purified photoisomers from bilirubin XIIIα (3) (Figure 4d) and mesobilirubin XIIIα (4). The photoisomer preparation derived from bilirubin IXα (1) contains two major components in the ratio of about 1:2 (Figure 2b). The NMR spectrum of this material (Figure 4b) was essentially a composite of the spectra of the corresponding IIIα and XIIIα photoisomers. Comparison of all of these spectra (Figures 3 and 4) showed unambiguously that the predominant photoisomer in the preparation derived from *Z,Z*-**1** is the 4*Z*,15*E* diastereomer, as designated in Figure 2b.

These data provide conclusive evidence that *E,Z* isomers are the main photoproducts formed on short-term irradiation of bilirubin in vitro,[14] and they confirm our previous structural assignments for "photobilirubin".[15] Clear evidence that they are the predominant yellow photoproducts excreted by the liver in vivo during blue-light irradiation of jaundiced rats is shown in Figure 2.[20]

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.). F.R.T. thanks the Caixa de Barcelona for a fellowship. The 360-MHz spectra were run at the University of California, Davis, NMR Facility with the assistance of Drs. J. L. Dallas and G. B. Matson. We are grateful to Diana Fedorchak and Michael Karasik for fast editorial aid.

(14) Assignment of the *E,E* configuration to the minor, most polar photoproducts from **1**–**4** follows from their thermal and acid lability, comparison with analogous *E,E* isomers,[3b,4a,b] and the absence of a similar product from the photolysate of (*Z*)-xanthobilirubinic acid.

(15) Previously we used the term "photobilirubin" to refer to the mixture of photoisomers obtained on irradiating *Z,Z*-**1** to photoequilibrium. Now that the individual isomers have been characterized, we recommend that the photobilirubin nomenclature be discontinued for the individual isomers. Several other "photobilirubins" have been reported and designated as configurational isomers of bilirubin. Many of these structural assignments are inconsistent with our data. Stoll et al.[16] isolated four substances by preparative TLC on silica and referred to them as photobilirubins IA, IB, IIA, and IIB. IA and IB were designated as 4*Z*,15*E*-**1** and 4*E*,15*Z*-**1**, respectively. IIA and IIB were considered to be conformational isomers of 4*E*,15*E*-**1**. We have been unable to separate or purify authentic *E,Z* isomers of **1** by TLC as described (see also: Sloper, R. W.; Truscott, T. G. *Photochem. Photobiol.* **1982**, *35*, 743–745). HPLC of IA and IB[9,11] revealed that both are complex mixtures containing only trace amounts of the designated *E,Z*/*Z,E* isomers and that neither IIA nor IIB is the 4*E*,15*E* diastereomer of **1**. Our data show unequivocally that none of the structural assignments in ref 16 is correct. These incorrect assignments have been perpetuated in subsequent papers.[8b,c,17] Onishi et al. detected more than 24 components after irradiating *Z,Z*-**1** anaerobically in $CHCl_3$.[18] At least seven of these were stated to be geometric isomers of **1**, but structures and adequate supporting data were not presented. Many of the observed products were probably secondary products and artifacts unrelated to the primary photochemistry of **1** and resulting from overirradiation and radical reactions. Isobe and Onishi have isolated three substances, designated as peaks 1, 2, and 3, from photolysis of *Z,Z*-**1** in aqueous serum albumin.[19] Peak 1 was not identified, peak 2 was attributed to *E,E*-**1**, and peak 3 was attributed to a mixture of the two *E,Z* isomers of **1**. On the basis of data described in this and the following communication, it is clear that peak 2 is not *E,E*-**1** and probable that peak 3 is almost exclusively 4*Z*,15*E*-**1**.

(16) Stoll, M. S.; Zenone, E. A.; Ostrow, J. D.; Zarembo, J. E. *Biochem. J.* **1979**, *183*, 139–146.

(17) Cohen, A. H.; Ostrow, J. D. *Pediatrics* **1980**, *65*, 740–750. Stoll, M. S.; Zenone, E. A.; Ostrow, J. D. *J. Clin. Invest.* **1981**, *68*, 134–141.

(18) Onishi, S.; Itoh, S.; Kawade, N.; Isobe, K.; Sugiyama, S. *Biochem. Biophys. Res. Commun.* **1979**, *90*, 890–896.

(19) Isobe, K.; Onishi, S. *Biochem. J.* **1981**, *193*, 1029–1031.

(20) Details of the animal experiments will be published separately. The structure of the minor photometabolite at R_f 6.1 min in Figure 2d is discussed in the following communication.

(21) Note **Added in Proof**: After this communication had been accepted, we were made aware of work in press on the ^1H NMR spectral analysis of the mixture of 4*Z*,15*Z*-**1** and 4*E*,15*E*-**1** obtained by anaerobic irradiation of 4*Z*,15*Z*-**1** (Falk, H.; Müller, N.; Ratzenhofer, M.; Winsauer, K. *Monatsh. Chem.*). In this work the authors also converted the mixture of *E* isomers of **1** to the corresponding, previously characterized, 4*Z*,15*E* and 4*E*,15*Z* biliverdins IXα, thus providing independent confirmation of the structural assignments made in the present communication.

Phototherapy for Neonatal Jaundice. Stereospecific and Regioselective Photoisomerization of Bilirubin Bound to Human Serum Albumin and NMR Characterization of Intramolecularly Cyclized Photoproducts

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Received July 30, 1982

The photochemistry of (*Z,Z*)-bilirubin IXα (**1**) is important because of the clinical use of phototherapy for treating neonatal hyperbilirubinemia (neonatal jaundice). It is now established that **1** undergoes rapid reversible configurational photoisomerization to *E* isomers in organic solvents, in serum, and in jaundiced mammals.[1] The *E* isomers of **1** are unstable and revert to the parent *Z,Z* isomer photochemically or thermally. At room temperature thermal reversal is highly solvent dependent, being slow in basic organic solvents, serum, or aqueous albumin and rapid in acidic or polar solvents. Previously, we described the formation of additional yellow products, more stable than the *E* isomers, on prolonged photolysis of **1**.[1,2] Small amounts of these products are formed in jaundiced rats exposed to blue light and in humans during phototherapy.[1,3] We now show that these compounds are structural isomers of **1**. We also show that complexation of **1** with serum albumin (SA) has a marked species-dependent influence on bilirubin photoisomerization and, in particular, on the regioselectivity of the configurational isomerization.

On photolysis of **1** in $CHCl_3$–Et_3N (1:1) (Figure 1a–d) the system reached a photostationary state in ca. 10 min.[4] HPLC revealed the expected *E,E* and *E,Z* isomers (R_f 4.8 and 7.4 min, respectively) and a minor peak at R_f 6.1 min.[4] On continued photolysis the 6.1-min peak grew slowly, without affecting the *E,Z*:*Z,Z* ratio, along with a smaller peak (R_f 4.7 min) that ran close to *E,E*-**1**. All peaks except the R_f 6.1-min peak and a minor unidentified peak at R_f 6.7 min disappeared on treatment of the photolysate with CF_3CO_2H[1] (Figure 1e). Similar rapid configurational isomerization accompanied by slow growth of the 4.7- and 6.1-min peaks was observed when complexes of **1** with SA were irradiated at pH 7.4.[5] However, the identity of the albumin

(1) McDonagh, A. F.; Palma, L. A.; Trull, F.; Lightner, D. A. *J. Am. Chem. Soc.*, preceding communication in this issue, and references cited therein. See the preceding communication for the structure of **1**.

(2) McDonagh, A. F. "Phototherapy in the Newborn: An Overview"; Odell, G. B., Schaffer, R., Simopoulos, A. P., Eds.; National Academy of Sciences, Washington, DC, 1974; pp 56–73. McDonagh, A. F. "Bilirubin Metabolism in the Newborn (II)"; Bergsma, D., Blondheim, S. H., Eds.; Excepta Medica: Amsterdam, 1976; pp 30–38.

(3) McDonagh, A. F.; Ennever, J. F.; Palma, L. A., manuscript in preparation.

(4) Solutions containing 1.25 mg of pigment/10 mL were photolyzed under argon with a 20-W blue fluorescent tube.[1] Albumin solutions were made up in 0.1 M phosphate buffer, and the albumin:pigment ratio was 1.1:1. The solvent for reverse-phase HPLC (Beckman-Altex Ultrasphere-IP or Ultrasphere-ODS column, 5 μm, C-18, 25 × 0.46 cm; with a Beckman-Altex ODS precolumn, 4.5 × 0.46 cm) was 0.1 M di-*n*-octylamine acetate in MeOH (0.75 mL/min, detector 450 nm). Retention times may vary with age and origin of column. Retention volume = $0.75R_t$. For HPLC, albumin solutions were diluted 1:4 or 1:9 with ice-cold mobile phase, and 20 μL of the supernatant was injected. Lumirubins were isolated by solvent extraction[2] and TLC on silica (solvent, 1% AcOH in 10% MeOH–$CHCl_3$). Nonphotochemical work was done under safelights. Mass spectra were field desorption (FD) spectra.

(5) (a) **1** forms strong association complexes ($K_a \simeq 10^7$–10^8 M^{-1}) with mammalian serum albumins (See: McDonagh, A. F. "The Porphyrins"; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 6, Chapter 6, pp 392–491). (b) Reactions were studied by using Cohn Fraction V serum albumins from horse, human, guinea pig, ox, and rat and also by using adult rat and human serum. (c) For photolysis of **1** in anaerobic conditions, see: McDonagh, A. F. *Ann. N.Y. Acad. Sci.* **1975**, *244*, 553–569.

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had a marked effect on the rates of product formation and on the composition of the photoproducts at a given irradiation time (cf. Figure 1f–j).

The isolated R_f 6.1-min product separated on TLC into two components that exhibited identical absorption spectra (λ_{max} 434 nm, 10% MeOH–$CHCl_3$) and readily interconverted in base (1% NH_4OH–MeOH), suggesting an isomeric (epimeric) relationship.[6] The most mobile component we call lumirubin IX (**2**); the least mobile, isolumirubin IX (**3**). Methylation (CH_2N_2) of an unresolved mixture of **2** and **3** gave a product with a FD MS[4] parent ion at 612 amu, the same value as for **1**-dimethyl ester, indicating that **1**, **2**, and **3** are isomeric. An analogous pair of compounds [lumirubin XIII (**4**) and isolumirubin XIII (**5**), λ_{max} 431 and 432 nm, respectively] was isolated from photolysis of (*Z*,*Z*)-bilirubin XIIIα (**6**) in aqueous human serum albumin or $CHCl_3$–Et_3N. However, similar compounds were not detected during photolysis of bilirubin IIIα (**7**), mesobilirubin IXα, mesobilirubin XIIIα, or xanthobilirubinic acid in the same solvents.[7] Since none of these compounds has a vinyl group at C-3, as in **1** or **6**, we conclude that this function is involved in lumirubin formation.

The structures of **2**–**5** were deduced from 360-MHz ^1H NMR. Figure 2 shows the olefinic region of the spectra of lumirubins IX (**2**) and XIII (**4**). (In this region, the spectrum of each isolumirubin resembles that of the corresponding lumirubin; i.e., **2** ≡ **3** and **4** ≡ **5**.) Striking features in the spectrum of **4**, compared to its symmetrical parent **6** are (a) a strong upfield shift from 6.09 to 5.77 ppm of one of the two, formerly equivalent, meso protons at C-5 and C-15, (b) loss of one of the two identical vinyl groups, and (c) emergence of a new broad doublet at 5.87 ppm corresponding to one olefinic proton. The olefinic regions of lumirubins IX (**2**) and XIII (**4**) are similar, except that in **2** the remaining unreacted (exo) vinyl group has the same chemical shift and appearance as that in bilirubin IIIα (**7**). Therefore, in the conversion of **1** and **6** to the corresponding lumirubins, half of each molecule remains essentially unchanged. Moreover, the unchanged halves of **2** and **4** differ only in the position of a vinyl substituent. Key evidence for the structure of the changed half of **4** is (a) the appearance of two new high-field CH_3 signals, one a singlet at 1.07 ppm (1.01 ppm for the iso-compound, **5**) and the other a doublet near 1.17 ppm (1.22 ppm in **5**), (b) loss of signals corresponding to the C-2 and C-7 CH_3 groups of the parent compound **6**, and (c) emergence of a single proton signal near 3.2 ppm coupled to the high-field CH_3 doublet. Similar data were obtained for **2** and **3**. These data are consistent with the structures formulated in Figure 2 for lumirubins IX and XIII.[8] The corresponding isolumirubins are diastereomers related by epimerization at C-2. Like other compounds with a dipyrrylmethenone chro-

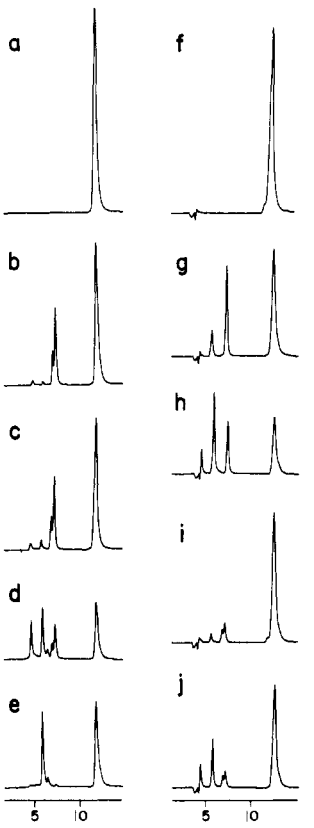


Figure 1. Photolysis of **1** and **1**-serum albumin complexes: photolysis of **1** in $CHCl_3$–Et_3N (1:1); HPLC of samples at (a) 0 min, (b) 10 min, (c) 30 min, (d) 4 h; (e) HPLC of sample d after treatment with CF_3CO_2H; photolysis of **1** in aqueous human serum albumin, pH 7.4, HPLC at (f) 0 min, (g) 30 min, (h) 4 h; photolysis of **1** in aqueous rat serum albumin HPLC at (i) 30 min, (j) 4 h. For experimental details see ref 4. Retention times are in minutes.

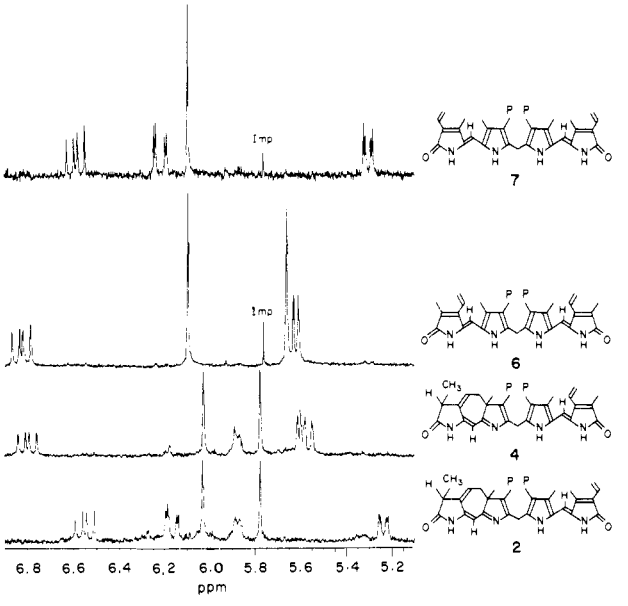
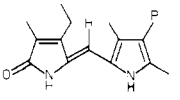


Figure 2. Olefinic region of the 360-MHz NMR (Me_2SO-d_6) of (top to bottom) bilirubin IIIα, bilirubin XIIIα, lumirubin XIII, lumirubin IX. Proton resonances are in ppm downfield from Me_4Si (P = $CH_2CH_2CO_2H$).

(6) These components remained homogeneous on two-dimensional TLC and coeluted on the HPLC systems used in this work.

(7) (*Z*)-Xanthobilirubinic acid:



Grunewald, J. O.; Cullen, R.; Bredfeldt, J.; Strope, E. R. *Org. Prep. Proc. Int.* **1975**, *7*, 103–110.

(8) These structures are among several that have been proposed previously for "photobilirubins IIA and IIB", prepared in 2% yield by UV–vis irradiation of **1** in Me_2SO.[9,10] It now seems likely that **2** and **3** are the same as "photobilirubin II" and "unknown pigment" or "peak 2".[9–11] However, we do not find, as reported for photobilirubin II,[9] that **2** and **3** readily revert to **1** on photolysis.[13] It is also likely that **2** and **3** account for the unidentified secondary product with relatively high fluorescence yield detected by Lamola et al. on photolysis of **1** in human serum albumin solution.[12]

(9) Stoll, M. S.; Zenone, E. A.; Ostrow, J. D.; Zarembo, J. E. *Biochem. J.* **1979**, *183*, 139–146.

(10) Stoll, M. S.; Vicker, N.; Gray, C. H.; Bonnett, R. *Biochem. J.* **1982**, *201*, 179–188.

(11) Isobe, K.; Onishi, S. *Biochem. J.* **1981**, *193*, 1029–1030.

(12) Lamola, A. A.; Flores, J.; Doleiden, F. H. *Photochem. Photobiol.* **1982**, *35*, 649–654.

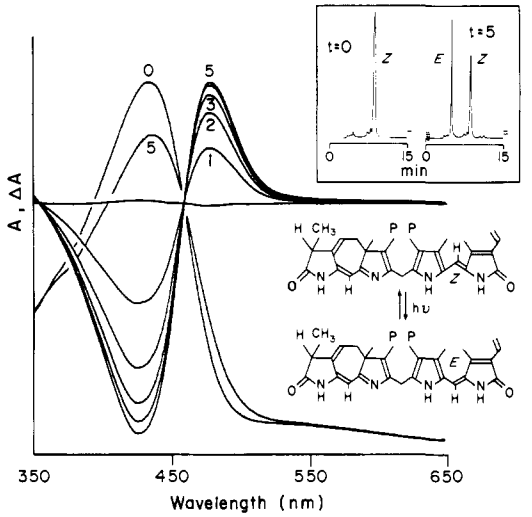


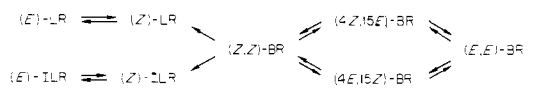
Figure 3. Absorbance and absorbance difference spectra on photolysis of isolumirubin IX and (inset) HPLC before and after photolysis. The numbers by the curves give the cumulative irradiation time in arbitrary units. The HPLC solvent was 0.1 M di-*n*-dodecylamine acetate in MeOH (1 mL/min) (P = $CH_2CH_2CO_2H$).

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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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-$MPH_4 \cdot (HCl)_2 \cdot H_2O$, 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.

(12) Archer, M. C.; Scrimgeour, K. G. *Can. J. Biochem.* **1970**, *48*, 278–287.

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