gazz Chim. Ital. 102*, 855.
Huang, C. Y., Max, E. E., & Kaufman, S. (1973) *J. Biol. Chem. 248*, 4235.
Irwin, W. J., & Wibberley, D. G. (1967) *J. Chem. Soc. C*, 1745.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, p 490, McGraw-Hill, New York.
Kaiser, F. E., Gehrke, C. W., Zumwalt, R. W., & Kuo, K. C. (1974) *J. Chromatogr. 94*, 113.
Kaufman, S. (1957) *J. Biol. Chem. 226*, 511.
Kaufman, S. (1975) in *Chemistry and Biology of Pteridines* (Pfleiderer, W., Ed.) p 291, Walter de Gruyter, Berlin.
Kaufman, S. (1979) *J. Biol. Chem. 254*, 5150.
Kaufman, S., & Fisher, D. B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) p 285, Academic Press, New York.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Mager, H. I. X., Addink, R., & Berends, W. (1967) *Recl. Trav. Chim. Pays-Bays 86*, 833.
Moad, G., Luthy, C., Benkovic, P. A., & Benkovic, S. J. (1979) *J. Am. Chem. Soc. 101*, 6068.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.
Pfleiderer, W., & Walter, H. (1964) *Justus Liebigs Ann. Chem. 677*, 113.
Rembold, H., & Schramm, H. (1963) *Chem. Ber. 96*, 2786.
Shiman, R., & Gray, D. W. (1980) *J. Biol. Chem. 255*, 4793.
Shiman, R., Gray, D. W., & Pater, A. (1979) *J. Biol. Chem. 254*, 11300.
Storm, C. B., Shiman, R., & Kaufman, S. (1971) *J. Org. Chem. 36*, 3925.
Viscontini, M., & Bieri, J. (1972) *Helv. Chim. Acta 55*, 21.
Waalkes, T. P., & Udenfriend, S. (1957) *J. Lab. Clin. Med. 50*, 733.
Whitely, J. M., Drais, J. H., & Huennekens, F. M. (1969) *Arch. Biochem. Biophys. 133*, 436.