Dopamine β-hydroxylase, we have prepared the (*R*)- and (*S*)-[β-^{2}H]phenethylamine and examined the absolute stereochemistry and primary and secondary isotope effects in its hydroxylation.

The (*R*)-[β-^{2}H]- and (*S*)-[β-^{2}H]-*N*-acetylphenylalanines were synthesized by the method of Kirby.[10] The proton magnetic resonance (pmr) spectra of these intermediate deuterated phenylalanines showed only an AX quartet, indicating the presence of only one diasteriomer. The enantiomers were esterified using diazomethane and the D,L-*N*-acetylphenylalanine methyl ester resolved using α-chymotrypsin.[11] This method of resolution offers a milder route to the enantiomeric deuterated compounds which is less likely to induce epimerization at the α center than the *N*-chloroacetyl method used earlier. After resolution, the free deuterated phenylalanines were generated by acid hydrolysis and decarboxylated using acetophenone.[12] Analysis of the final (*R*)- and (*S*)-[β-^{2}H]phenylethylamine by both pmr and mass spectroscopic techniques[13] showed greater than 92% deuterium at the one benzylic position. The Dopamine β-hydroxylase enzyme used was purified by the method of Kaufman through the calcium phosphate gel step.[4]

Hydroxylation of these enantiomeric deuterated substrates leads to complete loss of deuterium from the *R* isomer and complete retention of deuterium in the *S* isomer, demonstrating that the hydroxylation takes place with a net retention of configuration at the benzylic center.[13] This confirms the results of Taylor[9] obtained using a different substrate and an entirely different chemical approach to the creation of the isotopic asymmetry at the benzylic center.

A variety of anions stimulate the activity of Dopamine β-hydroxylase,[4] with fumarate being particularly effective. We examined the primary and secondary isotope effects on the rate of hydroxylation under saturating conditions both in the presence and absence of fumarate. In the presence of fumarate the k_H/k_D for the hydroxylation of the (*R*)-[β-^{2}H]-phenylethylamine is 2.0 and for the *S* isomer the secondary isotope effect is 1.0. In the absence of fumarate the primary isotope effect for the *R* isomer is 5.0 and the secondary isotope effect for the hydroxylation of the *S* isomer is 1.7.[14]

(10) G. W. Kirby and J. Michael, *Chem. Commun.*, 415 (1971).

(11) G. E. Clement and R. Potter, *J. Chem. Educ.*, **48**, 695 (1971). After complete hydrolysis of the L ester, the D-*N*-acetylphenylalanine methyl ester was extracted with methylene chloride. The pH of the aqueous phase was increased to 12 to denature the protein and then adjusted to 6 to recover the L isomer.

(12) G. Chatelus, *Bull. Soc. Chim. Fr.*, 2523 (1964).

(13) The retention and/or loss of deuterium was determined by mass spectrometric analysis of the product of the enzymic reaction on a LKB-9000 combined gas chromatograph–mass spectrometer utilizing a 1% OV-17 column on Supelcoport 60/80. We are grateful to Dr. H. Fales, Laboratory of Chemistry, National Heart and Lung Institute, for access to this insrument and measurement of deuterium incorporation in the substrate by chemical ionization mass spectroscopy.

(14) For the hydroxylation in the presence of fumarate the reaction mixture contained the following: phosphate buffer pH 6.3, 1.8 mmol; fumaric acid, 150 μmol; catalase, 1350 units; phenylethylamine, 9.9 μmol; 0.475 mg of Dopamine β-hydroxylase; ascorbate 18 μmol, in a total volume of 3 ml of H_2O. For the hydroxylation in the absence of fumarate the conditions were the same except that fumaric acid was omitted and 0.950 mg of Dopamine β-hydroxylase was used. The reaction mixture was incubated at 35° in open shaking tubes. Samples were taken every 10 min for 50 min, the phenylethanolamine product separated on a Bio-Rex 70 cation exchange resin using borate buffer, pH 9.9, and assayed using ninhydrin. The effect of phosphate on the fumarate stimulation of the hydroxylation of phenethylamine is evidently quantitatively different from its effect on Dopamine.[4]

In an earlier kinetic study of this system Goldstein[15] suggested that the rate limiting step in the overall hydroxylation reaction was the interconversion of the central ternary complex. The isotope effects in the absence of fumarate are consistent with this. It appears that the effect of the fumarate is to reduce the activation energy of the breaking of the benzylic C–H bond to the point where that step is only partially rate determining.

A number of isotope effects have been reported for the reaction of nitrenes and carbenes with C–H bonds. In these cases the direct insertion of the singlet nitrene into a C–H bond is characterized by an isotope effect of 1.3–1.7.[16] For triplet nitrenes, which react *via* a pathway of hydrogen abstraction followed by C–N bond formation an isotope effect of 4.2 is observed.[16] For carbene reactions isotope effects of 1.1–1.4 have been reported;[17] while for "carbenoid" species values of 1.4–2.0 are observed.[18] A substantial secondary isotope effect indicates that there is a large change in the geometry of the reacting site between the ground state and the transition state. The isotope effects observed for the hydroxylation in the absence of fumarate are only consistent with a mechanism that involves the abstraction of the benzylic pro-R hydrogen followed by the subsequent formation of a C–O bond. They are not consistent with the direct insertion of an "oxenoid" species into the C–H bond.

Further studies of this system using other stereospecifically deuterated substrates are being pursued.

(15) M. Goldstein, T. H. Job, and T. W. Garvey, *Biochemistry*, 7, 2724 (1968).

(16) W. Lwowski, Ed., "Nitrenes," Interscience, New York, N. Y., 1970.

(17) W. Kirmse, H.-D. v. Scholz, and H. Arold, *Justus Liebigs Ann. Chem.*, 711, 22 (1968).

(18) M. J. Goldstein and S. J. Baum, *J. Amer. Chem. Soc.*, **85**, 1885 (1963); M. J. Goldstein and W. R. Dolbier, *ibid.*, **87**, 2293 (1965).

(19) Recipient of Public Health Service Research Career Development Award GM-70586 from the National Institute of General Medical Sciences.

L. Bachan, C. B. Storm,*[19] J. W. Wheeler
Department of Chemistry, Howard University
Washington, D. C. 20059

S. Kaufman
Laboratory of Neurochemistry
National Institute of Mental Health
Bethesda, Maryland 20014

Received July 3, 1974

Reversible Oxygen Adduct Formation in Cobalt(II) "Picket Fence Porphyrins"

Sir:

Replacement of Fe(II) by Co(II) in hemoglobin, to give "cobalglobin," CoHb,[1] results in an oxygen carrying protein remarkably similar to the natural iron protein, Hb.[2–4] Small differences in the degree of co-

(1) The abbreviations used in this paper are: CoHb, cobalt substituted hemoglobin or "cobalglobin;" Hb, hemoglobin; DMF, dimethylformamide; TPP, tetraphenylporphine; 1-MeIm, 1-methylimidazole; H_2PDME, protoporphyrin IX dimethyl ester; Co(*p*-OCH_3)TPP, *meso*-tetra(*o*-methoxyphenyl) porphyrincobalt(II).

(2) B. M. Hoffman and D. H. Petering, *Proc. Nat. Acad. Sci. U. S.*, **67**, 637 (1970).

(3) C. A. Spilburg, B. M. Hoffman, and D. H. Petering, *J. Biol. Chem.*, **247**, 4219 (1972).

(4) G. C. Hsu, C. A. Spilburg, C. Bull, and B. M. Hoffman, *Proc. Nat. Acad. Sci. U. S.*, **69**, 2122 (1972).

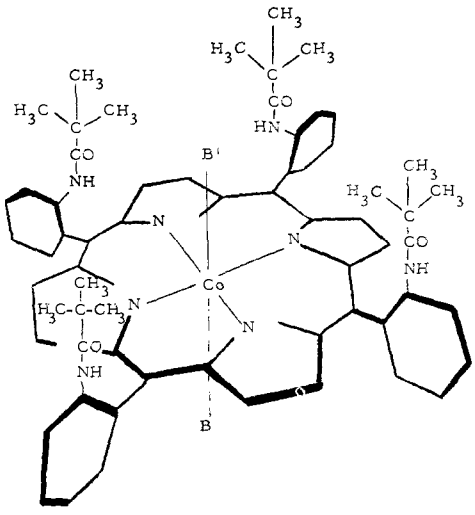


Figure 1. Co(II) complexes of $\alpha,\alpha,\alpha,\alpha$-$H_2$TpivPP: **1**, no axial ligands; **2**, B = 1-MeIm, B′ = vacant; **3**, B = 1-MeIm, B′ = O_2.

operativity[5] (decreased in CoHb) and in $p_{1/2}$[2] (7.5 mm for Hb, 20.5 mm for CoHb at 15°) have helped clarify possible structural mechanisms for the cooperativity of oxygen binding.[6-8] In contrast to CoHb simple Co(II) porphyrins show very little tendency to bind O_2 at ambient temperature although at reduced temperatures[9-13] their monomeric dioxygen complexes have been characterized. Thus the role of the protein in favoring oxygen binding in CoHb and its influence on the binding of oxygen in Hb are still to be clarified. Herein we report the reversible oxygenation of cobalt porphyrin complexes (Figure 1) at ambient temperature without the agency of a protein.

meso-Tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinocobalt(II), Co($\alpha,\alpha,\alpha,\alpha$-TpivPP) (**1**), ($\mu$ = 2.9 BM,[14] $\lambda_{C_6H_6}$ 528 nm, 25°) was prepared in good yield by heating the free porphyrin[15-17] and excess $Co(OAc)_2 \cdot 4H_2O$ in DMF (80–90°, 6 hr), under nitrogen, followed by silica gel column chromatography (10% ether in benzene as eluent) under nitrogen. The complex does not appear to form a dioxygen complex or to irreversibly oxidize, either in the solid state or in benzene solution, but gives an esr spectrum (undiluted solid sample, 25°) similar to that of Co(*p*-OCH_3)TPP.[1,9]

Recrystallization of **1** from toluene containing an excess of 1-MeIm, under N_2, yields the low spin, five-coordinate complex Co($\alpha,\alpha,\alpha,\alpha$-TpivPP)(1-MeIm) (**2**)

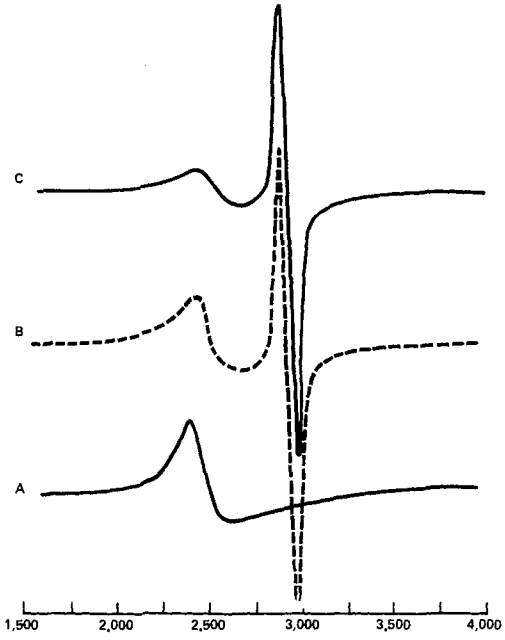


Figure 2. Esr spectra of (A) **2** under N_2, (B) **2** in air, and (C) **2** under 1 atm of O_2.

(μ = 2.9 BM,[14] λ_{DMF} 532 nm, 25°). The esr spectrum of **2** at 25° (Figure 2) both as an undiluted solid sample and in toluene shows a single broad absorption (g = 2.30) analogous to that found in the better defined spectra reported for frozen toluene glasses of Co[*p*-OCH_3)TPP]B[9] and Co(PDME)B.[13] Exposure of **2** either as a solid or in toluene to oxygen at 25° results in the appearance of a new, sharp peak at g = 2.03 and simultaneous diminution of the peak at g = 2.30. In addition the oxygenated toluene solution examined as a glass at 77°K demonstrated hyperfine splitting. These esr spectral changes parallel those reported for the formation of Co[(*p*-OCH_3)TPP](B)O_2[9] and Co(PDME)-(B)O_2[12] from the corresponding five-coordinate complexes and are thus attributed to the equilibrium (eq 1).

$$\underset{\mathbf{2}}{\text{Co}(\alpha,\alpha,\alpha,\alpha\text{-TpivPP})(1\text{-MeIm})} \overset{O_2}{\rightleftharpoons} \underset{\mathbf{3}}{\text{Co}(\alpha,\alpha,\alpha,\alpha\text{-TpivPP})(1\text{-MeIM})O_2} \quad (1)$$

λ_{DMF} 542 mμ, μ = 2.54 BM[14]

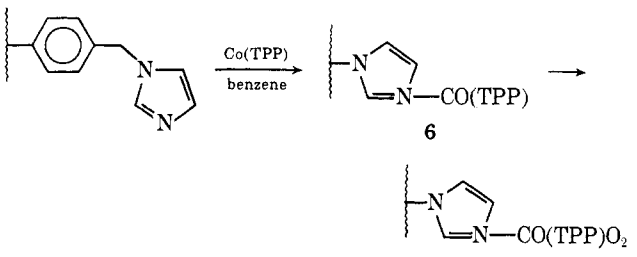
The precise extent of oxygenation of **2** has been difficult to measure. Electronic spectral changes accompanying the oxygenation of toluene, CH_2Cl_2, and DMF solutions of **2** are minor and inadequate for quantitative determination of the relative amounts of **2** and **3**. However, manometric oxygen uptake measurements on solutions of **1** containing 5 equiv of 1-MeIm show 52 ± 5% oxygenation in DMF and 29 ± 5% in toluene at 1 atm of O_2, 25°. That the equilibrium shown in eq 1 is dependent on the concentration of oxygen was demonstrated by comparing the esr spectra of solid **2** in air and under 1 atm of O_2 at 25° (Figure 2). The ratio of the peaks due to **3** and **2** (g = 2.03)/(g = 2.30), increased significantly at higher p_{O_2}.

The ir spectrum of **2** (at 25°) shows no bands ascribable to coordinated dioxygen, either as KBr pellets or in a difference study of CH_2Cl_2 solutions. This is surprising in view of the ir absorptions reported for the

(5) T. Yonetani, H. Yamamoto, F. Kayne, and G. Woodrow, *Biochem. Soc. Trans.*, 44 (1973).
(6) J. L. Hoard, *Science*, **174**, 1295 (1971).
(7) J. L. Hoard and W. R. Scheidt, *Proc. Nat. Acad. Sci. U. S.*, **70**, 3919 (1973).
(8) R. G. Little and J. A. Ibers, *J. Amer. Chem. Soc.*, **96**, 4440 (1974).
(9) F. A. Walker, *J. Amer. Chem. Soc.*, **92**, 4235 (1970).
(10) H. C. Stynes and J. A. Ibers, *J. Amer. Chem. Soc.*, **94**, 1559 (1972).
(11) F. A. Walker, *J. Amer. Chem. Soc.*, **95**, 1150 (1973).
(12) F. A. Walker, *J. Amer. Chem. Soc.*, **95**, 1154 (1973).
(13) D. V. Stynes, H. C. Stynes, J. A. Ibers, and B. R. James, *J. Amer. Chem. Soc.*, **95**, 1142 (1973).
(14) Magnetic moments were determined by the Faraday method and corrected for the diamagnetic susceptibilities of all ligands. All complexes have acceptable elemental analyses which have been provided to the referees.
(15) J. P. Collman, R. R. Gagne, T. R. Halbert, J.-C. Marchon, and C. A. Reed, *J. Amer. Chem. Soc.*, **95**, 7868 (1973).
(16) J. P. Collman, R. R. Gagne, and C. A. Reed, *J. Amer. Chem. Soc.*, **96**, 2629 (1974).
(17) J. P. Collman, R. R. Gagne, T. R. Halbert, G. Lang, and C. A. Reed, manuscript in preparation.

angular bonded oxygen in Co(acacen)BO_2[18] and Co(salen)BO_2.[19] However, any ν_{O_2} absorption may be obscured by strong porphyrin bands in the 900–1500-cm^{-1} region or may be greatly temperature dependent as has been found for Fe($\alpha,\alpha,\alpha,\alpha$-TpivPP)(1-MeIm)$O_2$.[20] Further studies to resolve this apparent paradox are underway.

Ambient temperature oxygenation of Co(II) porphyrins is not limited to the "picket fence" porphyrins. The five-coordinate complex, Co(TPP)(1-MeIm), **4**, in toluene solution does not appreciably oxygenate at 25°, though oxygenation has been detected in toluene glasses at −195°.[21] However, treatment of polystyrene-bonded imidazole, **5**,[22] with Co(TPP), afforded **6** whose



esr spectrum suggests five-coordinate Co(II) (g = 2.30). Exposing **6** to dry air or oxygen at 25° gives a new peak at g = 2.03 with corresponding decrease in the intensity of the broad peak at g = 2.30. As with **2** the degree of oxygenation seems dependent on p_{O_2} but quantitative determination of the extent of oxygenation has not been made.

The greater extent of oxygenation of the "picket fence" cobalt porphyrin, **1**, the polymer substituted cobalt porphyrin, **6**, and CoHb compared with the structurally similar cobalt porphyrins, Co[(p-OCH_3)-TPP]B and Co(PDME)B, at 25° is remarkable. We propose that these equilibrium changes arise from restrictions in the extent of solvation of the unoxygenated and/or oxygenated complexes within the "picket fence" cavity, the solid cross-linked polystyrene, and the globin cavity compared with simple cobalt porphyrins in solution. Whether the effect is enthalpic and/or entropic must await precise measurements of O_2 binding constants over a range of temperatures. It is apparent that the oxygen binding site of hemoproteins may experience an environment more like a solid than a solution. This is yet another way in which the protein influences the chemistry of the porphyrin.

Acknowledgment. We gratefully acknowledge helpful discussions with B. Hoffman, J. Brauman, and R. Drago. This work was supported by National Institutes of Health Grant GM-17880 and National Science Foundation Grant GP20273X.

(18) A. L. Crumbliss and F. Basolo, *J. Amer. Chem. Soc.*, **92**, 55 (1970).
(19) C. Floriani and F. Calderazzo, *J. Chem. Soc. A*, 946 (1969).
(20) J. P. Collman, R. R. Gagne, H. B. Gray, and J. Hare, *J. Amer. Chem. Soc.*, **96**, 6522 (1974).
(21) H. C. Stynes and J. Ibers, *J. Amer. Chem. Soc.*, **94**, 5125 (1972).
(22) J. P. Collman and C. A. Reed, *J. Amer. Chem. Soc.*, **95**, 2048 (1973).

James P. Collman,* Robert R. Gagne
Jay Kouba, Helena Ljusberg-Wahren
Department of Chemistry, Stanford University
Stanford, California 94305
Received July 22, 1974

Three-Electron Oxidations. VIII. Direct Evidence for the Synchronous Character of Three-Electron Oxidations[1,2]

Sir:

We have recently found[3] that a number of chromium(VI) oxidations proceeds through an intermediate formed from one molecule of chromic acid and two molecules of organic substrates. The reaction results in a one-electron oxidation of the second molecule, and we proposed that both of these reactions may proceed simultaneously in a rate-limiting step leading directly from chromium(VI) to chromium(III) without the necessity of forming an unstable chromium(IV) intermediate. We felt that the ability to avoid the formation of the chromium(IV) intermediate is the main reason for the unusual ease with which the oxidative decomposition of these termolecular complexes take place.

However, an alternate mechanism consisting of a rate-limiting two-electron oxidation followed by a rapid one-electron oxidation could not be completely excluded. Such a mechanism would be consistent with our results, provided that the lifetime of the chromium(IV) intermediate was too short to allow any ligand exchange with the solvent or with other substrates to occur. The plausibility of this alternate two-step mechanism has increased as a result of our recent observation that the chromic acid oxidation of alcohols is strongly catalyzed by picolinic acid,[4] and that of iodides by oxalic acid,[5] without any noticeable oxidation of the organic acid.

In all previously investigated cases the one-electron oxidation involved a carbon–carbon bond cleavage in oxalic acid[3a,b,d] or in a tertiary hydroxy acid (2-hydroxy-2-methylbutyric acid).[3c] We now found that the chromic acid oxidation of glycolic acid yields glyoxylic acid, formaldehyde, and carbon dioxide if the oxidation is carried out at relatively low concentrations of glycolic acid; however, only glyoxylic acid is formed at high glycolic acid concentrations. A kinetic study revealed that the reaction proceeds through a transition state containing one molecule of glycolic acid at low concentrations and two molecules of the acid at high concentrations.[6]

At high glycolic acid concentrations the reaction is best described as a three-electron oxidation.

$$2HOCH_2CO_2H + Cr(VI) \rightleftharpoons \text{complex}$$

$$\text{complex} \xrightarrow[\text{limiting}]{\text{rate}} OCHCO_2H + HO\dot{C}HCO_2H + Cr(III)$$

We also found that the chromic acid oxidation of a mixture of isopropyl alcohol and glycolic acid results in a cooxidation reaction leading to acetone and glyoxylic acid. When the reaction is carried out in the presence of acrylonitrile, which acts as a free radical scavenger, only acetone is isolated, indicating that

(1) Part VII: F. Hasan and J. Roček, *J. Org. Chem.*, in press.
(2) This investigation was supported by the National Science Foundation.
(3) (a) F. Hasan and J. Roček, *J. Amer. Chem. Soc.*, **94**, 3181 (1972); (b) *ibid.*, **94**, 9073 (1972); (c) *ibid.*, **95**, 5421 (1973); (d) *ibid.*, **96**, 534 (1974).
(4) J. Roček and T. Y. Peng, to be submitted for publication.
(5) G. Vandegrift and J. Roček, to be submitted for publication.
(6) F. Hasan and J. Roček, to be submitted for publication.