References and Notes

(1) For reviews on 1,1-diazene behavior see: (a) Lemal, D. M. In "Nitrenes", Lwowski, W., Ed.; Interscience: New York, 1970, Chapter 10. (b) Ioffe, B. V.; Kuznetsov, M. A. *Russ. Chem. Rev. (Engl. Trans.)* **1972**, *41*, 131.

(2) (a) Casewit, C.; Goddard, W. A. *J. Am. Chem. Soc.*, in press. This paper includes zero point and temperature effects (298 K). The estimate is based on a large basis GVB-CI calculation of the N-H bond energy of the parent H_2N_2 1,1-diazene and corrected for the difference (18.5 kcal/mol) between the N-H and N-CH_3 bond strengths. (b) Pasto, D. J.; Chipman, D. M. *Ibid.* **1979**, *101*, 2290. This calculation does not include zero point and temperature corrections.

(3) Hinsberg, III, W. D.; Dervan, P. B. *J. Am. Chem. Soc.* **1978**, *100*, 1608.

(4) Chromatography on deactivated basic alumina at −82 °C using dimethyl ether–propane as solvent removed the *tert*-butyl alcohol and substantial amounts of unreacted 1-amino-2,2,6,6-tetramethylpiperidine (**4**). A 1,1-diazene → tetrazene dimerization (**2** → **3**) on the column resulted in the loss of ~65% of the purple 1,1-diazene **2** upon chromatography. Importantly, the chromatography and subsequent addition of triethylamine[5] were necessary to obtain reproducible kinetics.

(5) The 1,1-diazene **2** is sensitive to trace acid.

(6) From the NMR experiment (EM-390) we measured the concentration of **2** against an internal standard (CH_2Cl_2). From this we calculate the extinction coefficient for the n-π^* electronic transition of **2**, $\epsilon \sim 18 \pm 3$.

(7) The tetrazene product **3** was isolated previously and characterized. The ratios of hydrocarbon products are sensitive to both solvent and temperature and will be reported in a full manuscript. At 0 °C in Et_2O we find hydrocarbons **5**–**8** in ratios of 61:10:24:5, respectively.

(8) The purple solution is introduced via Teflon tubing connected to sample injection parts into a specially designed copper-jacketed quartz cell attached to a cryogenic unit[9] maintained at −21 to +4 ± 0.2 °C. The optical density was monitored on a Cary Model 14 spectrophotometer.

(9) Air Products Laboratory cryogenic system, Model LC-1-100 liquid nitrogen Dewar assembly, Model WMX-1A optical shroud with injector ports.

(10) For *trans*-azo-*tert*-butane the enthalpy of activation $\Delta H^{\ddagger} = 42.2 \pm 0.8$ kcal/mol. Martin, J. C.; Timberlake, J. W. *J. Am. Chem. Soc.* **1970**, *92*, 978. For a recent discussion of the enthalpies of 1,2-diazene decompositions, see Engel, P. S.; Hayes, R. A.; Keifer, L.; Szilagyi, S.; Timberlake, J. W. *Ibid.* **1978**, *100*, 1876.

(11) (a) Schultz, A.; Rüchardt, C. *Tetrahedron Lett.* **1976**, 3883. Duismann, W.; Rüchardt, C. *Chem. Ber.* **1978**, *111*, 596. (b) Reichardt, C. *Angew. Chem., Int. Ed. Engl.* **1965**, *4*, 29, and references cited there.

(12) Hinsberg, III, W. D., Ph.D. Thesis, California Institute of Technology, 1979.

(13) Computer simulation performed using the MS1M4 Stochastic Mechanism Simulator developed by F. A. Houle and D. L. Bunker, Quantum Chemistry Program Exchange No. 293.

(14) On the basis of the computer simulation we estimate an E_a of ≤7 kcal/mol for the bimolecular process **2** → **3**.

(15) National Science Foundation Predoctoral Fellow, 1975–1978.

(16) Alfred P. Sloan Research Fellow, 1977–1979; Camille and Henry Dreyfus Teacher-Scholar, 1978–1983.

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Generation and Thiol Reduction of a "Quinonoid" Dihydropterin and an Oxidized Pyrimidine Analogue

Sir:

In an effort to determine the exact role of the tetrahydropterin cofactor utilized in the formation of tyrosine from phenylalanine and molecular oxygen in the reaction catalyzed by phenylalanine hydroxylase[1] (E.C. 1.14.16.1), we have been investigating the spectral and chemical properties of a "quinonoid" dihydropterin and an analogous "quinonoid" oxidized pyrimidine, the presumed products derived from the respective cofactors during turnover. The quinonoid compounds also may be generated by chemical oxidants including bromine, dichlorophenolindophenol,[2] and ferricyanide[3] as well as peroxidase.[4] These species are rapidly reduced to the parent cofactor by a variety of reagents, including thiols[5] and quinonoid dihydropteridine reductase.[6] Our results for the thiol reduction of the quinonoid compounds implicate the intermediacy of thiol adducts presumably at the 4a,8a- and 5,6 positions of the oxidized pterin and pyrimidine, respectively, during the reduction process and provide direct evidence for the existence of a hydroxylated derivative at the 5 position of 4-hydroxy-2,5,6-triaminopyrimidine during its oxidation by horseradish peroxidase (E.C. 1.11.1.7).

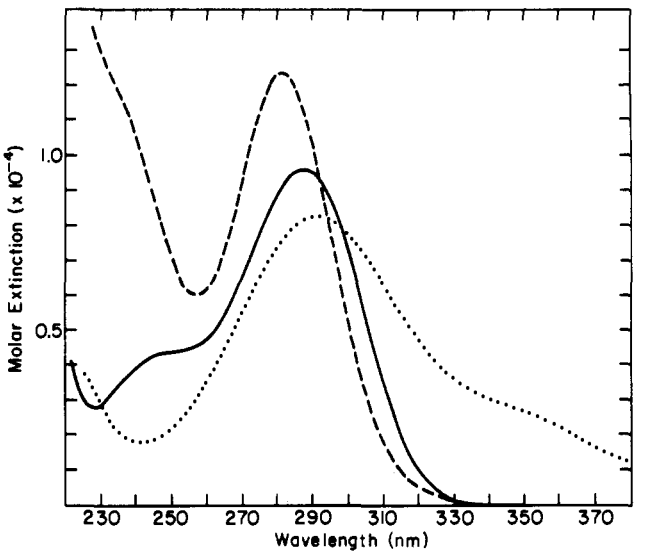
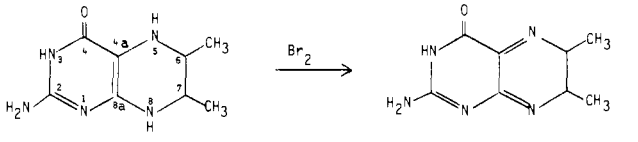


Figure 1. UV spectra of 4-hydroxy-2,5,6-triaminopyrimidine (**5**) (—), the product of bromine oxidation of **5** (···), and 2,6-diamino-4,5-dihydroxypyrimidine (**8**) in the presence of a 20-fold excess of dithiothreitol (- - -), in 0.2 M Tris·HCl, pH 8.10.

When 6,7-dimethyltetrahydropterin (**1**) is treated with 1 equiv of bromine, an oxidized pterin species (**2**)[7] is generated whose UV spectral characteristics are consistent with those attributed to the primary oxidation product of **1** in its phenylalanine hydroxylase catalyzed oxidation.[2] The reaction of **2** with excess 2-mercaptoethanol (pH 7.45–8.10, Tris buffer, μ = 0.2 KCl, 25 °C) gives two products, **1** and 7,8-dihydro-6,7-dimethylpterin (**3**). The reaction can be followed by the



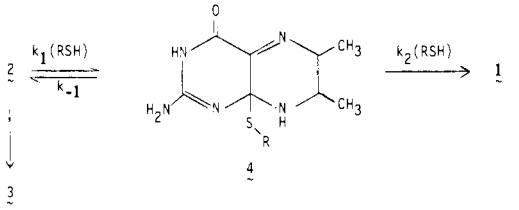
1 2

decrease in absorbance at 380 nm. The reductive process at a given pH is dependent on the second power of the total thiol concentration, independent of buffer, and gives rise to the following rate law as shown by the dependence of the rate on pH:

$$k_{obsd} = k_2'[RSH][RSH]$$

The kinetics are consistent with the processes outlined in Scheme I, where $k_2' = k_1k_2/k_{-1} = 2.2 \times 10^3\ s^{-1}\ M^{-2}$ and require the intermediacy of an adduct such as **4**, derived from attack by mercaptoethanol at the 8a carbon. We cannot rule out, on the basis of presently available data, formation of an analogous 4a adduct. The intermediate resembles that recently isolated by Sayer et al.[8] in the reaction of 1,3-dimethyl-5-(*p*-nitrophenylimino)barbituric acid with thiols and implicated

Scheme I



2 1 3 4

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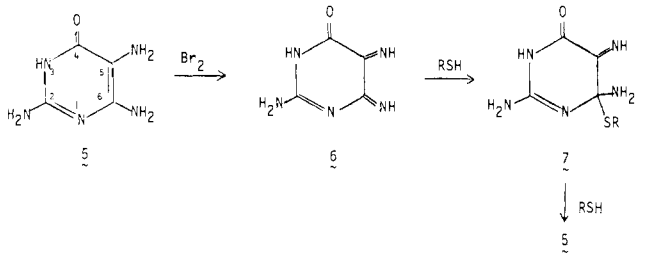
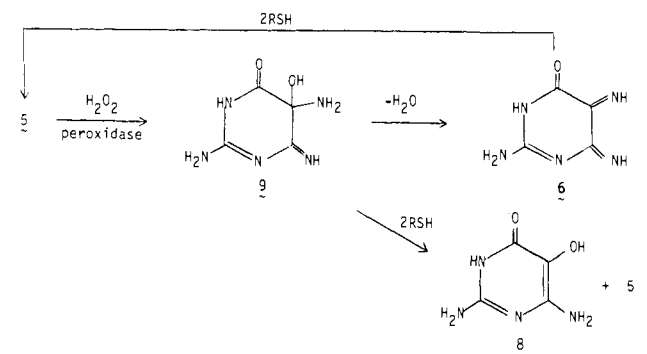
by kinetic evidence in the nonenzymic reduction of flavins and flavin analogues by thiols.[9-12] In the present case the decomposition of **4** to **1** apparently is rate limiting and **4** does not accumulate.

An analogous sequence of reactions was carried out with 4-hydroxy-2,5,6-triaminopyrimidine (**5**). The product of bromine oxidation, as well as hydrogen peroxide oxidation catalyzed by horseradish peroxidase, gave UV spectral changes similar to those observed for the conversion of the tetrahydropterin **1** into its quinonoid dihydropterin **2** suggesting the formation of **6**. It is also noteworthy that **5** has been shown to substitute for tetrahydropterin in the reaction catalyzed by phenylalanine hydroxylase.[13,14] Compound **6** generated by bromine oxidation gives rise to biphasic kinetics when treated with a large excess (~20-fold) of 2-mercaptoethanol in 0.2 M Tris·HCl (μ = 1.0 KCl) at 25 °C, pH 7.10, with the reaction being monitored at 300 nm, indicative of the buildup and decay of an intermediate (**7**).[15] The rate of accumulation of **7** measured at 350 nm follows a first-order dependence on mercaptoethanol[16] but is independent of buffer concentration yielding a second-order rate constant for the reaction of **5** with mercaptoethanol of 2.6×10^2 M^{-1} s^{-1}. The decay of **7** also shows a first-order thiol dependence yielding a second-order rate constant for the reaction of thiol with **7** of 1.5×10^1 M^{-1} s^{-1}. The accumulation of **7** contrasts with the greater instability of **4**. This behavioral difference might reflect a greater stability of **2** vs. **6** owing to the bridging ethylene moiety or a difference in the position of thiol addition to **2** vs. **6**.



When the hydrogen peroxide–peroxidase catalyzed reaction is carried out in 0.2 M Tris, pH 8.10, in the presence of excess hydrogen peroxide and a 20-fold excess of dithiothreitol, the triaminopyrimidine (**5**) is not recycled but is converted into a diaminodihydroxypyrimidine. The product is identical in its UV and GC-MS behavior with authentic 2,6-diamino-4,5-dihydroxypyrimidine (**8**) synthesized by the method of Chesterfield et al.[17] Compound **8** shows an absorbance maximum at 281 nm at both neutral and acidic pH values, whereas the λ_{max} for **5** is at 264 nm in 0.1 N HCl and 288 nm at pH 8.10 (Figure 1).[18] The formation of **8** requires the initial formation of a carbinolamine (**9**) at the 5 position (Scheme II),[19] which in the absence of the thiol at pH 8.10 eliminates water to form a quinonoid species but which can be trapped by thiol addition. According to this scheme, excess peroxide and dithiolthreitol would lead eventually to complete conversion into **8**, which is the observed phenomenon. It is noteworthy that oxidation prior to dithiothreitol addition does not generate **8**, mandating that the reversible hydration of **6** is negligible under these conditions. Thus, when the peroxidase catalyzed oxidation is quenched with CN^-, followed by addition of excess dithiothreitol, the quinonoid oxidation product is reduced back to **5**. The failure to observe an analogous ring-opened species during the oxidation of the tetrahydropterin (**1**) might be due to rapid recyclization or possibly to stereoelectronic factors that favor hydroxyl elimination. We note that **8** (10^{-4} M) is not converted into **5** under our conditions (pH 8.1) in the present of 10^{-1} M NH_4Cl. The finding that electrophilic attack occurs at the 5 carbon parallels our observation of electrophilic additions to the 4a carbon of 5-deaza-6-methyltetrahydropterin[20] and offers some support to an earlier hypothesis that species similar to **8** might occur as intermediates in the phenylalanine hydroxylase catalyzed reactions.[21,22]

Scheme II



Acknowledgment. This investigation was supported through National Science Foundation Grant No. PCM7803847.

References and Notes

(1) Kaufman, S.; Fisher, D. B. "Molecular Mechanisms of Oxygen Activation", O. Hayaishi, Ed.; Academic Press: New York, 1974; p 285.
(2) Kaufman, S. *J. Biol. Chem.* **1961**, *236*, 804.
(3) Archer, M. C.; Scrimgeour, K. G. *Can. J. Biochem.* **1970**, *48*, 278.
(4) Nielsen, K. H.; Simonsen, V.; Lind, K. E. *Eur. J. Biochem.* **1969**, *9*, 497.
(5) Bublich, C. *Biochim. Biophys. Acta* **1969**, *191*, 249.
(6) Kaufman, S. *J. Biol. Chem.* **1957**, *226*, 511.
(7) Compound **2** has been proposed (Kaufman, S. *J. Biol. Chem.* **1964**, *239*, 332) to exist in one of two tautomeric forms designated the ortho- and para-quinonoid structures, which at pH values below the pK_a of N-8 are resonance structures. We have depicted the ortho structure.
(8) Sayer, J. M.; Conlon, P.; Hupp, J.; Fancher, J.; Belanger, R.; White, E. J. *J. Am. Chem. Soc.* **1979**, *101*, 1890.
(9) Gascoigne, I. M.; Radda, G. K. *Biochim. Biophys. Acta* **1967**, *131*, 498.
(10) Yokoe, I.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 450.
(11) Loechler, E. L.; Hollocher, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 3235.
(12) Bruice, T. C. *Prog. Bioorg. Chem.* **1976**, *4*, 1.
(13) Bailey, S. W.; Ayling, J. E. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1614.
(14) Kaufman, S.; *J. Biol. Chem.* **1979**, *254*, 5150.
(15) As in the case of **4**, the structure of **7** is uncertain and may involve thiol addition at the 5 rather than 6 position.
(16) The lack of a positive intercept in the plot of k_{obsd} vs. total thiol is consistent with negligible return of **7** to **6**.
(17) Chesterfield, J. H.; Hurst, D. T.; McOmie, J. E. W.; Tufe, M. S. *J. Chem. Soc.* **1964**, 1001.
(18) For isolation of **8**, **5** (0.017 mmol) was treated with 0.051 mmol of H_2O_2 and 0.34 mmol of dithiothreitol in 100 mL of water (pH 8.1) in the presence of ~0.3 mg of horseradish peroxidase, and monitored by following the shift in λ_{max} of the reaction mixture aliquots acidified with 0.1 N HCl. The reaction was then quenched by treatment with 2 molar equiv of $Na_2S_2O_5$ followed by adjusting the pH to ~1 with 6 N HCl. After concentration of the product mixture in vacuo, the enzyme was removed by Sephadex G-25 gel filtration and the fractions containing **8** were combined and concentrated to dryness. Excess dithiothreitol was removed by ether extractions and **8** analyzed as its di-, tri-, and tetratrimethylsilyl derivatives by GC–MS on an OV-17 column.
(19) The possibility exists that in the absence of dithiothreitol a different peroxidase species is acting (Yamakazi, I. "Molecular Mechanisms of Oxygen Activation", O. Hayaishi, Ed.; Academic Press: New York, 1974; p 535) which does not generate **6** via **9**. However, when the reaction is carried out in phosphate buffer (0.2 M, pH 8.10, no Cl^- present) results identical with those in Tris·HCl were obtained (i.e., formation of **6** in the absence and **8** in the presence of dithiothreitol). Moreover, the lack of formation of **9** from **6** generated by bromine oxidation further points to the absence of **6** → **9** conversion under the present conditions.
(20) (a) Moad, G.; Luthy, C. L.; Benkovic, S. J. *Tetrahedron Lett.* **1978**, 2271. (b) Moad, G.; Luthy, C. L.; Benkovic, S. J. *Proc. Int. Symp. Chem. Biol. Pteridines, 6th 1979*, **1979**, 55.
(21) Kaufman, S. "The Chemistry and Biology of Pteridines", W. Pfleiderer, Ed.; de Gruyter: Berlin, **1975**; p 291.
(22) NOTE ADDED IN PROOF. Examination of the spectral changes during the horseradish peroxidase catalyzed oxidation of **1** and **5** in the presence of excess dithiothreitol and peroxide indicates that differing kinetic pathways are followed during the net two-electron oxidation of **1** and **5** (Kiel, J., personal communication).

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