Synthetic Oxygen Carriers of Biological Interest

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Certain metal–ligand combinations provide the delicate balance required to form a 1:1 dioxygen complex without the metal (M) and/or the ligand (L) being irreversibly oxidized. These systems, which are

$$M(L)_n + O_2 \rightleftharpoons M(L)_n(O_2) \quad (1)$$

called oxygen carriers, have long been recognized in vitally important biological processes, and as early as 1852 Fremy[1] reported that solid cobalt ammine salts absorb oxygen from air and release it again when dissolved in water. However, the significance of synthetic oxygen carriers was not apparent until 1938.

Since the initial discovery by Tsumaki[2] that Schiff base chelates of cobalt(II) are oxygen carriers, there has been a continued interest in this property of such metal complexes. Much of the research effort has been devoted toward the more fundamental aspects of the problem, such as the metal–ligand properties of a good oxygen carrier, the nature of the metal–dioxygen bonding, and the structure. However, considerable research has also been devoted to possible applications of these oxygen carriers. For example, Calvin and his students[3] extensively investigated the absorption–desorption of molecular oxygen on various solid cobalt(II)–Schiff-base chelates. These complexes were studied for the purpose of isolating pure oxygen from air, and oxygen so produced was used for several months aboard a destroyer tender for welding and cutting.[4a] Other applications of synthetic oxygen carriers include their use as catalysts for reactions of molecular oxygen.[4b] It is even possible that they may eventually find use in artificial blood.[5]

Twelve years ago, Vaska[6] reported that $IrCl(CO)(P(C_6H_5)_3)_2$ is an oxygen carrier, and the structure of its oxygen complex was determined.[7] Other low-valent transition-metal compounds of this type have since been found to be oxygen carriers and/or homogeneous catalysts for certain reactions of dioxygen.[8] Structural studies on these complexes have consistently revealed a triangular MO_2 arrangement with each oxygen atom equidistant from the metal and with the O–O bond lengthened over that in dioxygen. Because of structural differences[9] from

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(1) E. Fremy, *Justus Liebigs Ann. Chem.*, **83**, 227, 289 (1852).
(2) T. Tsumaki, *Bull. Chem. Soc. Jpn.*, **13**, 252 (1938).
(3) See, for example, A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds", Prentice-Hall, Englewood Cliffs, N.J., 1952, pp 336–357.
(4) (a) R. F. Stewart, P. A. Estep, and J. J. S. Sebastian, *U.S. Bur. Mines, Inf. Circ.*, **No. 7906** (1959); (b) A. E. Martell and M. M. Taqui Khan, *Inorg. Biochem.*, **2**, 654 (1973).
(5) R. P. Geyer, *Bull. Parenter. Drug Assoc.*, **28** (2), 88 (1974); J. E. Baldwin, *Newsweek*, 91 (March 24, 1975).
(6) L. Vaska, *Science*, **140**, 809 (1963).
(7) J. A. Ibers and S. J. La Placa, *Science*, **145**, 920 (1964).
(8) J. P. Collman, *Acc. Chem. Res.*, **1**, 136 (1968); J. Valentine, *Chem. Rev.*, **73**, 235 (1973).
(9) J. L. Hoard, "Structural Chemistry and Molecular Biology", A. Rich and N. Davidson, Ed., W. H. Freeman, San Francisco, Calif., 1968, p 573.

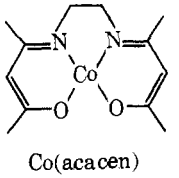
natural oxygen carriers, it became increasingly apparent that the study of low-valent second- and third-row transition-metal oxygen complexes, though interesting from many points of view, probably lacked significant biological relevance. These oxygen carriers will not be discussed in this Account.

Research on synthetic oxygen carriers at Northwestern University was largely prompted by the isolation and characterization of the monoadduct of dioxygen with cobalt Schiff-base (SB) complexes, $Co(SB)(O_2)$. Work was then extended to studies of the reversible reaction of molecular oxygen with cobalt(II) porphyrins, cobalt(II) hemoglobin, cobalt(II) myoglobin, and other metal-substituted hemoglobins as well. More recently this work has been extended to the interaction of O_2 with Fe, Mn, and Cr synthetic systems.

Cobalt(II)–Schiff-Base Complexes

Calvin and coworkers[3] conducted an exhaustive study of the oxygen-carrying properties of solid cobalt(II)–Schiff-base complexes. Floriani and Calderazzo[10] studied the reaction of oxygen with *N,N'*-ethylenebis(salicylideniminato)cobalt(II), (Co(salen)) and some of its derivatives in various solvents. Except for 3-methoxysalen the products were the usual diamagnetic peroxo-bridged species of the type $(B)(salen)Co^{III}-(O_2^{2-})-Co^{III}(salen)(B)$, where B stands for a nitrogenous base, such as pyridine. Most cobalt(II) complexes react with oxygen to yield such species, and these may also be readily oxidized to the corresponding superoxo-bridged complexes, $Co(III)-(O_2^-)-Co(III)$. Much more significant was their observation that Co(3-methoxysalen) in pyridine (py) solution reacts with oxygen to yield the monomeric complex Co(3-methoxysalen)(py)(O_2). This was the first report of the isolation of 1:1 dioxygen–cobalt complex, and it represents a significant development because oxyhemoglobin is a 1:1 dioxygen–iron complex.

Our independent experiments,[11] carried out concurrently with those of Floriani and Calderazzo,[10] made use of *N,N'*-ethylenebis(acetylacetoniminato)-cobalt(II) (Co(acacen)), which is more soluble than



Co(acacen)

Co(salen). The reaction of Co(acacen) in a coordinating solvent (e.g., dimethylformamide, DMF) or in a noncoordinating solvent (e.g., toluene) with added base (e.g., py) at room temperature results in a nonstoichiometric, continuous slow uptake of oxygen over a period of days (Figure 1). Under these conditions, it appears the cobalt(II) is catalyzing the oxidation of the organic ligand. However, at temperatures near 0° or below there is a rapid and reversible uptake of dioxygen, corresponding to the formation of the 1:1 $Co(O_2)$ complex (Figure 1). Various experimental techniques were then used to establish the

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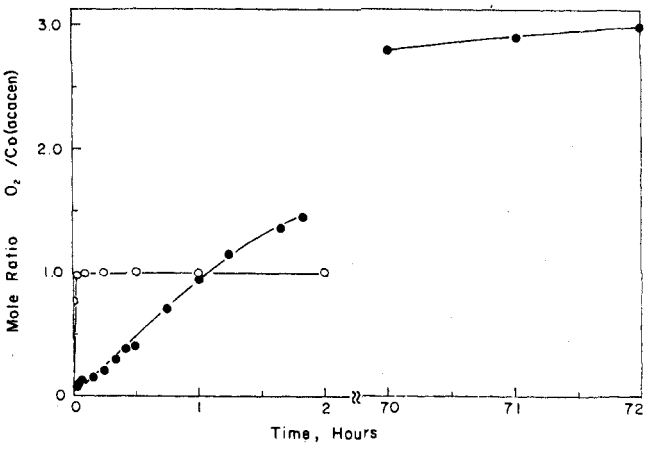


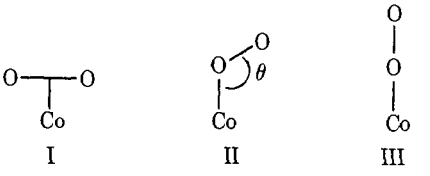
Figure 1. Oxygen absorption by DMF solutions of Co(acacen): ○, data collected at 6°C and 760 mmHg of O_2 pressure; ●, data collected at 25°C and 480 mmHg of O_2 pressure (from ref 11).

$$Co(acacen)(B) + O_2 \rightleftharpoons Co(acacen)(B)(O_2) \qquad (2)$$

presence of these 1:1 complexes (eq 2) in solution and in the solid state. The solid complexes have a very strong ir band in the region of 1140 cm^{-1} (which disappears when O_2 is removed and reappears when O_2 is returned). This we attribute to the O–O stretching vibration.

EPR spectra[12] of these systems in both liquid and frozen solution clearly demonstrate that the oxygen complexes are monomeric. These spectra exhibit interaction with but one ^{59}Co nucleus, in contrast with the two interacting ^{59}Co nuclei of μ-superoxo dimers.[13] The EPR technique has been used extensively to establish the presence of monomeric complexes of dioxygen in numerous cobalt systems.[14]

The spin Hamiltonian parameters for these monomeric oxygen complexes permit definite conclusions to be drawn about their electronic and geometric structures.[12] Our analysis shows that there is an ~90% transfer of spin density from Co(II) to O_2 upon complex formation. This result, in conjunction with the ir measurements, is compelling evidence for charge transfer upon complex formation. Thus the 1:1 cobalt dioxygen complexes should be *formally* described[12,14f] as $Co(III)-O_2^-$. Analysis[12] of magnetic resonance and magnetic moment data also permitted discrimination between the three plausible geometries of the $Co(III)-O_2^-$ linkage. We concluded that



only nonsymmetric bonding, as in structure II, is consistent with experiment, with the simplest model involving an angle θ of 120°.

(12) B. M. Hoffman, D. L. Diemente, and F. Basolo, *J. Am. Chem. Soc.*, **92**, 61 (1970).

(13) M. Mori, J. A. Weil, and J. K. Kinnaird, *J. Phys. Chem.*, **71**, 103 (1967).

(14) (a) H. Kon and N. Sharpless, *Spectrosc. Lett.*, **1**, 45 (1969); (b) J. H. Bayston, N. King, F. D. Looney, and M. E. Winfield, *J. Am. Chem. Soc.*, **91**, 2775 (1969); (c) F. A. Walker, *ibid.*, **92**, 4235 (1970); (d) S. A. Cockle, H. O. Hill, and R. J. P. Williams, *Inorg. Nucl. Chem. Lett.*, **6**, 131 (1970); (e) D. L. Diemente, B. M. Hoffman, and F. Basolo, *Chem. Commun.*, 467 (1970); (f) B. M. Hoffman, T. Szymanski, and F. Basolo, *J. Am. Chem. Soc.*, **97**, 673 (1975).

These conclusions about the electronic and geometric properties, made solely on the basis of our spectroscopic data, have recently been confirmed. Rodley and Robinson[15] have carried out an X-ray crystallographic study of a Co(SB)(py)(O_2) complex. They find that the nonsymmetric structure (II) is indeed correct, with $\theta = 125°$. Further, the O–O distance of 1.2 Å is different from that of the O_2^{2-} ion in K_2O_2 (1.49 Å). In addition, EPR studies[16] using ^{17}O-enriched dioxygen show that nearly 100% of the spin density is indeed located on dioxygen, and that the two oxygen atoms are nonequivalent as required by structure II.

Cobalt(II)–Porphyrin Complexes

With the discovery of the 1:1 Co(O_2)–Schiff-base complexes it was apparent that parallel studies on cobalt–porphyrin complexes were in order, since metalloporphyrins are utilized in biological systems. Although an extensive literature already existed on metalloporphyrin chemistry, essentially all of that chemistry had been carried out in aqueous systems.

By 1970, when this work was initiated, the significant result of Perutz[17] that the dioxygen molecule travels through a hydrophobic pocket to reach the coordination site on the Fe atom in hemoglobin was well known. Consequently we reasoned that one might simulate this hydrophobic environment on the protein by abandoning aqueous systems and working in aprotic solvents. Most of our early studies[18] were carried out in toluene which offered the advantage of reasonable spectral clarity down to about −70°. In order to stay as close as possible to biologically relevant systems, we chose to use the porphyrin protoporphyrin IX dimethyl ester (Figure 2a), which differs from the porphyrin in the heme group of hemoglobin through esterification of the two acid groups. The most common synthetic porphyrin, tetraphenylporphyrin (TPP) (Figure 2b), is less closely related to the natural porphyrins. Its popularity arises from its ease of synthesis and purification.

Initial experiments,[18,19] indicated that, if cobalt(II) protoporphyrin IX dimethyl ester (Co(PIXDME)) is dissolved in toluene and a base is added, the equilib-

$$\text{Co(PIXDME)} + \text{B} \rightleftharpoons \text{Co(PIXDME)(B)} \qquad (3)$$

rium is strongly to the right.[20] At room temperature in the presence of dioxygen no apparent reaction occurs, but if the solution is cooled and exposed to dioxygen reaction 4 takes place. These reactions are

$$\text{Co(PIXDME)(B)} + \text{O}_2 \rightleftharpoons \text{Co(PIXDME)(B)(O}_2\text{)} \qquad (4)$$

monitored readily by either EPR[19] or visible[18] spectroscopy, with the latter technique offering some advantages for quantitative measurements.

The EPR parameters[21] of Co(PIXDME)(B)(O_2)

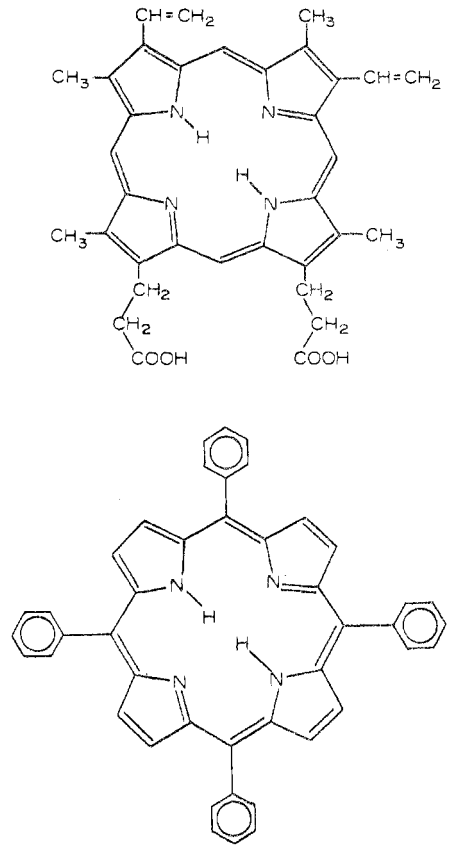


Figure 2. (a) Top structure is protoporphyrin IX. (b) Bottom structure is 5,10,15,20-tetraphenylporphyrin.

are not qualitatively different from those of the Co(SB)(B)(O_2) complexes. Thus, the formal description of the metal–dioxygen linkage as Co^{III}–O_2^- seems to be largely independent of the equatorial ligand.

Cobalt(II) and Other Metal Hemoglobins and Myoglobins

The successful preparation of Co–O_2 complexes suggested to us the possibility of introducing a functional paramagnetic probe into hemoglobin (Hb) and myoglobin (Mb) by removing the heme group and reconstituting the protein with a cobalt(II) porphyrin to form an oxygen-carrying cobalt metalloprotein. The study of such metal-substituted proteins would permit us to observe directly the influence of the protein environment upon the metalloporphyrin by comparing the oxygen-binding and spectroscopic properties with those of Co(PIXDME) in solution. Also, variation of the stereochemical properties of the functioning metal atom binding site of hemoglobin would allow us to examine the involvement of the heme in the conformational changes which constitute hemoglobin allosteric, or cooperative, ligand binding (see below).

We thus prepared globins from hemoglobin and myoglobin and reconstituted them with cobalt(II) protoporphyrin IX (Co(PIX)).[21] These *coboglobins*, the cobalt analogs of hemoglobin (CoHb) and myoglobin (CoMb), indeed function as oxygen-carrying proteins.[21,22] EPR studies showed that the cobalt

(15) G. A. Rodley and W. T. Robinson, *Nature (London)*, **235**, 438 (1972).
(16) (a) E. F. Vansant and J. H. Lunsford, *Adv. Chem. Ser.*, **No. 121**, 441 (1973); (b) E. Melamud, B. L. Silver, and Z. Dori, *J. Am. Chem. Soc.*, **96**, 4689 (1974).
(17) M. F. Perutz, "The Harvey Lecture", Academic Press, New York-London, 1969, p 213.
(18) H. C. Stynes and J. A. Ibers, *J. Am. Chem. Soc.*, **94**, 1559 (1972).
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(20) D. V. Stynes, H. C. Stynes, B. R. James, and J. A. Ibers, *J. Am. Chem. Soc.*, **95**, 1796 (1973).
(21) B. M. Hoffman and D. H. Petering, *Proc. Natl. Acad. Sci. U.S.A.*, **67**, 637 (1970).
(22) B. M. Hoffman, C. A. Spilburg, and D. H. Petering, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 343 (1971).

atom in deoxy-CoMb and CoHb is five-coordinate and, as is true for the iron in natural proteins, is coordinated to the imine nitrogen of the proximal histidine residue.[21] Further, Co(PIX) has the same orientation within the protein as does the heme.[23] Comparison with the spectrum of the monoimidazole complex of Co(PIXDME) in organic solvents shows that the five-coordinate Co(II) of CoHb exhibits no significant perturbations which are caused by its incorporation into the protein environment.[21] Exposing CoHb (CoMb) to oxygen gives oxy-CoHb (oxy-CoMb).[21-24] EPR studies again show that the Co–O_2 linkage within the protein is not significantly different from that of an oxygenated cobalt porphyrin in solution.[21]

Comparisons of the properties of the naturally occurring heme group both in and out of a protein environment were previously impossible, since heme cannot ordinarily be prepared in a five-coordinate state and heme in solution is oxidized to hemin upon exposure to oxygen. We reported[25] the first such comparison when we used Mössbauer emission spectroscopy to study heme complexes produced in frozen solution by nuclear decay from the isomorphous Co-labeled compound. The Mössbauer parameters of the daughter compound of ^{57}Co(PIX)(py)(O_2) agree well with the values for oxyhemoglobin,[26] suggesting that the electronic structure of the heme iron in oxy-Hb is not measurably influenced by the protein. This parallels the conclusions from EPR studies of oxy-CoHb.[21] Thus, the stabilization of the heme group in hemoglobin is probably not ascribable to protein-induced changes of the electronic state or iron in oxyheme.

From discussions of the implications of CoHb linkage properties, we realized that investigation of other metal-substituted hemoglobins (e.g., MnHb, ZnHb, and ZnMb[27]) could offer additional valuable insights into the nature of hemoglobin cooperativity. Such substitutions have provided new physical and chemical probes with which we can observe the influence of a protein environment on the physical and chemical properties of a prosthetic group. EPR studies of the paramagnetic (Co(II), Co(III)–O_2^-,[21,23] and Mn(II)[28]) metal-substituted proteins primarily give information about the influences of the protein environment on the metal center. However, EPR studies of zinc porphyrins and of ZnHb and ZnMb in the photoexcited triplet state provide a probe to examine the interactions between the porphyrin macrocycle itself and the protein environment.[29]

We have found[29] that the porphyrin–protein interactions in ZnHb differ from those in ZnMb; chain differences within hemoglobin are also observed. Stresses in hemoglobin which, for example, tend to stabilize a quasi-D_{2d} type of ruffling of the macrocycle could account for the observations. Indeed, it may be that the presence of such stresses is responsible for the unique properties of deoxy-Hb, and that changes in the ruffling of the porphyrin are an important component in the mechanism of cooperative ligand binding. (See section on hemoglobin cooperativity). Despite the fact that MnHb cannot reversibly bind molecular oxygen, there is a high degree of parallelism between the molecular properties of MnHb and those of Hb.[30-32] In particular, the redox reactions of MnHb are important to an understanding of hemoglobin cooperativity (see below). Preliminary X-ray studies show that Mn^{III}Hb resembles Fe^{III}Hb very closely.[33] Since Mn^{III}Hb undergoes a change in quaternary structure upon reduction, just as does Fe^{III}Hb, MnHb retains both the major functional and structural properties of Hb.

Thermodynamics of Dioxygen Binding to Cobalt(II) Complexes

Long before EPR experiments permitted the formulation $Co^{III}(O_2^-)$ for these complexes, it was suggested that the ease of oxygen uptake by metal complexes should depend on the ease of oxidation of the metal in a given complex.[34] This implies that metals which are readily oxidized (e.g., Fe(II)) react with oxygen irreversibly, metals difficult to oxidize (e.g., Ni(II)) do not react with oxygen, whereas metals between the two extremes (e.g., Co(II)) may react with oxygen reversibly. Furthermore, reversible dioxygen uptake ability of a complex depends not just on the metal but also on the ligand.

Equilibrium constants, K_{O_2}, have been determined for the reaction of dioxygen with cobalt(II) in many different ligand environments (eq 5). For a given co-

$$\mathrm{Co(L)(B)} + \mathrm{O_2} \overset{K_{O_2}}{\rightleftharpoons} \mathrm{Co(L)(B)(O_2)} \qquad (5)$$

balt(II)–Schiff-base chelate, Co(L), a linear correlation was found[35] between K_{O_2} and the ease of oxidation of cobalt(II) to cobalt(III) as B varies (Figure 3). There are enough data to show that the protonic base strength of the axial base does not correlate with oxygen uptake. For example, the values of K_{O_2} for changes in axial base decrease in the order n-butylamine > 1-methylimidazole > piperidine, although the pK_a's of these protonated bases are 10.6, 7.25, and 11.3, respectively. That 1-methylimidazole is much more effective in promoting complex formation than is expected from its protonic basicity may arise from its good π-electron-donating property.

This same type of behavior had been observed earlier[20,36,37] for cobalt porphyrins. Figure 4 shows a plot

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(27) (a) S. F. Andres and M. Z. Atassi, *Biochemistry*, **9**, 2268 (1970); (b) J. J. Leonard, T. Yonetani, and J. B. Calles, *ibid.*, **13**, 1461 (1974).

(28) T. Yonetani, H. Yamamoto, J. E. Erman, J. S. Leigh, and G. H. Reed, *J. Biol. Chem.*, **247**, 2447 (1972).

(29) B. M. Hoffman, *J. Am. Chem. Soc.*, **97**, 1688 (1975).

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(31) Q. H. Gibson, B. M. Hoffman, R. H. Crepeau, S. J. Edelstein, and C. Bull, *Biochem. Biophys. Res. Commun.*, **59**, 146 (1974).

(32) B. M. Hoffman, Q. H. Gibson, C. Bull, R. H. Crepeau, S. J. Edelstein, R. G. Fisher, and M. J. McDonald, *Ann. N.Y. Acad. Sci.*, **244**, 174 (1975).

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(36) D. V. Stynes, H. C. Stynes, J. A. Ibers, and B. R. James, *J. Am. Chem. Soc.*, **95**, 1142 (1973).

(37) J. A. Ibers, D. V. Stynes, H. C. Stynes, and B. R. James, *J. Am. Chem. Soc.*, **96**, 1358 (1974).

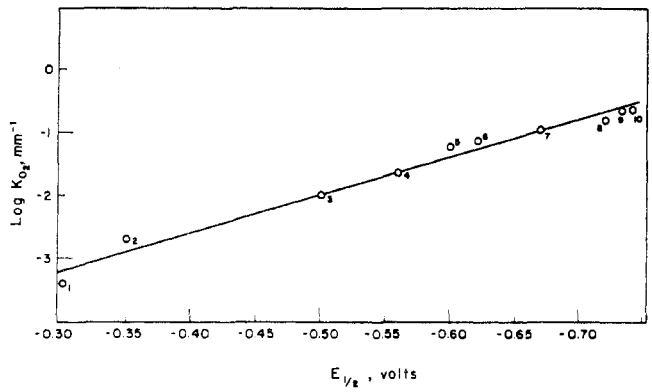

Figure 3. Comparison of oxygen uptake (log K_{O_2}) at −21°C for Co(benacen)B to the polarographic half-wave potentials ($E_{1/2}$) for $Co^{II \rightarrow III}$(benacen)B_2: 1,PPh_3; 2, CNpy; 3, py; 4, 3,4-lutidine; 5, piperidine; 6, *sec*-$BuNH_2$; 7, 5-Cl-N-MeIm; 8, N-MeIm; 9, *i*-$BuNH_2$; 10, *n*-$BuNH_2$. benacen = benzoylacetylacetonate ion, PPh_3 = triphenylphosphine, py = pyridine, and Bu = butyl (from ref 35).

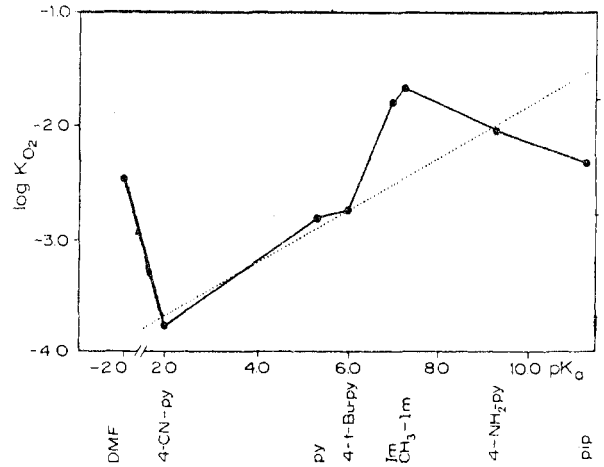

Figure 4. Comparison of oxygen binding of Co(PIXDME)(B) in toluene at −45°C as a function of the pK_a of the base, B. The dashed line shows the correlation of log K_{O_2} with pK_a for the pyridine systems (from ref 20).

of log K_{O_2} vs. pK_a for different axial bases on Co(PIXDME) in toluene solution. Neither for these equilibrium constants nor for the derived thermodynamic quantities, ΔH, ΔG, or ΔS, is there a simple, general relationship that is related to the pK_a's. However, as the dashed line indicates, there is a reasonably linear correlation of the equilibrium constant with the pK_a's of the substituted pyridines. Tetraphenylporphyrin and other synthetic porphyrins yield approximately the same results.[38]

We attribute such differences to the π-donor properties of these ligands. In the Pauling model[39] the bonding of an oxygen atom to a metal complex may be explained in terms of the σ donation from a nonbonding sp^2 lone pair on oxygen to the d_{z^2} orbital on the metal, accompanied presumably by synergistic π back-bonding from the filled d_{xz} (or d_{yz}) orbitals into the empty π^* orbitals of oxygen. Since ligands coordinated trans to oxygen will compete for π electron density on the metal, the binding of O_2 to Co(P)(B), where P is any porphyrin, will be sensitive to the π-donating ability or π-accepting ability of the axial ligand B. Good π acceptors will decrease π electron density on the metal, resulting in a poorer oxygen carrier, whereas good π donors will promote oxygenation by increasing the electron density available for back-bonding. Imidazoles are much better π donors than pyridines; the strong π-donor properties of DMF have also been demonstrated. On the other hand, the log K_{O_2} for piperidine is much less than that expected from basicity arguments. It is possible here that steric effects play a role through the interactions of the ring protons with parts of the porphyrin core, preventing close approach of the base to the metal. We also found[35,40] that a change from the essentially nonpolar solvent toluene to the polar solvent dimethylformamide (DMF) has a pronounced effect on the equilibrium of eq 5, leading to much greater degree of oxygenation. We ascribe this large solvent effect to the stabilization of the polar Co(III)–O_2^- species in the more polar solvents. Although cobalt porphyrins are reversibly oxygenated at low temperatures in aprotic solvents, it is known[36] that in the presence of a protonic base irreversible oxidation occurs.

Table I
Representative Thermodynamic Data for Reversible Oxygen Binding to Cobalt(II) Complexes, to Myoglobin, and to a Modified Silica Gel

System[a]	log K_{O_2}, mm^{-1}	ΔH, kcal/mol	ΔS, eu	$P_{1/2}$, Torr[e]	Ref
Co(benacen)-(MeIm)[b]	−2.98	−17.5	−73.5	955	35
Co(PIXDME)-(MeIm)[b]	−4.10	−11.8	−59	12,500	20
CoMb[c] (sperm whale)	−1.65	−13.3	−53	45	41
Mb[c] (sperm whale)	−0.3	−18.1	−60	0.50	*f*
(IPG)Fe(TPP)[d]				900	72

[a] Data in the table are from literature values extrapolated to 20°C. [b] Solvent is toluene. [c] Solvent is water, phosphate buffer at pH 7. [d] Solid-gas phase. [e] $P_{1/2}$ is the pressure at which one-half of the complex is present in the oxygenated form. [f] M. H. Keyes, M. Falley, and R. Lumry, *J. Am. Chem. Soc.*, 93, 2038 (1971).

For changes in the quadridentate equatorial ligand, keeping the same axial base, there is also a linear correlation[35] between the log K_{O_2} and the $E_{1/2}$ values for various cobalt(II) complexes. In general, cobalt(II) porphyrins have a smaller tendency to form dioxygen complexes than do cobalt(II)–Schiff-base chelates. This is perhaps the result of the porphyrin ligand having a greater tendency to delocalize π electron density away from cobalt atom than does the less aromatic Schiff-base ligand. Representative thermodynamic data for dioxygen uptake by cobalt(II) complexes of Schiff bases and of porphyrins are given in Table I. Also shown in this table for comparison are corresponding values for CoMb and Mb.

The protein environment, although producing no major effect on the electronic structure of the oxygen adduct, does produce a large effect on its stability.[22,41] We can directly observe the influence of the globin (apoprotein) upon the reactivity of the metalloporphyrin (prosthetic group) by comparing oxygen

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binding of the five-coordinate Co(PIXDME)(MeIm) complex with that of CoMb. For example, at 25°, the incorporation of Co(PIX) into globins of whale, horse, or harbor-seal myoglobins increases the K_{O_2} by a factor of ~300. Through the use of a formal thermochemical cycle oxygen binding to protein and to solution porphyrin can be related, and the protein contribution to the thermodynamics of dioxygen binding to the metalloprotein can be determined. We find that the protein provides a significantly favorable (positive) entropic contribution to O_2 binding.[22,41] The enhancement of oxygen binding by horse CoMb is entirely entropic. Binding to the harbor-seal CoMb is actually disfavored enthalpically compared with the free porphyrin, but this is compensated by a further favorable change in the entropy. The whale CoMb does exhibit a rough balance between favorable entropy and enthalpy contributions. Thus, by combining the results for model compound and protein, we see how these three myoglobins of identical function and similar structure achieve similar O_2 binding properties near physiological temperatures through a different balance of enthalpic and entropic factors.[42]

The molecular basis of the more favorable entropy of dioxygen binding in the protein environment has been discussed,[41] and at least in part is related to the effects of polar solvents on binding to Co(PIX) described above. Formation of the strongly dipolar Co(III)–O_2^- linkage from a neutral cobalt(II) porphyrin and a neutral oxygen molecule causes solvent reorganization which accommodated the charge separation and thus stabilizes the metal–dioxygen bond. This reorganization would appear thermodynamically as a negative contribution to the entropy of oxygen binding to solution porphyrin. If the hydrophobic protein crevice is already organized to accommodate a polar metal–oxygen group, the negative entropy associated with the formation of the crevice would appear as part of the entropy of formation of the metalloprotein itself, not as a contribution to the entropy change upon oxygen binding.

Hemoglobin Cooperativity

A chief source of the ongoing interest in ligand binding to hemoglobin rests in the following allosteric properties which result from its tetrameric nature.[43] The dioxygen binding curve is "cooperative" (autocatalytic), not hyperbolic. The degree of cooperative binding can be expressed by the Hill constant, n, which has the value $n \approx 2.8$. For comparison, independent O_2 binding by the four hemes of Hb would require $n = 1.0$, whereas all-or-none binding to the four hemes would require $n = 4$. Hemoglobin exhibits two other so-called linkage properties: first, the binding of O_2 is pH dependent, although there is no ionizable group at the heme (Bohr effect); second, the oxygen affinity is altered by the binding of an organic phosphate to Hb, although the binding site is far removed from the hemes (phosphate effect).

The ligated and unligated forms of Hb, respectively denoted as R and T, differ both in the conformation of the individual chains (tertiary structure) and in the relative orientation of the chains (quaternary structure). The linkage effects exhibited by Hb arise from the reversible transition between these two forms. Perutz and Ten Eyck[44] concluded that ligand binding can be accompanied by Bohr and phosphate effects and the normal quaternary structure transition, even though cooperativity ($n > 1$) is weak or absent; thus, these linkage effects are diagnostic of a quaternary structure change. The degree of cooperativity, as measured by the n value, appears to reflect the details of the ligation process and thus the nature and type of intermediates which occur as the four hemes of Hb react.

Table II
Effect of pH on Oxygen Binding to CoHb and Hb[a]

	DPG,[d] mM	log $P_{1/2}$ pH 7.3[b]	log $P_{1/2}$ pH 9.4[c]	Δ log $P_{1/2}$
CoHb	0.2	2.04	0.96	1.08
	0	1.56	0.96	0.60
Hb	0.2	0.63	−0.47	1.10
	0	0.21	−0.47	0.68

[a] From ref 45. [b] 0.05 M Bis-Tris; 4°C. [c] 0.05 M Tris; 4°C. [d] DPG, diphosphoglycerate.

In constructing a detailed scheme for the ligation process of Hb, a question can be formulated as to the nature of an "allosteric trigger",[44] the mechanism by which the ligation (or oxidation) of five-coordinate, iron(II) heme induces conformational changes within the protein. Our preparation of cobalt- and manganese-substituted hemoglobins has provided a unique opportunity to help answer this question. We have found that CoHb retains the full Bohr and phosphate effects upon oxygen binding,[22,45] and that the binding of O_2 is cooperative.[21] The Hill constant is at least 2.3[23,24] and probably is as great as 2.5,[46] whereas that of Hb is 2.8. Thus, following Perutz's arguments,[44] CoHb must undergo a quaternary structure change upon oxygenation, and the stereochemical changes that occur as the five-coordinate, low-spin cobalt(II) protoporphyrin IX of CoHb binds oxygen must be compatible with the mechanism that triggers this quaternary structure transition.

This phenomenon of "heme–heme interaction" or cooperativity must have its basis at the molecule level, even though the Fe atoms are separated by ≈25 Å. The problem has been the subject of considerable speculation that is not appropriate to review here. Suffice it to say that the "trigger mechanism" proposed by Perutz, which is based on ideas of Williams[47] and Hoard,[48] may be illustrated in the top of Figure 5. In deoxy-Hb the high-spin Fe(II) atom is about 0.8 Å above the mean plane of the porphyrin. Upon oxygenation the Fe atom, which is variously

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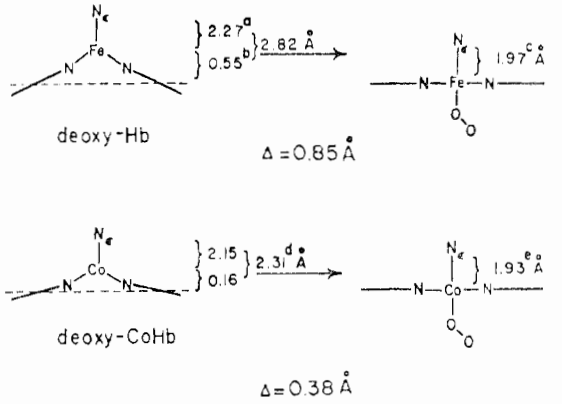

Figure 5. Illustration of the calculation of the total movement of the proximal histamine in Hb (top) and CoHb (bottom). The displacements are taken relative to the 24-atom porphyrin core (from ref 52).

thought to be Fe(II) or Fe(III), is now low spin, and it is believed to move into the mean plane of the porphyrin. Perutz proposes that the high-spin Fe(II) ion is held in a stressed five-coordinate state by the protein and that this stress or tension is released upon oxygenation. The resultant movement triggers a relaxation throughout a subunit which is ultimately transmitted to salt bridges.

However, the cobalt atom of CoHb is low spin even in the deoxy state.[21] Since low-spin Co(II) exhibits a smaller covalent radius than does high-spin Fe(II), we[21,22,45] predicted that the displacement of the Co(II) atom in CoHb would be relatively small compared with that of the Fe(II). Although CoHb has not been crystallized, our studies[49–52] of the stereochemistry of cobalt porphyrins have given considerable evidence regarding this prediction and have permitted reliable estimates of the possible motion to be expected in CoHb. As the model for deoxy-CoHb we have studied the structure of Co(OEP)(MeIm)[52] (OEP = octaethylporphyrin; MeIm = methylimidazole). On the basis of spectroscopic data similar to that discussed above, it is clear[21] that the cobalt in oxy-CoHb is low-spin six-coordinate Co(III) and thus a possible model is $Co(TPP)(Im)_2^{+}$ [51] (Im = imidazole). Whereas the proposed movement of the Fe atom in Hb is about 0.85 Å on oxygenation, an upper limit of about 0.38 Å can be placed on the similar movement of the Co atom in the CoHb. Since CoHb nevertheless displays cooperativity, it does not appear that cooperativity depends primarily upon the spin state and stereochemistry of the metal in the deoxy protein.[46,52] We thus concluded[22,52] that there are inherent shortcomings, or at least unresolved difficulties, in the trigger mechanism of Perutz.

A counterproposal has been advanced[53] in which it is suggested that in deoxy-CoHb tension within the protein increases the Co–histidine bond length and deforms the porphyrin in such a way that the net motion of the proximal histidine upon ligation is made equal to that in Hb. Direct evidence against this tension model in CoHb comes from resonance Raman studies of Hb and CoHb[54] and from EPR experiments of CoHb and model systems.[55,56] Since none of these experiments gives indications of tension, there appears to be no convincing evidence that the globin exerts a force which greatly increases the out-of-plane displacement of the Co atom or stretches the Co–N (of imidazole) bond in CoHb.

A second mechanism for cooperative effects in Hb is Hopfield's "linearly distributed energy model", in which the free energy of cooperativity is stored as small strains in many bonds.[57] Although attractive, this model may not account for the fact that deoxy-CoHb has the T quaternary structure, whereas met-Hb has the R structure since the metalloporphyrin geometries should be approximately the same in both molecules. Furthermore, the application of the model employs the specific value $n = 2.3$ for CoHb to relate Co(PIX) structures to CoHb function. However, as noted above, the Hill constant for CoHb of 2.3 cannot be considered as firm, and is probably not even the correct lower limit.

The implications of CoHb cooperativity for the nature of the Hb allosteric mechanism can be widened by using other metal-substituted hemoglobins. Met-Hb is in the high-spin, aqua form at low pH, and undergoes an ionization to the largely low-spin hydroxo form at $pK \sim 8$. This transition has been suggested to contribute to an observed increase of the n value for the redox reaction with pH.[44] We have observed that MnHb also exhibits an increase from $n = 1.4$ at pH 6.5 to $n = 2$ at pH 9, mimicking the behavior of Hb.[32,46] This observation is particularly significant, for Mn^{III}Hb does *not* exhibit a heme-linked ionization in the pH range of 5–10, and, therefore, Mn^{III}Hb does not display a "met-aqua" to "met-hydroxo," high–low spin transition.[32] Prompted by these results, we have directly addressed the question of the coupling of spin-state and cooperative redox behavior by examining the Hb redox equilibrium in the presence of F^- and of N_3^-.[58] In this way the oxidized heme is either constrained to remain high spin through binding of F^- or low spin through binding of N_3^-. The results are completely consistent with those for MnHb.

These experiments prove that the pH-induced changes in redox cooperativity do not arise from the pH dependence of the oxidized-heme spin state. The changes thus appear to reside in a pH dependence of the allosteric equilibrium; in addition, chain nonequivalence which depresses n at low pH[59] may diminish with increasing pH. On the other hand, a value of n of 2.2 appears to be the maximum obtainable with the high-spin forms, whereas azidomet-Hb exhibits an n of 2.7.[58] Whether this modest increase results from an effect of spin-state change on the position of the proximal histidine and to other ligand-induced changes in heme conformation[29] or whether

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it arises from an interaction of the N_3^- ligand with amino acid residues on the distal side of the heme and to an abolition of chain differences in the R state cannot be inferred from present data. However, since the results for coboglobin[21,22,45,50,56] and other studies[59,60] of Hb redox reactions do not support a simple coupling between spin state, the position of the heme-linked histidine, and cooperativity, the idea of an "allosteric trigger" thus seems to be either inappropriate or at least limited in its applicability.

Iron(II) Porphyrin Complexes

Research on synthetic oxygen carriers has very recently experienced the significant experimental development that iron(II) porphyrins can react reversibly with dioxygen (eq 6). One of the major difficul-

$$Fe(P)(B)_2 + O_2 \rightleftharpoons Fe(P)(B)(O_2) + B \quad (6)$$

ties encountered in attempts to obtain oxygen carriers of iron(II) complexes is the large driving force toward the irreversible formation of the stable μ-oxo dimer (eq 7). Considerable recent work has been

$$Fe^{II} + O_2 \rightleftharpoons Fe(O_2) \xrightarrow{Fe^{II}} Fe^{III}-O-Fe^{III} \quad (7)$$

aimed at overcoming this problem, and three approaches have been successful: (1) *steric*—in such a fashion that dimerization is inhibited; (2) *low temperature*—in order that reactions leading to dimerization are very slow; and (3) *rigid surfaces*—attachment of the iron complex on the surface in a manner that dimerization is prevented. The elegant steric approaches[61-64] were not done in our laboratories and are not discussed here. However, the low-temperature and the rigid-surface approaches which we have used are discussed.

One of the first synthetic iron(II) oxygen carriers[65] was an iron porphyrin with a built-in imidazole. The fact that it is sterically capable of forming a μ-oxo dimer and yet reversibly oxygenates at −45° suggested to us that the low temperature and not the neighboring group effect of an attached imidazole was responsible. Indeed the simple ferrous porphyrins, $Fe(TPP)(B)_2$, in methylene chloride solution at −79° are excellent oxygen carriers.[66] Similar observations were made independently in other laboratories using different iron(II) porphyrins.[67] The spectral changes show that $Fe(TPP)(py)_2$ is oxygenated in the presence of dioxygen and deoxygenated when the solution is purged with dinitrogen. Several oxygenation–deoxygenation cycles are possible at −79° with a minimum of irreversible oxidation occurring, and at this temperature a solution of the complex in one atmosphere of oxygen is stable for at least 1 day. The stoichiometry of the reaction corresponds to 1 mol of dioxygen per mol of iron (eq 8).

$$Fe(TPP)(B)_2 + O_2 \rightleftharpoons Fe(TPP)(B)(O_2) + B \quad (8)$$

Under 1 atm of oxygen, complete complex formation occurs in methylene chloride; only a small amount of complex is formed in toluene, whereas the degree of complex formation in ethyl ether lies between these extremes. Similarly, dioxygen complexes of cobalt are stabilized by polar solvents. The behavior in polar solvents supports formulation of the iron complex as $Fe^{III}(O_2^-)$. This formulation is in accord with the ir spectrum[68] of HbO_2.

Kinetic and equilibrium studies have been made on the reactions of $Fe(TPP)(B)_2$ with oxygen and with carbon monoxide in methylene chloride at −79°. The results of this investigation[69] are in accord with a rate-determining dissociation process (eq 9), followed by the rapid reaction of the five-coordinated intermediate, Fe(TPP)(B), with either oxygen (eq 10) or carbon monoxide (eq 11).

$$Fe(TPP)(B)_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe(TPP)(B) + B \quad (9)$$

$$Fe(TPP)(B) + O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Fe(TPP)(B)(O_2) \quad (10)$$

$$Fe(TPP)(B) + CO \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Fe(TPP)(B)(CO) \quad (11)$$

Some of the data obtained are given in Table III, along with corresponding data for Mb and for the isolated α and β chains of Hb. The simple five-coordinate ferrous porphyrin Fe(TPP)(MeIm) displays the same trends in reacting with dioxygen and carbon monoxide as do the more complicated heme proteins. This suggests that the simple system is a satisfactory model for the active site of heme proteins. Although the carbon monoxide complex is more stable than the corresponding oxygen complex, dioxygen reacts more rapidly than does carbon monoxide with the five-coordinate iron species. Nitric oxide, which contains

Table III
Comparisons between Kinetic and Thermodynamic Parameters for Reactions of O_2 and CO with a Simple Ferrous Porphyrin and with Heme Proteins

Ferrous system[a]	k_3/k_2	k_{-3}/k_{-2}	K_{CO}/K_{O_2}
Fe(TPP)(MeIm)	0.031	$<1.1 \times 10^{-3}$	>30
Myoglobin[b] (horse)	0.016	1.08×10^{-3}	14.4
Myoglobin[c] (aplysia)	0.033	0.29×10^{-3}	114
Isolated α chains[d,e]	0.071	0.52×10^{-3}	133
Isolated β chains[d,e]	0.030	0.17×10^{-3}	180

[a] The values for the heme proteins are at 20°C in aqueous solution (pH 7.0–7.4), whereas for Fe(TPP)(MeIm) the conditions are −79°C and methylene chloride solution (from ref 69). [b] G. A. Millikan, *Proc. Roy. Soc., Ser. B*, **120**, 366 (1936). [c] B. A. Wittenberg, M. Brunori, E. Antonini, J. B. Wittenberg, and J. Wyman, *Arch. Biochem. Biophys.*, **111**, 576 (1965). [d] M. Brunori, R. W. Noble, E. Antonini, and J. Wyman, *J. Biol. Chem.*, **241**, 5238 (1966). [e] R. W. Noble, Q. H. Gibson, M. Brunori, E. Antonini, and J. Wyman, *J. Biol. Chem.*, **244**, 3905 (1969).

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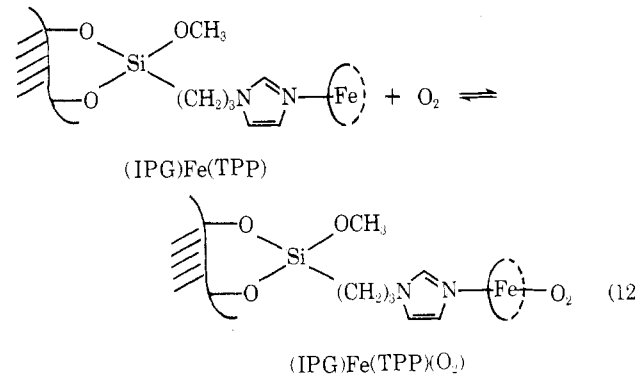
an unpaired electron, also reacts with heme proteins faster[43] than does CO. Thermodynamically Fe(porphyrin)(B)(NO) is more stable than Fe(porphyrin)(B)(CO), and this stability is augmented in the protein.[70]

The ratio k_2/k_{-1} (see eq 9 and 10) is approximately equal to 1 for different axial bases, which shows that there is no large kinetic preference of iron porphyrin for oxygen compared with nitrogenous bases. This illustrates the subtlety of the heme environment in the oxygen-carrying proteins. The distal imidazole can stabilize the oxygen complex through hydrogen bonding, but geometric constraints fortunately prevent its coordination to the iron center. The values of k_2/k_{-1} show that if the distal imidazole could bind to heme, it would seriously compete with oxygen for the available site, and the protein would cease to function as an effective oxygen carrier.

The third successful method of obtaining an oxygen carrying iron complex is to attach it to the surface of a solid so that two iron atoms cannot approach each other to form a dimer. In a classic experiment Wang[71] reported the first synthetic Fe(II) oxygen carrier. He showed that oxygen binds reversibly to 1-(2-phenylethyl)imidazole heme diethyl ester embedded in a matrix of an amorphous mixture of polystyrene and 1-(2-phenylethyl)imidazole. We have found[72] that the attachment of Fe(TPP) to a rigid modified silica gel support also inhibits dimerization and produces an efficient oxygen carrier. Collman and Reed[73] reported that cross-linked polystyrene, containing imidazole ligands coordinated to Fe(TPP), was not a rigid enough support to prevent dimerization upon treatment with oxygen.

The modified silica gel employed is one prepared earlier for other purposes[74] and contains 3-imidazolylpropyl (IPG) groups bonded to surface atoms of silicon. This then reacts with Fe(TPP)(B_2) to coordinate the iron(II) porphyrin to imidazole groups attached to the silica surface. Heating (IPG)Fe(TPP)(B) in flowing helium removes the axial base (pyridine or piperidine) and generates the active coordination site, (IPG)Fe(TPP) (see eq 12), resembling that of the five-coordinated iron(II) porphyrin in hemoglobin and myoglobin. The open coordination site can then reversibly bind dioxygen.

The chemisorption of oxygen is weak. It is irreversible at −127° and has a $P_{1/2}$ of 0.4 Torr at −78°, and a $P_{1/2}$ of 230 Torr at 0°. Data for human myoglobin[43] extrapolated to 0° give a $P_{1/2}$ of 0.14 Torr. Successful



(12)

O_2 binding on the modified silica gel, however, confirms the utility of the rigid-surface approach.

Conclusion

During the last 5 years research on synthetic oxygen carriers in our laboratories has been devoted almost entirely to systems related to the natural oxygen carriers. The discovery of 1:1 O_2–Co(Schiff base) complexes, followed by similar observations on 1:1 O_2–Co(porphyrin) complexes, and the reconstitution of hemoglobin with Co in place of Fe have afforded an excellent opportunity to attack some of the central problems in biological oxygen carriers. For example, various experiments enabled us to assess the role of the globin in the uptake of oxygen and other small molecules by biological molecules. Moreover, the combination of results from the model cobalt porphyrin systems and cobalt-substituted hemoglobin has enabled us to test critically current theories for the allosteric properties of hemoglobin. More recently with the discovery of stable, reversibly oxygenated iron porphyrin systems we anticipate an even greater opportunity to test critically the role of the globin in hemoglobin chemistry. The current efforts to study hemoglobin substituted with other metals, such as Mn and Zn, and to study analogous porphyrin systems has already begun to provide further insight into the biological systems. Extension of the techniques used to obtain oxygen carriers with Fe complexes to corresponding Cr, Mn, and V systems has now produced new synthetic oxygen carriers.[75]

We thank all of our coworkers whose names appear in the references and we gratefully acknowledge that without their efforts the research on synthetic oxygen carriers done at Northwestern University would not have been accomplished. This research was in part financially supported by National Institutes of Health Grants HL 13412, HL 13531, and HL 13157, by National Science Foundation Grants GP-27626, MPS 71-02605 and BMS-00478, by the Petroleum Research Fund, administered by the American Chemical Society, and by the Materials Research Center, Northwestern University.

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Additions and Corrections

Page 390. In the legend to Figure 5, line 2, histamine should read histidine. In column 1, the sentence beginning on line 26 of the text should read: Whereas the proposed movement of the N atom of the proximal histidine group in Hb is about 0.85 Å on oxygenation, an upper limit of about 0.38 Å can be placed on the similar movement in CoHb.

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