*METALLOENZYMES: THE ENTATIC NATURE OF THEIR ACTIVE SITES**,†

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Selective chemical modifications of specific amino acid side chains have been shown to affect the function of a large number of enzymes and constitute an apparent link between their chemical and catalytic properties.[2-4] The modifications indicate that particular side chains are exceptionally reactive toward certain reagents. This unusual reactivity has led to the hypothesis that side chains present in active, enzymatic sites are chemically atypical when compared to similar residues in analogous model substances or in catalytically inert regions of the protein. This view suggests the search for unusual physical features of these functional enzyme side chains which would define "atypical" character in terms of structural or electronic abnormalities of the groups or of their environment. In the case of most (nonmetallo-)enzymes this search has proved difficult since *alterations of catalytic activity* attending chemical modifications have usually served as the relative indices of the condition of the side chains. In certain cases anomalous pK_a values have also been reported but the difficulties associated with their interpretations are well known.[5] Thus, with this class of enzymes it has not been feasible to examine the possibility that enzymes might be *poised for catalytic action in the absence of substrate*, i.e., are in an *entatic state*.[1]

Metalloenzymes would seem exceptionally well suited for the examination of the physicochemical basis of enzymatic properties, for the physical and chemical characteristics of certain metals constitute *intrinsic* probes of their protein environments.[6-8] In fact, the systematic examination of the absorption, optical rotatory dispersion (ORD), and electron paramagnetic resonance (EPR) spectra, magnetic properties, redox potentials, and ligand binding has increasingly revealed that the properties of the intrinsic catalytically active metals of metalloenzymes are quite unusual when compared to those of well-defined model coordination complexes.[7, 8] Importantly, these properties are discernible *in the absence of substrate*. On the other hand, the features of the vast majority of metalloproteins presently thought to be enzymatically inert are comparable to those of known models emphasizing the unusual nature of metalloenzymes.[7, 9] The following discussion will summarize pertinent characteristics of some of the metals of metalloenzymes, here viewed as probes, and draw deductions as to their relevance to the catalytic potential of metalloenzymes.

Spectral Properties of Metalloenzymes.—Both the absorption and EPR spectra of copper and nonheme iron enzymes are readily accessible and reveal unusual properties when compared with the simple complexes of the same metals (Tables 1 and 2). Their features indicate an unusual environment, either by virtue of their intensities or by the location of the maxima and fine structure, jointly reflecting the unusual geometry of the metal site. In particular, the spectra of

the numerous typical "copper blue enzymes" have no known parallel with the spectra of any type of copper compounds, including all copper proteins currently known not to be enzymes, copper protein complexes, and complex ions (models).[9, 10] The absorption bands differ strikingly in intensity, i.e., transition

TABLE 1. *Spectral characteristics of copper (A), iron (B), and cobalt (C) complexes and enzymes.*

	Compound	λ, mμ (molar absorbance, M^{-1} cm^{-1})			Notes	Source
A.	*1. Simple Cu(II) Complexes:*					
	$[Cu(NH_3)_4]^{2+}$	600(~20)	660(~40)	750(~25)	Tetragonal	(1)
	Bis(3-phenyl-2,4 pentanedionato) copper(II)	490(~50)	520(~25)	580(~50) 650(~50)	Twofold axis	(2)
	$[Cu(H_2O)_2]^{2+}$	700(~5)	790(~10)	950(~5)	Tetragonal	(3)
	2. Cu-Protein Complexes:					
	Cu(II) carbonic anhydrase		760 broad	(120)	Unknown low symmetry	(4)
	Cu(II) serum albumin		570 broad	(90)	?	(5)
	3. "Inert" Cu Proteins:					
	Erythrocuprein		655 broad	(280)	Unknown symmetry, probably tetragonal	(6)
	Cerebrocuprein		655 broad	(400)		(7)
	4. "Active" Cu Proteins:					
	Plastocyanin	460(500)	597(4400)	770(1600)		(8)
	Laccase	450(970)	608(4000)	850(700)	Unknown but low symmetry	(9)
	Pseudomonas aeruginosa blue	500(weak)	625(~3000)	725(weak)		(10)
	Pseudomonas dentrificans blue	450(weak)	594(~3000)	800(1000)		(11)
B.	$[Fe(CN_6)]^{3-}$	General rise in absorption with but one or two discrete bands			Octahedral	(12)
	$[Fe(H_2O)_5OH]^{2+}$					
	Ferredoxin (spinach)*	325(5160)	420(5160)	463(4650) 570(shoulder)	?	(13)
C.	$[Co(H_2O)_6]^{2+}$	510(10)	1200(2)		Octahedral	(14)
	$[CoCl_4]^{2-}$	685(700)	1700(100)		Tetrahedral	(15)
	Co carbonic anhydrase	510(280)	550(380)	615(300) 640(280)	Irregularly tetrahedral or five coordinate	(16)
	Co alkaline phosphatase	515(265)	555(350)	605(180) 640(230)		(17)

* Exemplifying plant and bacterial ferredoxins and adrenodoxin.

(1) Smith, D. W., *Inorg. Chem.*, **5**, 2236 (1966).
(2) Belford, R. L., and J. W. Carmichael, *J. Chem. Phys.*, **46**, 4515 (1967).
(3) Holmes, O. G., and D. S. McClure, *J. Chem. Phys.*, **26**, 1686 (1957).
(4) Lindskog, S., and B. G. Malmström, *J. Biol. Chem.*, **237**, 1129 (1962).
(5) Klotz, I. M., and H. A. Fiess, *J. Phys. Chem.*, **55**, 101 (1951).
(6) Markowitz, H., G. E. Cartwright, and M. M. Wintrobe, *J. Biol. Chem.*, **234**, 40 (1959).
(7) Porter, H., and A. Ainsworth, *J. Neurochem.*, **5**, 91 (1959).
(8) Katch, S., I. Shiratori, and A. Takamiya, *J. Biochem. Japan*, **51**, 32 (1962).
(9) Omura, T., *J. Biochem. Japan*, **50**, 389 (1961).
(10) Horio, T., *J. Biochem. Japan*, **45**, 267 (1958).
(11) Iwasaki, M., S. Shidara, H. Suzuki, and T. Mori, *J. Biochem. Japan*, **53**, 299 (1963).
(12) Ballhausen, C. J., *Ligand Field Theory* (New York: McGraw-Hill, 1962), p. 253.
(13) Whatley, F. R., K. Tagawa, and D. I. Arnon, these PROCEEDINGS, **49**, 266 (1963).
(14) Ballhausen, C. J., and C. K. Jørgensen, *Acta Chem. Scand.*, **9**, 397 (1955).
(15) Cotton, F. A., D. M. L. Goodgame, and M. Goodgame, *J. Am. Chem. Soc.*, **83**, 4690 (1961).
(16) Lindskog, S., and P. O. Nyman, *Biochim. Biophys. Acta*, **85**, 462 (1964).
(17) Simpson, R. T., and B. L. Vallee, *Federation Proc.* (April 1968).

TABLE 2. *EPR parameters of Cu(II) and Fe(III) models, "copper blue enzymes" and ferredoxins.*

Compound	High field (g)	Low Field g	Low Field A_{cm}^{-1}	Source
$Cu(NH_3)_4^{2+}$	2.05	2.4–2.15	0.015	(1)
Cu(II) EDTA (1:10)	2.09	2.40	0.015	(2)
Pseudomonas aeruginosa blue protein	2.055	2.26	0.006	(3)
Ceruloplasmin	2.06	2.21	0.008	(4)
Laccase (*Polyphemus*)	2.05	2.20	0.009	(5)
Laccase (*Rhus*)	2.09	2.27	0.004	(6)
Rhus vernicifera (stellacyanin)	2.04	2.30	0.004	(6)
Plastocyanin	2.05	2.23	0.006	(7)
	g_x	g_y g_z		
Fe(III)	1.7,	2.1, 2.8		(8)
Ferredoxin (spinach)*	1.89,	1.96, 2.04		(9)

* Exemplifying plant and bacterial ferredoxins and adrenodoxin.

(1) McGarvey, B. R., *Transition Metal Chem.*, **3**, 180 (1966).
(2) Malmström, B. G., and T. Vänngård, *J. Mol. Biol.*, **2**, 118 (1960).
(3) Mason, H. S., *Biochem. Biophys. Res. Commun.*, **10**, 11 (1963).
(4) Malmström, B. G., and T. Vänngård, *J. Mol. Biol.*, **2**, 118 (1960).
(5) *Ibid.*
(6) Blumberg, W. E., W. G. Levine, S. Margolis, and J. Peisach, *Biochem. Biophys. Res. Commun.*, **15**, 277 (1964).
(7) Blumberg, W. E., and J. Peisach, *Biochim. Biophys. Acta*, **126**, 269 (1966).
(8) Baker, J. M., B. Bleaney, and K. D. Bowers, *Proc. Phys. Soc. London*, **B69**, 1205 (1956).
(9) Brintzinger, H., G. Palmer, and R. H. Sands, these PROCEEDINGS, **55**, 397 (1966).

probability, and in fine structure, i.e., energy distribution of transitions (Table 1, *A*). In instances studied so far, extremes of pH, but without removal of copper, followed by neutralization, reversibly remove and restore these spectral or EPR features concomitantly with activity. Hemocyanin, which functions in oxygen transport, also has an abnormal though different absorption spectrum.[9, 10]

The spectra of nonheme iron enzymes also differ from those of iron complex ions.[11] In this instance the spectra consist of at least four intense but discrete absorption bands between 320 and 600 mμ, a pattern not yet observed in other iron complexes known to us (Table 1, *B*).

The zinc ion, also common in metalloenzymes, has neither intrinsic color nor unpaired electrons, rendering it a poor probe in this context. However, cobalt has been substituted for zinc in carboxypeptidase,[12] carbonic anhydrase,[13] and alkaline phosphatase of *E. coli*,[14] resulting in enzymatically active products which now exhibit visible spectra. Again, these differ significantly from those of model cobalt(II) complexes[8] in that there are as many as *four* bands of high intensity, spread over the region of energy from 450 to 700 mμ (Table 1, *C*).[12–14]

Alteration of the coordination sphere by reaction with an added ligand, e.g., inhibitor anions like cyanide, sulfide, and sulfonamides, converts the irregular spectra of cobalt(II) carbonic anhydrase to one recognizably that of a tetrahedron.[13] Similarly, addition of azide, cyanate, and thiocyanate to "copper blue enzymes" removes the unusual features of their spectra.[15, 16] Modification of the absorption spectra through substitution of ligands at the metal ion therefore helps to demonstrate an unusual condition of the active metal-ligand site, an experimental approach which is capable of considerable expansion.

Electron spin resonance spectra of copper and iron enzymes are similarly

unusual (Table 2). Again, prototypes for the EPR spectra of the "copper blue enzymes" have not as yet been found. The small g values, just at the extreme limit of what has been observed in conventional models, together with the unique, small values of A, the nuclear hyperfine splitting constant, have been interpreted to denote an unusual distortion around the cupric ion.[9, 10, 15, 17] In the ferredoxins, the three g values, centered on $g = 1.94$, are equally unusual in iron chemistry.[18] High g and low A values, which could well denote a low symmetry environment, have also been seen in the few molybdenum enzymes examined so far, but their detailed interpretation has not been recorded as yet.[19] Thus, jointly the absorption and EPR spectra of virtually all metalloenzymes containing a suitable metal are unusual and characteristic of low symmetry, apparently a feature common to their metal binding sites.

Prosthetic Groups.—Metal-containing heme or corrin prosthetic groups can be valuable probes also, though the physical characteristics of the metals become intertwined with those of the organic moiety. Hence, the spectral contribution of the metal probe can be reduced considerably or even obscured completely. Unusual features could appear now either in the physical properties of the metal or in those of their organic moiety, but not necessarily in both.

On the basis of a comparison with model heme complexes the spectra of cytochromes cc',o, and P_{450} deviate markedly both in the wavelength and intensity of the Soret band region, reflecting an unusual condition of the organic moiety.[20] Though not so markedly anomalous, the cytochrome oxidase spectrum has a split Soret band, and an unusual position and intensity of the α-band, showing that its heme moiety, too, differs considerably from those of model compounds.[20, 21] Although other cytochromes have often been reported to have conventional hemochrome absorption, spectral evidence is accumulating that some of their properties are also unusual. In addition to the absorption bands of other heme proteins, the cytochromes c in the oxidized state exhibit one band at 695 mμ of unknown origin and have weak absorption bands beyond 800 mμ. The long-wavelength bands and the studies of magnetic susceptibility measurements have shown that these cytochromes are in fact about 5 per cent high spin, implying that their "low spin" $\rightleftharpoons$ "high spin" equilibrium is finely balanced in a manner unusual in models. Electron spin resonance and magnetic methods have recently shown that cytochromes a_3 and b are spin-state mixtures rather than simple hemochromogens, as had long been supposed.[22]

Redox Potentials.—A number of atypical thermodynamic and kinetic properties of these same metalloenzymes are also indicative of unusual sites of the metal ions. Thus, the redox potentials of the "copper blue enzymes" are much higher than those of all copper complexes except those which are strongly distorted from tetragonal symmetry, viz. $+0.3$ to $+0.4$ volt, as compared with -0.5 to $+0.2$ volt.

The redox potentials of a number of cytochromes are illustrated in Figure 1 and compared with model heme complexes. While the potentials of most model heme complexes are below 0.0 volt, those of cytochromes b, c, and a all lie higher, many of them by 0.3 to 0.4 volt. These data indicate that in the electron-transfer proteins the environment destabilizes the Fe(III) with respect to the Fe(II)

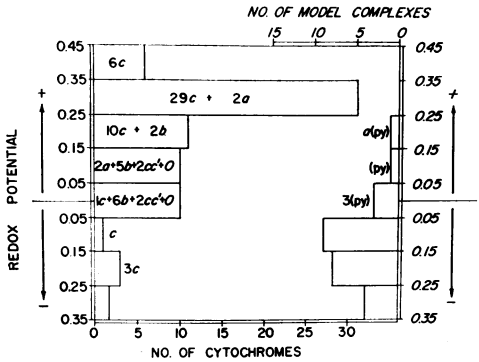

FIG. 1.—The redox potentials of cytochromes (*left-hand side*) compared with those of models (*right-hand side*). The cytochromes are given their conventional labels and the numbers in the boxes indicate the number of examples of a particular cytochrome type.

On the right, the lower boxes give the number of model heme complexes with imidazole bases, while the higher three boxes refer to pyridine heme complexes. The top box is for a pyridine heme *a* complex.

state to a degree unknown in models. It has been conjectured that the absorption and EPR spectra and the spin-state equilibria jointly suggest that in these instances the axial bonds to the iron from the proteins are longer than is normal in model-heme complexes (*vide infra*). This is entirely consistent with the high redox potentials observed. In corrin enzyme complexes a similar situation seems to pertain, since there is now evidence that the nominally cobalt(III) atom can be 5-coordinate, bringing the three valence states of cobalt, i.e., (I), (II), and (III), closer together in energy than is conventional.[23]

Implications to Enzymatic Catalysis.—The principal biological functions of the metalloenzymes studied so far relate either to electron transfer or hydrolysis. Intuitively, one might conjecture that the atypical properties are connected to these catalytic functions. Present treatments of the mechanism of enzymatic catalysis postulate an active intermediate, and the rate of a reaction depends on the ease with which the related transition state is reached. In our terms, this would be facilitated by *a state of entasis: the existence in the enzyme of an area with energy, closer to that of a unimolecular transition state than to that of a conventional, stable molecule, thereby constituting an energetically poised domain.* On this basis the differences between the physical properties of metals in metalloenzymes and models could be understood, since conventional metal complexes, which generally serve as models, do not have geometries of the proposed unstable intermediates of chemical reactions but relax to conventional, stable geometries. It appears that the future design of suitable models should incorporate the emerging structures of metal complexes in enzyme centers, which may deviate significantly from simpler systems currently known.

Electron Transfer.—It is generally assumed that the intermediates of electron transfer reactions involving complex ions require a compromised geometry between those normally assumed by the two valence states, i.e., the transition state is thought to be a distorted complex. Copper(I) and copper(II) demand different site symmetries, i.e., tetrahedral and tetragonal. Therefore, a suitable intermediate of irregular symmetry would result in an entatic state. Precisely such geometry would appear to exist in the "copper blue enzymes" (*vide supra*). On the other hand, the two valence states of iron, Fe(II) and Fe(III), demand the same, i.e., octahedral, symmetry but different bond distances. Thus, a compromise would result in lengthened bonds to iron(III), as postulated for the

cytochrome (*vide supra*). Thus, in many enzymes concerned with biological electron transfer the properties of the catalytically active metal are consistent with those to be expected if the metal had the distorted geometry approximating that of the plausible transition state for the very reaction in which it is involved. The active enzymatic site itself would thus be designed to achieve a condition energetically favorable to catalysis.

Hydrolytic Reactions.—When a metal participates in a catalytic step involving transfer of an atom, radical, or a group rather than of an electron, ligand substitution is required. The active intermediates in substitution reactions of simple metal chelates either are presumed to have an open coordination position[24] or a distorted coordination sphere. Just such energetically unfavorable, distorted environments seem to exist, for example, in cobalt (II) carbonic anhydrase, carboxypeptidase, and phosphatase prior to substrate entry. Among the elements of the first transition series, Co^{2+} and Zn^{2+} atoms readily accept distorted geometries in model complexes. Strikingly, they are most effective in enzymes catalyzing substitution reactions as for instance in carbonic anhydrase,[13] carboxypeptidase,[12] and alkaline phosphatase.[14] Nickel and copper (II) both have more regular geometries in model complexes and are either less active or inactive when substituted into these same proteins. In model systems, by contrast, both of these cations are more active catalytically than either cobalt or zinc.

Thus in these hydrolytic enzymes the particular metal present and its structural condition would appear to be suitably chosen to assist reaction, for they are such as to permit addition (or substitution) with very low activation energy. Thus the entatic state is again closely allied to the required intermediate of a conventional chemical reaction.

Entatic Active Sites and Active Centers.—Now it might be asked in what manner a protein might generate a center resulting in irregular metal geometries and whether or not analogous centers might be encountered in enzymes other than metalloenzymes. The identities of the amino acid side chains of metalloenzymes which serve as ligands for the metal atom are not known in their entirety at present in any instance. Moreover, on the basis of values derived from models it has not been possible to assign readily the apparent pK's to specific amino acid side chains of the apoenzyme which become the ligands of the metalloenzymes in the few instances which have been examined with this objective in mind. This contrasts with the relatively extensive knowledge on the identity of metal binding side chains of catalytically inactive peptides or proteins; these correspond quite well with those of amino acid and peptide models.[4, 25]

The difference between the properties of metalloenzymes and simple metal complexes might be thought to reflect the constraints imposed in the former by the specific secondary and tertiary structure on amino acid side chains serving as their *multidentate* metal ligands. Further, in metalloenzymes unusual disposition of the ligands may force on the metal atoms irregular geometries which may or may not be encountered in nonfunctional metalloproteins. Hence, in metalloenzymes the metal and its ligands should be considered to generate the entatic state jointly. For example, the metal-ligand pair could well be an extremely effective acid-base system for cooperative attack—both being in a condition close

to that of the intermediate state for their normal reactions and removed from a position of minimum free energy, as seen in models. Similar considerations might be pertinent for substrate binding sites as well.

The ligand-metal interaction of metalloenzymes might be compared with, e.g., that of reactive histidyl, seryl, tyrosyl, or sulfhydryl residues of certain enzymes with protons, the proton being the simplest cation. This unusual reactivity has been related to unusual microscopic, chemical, and conformational environments which might predispose the ligands of apometalloenzymes to the formation of metal complexes of distorted geometries and the organic residues of other (non-metallo-) enzymes to selective reactivity with organic reagents. At present any abnormalities of proton-ligand conditions would be hard to demonstrate by physical methods since the proton is a relatively poor probe, being at present accessible largely to kinetic or chemical methods.

In view of the curious properties of metals at active sites it is important to search for other systems and methods which might permit the demonstration of the presence or absence of "anomalous" properties of the groups at an active site. Thus, the spectral alterations which are seen on interaction of coenzymes, e.g., FAD, NADH, PPL, etc., with their respective apoenzymes might be indicative of an entatic state in such systems, and hence, coenzymes might be employed as probes in these instances. Already it is known that many of these coenzymes exhibit unusual spectroscopic properties when bound to enzymes. Only when more searching experiments on these and other systems are performed will it be clear to what degree metalloenzymes are a very special class of enzymes or examples of a very general state of biological catalysts which happen to be demonstrable in this group by virtue of the properties of the metal probe. These considerations should further stimulate the search for new complex ion models and others which might exhibit features suggested by the entatic state of enzymes. Additional kinetic, thermodynamic, and physical-chemical approaches for the characterization of this state might also be discerned.

A substrate entering this poised domain would find itself under attack by unusually activated groups, and, consequently, the activation energy for reactions would be small.[26] The view presented in no way precludes, of course, that the entry of the substrate itself would lower the energy of activation further. The ideas here suggested rest upon the properties of the *enzyme itself* and are not intended to bear upon the role of substrate; hence, they make no contribution to the manner in which the substrate will subsequently affect its complex with the enzyme or lower the activation energy further, a problem which has been considered.[3, 27–29]

* For the purposes of our discussion the designation "active site" will refer specifically to atom(s) or groups essential to the catalytic step, i.e., the metal atoms, their ligands, and amino acid side chains demonstrably involved in activity. "Entatic," from the Greek "entasis," *ἔντασις*, literally meaning in a stretched state or under tension, implies a catalytically poised state *intrinsic to the active site.* "Active centers" will refer to all those features of primary, tertiary, and quaternary structure of enzymes—including the active site—which are required for substrate binding, specificity, and catalysis.

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[1] "Entatic," *see* asterisk footnote above.

[2] Linderstrom-Lang, K. U., and J. A. Schellman, in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (New York: Academic Press, 1960), vol. 1, 2d ed., p. 443.

[3] Koshland, D. E., *Advan. Enzymol.*, **22**, 45 (1960).

[4] Sri Ram, J., M. Bier, and P. H. Maurer, *Advan. Enzymol.*, **24**, 105 (1962).

[5] Tanford, C., *Advan. Prot. Chem.*, **17**, 69 (1962).

[6] Vallee, B. L., and J. E. Coleman, in *Comprehensive Biochemistry*, ed. M. Florkin and E. H. Stotz (Amsterdam: Elsevier Publ. Co., 1964), vol. 12, p. 165.

[7] Vallee, B. L., and W. E. C. Wacker, "Metalloproteins," in *The Proteins*, ed. H. Neurath (New York: Academic Press, in press), vol. 5.

[8] Dennard, A. E., and R. J. P. Williams, in *Transition Metal Chemistry*, ed. R. L. Carlin (New York: Marcel Dekker, Inc., 1966), vol. 2, p. 115.

[9] Brill, A. S., R. B. Martin, and R. J. P. Williams, in *Electronic Aspects of Biochemistry*, ed. B. Pullman (New York: Academic Press, 1964), p. 519.

[10] Frieden, E., S. Osaki, and H. Kobayashi, *J. Gen. Physiol.*, **49**, 213 (1965).

[11] Beinert, H., in *Non-heme Iron Proteins*, ed. A. San Pietro (Yellow Springs, Ohio: The Antioch Press, 1965), p. 23.

[12] Coleman, J. E., and B. L. Vallee, *J. Biol. Chem.*, **235**, 390 (1960).

[13] Lindskog, S., *J. Biol. Chem.*, **238**, 945 (1963).

[14] Simpson, R. T., and B. L. Vallee, *Federation Proc.*, (April 1968).

[15] Gould, D.; Ehrenberg, A.; and Williams, R. J. P.; all in *Hemocyanin*, ed. H. Ghiretti (New York: Academic Press, in press).

[16] Laurell, C. B., in *The Plasma Proteins*, ed. F. W. Putnam (New York: Academic Press, 1960), vol. 1, p. 349.

[17] Blumberg, W. E., in *The Biochemistry of Copper*, ed. J. Peisach, P. Aisen, and W. E. Blumberg (New York: Academic Press, 1966), p. 67.

[18] Beinert, H., and G. Palmer, *Advan. Enzymol.*, **27**, 105 (1965).

[19] Bray, R. C., P. F. Knowles, and L. S. Meriwether, in *Magnetic Resonance in Biological Systems*, ed. A. Ehrenberg, B. G. Malmström, and T. Vänngård (Oxford: Pergamon Press, 1967), p. 249.

[20] Lemberg, R.; Ehrenberg, A.; and Williams, R. J. P.; all in *Hemes and Hemeproteins*, ed. B Chance, R. Estabrook, and T. Yonetani (New York: Academic Press, 1966), pp. 79, 331, 462, 552.

[21] Williams, R. J. P., in *Hematin Enzymes*, ed. J. E. Falk, R. Lemberg, and R. K. Morton (Oxford: Pergamon Press, 1961), p. 41.

[22] Ehrenberg, A., and R. Bois-Poltoratsky, in *Symposium on Cytochromes*, ed. K. Okunuki and M. D. Kamen (Tokyo: University of Tokyo Press, 1967), p. 309.

[23] Firth, R. A., M. A. D. Hill, J. M. Pratt, R. G. Thorpe, and R. J. P. Williams, *Chem. Comm. (London)* (1967), p. 127.

[24] Hemoglobin and myoglobin have normal heme spectra. Their crystal structure, however, reveals the Fe(II) atom to be 5 coordinate (Bluhm, M. D., G. Bodo, H. M. Dintzis, and J. C. Kendrew, *Proc. Roy. Soc. (London)*, **A 246**, 369 (1958)). This type of high spin Fe(II) complex, not yet observed in models, would have been revealed in the pure iron d-d absorption or in the EPR spectra, could these have been observed. Such an open coordination position allowing substitutions involving transport or storage, e.g. of O_2, is clearly an example where biological requirement is precisely met by the unusual geometry of a complex. It is of great interest to anticipate whether or not such enzymes as peroxidase, tryptophan pyrrolase, and other heme oxygenases which have spectra, etc., similar to Hgb and Mgb, but very different from the heme electron transfer proteins, also contain 5 coordinate iron (II).

[25] Gurd, F. R. N., and P. E. Wilcox, *Advan. Prot. Chem.*, **11**, 311 (1956).

[26] Bruice, T. C., and S. J. Benkovic, *Bioorganic Mechanisms* (New York: W. A. Benjamin, Inc. 1966), vol. 1, p. 119.

[27] Smith, E. L., *Advan. Enzymol.*, **12**, 191 (1951).

[28] Eyring, H., R. Lumry, and J. D. Spikes, in *The Mechanism of Enzyme Action*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1954), p. 123.

[29] Koshland, D. E., these PROCEEDINGS, **44**, 98 (1958).