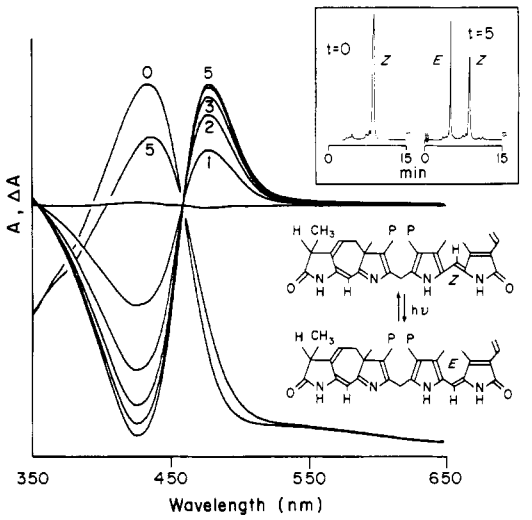


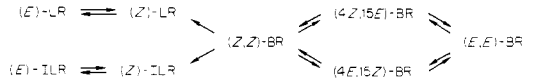
Figure 3. Absorbance and absorbance difference spectra on photolysis of isolumirubin IX and (inset) HPLC before and after photolysis. The numbers by the curves give the cumulative irradiation time in arbitrary units. The HPLC solvent was 0.1 M di-*n*-dodecylamine acetate in MeOH (1 mL/min) (P = $CH_2CH_2CO_2H$).

mophore,[1] lumirubins **2–5** undergo facile reversible configurational isomerization to more polar and thermally unstable *E* isomers on exposure to light, reaching $Z \rightleftarrows E$ equilibrium without detectable reversion to the parent bilirubins.[13] This reaction is readily detectable by difference spectroscopy[1] and HPLC (Figure 3), and it accounts for the minor HPLC peak at R_f 4.7 min appearing on prolonged photolysis of **1** (Figure 1).

Configurational isomerization of **1** bound to horse, rat, guinea pig, or bovine serum albumin generates 4*E*,15*Z*-**1** *and* 4*Z*,15*E*-**1**, with a slight preference for the latter (cf. Figure 1). In contrast, **1** bound to human serum albumin yields, under the same conditions, only one of the two diastereomeric *E*,*Z* isomers, 4*Z*,15*E*-**1** (Figure 1). Interestingly, similar marked stereoselectivity and regioselectivity is observed in humans during phototherapy,[3] whereas in jaundiced rats both *E*,*Z* isomers are formed.[1] Therefore the in vivo photochemistry appears to resemble the in vitro photochemistry very closely.

Acknowledgment. These studies were supported by Grants AM-26307, AM-11275, and AM-26743 (A.F.M.) and HD-09026 (D.A.L.) from the National Institutes of Health, Grant CHE 79-10133 (D.A.L.) from the National Science Foundation, and a research grant (MSC Springer No. 34) from the University of California, San Francisco (A.F.M.). The 360-MHz NMR spectra were run at University of California, Davis, NMR Facility with the assistance of Drs. J. L. Dallas and G. B. Matson. We thank Dr. K. L. Rinehart, University of Illinois, for running the FD MS of lumirubin IX dimethyl ester and Dr. Kenneth W. Ehler, University of Nevada, for preliminary studies on lumirubin IX and the synthesis of its ester. The editorial assistance of Diana Fedorchak and Michael Karasik also is acknowledged.

(13) (a) Photoreversion of **2** and **3** to **1** and of **4** and **5** to **6** was observed on relatively prolonged irradiation or at high irradiances. Reversion was accompanied by overall loss of pigment, and isosbestic points were not observed in the difference spectra. (b) Lumirubin formation from **1** is not inhibited by O_2. It is probably a concerted single-photon process proceeding via singlet **1**. Our unpublished data suggest that (*E*,*Z*)-bilirubins are not intermediates in lumirubin formation. Therefore the "fast" anaerobic photochemistry of bilirubin (**BR**, **1**) may be summarized by



where LR and ILR represent lumirubin and isolumirubin IX, respectively, and each step is a one-photon process. On the time scale of the $Z \rightleftarrows E$ conversions, lumirubin formation is slow and irreversible.

Phenylalanine Hydroxylase: Structural Determination of the Tetrahydropterin Intermediates by ^{13}C NMR Spectroscopy

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Received August 12, 1982

We have recently demonstrated[1a] that a tetrahydropterin-derived intermediate with a UV spectrum similar to the 4a-hydroxy-6-methyl-5-deazatetrahydropterin is formed during the turnover of L-phenylalanine to L-tyrosine catalyzed by phenylalanine hydroxylase (PAH; E.C. 1.14.16.1). The structure was postulated to be the 4a-hydroxy adduct, an intermediate proposed originally by Kaufman.[1b] In addition, when pyrimidine cofactors are used, the 5-amino substituent is cleaved to give, after reduction, a 5-hydroxypyrimidine.[2] These results implicate the 4a-carbon of the pterin as the site of electrophilic addition of oxygen. In this communication we report direct evidence for the enzyme-catalyzed addition of oxygen to the 4a-carbon of 6-methyltetrahydropterin (6-MPH_4) by using ^{13}C NMR at subzero temperatures and 90% selectively enriched [4a-^{13}C]-6-MPH_4 (**1**)[3] to detect the enzymatic reaction products.[4,5]

(1) (a) Lazarus, R. A.; Dietrich, R. F.; Wallick, D. E.; Benkovic, S. J. *Biochemistry* **1981**, *20*, 6834–6841. (b) Kaufman, S. In "Chemistry and Biology of Pteridines"; Pfleiderer, W., Ed.; Walter de Gruyter: Berlin, 1975; 291–304.

(2) Bailey, S. W.; Ayling, J. E. *J. Biol. Chem.* **1980**, *255*, 7774–7781.

(3) Lazarus, R. A.; Sulewski, M. A.; Benkovic, S. J. *J. Labelled Comp. Radiopharm.*, in press.

(4) The generation of the initial intermediate was carried out as follows. A solution of 0.02 M Tris, pH 8.5, containing 4 mM L-phenylalanine, catalase (0.67 mg/mL), 1.67 mM EDTA, saturated with oxygen[6] at 0 °C, was incubated under O_2 with PAH[7] (1.2 mg/mL) for 15 min at 0 °C. At t = 0, the activated enzyme solution (1.4 mL) at 0 °C was added to 0.85 mg of [4a-^{13}C]-6-MPH_4·$(HCl)_2$·H_2O, giving a pH of 8.0 and a tetrahydropterin concentration of 2.2 mM.[8] After O_2 was bubbled into the reaction, aliquots were taken and analyzed for the remaining tetrahydropterin,[9] for the formation of the intermediate,[10] and for the amount of tyrosine produced.[11] After 2 min only 35% of the [4a-^{13}C]-6-MPH_4 remained, which was confirmed by the generation of the UV spectrum of the initial intermediate.[1a] Tyrosine assays indicated that ca. 2.0 mM tyrosine (90%) had formed after 3.5 min. The solution containing the intermediate was slowly mixed at t = 3.5 min with 0.93 mL of precooled CD_3OD (−30 °C; bromobenzene/N_2 bath), taking care to keep the contents cold due to the high heat of solution. The unfiltered solution (−30 °C) was transferred to an NMR tube, and the ^{13}C NMR spectrum was recorded at −30 °C. Similar results (vide infra) were obtained in the absence of catalase and EDTA.

(5) ^{13}C NMR spectra for the [4a-^{13}C]-6-MPH_4 products were measured at 90.56 MHz. The samples were maintained at −30 °C, in order to slow the rates of dehydration[8] and rearrangement[12] of the intermediates and to shorten the relatively long spin–lattice relaxation times (T_1 = 43.2 s at 44 °C for C4a in the oxidized pterin) for these quaternary carbons, making it possible to collect spectra with adequate signal to noise ratios in 100–200 scans. During the time course of the reaction, the spectra were acquired by using a time-sharing data collection sequence, which alternately stored one scan under conditions of complete proton decoupling and one scan with the decoupler gated off. In this way it was possible to monitor continuously the long-range couplings between the 4a-carbon and the 6-proton in the products. The repetition rate for the entire sequence was approximately 16 s.

(6) An O_2-saturated solution of this buffer solution is 2.2 mM O_2 at 0 °C.

(7) PAH was purified through step IIB and had a specific activity of 10.0 μmol tyrosine min^{-1} mg^{-1}: Shiman, R.; Gray, D. W.; Pater, A. *J. Biol. Chem.* **1979**, *254*, 11300–11306.

(8) The generation of the initial intermediate was performed at 0 °C because of its instability at higher temperatures. The intermediate exhibits a first-order rate of decay (followed by decrease at 244 nm) with $t_{1/2}$ = 2.0 min at 23.2 °C. An Arrhenius plot yields E_a = 18.2 kcal/mol; therefore, at 0 °C $t_{1/2} \simeq$ 29.6 min. The activation energy for the rate of decay of the intermediates in 40% MeOH–0.02 M Tris, pH 8.0, is 17.0 kcal/mol.

(9) 6-MPH_4 was assayed by adding 20 μL of the solution to 1 mL of 75 μM 2,6-dichlorophenol–indophenol in 0.1 M potassium phosphate, pH 6.8. The decrease in OD_{600} (ϵ_{600} 16 100) of the dye, read immediately after addition, corresponds to the amount of 6-MPH_4 in solution.

(10) The formation of the intermediate was monitored by the appearance of its UV spectrum ($\epsilon_{245} \simeq$16 000; $\epsilon_{290} \simeq$8000) in 0.02 M Tris, pH 8.2.

(11) Aliquots of 10 μL were quenched into 1 mL of 3% trichloroacetic acid and analyzed for tyrosine from the fluorescence of the nitrosonapthol derivative. Waalkes, T. P.; Udenfriend, S. J. *Lab. Clin. Med.* **1957**, *50*, 733–736.

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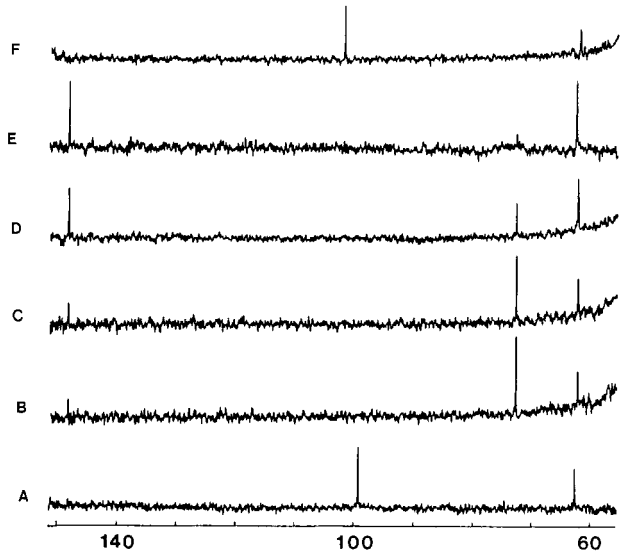
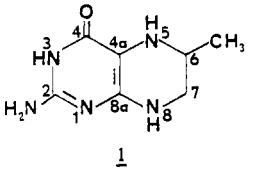


Figure 1. ^{13}C NMR spectra for the 4a-^{13}C intermediates described above, at −30 °C. Chemical shifts are referenced to internal CD_3OH (49.0 ppm). The signal at 61.5 ppm is due to Tris buffer (20 mM): (A) [4a-^{13}C]-6-methyltetrahydropterin (**1**) prior to addition of PAH and phenylalanine; (B) spectrum acquired after mixing with PAH[4] t = 19–54 min (100 scans); (C) t = 64–170 min (260 scans); (D) t = 228–258 min (200 scans); (E) t = 360–400 min (100 scans); (F) solution after 580 min, allowed to warm to 30 °C for 3 h (100 scans).



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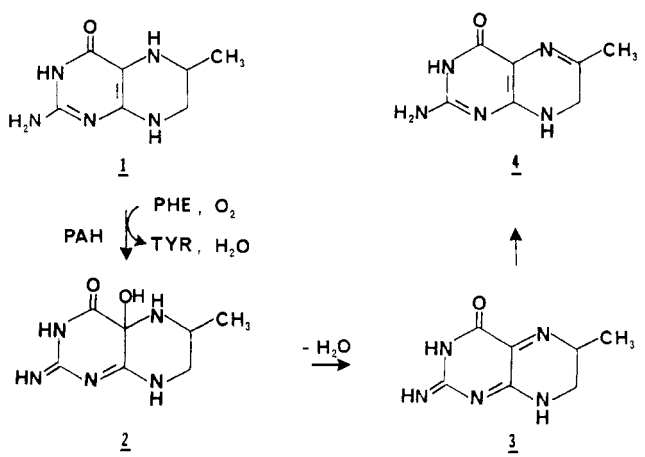
The ^{13}C NMR spectrum of [4a-^{13}C]-6-MPH_4 exhibits a resonance of 98.9 ppm at pH 8.0 (Figure 1A). When this compound is allowed to air oxidize to 7,8-6-MPH_2 (**4**), a resonance at 101.5 ppm appears (not shown).[13] The initial spectrum of the PAH-generated intermediate shows a new resonance at 72.3 ppm (Figure 1B). At longer times a resonance at 148.0 ppm grows in as the resonance at 72.3 ppm disappears (Figure 1B–E). After the contents are allowed to warm to 30 °C for 3 h, the resonance at 148.0 ppm is replaced by one at 101.5 ppm (Figure 1F).

The observed chemical shifts of the 4a-^{13}C resonance can readily be explained by Scheme I. The resonance at 72.3 ppm can be assigned confidently to the 4a-hydroxy adduct **2**, since ca. 90% of the tyrosine has already been produced, thus eliminating a 4a-peroxy adduct, which should have a similar ^{13}C NMR spectrum. A similar change in the chemical shift was observed for the 4a-carbon of the reduced flavin mononucleotide (104 ppm)

Scheme I



1 **4** **2** **3**

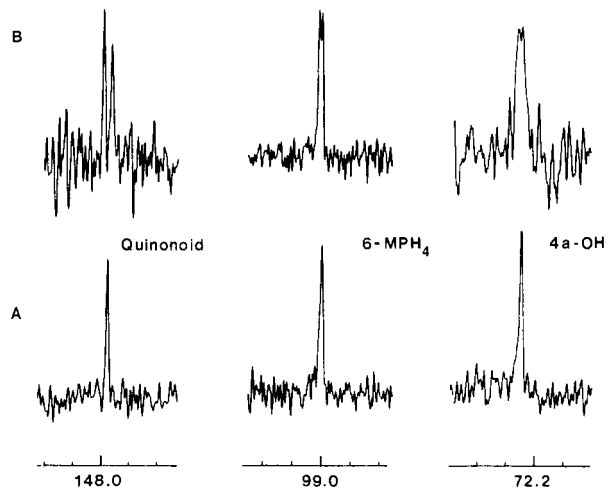
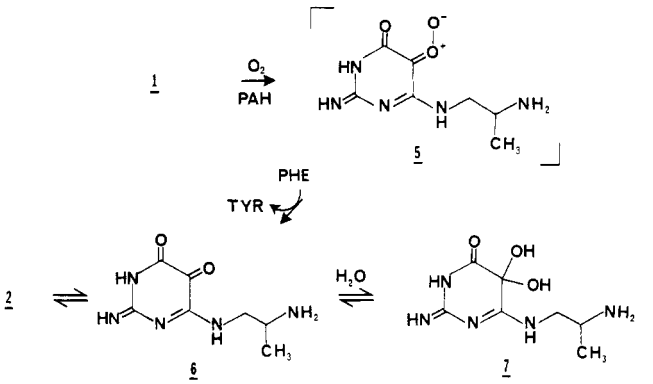


Figure 2. ^{13}C NMR spectra for the 4a-^{13}C intermediates described in the text: (A) broad-band ^1H decoupled; (B) fully ^1H coupled.

Scheme II



1 **5** **2** **6** **7**

to its oxygen adduct (74 ppm) in the bacterial luciferase reaction.[14] In addition the 3-methyl-4a-hydroxy-5-ethyl-4a,5-dihydroflavin pseudobase exhibits a 4a-^{13}C resonance at 74 ppm,[14] and Wessiak and Bruice have recently observed a resonance at 71.0 ppm, which they have ascribed to a 5-acetyl-4a-hydroxy-4a,5-dihydroflavin.[15] Structures such as $N(5)$-hydroxy-[16,17] or 8a-hydroxytetrahydropterins can now be eliminated as possible stable intermediates involved in the PAH-catalyzed hydroxylation of phenylalanine. Since only one intermediate having a ^{13}C resonance attributable to an sp^3 carbon was detected, it is unlikely that a 4a,8a-epoxide,[18] which should decompose through a hydroxy adduct, is present.[19]

(13) The 4a-^{13}C resonance for tetrahydrofolate and 7,8-dihydrofolate are 101.8 and 104.4 ppm, respectively, in NaOD as reported: Frick, W.; Weber, R.; Viscontini, M., *Helv. Chim. Acta* **1974**, *57*, 2658–2661. An upfield shift of ca. 2 ppm is observed for the 4a-carbon of folic acid upon a pH jump from 12.4 to 7.8: Ewers, U.; Günther, H.; Jaenicke, L. *Chem. Ber.* **1973**, *106*, 3951–3961.

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(17) For example, the ^{13}C chemical shift of the α carbon of dipropylamine is 52.3 ppm, whereas the α carbon of N-hydroxydiethylamine is 54.1 ppm. Similarly the carbon of 1*H*-benzotriazole resonates at 139.1 ppm whereas the carbon of 1-hydroxy-1*H*-benzotriazole comes at 142.5 ppm (Sadtler Standard Spectra, Sadtler Research Laboratories, Inc., 1978).

(18) Dmitrienko, G. I.; Snieckus, V.; Viswanatha, T. *Bioorganic Chem.* **1977**, *6*, 421–429. We have assumed that both such an epoxide and its ring-opened 4a-carbinolamine would have lifetimes sufficient for their observation.

(19) A hydroxylating species such as the 4a,5-oxaziridine proposed by Orf and Dolphin could give rise to a ^{13}C resonance similar to that we have assigned to the 4a-hydroxy adduct: Orf, H. W.; Dolphin, D. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2646–2650. However, if this is the case tyrosine formation would not accompany the appearance of the intermediate, contrary to what we observed. Similar arguments would eliminate a $N(5)$-nitroxyl radical as the hydroxylating species: Frost, J. W.; Rastetter, W. H. *J. Am. Chem. Soc.* **1981**, *103*, 5242–5245.

In summary, hydroxylating agents or products derived from addition of O_2 at any position other than the 4a-carbon are excluded.

Within the instrumental resolution (0.02 ppm) a single ^{13}C resonance is observed for **2**. Since hydroxylation at C4a generates a chiral center and racemic **1** (at C6) is employed, the absence of a second resonance is due to either magnetic equivalence at C4a in the two diastereomers or the less likely formation of a *single* geometrical isomer from *either* enantiomer. The latter hypothesis excludes nonenzymatic formation of **2**.

It has been suggested that an oxenoid species, **5**, which is formed at the 4a-carbon by addition of oxygen to the tetrahydropterin (or dihydroflavin), may be the actual hydroxylating species (Scheme II).[20] Support for this has been presented by Ayling[2] who observed that cleavage of the C4a-N5 bond occurs when pyrimidine cofactors are used, giving rise to an amine and, after reduction with β-mercaptoethanol, a 5-hydroxy pyrimidine (divicine). This raises the possibility that the resonance at 72.3 ppm might be the hydrated ketone **7** and the one at 148.0 ppm is due to **6**. However, structures **6** and **7** can be eliminated since all of the species involved must be ring closed, i.e., tetrahydropterins. Figure 2 shows the coupled and ^1H decoupled ^{13}C NMR spectra for the intermediates.[21] The observed coupling constants between C4a and H6 for the species are as follows: 6-MPH_4 (**1**), 1.8 Hz; 4a-OH (**2**), 2.3 Hz; quinonoid-6-MPH_2 (**3**), 5.2 Hz. It is clear that the long-range coupling from C4a to the proton at C6 is preserved in all the species, thus eliminating **6** and **7**[22] and supporting **2** and **3**. The tautomeric state of the quinonoid dihydropterin **3** is the subject of the following communication.

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Registry No. 1, 942-41-6; **2**, 83387-39-7; **3**, 70786-93-5; **4**, 17377-13-8; phenylalanine hydroxylase, 9029-73-6.

(20) Hamilton, G. A. *Prog. Bioorg. Chem.* **1971**, *1*, 83-157.

(21) The coupling between C4a and H6 is confirmed in the 360-MHz ^1H spectra of both **1** and **3**, when compared with those of their unlabeled counterparts.

(22) In addition the chemical shift of a ketone and its hydrate would be expected to resonate at a lower field than an imine or its hydrate. For example, the chemical shifts for the C5 of alloxan and its hydrate are 167.0 and 85.0 ppm, respectively.

Structural Determination of Quinonoid Dihydropterins

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Tetrahydropterins, which serve as biological cofactors for the aromatic amino acid hydroxylases,[1,2] are oxidized during the course of enzyme turnover to quinonoid dihydropterins.[2,3] The quinonoid dihydropterins may also be generated by a wide variety of chemical oxidants including bromine, 2,6-dichloroindophenol,[4] and ferricyanide[5] as well as H_2O_2 and peroxidase.[6] The unstable quinonoid compounds can either rearrange nonenzymatically to inactive 7,8-dihydropterins[5] or be reduced back to their tetrahydro form by NADH in a reaction catalyzed by dihydropteridine reductase (E.C. 1.6.99.7).[6-8]

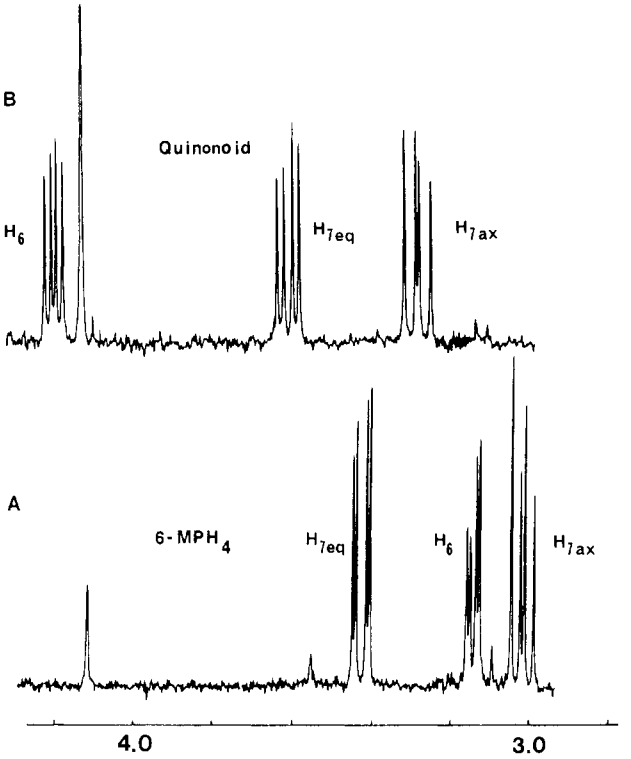
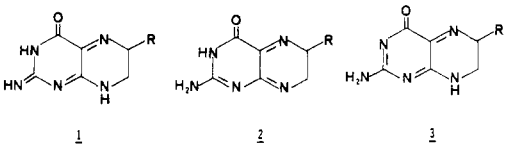


Figure 1. 360-MHz ^1H NMR spectra of 6-MPH_4 and quinonoid 6-MPH_2 at 5 °C; 32 transients were averaged per spectrum. Spectral width used; 5K, 0.305 Hz/pt resolution. The singlet at δ 4.13 is due to the protons at C7 of the rearranged 7,8-6-MPH_2 product: (A) 5 mM 6-MPH_4 in D_2O, 30 mM imidazole, 0.4 mM DSS, pD 8.4 (the 6-CH_3 at δ 1.18 was decoupled); (B) solution A, 10 min after addition of 1.1 equiv of Br_2 in D_2O, then adjusting pD to 8.4 (the 6-CH_3 at δ 1.40 was decoupled).

Postulated tautomeric structures for the quinonoid dihydropterins are shown in **1-3**. The UV spectra for quinonoid dihydropterins,[3,9] the rates for oxidation of various N-methylated tetrahydropterins,[10] and molecular orbital calculations[11] were interpreted to support the exocyclic para isomer (**1**). Support for the ortho isomer (**2**) is based on electrochemical data,[12] and arguments favoring the endocyclic para tautomer (**3**) have been derived from acid-base behavior.[13,14] The purpose of this communication is to demonstrate that the dihydropterin formed from both phenylalanine hydroxylase[15] and chemical oxidants exists as a para quinonoid isomer (**1** or **3**).



1 **2** **3**

Dehydration of 4a-hydroxy-6-methyltetrahydropterin, the initial product from phenylalanine hydroxylase,[15] gives quinonoid-6-

(1) Kaufman, S.; Fisher, D. B. "Molecular Mechanisms of Oxygen Activation"; Hayaishi, O., Ed.; Academic Press: New York, 1974; pp 285-369.

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(8) Webber, S.; Deits, T. L.; Snyder, W. R.; Whiteley, J. M. *Anal. Biochem.* **1978**, *84*, 491-503.

(9) Hemmerich, P. "Pteridine Chemistry"; Pfleiderer, W., Taylor, E. C., Eds.; Pergamon Press: London 1974; pp 143-169, 323.

(10) Viscontini, M.; Bobst, A. *Helv. Chim. Acta* **1965**, *48*, 816-822.

(11) Bobst, A. *Helv. Chim. Acta* **1967**, *50*, 1480-1491.

(12) Lund, H. "Chemistry and Biology of Pteridines"; Pfleiderer W., Ed.; Walter de Gruyter: Berlin, 1975; pp 645-670.

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(14) Scrimgeour, K. G. "Chemistry and Biology and Pteridines"; Pfleiderer W., Ed.; Walter de Gruyter: Berlin, 1975, pp 731-751.

(15) Lazarus, R. A.; DeBrosse, C. W.; Benkovic, S. J., previous communication in this issue.

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