KINETIC STUDIES OF THE REDUCTION OF *Rhus vernicifera* LACCASE

Thesis by

Robert Alan Holwerda

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ABSTRACT

Anaerobic stopped flow kinetic studies of the reactions of hydroquinone, $Fe(CN)_6^{4-}$, and $Fe(EDTA)^{2-}$ with Rhus vernicifera laccase have been performed with the objective of elucidating the mechanisms by which the three enzymatic copper sites are reduced.

Second order rate constants characterizing the hydroquinone reduction of laccase Type 1 and Type 3 copper sites at 25.6°, pH 7.0, $\mu = 0.1$ (phosphate) are 325 and 457 M^{-1} sec^{-1}, respectively. All of the experimental evidence points to the involvement of a common intermediate in the reduction mechanisms of these two functional units, and pH dependence results indicate that the phenolate monoanion HQ^- is the active reductant interacting with the metalloprotein in the shared slow step. A requirement for penetration of HQ^- into the first coordination sphere of Type 2 Cu(II) is strongly suggested by pH 6.0 fluoride inhibition results, and activation parameters (Type 1: $\Delta H^{\ddagger} = 15.0$ kcal/mole, $\Delta S^{\ddagger} = +13$ cal/mol-deg; Type 3: $\Delta H^{\ddagger} = 13.6$ kcal/mole, $\Delta S^{\ddagger} = +15$ cal/mol-deg; pH 7.0, $\mu = 0.1$) are compatible with rate-limiting reduction of an essentially axial copper(II) species.

Decolorization of the intense peak with maximum at 405 nm characteristic of the Type 2 Cu(II)-N_3^- laccase derivative is first order in $[N_3^-]$, zeroth order in [hydroquinone], suggesting that azide bridging is involved in an intramolecular Type 1 Cu(I) to Type 2 Cu(II) electron transfer step. Attack at the Type 2 copper is not a prerequisite to reduction of the Type 1 site in the laccase-azide complex,

but the reactivity of the "blue" copper is considerably altered from that in the native enzyme.

Ferrocyanide reduces laccase by a mechanism much different than that preferred by hydroquinone. The reduction rates of the Type 1 and Type 3 sites are identical ($k = 42\ M^{-1}\ sec^{-1}$, 25°, pH 7.0, $\mu = 0.1$; $\Delta H^{\ddagger} = 18.4$ kcal/mole, $\Delta S^{\ddagger} = +10$ cal/mol-deg), and only three electron equivalents are transferred to the metalloprotein anaerobically. The Type 2 copper atom is not reduced by $Fe(CN)_6^{4-}$. Ionic strength and pH dependence studies suggest that electrostatic factors direct $Fe(CN)_6^{4-}$ to its initial attack site. At least one histidine residue appears to be involved in outer-sphere complex formation between laccase and the highly anionic reducing agent. The rate-limiting step for ferrocyanide reduction of laccase is reversible; calculations based on the forward and reverse rate constants indicate that the "blue" copper is probably the initial attack site preferred by ferrocyanide. The unusually high activation enthalpy for the reduction of laccase "blue" copper by $Fe(CN)_6^{4-}$ is attributed to the inaccesibility of this site to direct attack by external reducing agents.

Preliminary experiments with $Fe(EDTA)^{2-}$ as reductant have shown that the laccase "blue" copper site is reduced over 1000 times slower than that of stellacyanin ($k = 2.31 \times 10^2\ M^{-1}\ sec^{-1}$ (laccase), $4.2 \times 10^5\ M^{-1}\ sec^{-1}$ (stellacyanin); 25.1°, pH 6.0, $\mu = 0.5$). Activation parameters for the reduction of laccase Type 1 copper by $Fe(EDTA)^{2-}$ are: $\Delta H^{\ddagger} = 13.0$ kcal/mole, $\Delta S^{\ddagger} = -4$ cal/mol-deg; pH 7.0, $\mu = 0.1$.

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INTRODUCTION

Laccase, a copper-containing glycoprotein, was discovered in the latex of the Japanese lacquer tree (Rhus vernicifera) by Yoshida in 1883.[1] Early preparations were found to cause the darkening of a polyphenolic latex fraction with the uptake of oxygen. Further details of early laccase studies leading to the proof that protein-bound copper is responsible for enzymatic polyphenoloxidase activity may be found in Levine's interesting review.[2]

Although laccase was named for its occurrence in the lacquer tree, it has been isolated from many other plant sources as well.[3] The best substrates for laccase are para- and ortho- aryl diamines and diphenols, oxidation products being the corresponding quinones. One of the most reactive substrates for Rhus laccase is p-phenylene-diamine, and hydroquinone is turned over about one-third as fast as this.[4] Unlike tyrosinase, another copper-containing polyphenol-oxidase, laccase is unreactive with monophenols and meta- diamines and diphenols.

Nakamura's work in the late 1950's marks the beginning of modern understanding of laccase. He found the molecular weight of the enzyme to be 120,000 with 4 Cu/mole[5] and established the stoichiometry of the laccase-catalyzed oxidation of hydroquinone (H_2Q) by O_2: $2H_2Q$ are consumed per O_2 reduced. Water is probably the only oxygen reduction product, as H_2O_2 or other intermediates have never been detected. Nakamura's suggested mechanism[6]:

$$2\,Cu^{2+} + H_2Q \rightarrow 2\,Cu^{2+} + Q + 2H^+$$

$$2\,Cu^{+} + \tfrac{1}{2}\,O_2 + 2H^+ \rightarrow 2\,Cu^{2+} + H_2O$$

is still thought to reflect the basis for laccase enzymatic activity.

Nakamura subsequently published ESR experiments[7] demonstrating that semiquinone (SQ) is quantitatively produced from hydroquinone during the laccase-catalyzed oxidation of H_2Q by O_2. Apparently semiquinone is enzymatically produced from H_2Q via one-electron transfer steps; rather than reacting directly with O_2 or remaining oxidized laccase copper sites, SQ was found to disproportionate to H_2Q and Q. Free radical formation and decay has also been observed in the fungal laccase-catalyzed oxidation of p-phenylenediamine.[8]

Reinhammar's recently published tree laccase purification procedure[9] includes a molecular weight estimate (110,000) in good agreement with Nakamura's value and a complete amino acid analysis of the protein. Laccase contains an excess of 38 basic amino acid residues over acid residues (arginine + histidine + lysine = 56; aspartic acid + glutamic acid - NH_3 = 18), explaining its high isoelectric point of 8.55. The enzyme contains only about 55% protein by weight, the remaining 45% being carbohydrate.

Laccase is just one of a number of biologically important copper-containing oxidases.[10,11] Others include tyrosinase, cytochrome oxidase, ascorbate oxidase, and possibly ceruloplasmin. Within the past ten years it has become evident that the copper sites within a large number of metalloproteins may be classified into three categories

(named, for lack of more original titles, Type 1, Type 2, and Type 3) on the basis of their visible and ESR spectra. Malkin and Malmström,[12] in reviewing the characteristics of these sites, point out that the classifications are best used when comparing proteins containing the "blue" (Type 1) copper structural unit. The spectroscopic properties of cupric proteins are often much different from those of simple Cu(II) complexes.[13,14]

Type 1 copper gives rise to the remarkably intense blue color of stellacyanin[15] (ϵ_{604} = 4080 $M^{-1}cm^{-1}$), azurin[16] (ϵ_{625} = 3500 M^{-1} cm^{-1}), spinach plastocyanin[17] (ϵ_{597} = 4900 $M^{-1}cm^{-1}$) and several other metalloproteins which contain this type of copper alone. Extinction coefficients are expressed per mole of "blue" copper. The Type 1 copper site is also found in the multicopper oxidases Rhus (ϵ_{614} = 5700 $M^{-1}cm^{-1}$)[15] and Polyporus versicolor (ϵ_{610} = 4600 $M^{-1}cm^{-1}$)[18] laccase, and ceruloplasmin (ϵ_{610} = 5600 $M^{-1}cm^{-1}$).[19] The most widely accepted theories[12] concerning the structure of the "blue" copper site and the origin of the intense absorption band suggest strong distortion of the copper coordination environment from axial symmetry to a more nearly tetrahedral configuration. Charge transfer and d-d transition assignments have both been proposed for the "blue" copper band.[20]

Another characteristic of the "blue" copper site is an ESR spectrum with an unusually small hyperfine constant (A_{11}). Typical low molecular weight Cu(II) complexes exhibit $|A_{11}|$ values in the range 0.015 to 0.020 cm^{-1}[12]; Rhus laccase Type 1 copper shows

$|A_{11}| = 0.0043\ cm^{-1}$.[15] Model studies[21,22] support the suggestion that small hyperfine constants are associated with a near-tetrahedral coordination environment about the copper atom. The low symmetry of the "blue" copper site is also indicated by the finding of three different g-values for laccase and stellacyanin Type 1 copper.[15]

Progress towards the determination of the ligand environment of the "blue" copper has been made very recently. An ENDOR study of stellacyanin indicated nitrogen coordination to copper at a solvent-inaccessible site. Fluorescence quenching and p-mercuribenzoate titration studies have suggested the presence of cysteine and tryptophan in stellacyanin[23] and azurin[24,25,26] coordination environments. Unfortunately, no data elucidating the coordination environments of Type 2 and Type 3 copper atoms appear to be available.

A second form of copper found in the "blue" oxidases, Type 2, has spectral properties resembling those of typical axial Cu(II) complexes. The presence of strong bands in the visible spectrum has prevented the assignment of comparatively weak Type 2 d-d transitions in laccase. The existence of Type 2 copper in this metalloprotein has been inferred from the appearance of two overlapping components in the ESR spectrum; one (Type 1) has narrow and the other (Type 2) has broad hyperfine structure. Computer simulation of composite spectra assuming two forms of copper are present in equal amounts was successful for both Rhus[15] and Polyporus[27] laccase.

The importance of the Type 2 copper site has been shown in experiments relating anion binding to this copper atom to losses in

oxidase activity. In the presence of 0.1 M NaF or NaN_3, changes are observed in the Type 2 ESR spectrum of laccase, but not in the "blue" copper component.[15] Fluoride, azide, and other anions have also been shown to cause dramatic inhibitions in Rhus laccase oxidase activity.[4] Fluorine superhyperfine splitting is found in the Type 2 spectrum when F^- is added to fungal laccase,[28] and again inhibition of activity accompanies anion binding. Ceruloplasmin Type 2 copper also functions as a fluoride and azide binding site.[29]

That the Type 2 copper atom is essential to enzymatic activity is perhaps most clearly shown in a study[30] with Polyporus laccase in which the Type 2 copper was specifically removed with bathocuproine disulfonate. The visible spectrum of the protein is essentially unchanged with Type 2 removal, but the broad ESR hyperfine component and oxidase activity are destroyed. Restoration of one copper atom per mole to the copper-depleted protein brings back the Type 2 ESR signal and oxidase activity.

Type 3 copper, called "ESR-nondetectable", refers, logically enough, to protein-bound Cu giving rise to no ESR signal. As Malkin and Malmström point out,[12] ESR-nondetectable copper need not be diamagnetic since extensive line broadening may obscure ESR signals from paramagnetic species. Fungal[27] and Rhus[15] laccases both contain 50% of their total copper, or 2Cu/mole, in an ESR-nondetectable form. Anaerobic redox titrations of laccase[31,33] have shown that the Type 3 site accepts two electrons and is associated with a near-ultraviolet absorption band near 330 nm ($\epsilon_{333} = 4500\ M^{-1}cm^{-1}$ for

Rhus laccase[15]). Approximately four electron equivalents are required to fully reduce laccase; Rhus laccase 614 nm and 330 nm absorption bands disappear and the ESR spectrum vanishes upon full reduction.

Fee, et al.[31] have discussed some of the structural alternatives for Type 3 copper. Two cuprous atoms in conjunction with a reducible disulfide bond was ruled out because Type 3 copper appears to be too strong an oxidant to be associated with the disulfide-sulfide couple. Still, recently completed photoelectron spectra of Rhus laccase indicate the protein may indeed contain both cupric and cuprous copper.[33] Another structural alternative, favored by the Swedish workers, is two antiferromagnetically coupled cupric atoms. The existence of many low molecular weight copper(II) compounds[34] showing spin-pairing is pointed out.[31]

The origin of the 330 nm Type 3 absorption band is by no means certain. One suggestion is that its position and intensity reflect ligand to Cu(II) charge transfer.[18] However, crystal spectra of copper acetate dihydrate[35] and other dimeric cupric carboxylates[36] reveal bands in this same region characteristic of the dimeric Cu(II)-Cu(II) unit.

The electrochemical properties of laccase are worthy of special note. The standard reduction potential of the Cu(II)/Cu(I) couple in aqueous solution is given by Latimer[37] to be +153 mv. The wide variation in reduction potentials among Type 1 copper sites in the "blue" copper proteins in spite of strong spectral similarities is an unexplained and puzzling phenomenon.

Reinhammar[38] has recently titrated laccase and stellacyanin with ascorbate in an anaerobic optical cell equipped with a combined metal electrode for simultaneous optical and potentiometric measurements. Potassium hexacyanoferrate was used as an electron mediator. The reduction potentials determined for Rhus laccase Type 1 and Type 3 copper sites are +394 mv and +434 mv, respectively, at pH 7.5, μ = 0.2, 0.1 mediator to protein ratio. Unfortunately, the observed reduction potentials depend somewhat on the concentration of hexacyanoferrate. With a mediator to protein ratio of 3 instead of 0.1, the potentials of Type 1 and Type 3 sites are +434 mv and +483 mv, respectively. ESR evidence was obtained for an interaction, possibly Type 2 reduction, between $Fe(CN)_6^{4-}$ and the Type 2 copper site. Reinhammar's results are in good agreement with those found earlier[32] by computer simulation of reductive titration curves. The Type 2 reduction potential was estimated at ~ 390 mv in this earlier study.

The fungal laccase "blue" copper (E_0' = +785 mv, pH 5.5, μ = 0.2, mediator ratio = 0.3)[38] is appreciably more oxidizing than its Rhus analog, whereas stellacyanin Type 1 copper is reduced at a potential (E_0' = +184 mv, pH 7.1, μ = 0.3)[38] similar to that of aqueous Cu(II). The unusual E_0' values for laccase "blue" copper are attributed[38] to interactive communication of this site with other copper atoms in the molecule. Also, coordination of unsaturated groups, the presence of reducing ligands such as sulfhydryl, and strong distortions from tetragonal symmetry all may stabilize cuprous copper relative to cupric, thus enhancing the value of the Cu(II)/Cu(I) couple.[14]

A Cu(II) - Cu(I) "valence-shuttle" has been suggested many times as the key to activity in the "blue" oxidases. Thus, ceruloplasmin is pictured[39,40] as being reduced by one-electron steps to the fully reduced form and then reoxidized by O_2 in a concerted process involving all four valence-changing copper atoms. Malkin and Malström[12] point out that the energetic unfavorability of the one-electron reduction of oxygen to superoxide ion argues against the existence of this kind of step in the reoxidation mechanism. Indeed, the reoxidation of fully reduced laccase is so fast[41,42] that cooperativity among the copper atoms almost certainly must lead to multi-electron reduction steps. This hypothesis is supported by the observation[41] that partially reduced laccase is not attacked by oxygen. "Blue" copper proteins such as stellacyanin which lack multi-electron transfer capability also are very slowly reoxidized by O_2.[42]

Only a very few studies dealing with the reduction of laccase copper sites are available. Ferrocyanide reacts rapidly with fungal laccase A Type 1 copper ($k = 1.1 \times 10^6\ M^{-1}sec^{-1}$, 25°, pH 5.4, 0.1 M acetate buffer),[41] and two more electron equivalents subsequently transfer to laccase at a somewhat slower rate. A second order rate constant of $7.7 \times 10^6\ M^{-1}sec^{-1}$ was obtained[41] for hydroquinone reduction of Type 1 copper under the same conditions. Fluoride ion does not inhibit initial reduction of Type 1 copper by $Fe(CN)_6^{4-}$, but does slow subsequent steps.

Large second order rate constants have been reported recently for fungal laccase B Type 1 copper reduction by H_2Q and $Fe(CN)_6^{4-}$.[43] The reduction rate of the 330 nm chromophore is independent of substrate concentration at high concentrations and is equal to 1.0 sec^{-1} (pH 5.5, 25°) for both reducing agents. Consequently, at least one of the electrons necessary for reduction of Type 3 copper is thought to be transferred intramolecularly from Type 1 Cu(I).[43] As for fungal laccase A, F^- does not inhibit the rate of "blue" copper reduction, but does slow the rate of electron transfer to the Type 3 site to 8×10^{-3} sec^{-1}.

Another recent paper[44] describes stopped flow kinetic studies of the reactions of reduced azurin and horse heart cytochrome c with fungal laccase. Again, large second order rate constants were measured for decolorization of Type 1 copper at room temperature ($k = 1.7 \times 10^4$ $M^{-1}sec^{-1}$ (cyt c(II)), 8.2×10^4 $M^{-1}sec^{-1}$ (azurin); 0.1 M acetate, pH 5.3).

The Cu(II)-Cu(II) pair may function not only as a two-electron acceptor, but also as the oxygen binding site.[12] Reduction of the Type 3 site presumably might produce a copper configuration similar to that proposed[45] for the oxygen carrier oxyhemocyanin. Reoxidation of fully reduced laccase then might involve intramolecular electron transfer steps from Type 1 and Type 2 copper to Type 3.

Malkin and Malmström[12] appear to minimize the importance of the Type 2 copper as an electron acceptor, and suggest it may be present to help stabilize oxygen intermediates as O_2 is reduced to

H_2O. Thus F^-, which blocks the Type 2 site, may prevent the stabilization of an anionic oxygen intermediate, but should not affect the initial reduction of Type 1 copper by substrate. Shifts in the Type 2 ESR spectrum and the appearance of a new spectral band at 400 nm when H_2O_2 is added to fungal laccase suggest that binding of HOO^- to Type 2 copper is indeed possible.[46]

EXPERIMENTAL OBJECTIVES

Spectroscopic characterization of laccase has been the subject of many papers. Comparatively little attention, however, has been paid to the mechanisms by which the copper sites cooperate to accomplish enzymatic oxidase activity. Existent kinetic studies often treat laccase activity as a whole, reporting apparent Michaelis-Menten parameters for substrate turnover. These values provide little information about the function of specific copper atoms in reduction or reoxidation of the enzyme, and, more importantly, assume a simple mechanism which may not apply to a two-substrate multi-site enzyme such as laccase. Specific reduction rate constants, when reported, are measured only for a limited variety of conditions and thus are of little use in discussing mechanisms.

The principal objective of studies described in this thesis is to provide an experimental background extensive enough to permit meaningful discussion of the anaerobic reduction mechanisms preferred by Rhus laccase. To achieve an integrated understanding of the mechanistic relationships among individual copper sites, experiments were designed to follow reduction rates of all three laccase functional units. Most of the data pertains to the optically observable Type 1 and Type 3 copper atoms, but anion inhibition experiments were included to clarify the function of the Type 2 site as well.

Hydroquinone, ferrocyanide ion ($Fe(CN)_6^{4-}$), and the ethylenediaminetetraacetate complex of iron(II) ($Fe(EDTA)^{2-}$) are the three

reducing agents considered in this work. A preliminary laccase kinetic study using chromous ion as reductant has been published,[47] and is not presented in this thesis. Hydroquinone was chosen because of its obvious similarity to physiological laccase substrates. The iron(II) complexes are well-characterized inorganic reductants with properties quite different from those of hydroquinone. Their laccase reduction mechanisms may be quite different from those preferred by physiological substrates, and it is hoped that comparison between H_2Q and Fe(II) reduction data will serve to emphasize the mechanistic aspects which enable laccase to interact particularly well with aryl two-electron donors.

The objectives sought in this thesis are based on a fundamental assumption which should be stated. Anaerobic reduction experiments were performed in the hope that oxygen is not typically involved in promoting reduction of the enzyme. In other words, it is assumed that the overall mechanism of laccase action may be treated as the sum of separable anaerobic substrate reduction and oxygen reoxidation steps. One experiment with ferrocyanide was performed in the hope of supporting this assumption, but more detailed reoxidation experiments will have to be performed before it can ever be fully justified.

Finally, it should be noted that the mechanisms stated in this thesis stress <u>functional</u> relationships among laccase copper sites. Chemical arguments have been of necessity limited in view of the absence of data pertaining to copper coordination environments, distances between metal atoms, and protein conformation. The

nomenclature used is as follows. The symbol (2, 2, Ox) refers to fully oxidized laccase. The first and second entries give the oxidation states of the Type 1 and Type 2 copper atoms, respectively. The final notation, either "Ox" or "Red", indicates whether the two-electron acceptor is oxidized or reduced.

References

1. H. Yoshida, J. Chem. Soc., 43, 472 (1883).
2. W. G. Levine, in The Biochemistry of Copper, J. Peisach, P. Aisen, and W. E. Blumberg, eds., Academic Press, New York, 1966, p. 371.
3. W. Franke, Handbuch Pflanzen-physiologie, 12, 401 (1960).
4. J. Peisach and W. G. Levine, J. Biol. Chem., 240, 2284 (1965).
5. T. Nakamura, J. Biochem. (Tokyo), 30, 44 (1958).
6. T. Nakamura, Biochim. Biophys. Acta, 30, 538 (1958).
7. T. Nakamura, in M. S. Blois, Jr., H. W. Brown, R. M. Lemmon, R. O. Lindblom, and M. Weissbluth, eds., Free Radicals in Biological Systems, Academic Press, New York, 1961, p. 169.
8. C. Broman, B. G. Malmström, R. Aasa, and T. Vänngård, Biochim. Biophys. Acta, 75, 365 (1963).
9. B. Reinhammar, Biochim. Biophys. Acta, 205, 35 (1970).
10. H. S. Mason, Ann. Rev. Biochem., 34, 595 (1965).
11. B. L. Vallee and W. E. C. Wacker, The Proteins, H. Neurath, ed., Vol. 5, Academic Press, 1970.
12. R. Malkin and B. G. Malmström, Advan. Enzymol., 33, 177 (1970).
13. C. K. Jørgensen, in ref. 2, p. 1.
14. A. S. Brill, R. B. Martin, and R. J. P. Williams, in Electronic Aspects of Biochemistry, B. Pullman, ed., Academic Press, New York, 1964, p. 519.
15. B. G. Malmström, B. Reinhammar, and T. Vänngård, Biochim. Biophys. Acta, 205, 48 (1970).

16. A. S. Brill, G. F. Bryce, and H. Maria, Biochim. Biophys. Acta, 154, 342 (1968).

17. S. Katoh, F. Shiratori, and A. Takamiya, J. Biochem. (Tokyo), 51, 32 (1962).

18. R. Malkin, B. G. Malmström, and T. Vänngård, Eur. J. Biochem., 10, 324 (1969).

19. W. E. Blumberg, J. Eisinger, P. Aisen, A. G. Morell, and I. H. Scheinberg, J. Biol. Chem., 238, 1675 (1963).

20. R. J. P. Williams, Endeavour, 26, 96 (1967).

21. D. C. Gould and A. Ehrenberg, Eur. J. Biochem., 5, 951 (1968).

22. D. Forster and V. W. Weiss, J. Phys. Chem., 72, 2669 (1968).

23. L. Morpurgo, A. Finazzi-Agrò, G. Rotilio, and B. Mondovi, Biochim. Biophys. Acta, 271, 292 (1972).

24. A. Finazzi-Agrò, G. Rotilio, L. Avigliano, P. Guerrieri, V. Boffi, and B. Mondovi, Biochemistry, 9, 2009 (1970).

25. G. Rotilio, A. Finazzi-Agrò, L. Avigliano, A. Lai, F. Conti, C. Franconi, and B. Mondovi, FEBS Lett., 12, 114 (1970).

26. A. Finazzi-Agrò, C. Giovagnoli, L. Avigliano, G. Rotilio, and B. Mondovi, Eur. J. Biochem., 34, 20 (1973).

27. B. G. Malmström, B. Reinhammar, and T. Vänngård, Biochim. Biophys. Acta, 156, 67, (1968).

28. R. Malkin, B. G. Malmström, and T. Vänngård, FEBS Lett., 1, 50 (1968).

29. L. E. Andréasson and T. Vänngård, Biochim. Biophys. Acta, 200, 247 (1970).

30. R. Malkin, B. G. Malmström, and T. Vänngård, Eur. J. Biochem., 7, 253 (1969).

31. J. A. Fee, R. Malkin, B. G. Malmström, and T. Vänngård, J. Biol. Chem., 244, 4200 (1969).

32. B. R. M. Reinhammar and T. Vänngård, Eur. J. Biochem., 18, 463 (1971).

33. F. Grunthaner, unpublished observations.

34. M. Kato, H. B. Johassen, and J. C. Fanning, Chem. Rev., 64, 99 (1964).

35. S. Yamada, H. Nakamura, and R. Tsuchida, Bull. Chem. Soc. (Japan), 30, 953 (1957).

36. S. Yamada, H. Nakamura, and R. Tsuchida, Bull. Chem. Soc. (Japan), 31, 303 (1958).

37. W. M. Latimer, Oxidation Potentials, 2nd. ed., Prentice-Hall Inc., Englewood Cliffs, New Jersey, 1952, p. 185.

38. B. R. M. Reinhammar, Biochim. Biophys. Acta, 275, 245 (1972).

39. G. Curzon and J. N. Cummings, in ref. 2, p. 545.

40. E. Frieden and S. Osaki, in Heavy Metals and Cells 2nd. Roch. Conf. on Toxicity, Wiley, New York, 1970, p. 39.

41. B. G. Malmström, A. Finazzi-Agrò, and E. Antonini, Eur. J. Biochem., 9, 383 (1969).

42. T. Nakamura and Y. Ogura, J. Biochem. (Tokyo), 64, 267 (1968).

43. L.-E. Andréasson, B. G. Malmström, and T. Vänngård, Eur. J. Biochem., 34, 434 (1973).

44. B. G. Malmström, A. Finazzi-Agrò, C. Greenwood, E. Antonini, M. Brunori, and B. Mondovi, Arch. Biochem. Biophys., 145, 349 (1971).

45. K. E. van Holde, Biochemistry, 6, 93 (1967).

46. R. Bränden, B. G. Malmström, and T. Vänngård, Eur. J. Biochem., 18, 238 (1971).

47. J. W. Dawson, H. B. Gray, R. A. Holwerda, and E. W. Westhead, Proc. Nat. Acad. Sci. (U.S.), 69, 30 (1972).

EXPERIMENTAL SECTION

I. Materials

Reagent grade chemicals were used without further purification, as were Sephadex ion-exchange and gel filtration resins. Triply distilled water was used in preparing solutions for kinetic measurements. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities.

Alfa Inorganics crude $K_3Cr(CN)_6$ was suspended in water, filtered to remove a large amount of green insoluble material, recrystallized from water-ethanol, washed with ethanol, and dried under vacuum. For the recrystallized material: % Cr (calc) = 15.98; % Cr (found) = 15.34.

Practical p-benzoquinone was recrystallized from absolute ethanol, thoroughly dried under suction on a sintered glass filter, and doubly sublimed. High yields of bright yellow needles with the correct melting point were obtained.

Chelex 100 resin, obtained from Bio-Rad, was purified by successive washings with concentrated NH_4OH, HCl, NaOH, and was rinsed thoroughly with water. Before use it was equilibrated with pH 7 phosphate buffer.

Union Carbide dialysis membrane was boiled extensively in several changes of distilled water to remove sulfurous impurities, and was stored immersed in cold distilled water.

Rubber serum caps were soaked in concentrated base before use, removing material which might be reactive with proteins or reducing agents.

M.C. and B. "fine chemical" grade NaN_3 was used without further purification after its azide content was found to be better than 99% of the theoretical value. Aldrich 1,4-cyclohexanediol (mixture of cis and trans) and M. C. and B. resorcinol were used as received. Sigma Grade III α-D(+) glucose and Type II glucose oxidase (from Aspergillus Niger) were also used as supplied.

II. Analyses

The purity of commercial hydroquinone[1] and potassium ferrocyanide preparations was verified by ceric titration using ferroin as indicator. Ceric titrations also showed that ferrous ammonium sulfate could be regarded as a primary standard in preparing solutions for use in $Fe(EDTA)^{2-}$ experiments. Arnold's ceric procedure[2] was used for the analysis of sodium azide.

Laccase concentrations were evaluated from total copper analyses of protein solutions by the spectrophotometric biquinoline method,[3] assuming 4 Cu/mole. Varian Techtron 1000 ppm aqueous $CuSO_4$ was used in the preparation of standard solutions, and was diluted with dithiazone-extracted pH 6 0.01 M phosphate buffer. Traces of non-enzymatic Cu were removed from protein solutions by stirring them in the presence of Chelex 100 resin for approximately 30 min.

A chromium analysis of recrystallized $K_3Cr(CN)_6$ was performed by the spectrophotometric peroxide procedure.[4]

III. Protein Purification

Laccase and stellacyanin were extracted and purified essentially by the method of Reinhammar.[5] Lacquer acetone powder was obtained from Saito and Co., Ltd., Tokyo. Acetone powder (100-200 g) was suspended in 0.01 M pH 6 potassium phosphate buffer, homogenized for several minutes, and stirred overnight at 5° to liberate the proteins into solution. Insoluble materials were then removed by passing the suspension through Whatman No. 1 filter paper on a Büchner funnel. The green filtrate was applied to a CM-Sephadex C-50 cation exchange column (typically 25 × 6 cm) equilibrated with 0.01 M buffer. All column work was performed at 5° either in a deli case or in a cold room.

A large amount of brown-yellow pigments passed directly through the column while the copper proteins formed a tight blue band at the top. The column was washed successively with 0.01, 0.05, 0.10, 0.15, and 0.20 M pH 6 buffers. Laccase was eluted in several liters with 0.10 M buffer, although occasionally it started to elute with 0.05 M buffer. At this point, stellacyanin remained at the top of the column. Small amounts of greenish material (perhaps a second form of laccase[5]) were eluted with 0.15 M buffer, followed by extremely dilute stellacyanin. Finally, stellacyanin was completely eluted with 0.2 M buffer.

The dilute laccase and stellacyanin fractions were dialyzed against 0.01 M buffer and passed through short DEAE-Sephadex A-50 columns equilibrated with the same buffer to remove small amounts of brown anionic pigments. Laccase (isoelectric point 8.55[5]) and stellacyanin (isoelectric point 9.86[5]) passed directly through DEAE columns.

Finally, laccase was added to a short CM-Sephadex C-50 column equilibrated with 0.01 M buffer, and was eluted much more concentrated with 0.2 M buffer. At this point, laccase was generally judged to be chromatographically pure according to the A_{280}/A_{614} absorbance ratios of 15.2-15.6 observed. Reinhammar[5] reports $A_{280}/A_{614} = 15.2$ for chromatographically pure laccase.

Much of the laccase was further subjected to gel filtration as a test of its chromatographic purity and as a final purification step. Sephadex G-150 gel was equilibrated with 0.01 M buffer, and the protein was run through a ca. 85 × 3 cm column with upward flow. Much of the laccase so treated did indeed pass through the column in a single band; excellent separation was achieved with laccase samples contaminated with stellacyanin. Following the final purification step, laccase was shell frozen in polyethylene vials and stored in liquid nitrogen.

A kinetic criterion was also applied for the purity of each new batch of laccase extracted. A room temperature kinetic measurement was performed with either ferrocyanide or hydroquinone, and the observed rate constant was checked against those previously determined under the same conditions. Remarkably good agreement was

found among rate constants for samples from all six batches of laccase used.

Stellacyanin also was usually pure after the DEAE-Sephadex procedure, A_{280}/A_{604} ratios of 5.6-6.1 being observed (literature value 5.6).[5] The dilute material was further diluted 1:2 with distilled water, concentrated on a short CM-Sephadex C-50 column using 0.2 M Na_2HPO_4, and shell frozen and stored in liquid nitrogen.

IV. Kinetics Measurements

Kinetic measurements were performed on a Durrum Model D-110 stopped flow spectrophotometer. This instrument contains a Kel-F flow subsystem leading to a 2 cm path length observation chamber, a tungsten visible light source, and a grating monochromator. Kepco Model ABC 1500(M) and Power/Mate power supplies were used for the photomultiplier tube and tungsten lamp, respectively. Samples to be mixed are contained in glass drive syringes submerged in water circulated from a Forma Scientific temperature control unit (temp. control ± 0.2°C). Viton o-rings fitted to the plunger heads provide a reasonable gas-tight seal against oxygen leakage for anaerobic solutions contained in the drive syringes. It should be noted, however, that solutions maintained in the drive syringes for longer than about 90 minutes are likely to be significantly contaminated with O_2. The syringes were soaked in concentrated HCl before the start of a set of runs to remove trace metal contaminants left from previous use.

Output voltages proportional to absorbance are obtained from the stopped flow apparatus; these were displayed vs. time on a Tektronix 564 B oscilloscope, or, for slower reactions, on a Hewlett-Packard Model 7004 B X-Y recorder.

The reactant inlet ports on the Durrum instrument were modified to accept gas-tight Hamilton fittings for a teflon needle, through which anaerobic solutions were drawn directly into the drive syringes. Solutions resting in the syringes were allowed at least 20 minutes to come to temperature equilibrium with the bath water before a kinetic determination was made.

One of the dangers in the stopped flow method[6] is that mixed solutions resting in the observation chamber may diffuse back through the flow system to interact with unmixed reactants in the small "dead space" between the drive syringes and the mixing jet. Over short time intervals, this problem need not be considered, but for reactions requiring over five minutes to be complete, the observation chamber was isolated by closing valves permitting flow between the drive syringes and the mixing jet. Even observing this precaution, it was found that best results were obtained if the "dead space" was routinely filled with fresh solution from the syringes immediately prior to performing a run. Since the solution in the "dead space" is moved to the observation chamber with the subsequent shot, contamination by back-diffusion will lead to spurious results.

As a test of the reliability of the stopped flow apparatus, several runs were performed using the redox reaction between

chromous ion and the glycinatopentaammine cobalt(III) ion as a standard. Room temperature rate constants for $\mu = 1.0$ were found to be in good agreement with the literature values[7] measured on a Cary 14 spectrophotometer.

V. Preparation of Solutions

After removal from liquid nitrogen storage, copper protein solutions were dialyzed against two changes of distilled water, the last being triply-distilled. Phosphate buffer and other solid components were then added, and a final minor adjustment of pH, if necessary, was made with sodium hydroxide or phosphoric acid. Buffered laccase solutions are stable to decomposition for at least several weeks at 5°, but most measurements were performed on freshly prepared solutions.

Protein solutions were deoxygenated by evacuation through a needle inserted into a serum-capped bottle. Freeze-pump and N_2 bubbling techniques were avoided; repeated freezing and thawing causes denaturation, as does the foaming induced by bubbling a gas through the solution. Evacuated protein samples were packed in an ice bath and purged with a stream of nitrogen flowing over the top of the liquid.

Hydroquinone and potassium ferrocyanide solutions were made up in serum bottles either by weight, or by diluting fresh stock solutions using Hamilton gas-tight syringes and stainless steel needles. Before introduction of the reducing agents, buffer solutions were purged with nitrogen for at least 30 minutes. Reducing agent solutions were

always used on the same day as their preparation.

Ferrous (ethylenediaminetetraacetate) solutions were prepared in similar fashion. A stock solution was prepared in a bubbler with an inlet at the bottom to allow continuous purging of the air-sensitive material. A Brinkman glass combination electrode was placed in one neck of the flask and a rubber serum cap covered the other. The bubbler initially contained Na_2H_2EDTA (10% excess over stoichiometric amount), phosphate buffer, and enough standard NaOH to neutralize hydrogen ions liberated upon the reaction of H_2EDTA^{2-} with Fe^{2+}. Iron(II) was introduced from a deoxygenated ferrous ammonium sulfate solution, and the pH of the bubbler contents was compared with the expected value using a Brinkman pH 101 meter. In no case was further adjustment of the pH necessary.

VI. Other Equipment

Visible and ultraviolet spectra reported in this thesis were recorded on a Cary 17 spectrophotometer. Routine measurements on protein solutions and for analyses were performed on a Cary 14 instrument.

Corning Model 12 and Brinkman pH 101 meters were used to make pH measurements. The combination electrode used in conjunction with the Brinkman instrument was fitted with a ground glass joint so it could be seated tightly in the vessel used to prepare $Fe(EDTA)^{2-}$ solutions.

VII. Data Analysis

Pseudo first order conditions for the metalloprotein were used in all kinetic experiments, 50- to 10,000-fold excesses of reducing agent typically being present. Laccase concentrations usually were ca. 1×10^{-5} M, yielding about a 0.1 absorbance change at 614 nm in the 2 cm stopped flow observation chamber.

Analysis of laccase absorbance decay curves is complicated by the presence of small amounts of oxygen in solutions mixed in the stopped flow apparatus. Oxygen slowly leaks into the drive syringes in spite of precautions against this occurrence. An initial steady-state period is observed when laccase and substrates are mixed aerobically. This phenomenon has already been documented for the laccase-catalyzed oxidation of hydroquinone.[8] Since reoxidation of Rhus laccase is much more rapid than reduction,[9] the steady-state absorbance levels at 614 and 330 nm are close to that expected for fully oxidized enzyme.

Following the steady-state period, net reduction of the copper sites commences. As oxygen was completely consumed, plots of log $(A_t - A_\infty)$ vs. time invariably became linear, indicating reduction reactions first order in the total oxidized protein concentration. A_t and A_∞ represent the absorbance at time t and after completion of the reaction, respectively. A set of typical analytical plots is included in the next section of this thesis.

Observed first order rate constants (k_{obs}) were obtained routinely by performing least squares analyses on the linear regions of

log $(A_t - A_\infty)$ *vs.* time plots. Two duplicate runs at least were performed and analyzed for each solution considered; agreement between duplicate runs was generally good.

To determine the effect of steady-state substrate consumption and product formation on observed rate constants, rigorously anaerobic conditions were achieved in several experiments through the addition of trace amounts of glucose and glucose oxidase to both reductant and oxidant solutions. No steady-state period for H_2Q or $Fe(CN)_6^{4-}$ reduction of laccase was observed, as expected, and k_{obs} values were found to be in good agreement with those calculated from runs where no oxygen scavenger other than laccase itself was present. The large excesses of reducing agent employed thus are sufficient to make the effect of steady-state substrate concentration depletion on k_{obs} negligible.

The stopped flow system was modified in June 1973 to accumulate voltage *vs.* time data in an analog input buffer for transmission to the Caltech PDP-10 computer. Data readouts, analytical plots, and observed rate constants all were obtained through the program "IDC" developed by Tom Dailey of the computing center staff. Very few of the data contained in this thesis were analyzed through this program (unfortunately), so further discussion of it will not be included here.

An important point to consider in analyzing 614 and 330 nm kinetic results is whether or not the absorbance changes at the two

wavelengths are truly independent. Redox titrations have demonstrated that 330 nm and 614 nm absorptions correspond to distinct copper sites, and the separation between the two peaks is large enough that changes in protein absorbance at one wavelength should not affect absorbance at the other. Absorbance changes accompanying oxidation of the reducing agent may serve to indirectly couple 614 and 330 nm rate processes, however. Consider ferrocyanide as an example. The oxidized minus reduced difference spectrum for hexacyanoferrate ions[10] is essentially zero at 614 nm, but the quantity ($\epsilon_{Fe(CN)_6^{3-}} - \epsilon_{Fe(CN)_6^{4-}}$) is approximately 500 $M^{-1}cm^{-1}$ at 330 nm. Observations at 614 nm will pertain to "blue" copper reduction alone, but the overall 330 nm absorbance change will include an increase originating from ferricyanide produced in the reduction of Type 1 copper. The observed rate of absorbance decrease at 330 nm thus should be influenced significantly by simultaneous changes in 614 nm absorbance. The ferrocyanide kinetic results have been interpreted with this effect in mind.

Hydroquinone is an ideal reductant for use in this laccase study because the benzoquinone minus hydroquinone absorbance is zero at 614 nm and also is negligibly small at 330 nm ($\Delta\epsilon < 100\ M^{-1}\ cm^{-1}$)[11] compared with the protein absorbance change at this wavelength. Consequently, no absorbance correlation between 614 and 330 nm results need be considered in interpreting the hydroquinone data.

Only 614 nm measurements of the $Fe(EDTA)^{2-}$ reduction of laccase are reported in this thesis. The near-ultraviolet absorbance of $Fe^{III}(EDTA)^-$ is such that it poses a major interference to the evaluation of Type 3 reduction kinetics.[12]

References

1. N. H. Furman and J. H. Wallace, J. Amer. Chem. Soc., 52, 1443 (1930).
2. J. W. Arnold, Ind. Eng. Chem., Anal. Ed., 17, 215 (1945).
3. L. Broman, B. G. Malmström, R. Aasa, and T. Vänngård, J. Mol. Biol., 5, 301 (1962).
4. E. Deutsch and H. Taube, Inorg. Chem., 7, 1532 (1968).
5. B. Reinhammar, Biochim. Biophys. Acta, 205, 35 (1970).
6. Q. H. Gibson, in Methods in Enzymology, K. Kustin, ed., Vol. 16, Academic Press, New York, 1969, p. 187.
7. R. Holwerda, E. Deutsch, and H. Taube, Inorg. Chem., 11, 1965 (1972).
8. T. Nakamura and Y. Ogura, J. Biochem. (Tokyo), 59, 449 (1966).
9. T. Nakamura and Y. Ogura, J. Biochem. (Tokyo), 64, 267 (1968).
10. J. H. Baxendale, H. R. Hardy, and L. H. Sutcliffe, Trans Farad. Soc., 47, 963 (1951).
11. B. N. Figgis, Introduction to Ligand Fields, Interscience, New York, 1966, p. 246.
12. H. J. Schugar, A. T. Hubbard, F. C. Anson, and H. B. Gray, J. Amer. Chem. Soc., 91, 71 (1969).

HYDROQUINONE REDUCTION OF LACCASE: NATIVE PROTEIN

I. Hydroquinone Concentration Dependence

Typical 614 and 330 nm absorbance-time curves for the reaction between hydroquinone and laccase at pH 7.0, $\mu = 0.1$ are illustrated in Figure 1; corresponding plots of log $(A_t - A_\infty)$ *vs.* time are given in Figure 2. Logarithmic plots were usually found to be linear for at least the last 50% of the total absorbance change at both wavelengths. In no case was evidence detected for apparent autocatalysis due to the reaction of semiquinone with the protein.

The relationship between 614 and 330 nm observed rate constants and the hydroquinone concentration was evaluated at several temperatures using phosphate buffer (0.0463 M, pH 7.0) alone to maintain the ionic strength at 0.1. Observed rate constants are collected in Tables 1 and 2, and the room temperature results are pictured in Figure 3.

The variation of 614 and 330 nm k_{obs} values with $[H_2Q]$ is first order over the range $5 \times 10^{-4} \leq [H_2Q] \leq 1 \times 10^{-2}$ M and for all temperatures considered. The rate law for laccase reduction at pH 7.0 thus is:

$$\frac{-d[\text{Cu(330) or 614)}]_{tot}}{dt} = k_1[H_2Q][\text{Cu(330 or 614)}]_{tot}$$

where $[\text{Cu(330)}]_{tot}$ and $[\text{Cu(614)}]_{tot}$ refer, respectively, to total concentrations of oxidized protein species absorbing at 330 and 614 nm.

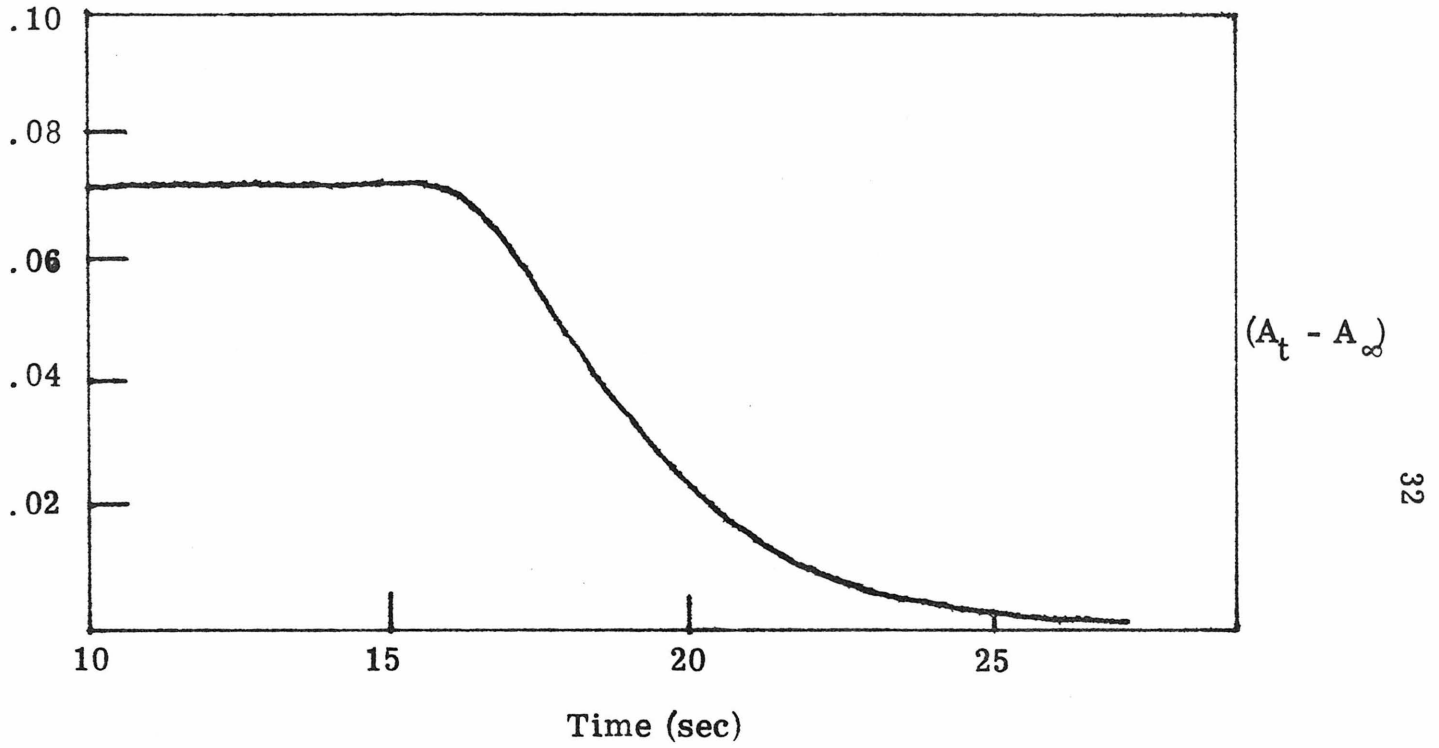

Figure 1a. Typical absorbance-time curve for the reaction of hydroquinone with laccase; 614 nm, 25.6°, pH 7.0, $\mu = 0.1$, $[H_2Q] = 1.25 \times 10^{-3}$ M.

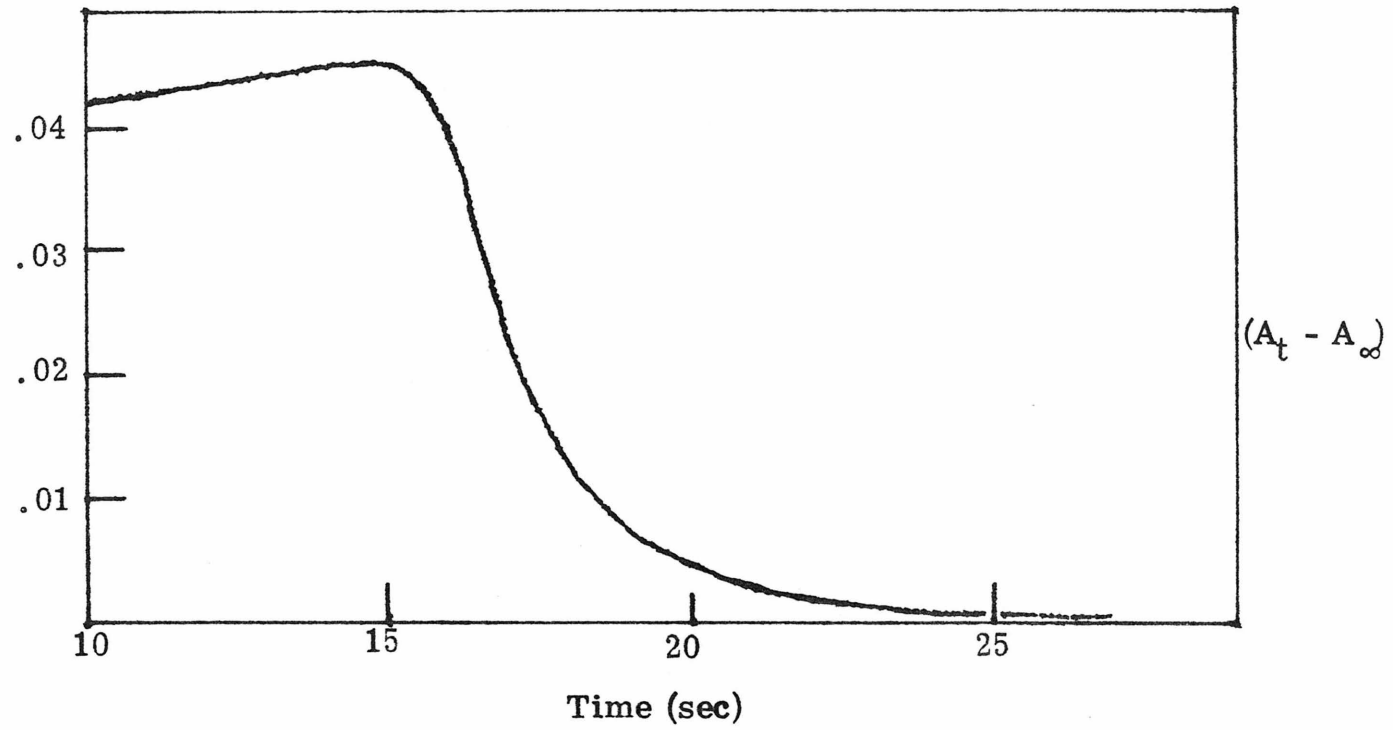

Figure 1b. Typical absorbance-time curve for the reaction of hydroquinone with laccase; 330 nm, 25.6°, pH 7.0, μ = 0.1, $[H_2Q] = 1.25 \times 10^{-3}$ M.

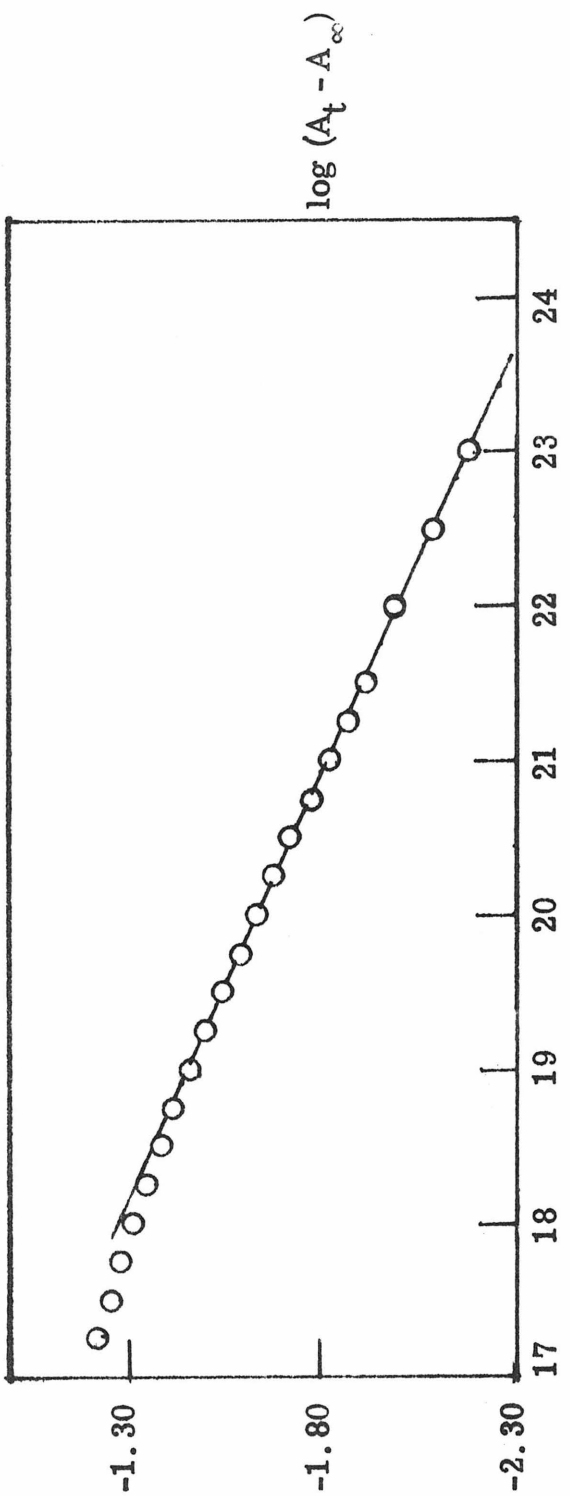

Figure 2a. First order plot based on the data in Figure 1a.

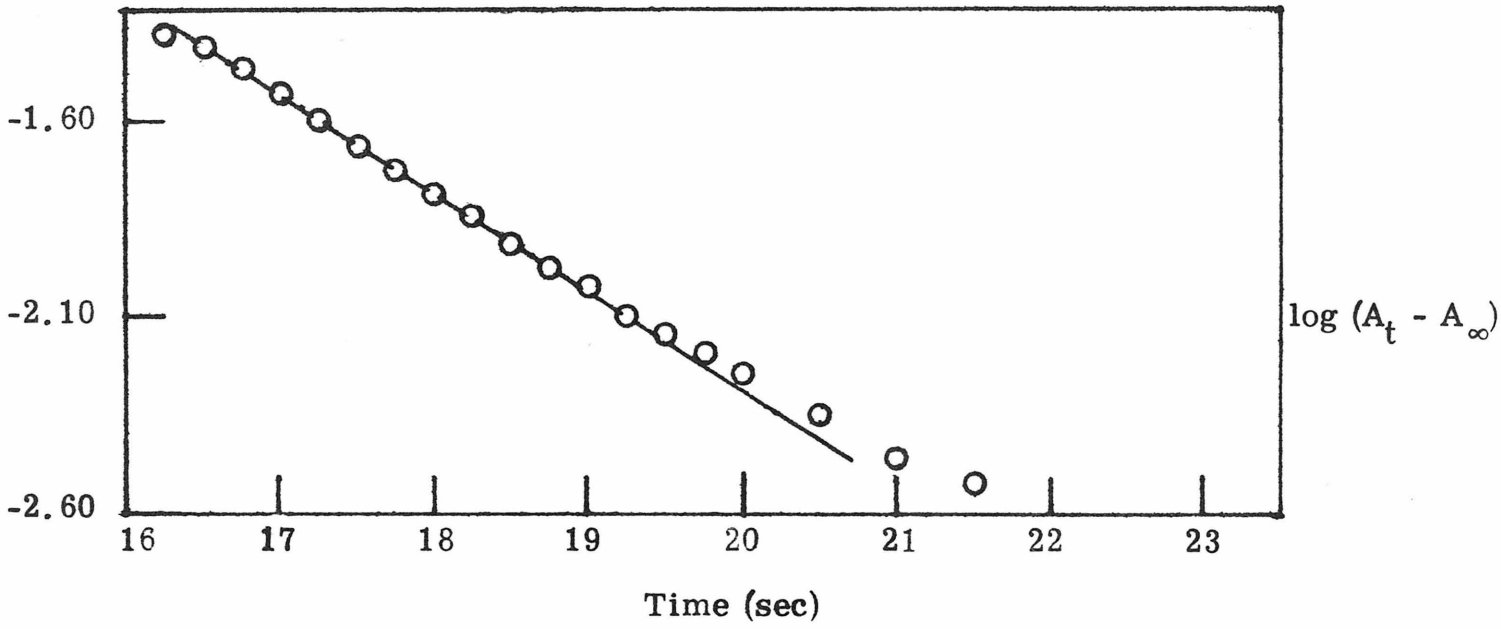

Figure 2b. First order plot based on the data in Figure 1b.

Table 1

Observed Rate Constants for the Reaction of Hydroquinone with Laccase; 614 nm, pH 7.0, $\mu = 0.1$

	$k_{obs}(sec^{-1})$			
$[H_2Q] \times 10^3(M)$	11.7°	17.1°	25.6°	35.7°
0.50			0.167	
			0.167	
0.75			0.247	
			0.246	
1.25	0.072	0.156	0.418	0.951
	0.074	0.156	0.430	0.962
2.50	0.142		0.818	1.702
	0.145		0.806	1.752
5.00	0.292	0.614	1.692	3.530
	0.295	0.637		
7.50	0.450			5.150
	0.423			
10.00	0.576	1.239	3.186	
	0.577	1.180	3.319	

Table 2

Observed Rate Constants for the Reaction of Hydroquinone with Laccase; 330 nm, pH 7.0, $\mu = 0.1$

	$k_{obs}(sec^{-1})$		
$[H_2Q] \times 10^3(M)$	11.7°	17.1°	25.6°
0.50			0.223
			0.225
0.75			0.343
			0.334
1.25	0.125		0.582
	0.125		0.599
2.50	0.233	0.476	1.135
	0.232	0.471	1.189
5.00	0.464		2.261
	0.457		2.301
7.50	0.690	1.377	
	0.700	1.378	
10.00	0.923		4.021
	0.919		4.142

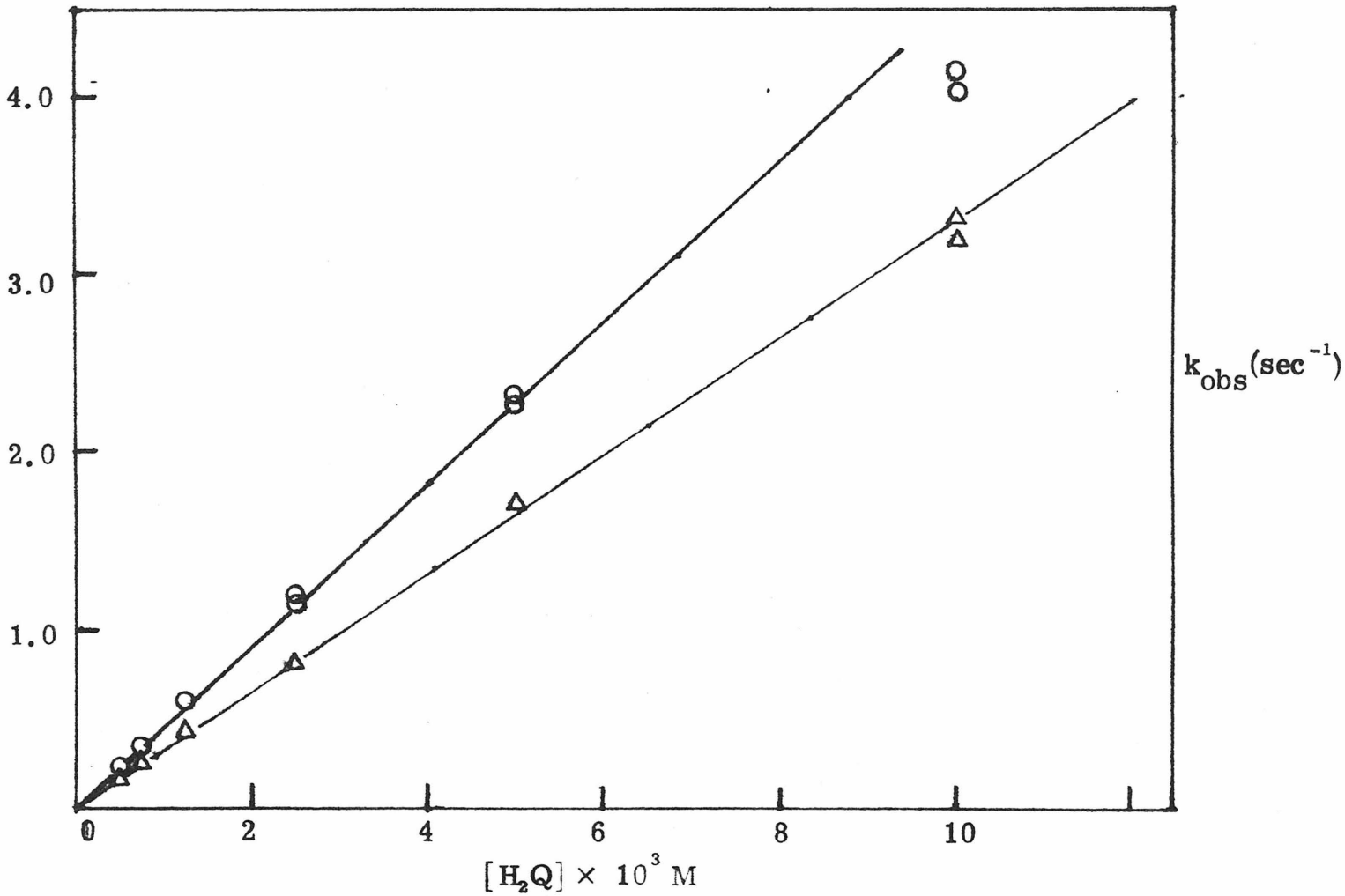

Figure 3. Plot of k_{obs} vs. $[H_2Q]$ for the reduction of laccase by hydroquinone; 25.6°, pH 7.0, $\mu = 0.1$; ○, 330 nm; Δ, 614 nm.

Values of the second order rate constant k_1 evaluated as the slopes of 25.6° k_{obs} *vs.* $[H_2Q]$ plots are:

$$k_1(614) = 325 \pm 16 \quad M^{-1} sec^{-1};$$

$$k_1(330) = 457 \pm 23 \quad M^{-1} sec^{-1}.$$

II. pH Dependence

A series of ionic strength 0.1 phosphate buffers in the range pH 5–8 was developed and employed in determining the hydrogen ion dependence of laccase reduction rates. Rate constants were measured at room temperature for two different hydroquinone concentrations at each pH. Separate protein solutions were made up in the appropriate buffer for each pH considered to be sure that hydrogen ion equilibration would not compete with the redox steps of interest. Results are illustrated in Figure 4 as a plot of log k_1 *vs.* pH and are tabulated in Table 3. The two k_1 values for each pH (calculated as $k_{obs}/[H_2Q]$ for each hydroquinone concentration) are in good agreement, indicating a first order $[H_2Q]$ dependence is followed over the entire pH range.

Type 3 reduction follows on inverse hydrogen ion dependence in the region pH 5-7. Measurements at higher pH were complicated by the appearance of slow downward instability in the baseline following fast decay of most of the 330 nm absorbance. To check the possibility that the downward drift might correspond to a slowly-reacting high pH form of laccase, a series of runs was performed at pH 7.8. Native laccase was reduced quickly enough to allow observation of most of the absorbance change for the slow component. Linear log $(A_t - A_\infty)$ *vs.*

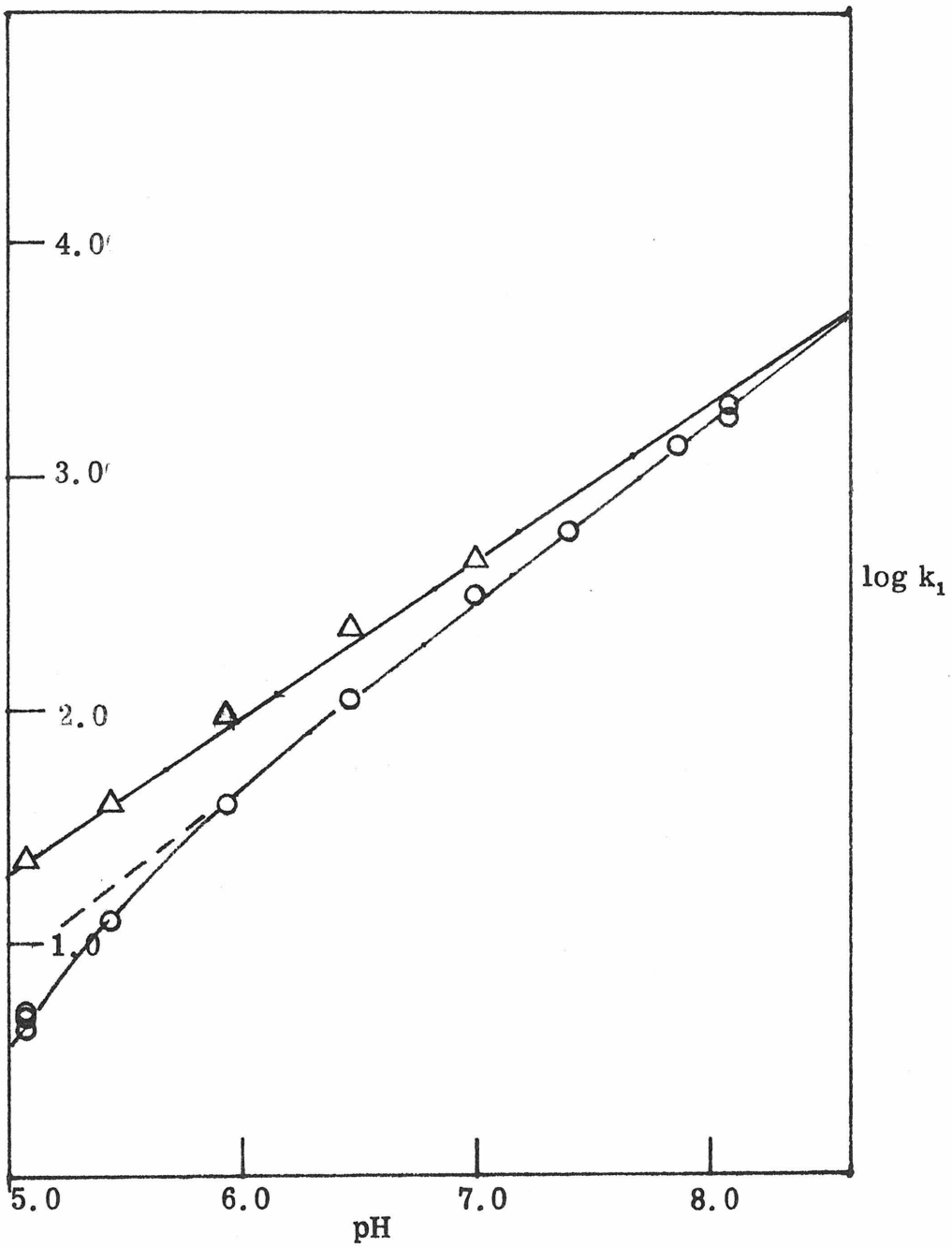

Figure 4. Plot of log k_1 vs. pH for the reaction of hydroquinone with laccase; 25.1°, μ = 0.1; ○; 614 nm; Δ, 330 nm.

Table 3

pH Dependence of Observed Rate Constants for the Reduction of Laccase by Hydroquinone; 25.1°, $\mu = 0.1$*

	$k_{obs}(sec^{-1})$			
	614 nm		330 nm	
pH	A	B	A	B
5.08	0.0056	0.0097	0.028	0.054
	0.0062	0.0100	0.029	0.052
5.44	0.015	0.029	0.050	0.093
	0.015	0.029	0.051	0.097
5.93	0.048	0.094	0.120	0.211
		0.091	0.120	0.212
6.46	0.140	0.261	0.284	0.510
	0.141	0.253	0.291	0.511
7.39	0.726	1.321		
	0.713	1.384		
7.86	1.594	3.225		
	1.675	3.243		
8.08	2.380	4.252		
	2.665	4.227		

*$[H_2Q] = 1.22 \times 10^{-3}$M (A), 2.38×10^{-3}M (B).

time plots were obtained for the slow process, suggesting it may correspond to reduction of an alternative form of laccase which interconverts slowly with the native enzyme. Results of several runs at varying $[H_2Q]$ (Table 4) indicate that the reduction rate of the high pH form of laccase is independent of the reducing agent concentration over the range $2.5 \times 10^{-3} \leq [H_2Q] \leq 1.0 \times 10^{-2}$ M ($k_{obs} = 0.161 \pm 0.008$ sec^{-1}; 25.1°, pH 7.8, $\mu = 0.1$). Approximately 30% of the overall 330 nm absorbance change is attributable to the high pH form at pH 7.8. The extent of this component increases with increasing pH, and its reduction rate appears at first inspection to be nearly pH-independent.

The rate of reduction of laccase "blue" copper by H_2Q also is essentially inverse first order in $[H^+]$. Between pH 6.5 and 8.1 a linear log k vs. pH relationship holds; below pH 6.5 hydrogen ion inhibition is even stronger. At high pH, no indication of a slower-reacting form of laccase was found in 614 nm measurements.

It is suggested that ionization of hydroquinone is responsible for the kinetic inverse hydrogen ion dependences of Type 1 and Type 3 reduction:

$$H_2Q \rightleftharpoons HQ^- + H^+ \quad K_{H_2Q}$$

The phenolate anion then is considered to be the actual reductant interacting with laccase. The rate law expected for this circumstance is:

$$\frac{-d[Cu(II)]}{dt} = k_1' [HQ^-][Cu(II)]$$

Table 4

Rate Data for the Reaction of Hydroquinone with the High pH Form of Laccase; 330 nm, 25.1°, pH 7.8, $\mu = 0.1$

$[H_2Q] \times 10^3 M$	$k_{obs}(sec^{-1})$
2.50	0.164
	0.153
5.00	0.141
	0.173
7.50	0.167
	0.162
10.00	0.172
	0.158

$$= \frac{k_1' K_{H_2Q} [H_2Q][Cu(II)]}{[H^+]}$$

$$= k_1'' [Cu(II)]/[H^+]$$

so $k_1 = k_1' K_{H_2Q}/(H^+)$. The value of K_{H_2Q} under conditions similar to those employed here (25°, $\mu \sim 0.04$) is 9.95×10^{-11}.[1] Plotting k_1 vs. $1/[H^+]$ to obtain $k_1' K_{H_2Q}$ and using Baxendale et al.'s K_{H_2Q} value yields the following estimates of k_1' (25.1°, $\mu = 0.1$):

$$k_1' \text{ (614)} = 1.48 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$$

$$k_1' \text{ (330)} = 4.04 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$$

III. Temperature Dependence

The data in Tables 1 and 2 were analyzed to yield k_1 values at each temperature as previously described for the 25.6° C data. These k_1 values were then used in constructing an Eyring plot (Figure 5) to obtain activation parameters for laccase reduction. The slopes of the lines obtained are proportional to the enthalpy of activation, $\Delta H^{\ddagger}$. Knowing $\Delta H^{\ddagger}$ and the hydrogen ion-independent quantity k_1'' from the pH dependence study, it is possible to evaluate the entropy of activation $\Delta S^{\ddagger}$ (k_1'') referred to the $(H^+) = 1.0$ M standard state. In addition, if it is assumed that $k_1'' = k_1' K_{H_2Q}$, activation parameters pertaining to the reaction of the hydroquinone anion with laccase may be obtained. Table 5 lists activation parameters based on both k_1'' and k_1'. The latter figures were calculated from the equations:

$$\Delta H^{\ddagger} (k_1'') = \Delta H^{\circ}_{H_2Q} + \Delta H^{\ddagger} (k_1')$$

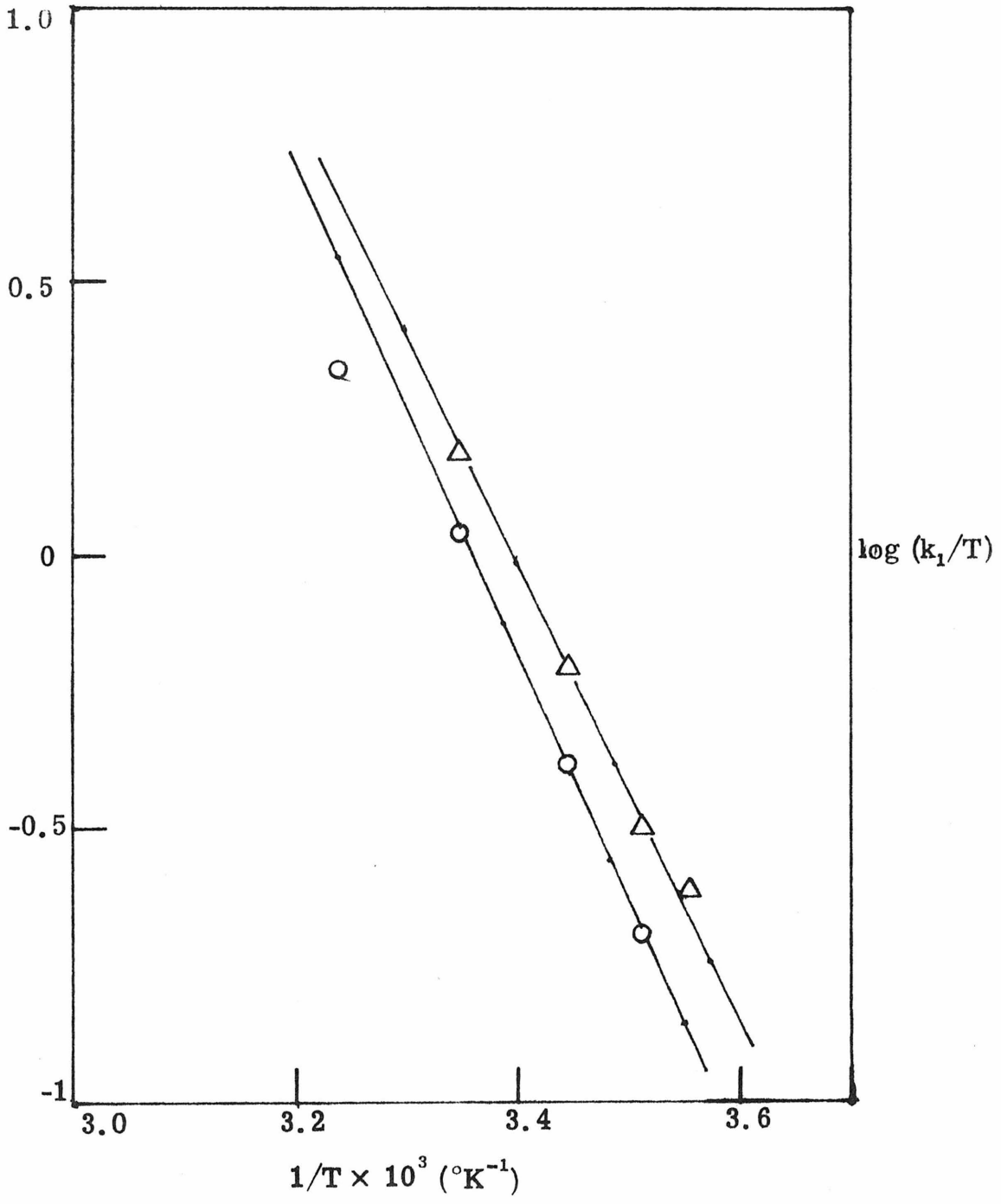

Figure 5. Eyring plot of rate data for the reaction of hydroquinone with laccase; pH 7.0, $\mu = 0.1$; ○, 614 nm; △, 330 nm.

Table 5

Activation Parameters for the

Hydroquinone Reduction of Laccase*

	$\Delta H^{\ddagger}(k_1'')$	$\Delta S^{\ddagger}(k_1'')$	$\Delta H^{\ddagger}(k_1')$	$\Delta S^{\ddagger}(k_1')$
330 nm	18.9 ± 0.5	-15.4 ± 2.0	13.6 ± 1.3	15 ± 4
614 nm	20.3 ± 0.5	-12.7 ± 1.8	15.0 ± 1.3	13 ± 4

*$\Delta H^{\ddagger}$ in kcal/mol; $\Delta S^{\ddagger}$ in cal/mol-deg. Values pertain to $\mu = 0.1$, laccase in its structural state at pH 7.0.

$$\Delta S^{\ddagger}(k_1'') = \Delta S^{\circ}_{H_2Q} + \Delta S^{\ddagger}(k_1')$$

where $\Delta H^{\circ}_{H_2Q}$ and $\Delta S^{\circ}_{H_2Q}$ are standard enthalpy and entropy changes, respectively, for the ionization of hydroquinone. Values for $\Delta H^{\circ}_{H_2Q}$ (5.5 kcal/mole) and $\Delta S^{\circ}_{H_2Q}$ (-28 cal/mol-deg) were taken from the study mentioned earlier.[1]

IV. Effect of Benzoquinone

A series of runs at $\mu = 0.1$, pH 7.0 was performed to determine the effect of added benzoquinone (Q) on laccase reduction rates with hydroquinone. Reoxidation of reduced copper sites by benzoquinone is not expected on the basis of the comparatively low (ca. 0.27 volt)[2] reduction potential for the Q/H_2Q couple at pH 7. Benzoquinone may, however, compete for enzymatic binding sites needed by hydroquinone, and the rapid equilibrium between H_2Q and Q producing semiquinone (SQ) must also be accounted for in interpreting kinetic results[3]:

$$H_2Q + Q \rightleftharpoons 2\,SQ \qquad K_{SQ}$$

Pseudo first order conditions for semiquinone as reductant are established by maintaining constant high concentrations of benzoquinone and hydroquinone simultaneously.

Observed rate constants obtained holding $[H_2Q]$ constant at 2.5×10^{-3} M and varying [Q] are included in Table 6. Benzoquinone causes modest increases in both 614 nm and 330 nm rate constants; inhibition at high [Q] was found at 330 nm but not at 614 nm.

Table 6

Effect of Benzoquinone on Observed Rate Constants for the Reaction of Hydroquinone with Laccase; 26.1°, pH 7.0, $\mu = 0.1$, $[H_2Q] = 2.5 \times 10^{-3}M$

$[Q] \times 10^3$(M)	$k_{obs}(sec^{-1})$ 614 nm	330 nm
0.00	0.871	1.483
	0.872	1.478
0.376	0.970	1.557
	0.952	1.527
	0.975	1.584
0.941	1.132	1.646
	1.087	1.623
	1.127	
	1.070	
1.88	1.199	1.630
	1.248	1.674
		1.562
		1.564
3.74	1.333	1.613
	1.325	1.653
7.48	1.540	1.448
	1.522	1.423
		1.479

The "blue" copper reduction results may be interpreted attributing benzoquinone-induced rate increases to the reaction of semiquinone with laccase. The rate law expected for this circumstance is:

$$\frac{-d[\,Cu(614)\,]}{dt}_{tot} = (k_1[\,H_2Q] + k_2[\,SQ])[\,Cu(614)\,]_{tot}$$

$$= (k_1[\,H_2Q] + k_2K_{SQ}^{\frac{1}{2}}[\,H_2Q]^{\frac{1}{2}}[\,Q]^{\frac{1}{2}})[\,Cu(614)\,]_{tot}$$

For constant $[\,H_2Q]$, a plot of k_{obs} *vs.* $[\,H_2Q]^{\frac{1}{2}}[\,Q]^{\frac{1}{2}}$ should be linear with slope $k_2K_{SQ}^{\frac{1}{2}}$ and intercept $k_1[\,H_2Q]$. A linear plot is indeed obtained with the experimental data (Figure 6) and the intercept ($0.874\ sec^{-1}$) agrees very closely with the $k_1[\,H_2Q]$ value predicted from earlier measurements ($0.872\ sec^{-1}$). From the slope of the line ($152\ M^{-1}sec^{-1}$; 26.1°, pH 7.0, $\mu = 0.1$) and the literature value[3] of K_{SQ} at pH 7.1, room temperature (*ca.* 1×10^{-6}) k_2 is estimated to be $1.5 \times 10^5\ M^{-1}sec^{-1}$.

The 330 nm data show an initial rise in rate as benzoquinone is added followed by significant inhibition at larger concentrations (Figure 6). The overall quinone dependence may reflect a combination of rate enhancement from semiquinone contributions to laccase reduction and rate inhibition from association of Q with the Type 3 site. A rate law may be derived for this situation, but no attempt was made to fit the data to it quantitatively in view of the small magnitude of the benzoquinone effect at 330 nm (10% maximum acceleration). Error limits in the data may easily amount to 25-50% of rate changes caused

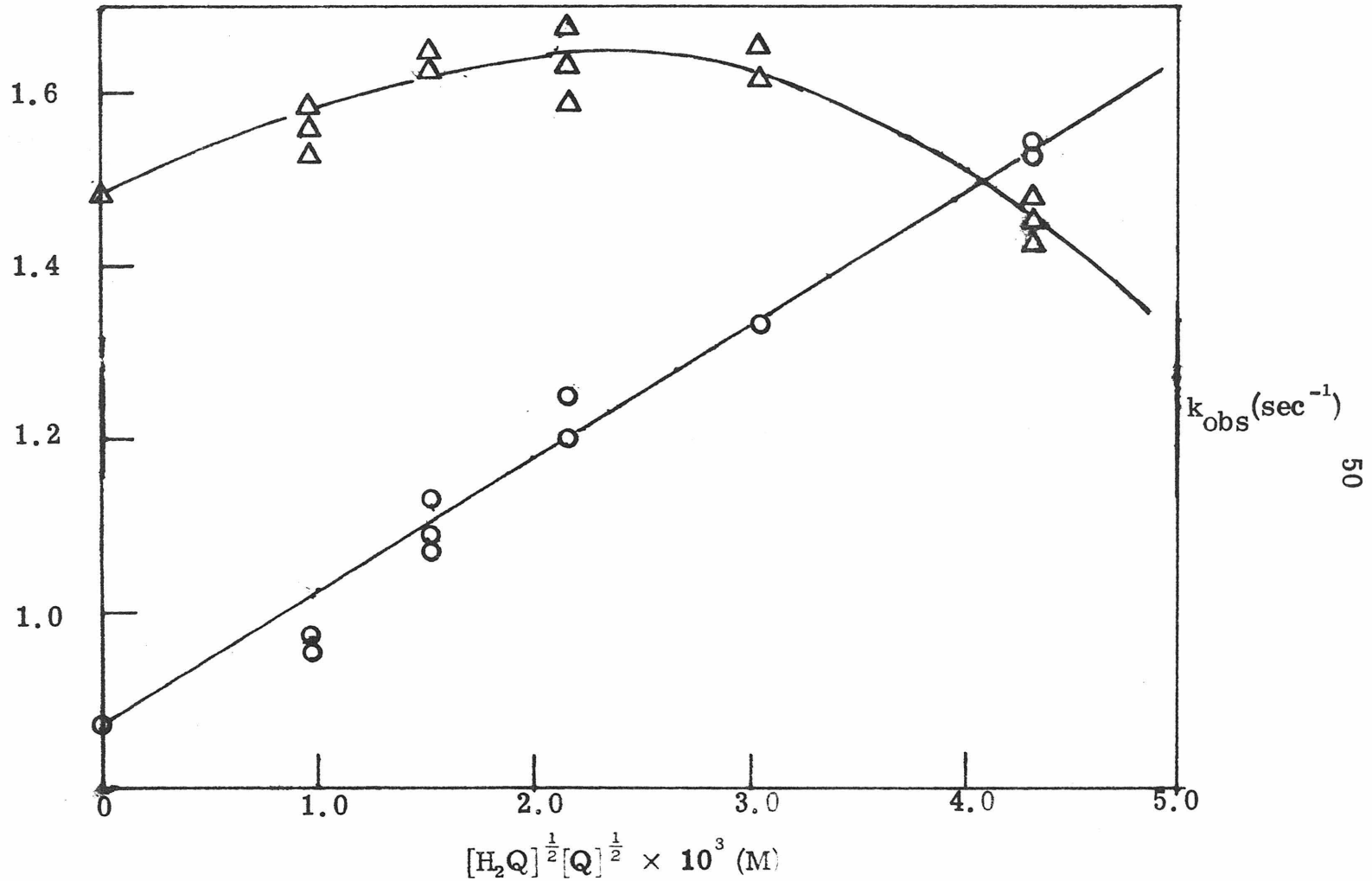

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Figure 6. Plot of k_{obs} vs. $[H_2Q]^{\frac{1}{2}}[Q]^{\frac{1}{2}}$ for the reaction of hydroquinone with laccase in the presence of added benzoquinone; 26.1°, pH 7.0, $\mu = 0.1$, $[H_2Q] = 2.50 \times 10^{-3}$ M; ○, 614 nm; △, 330 nm.

by benzoquinone. Again, the apparent intercept of the k_{obs} vs. $[H_2Q]^{\frac{1}{2}}[Q]^{\frac{1}{2}}$ plot agrees with the predicted $k_1[H_2Q]$ value.

The benzoquinone experiments show that small concentrations of Q generated in kinetic runs with large excesses of H_2Q have negligible influence on observed rates. This is indicated by the excellent agreement between $k_1[H_2Q]$ values evaluated from runs with no added benzoquinone and from extrapolating the kinetic benzoquinone dependence data to $[Q] = 0$. Furthermore, it is evident that semiquinone does not contribute significantly to the reduction of laccase "blue" and ESR-nondetectable copper sites in experiments with no added benzoquinone. This confirms Nakamura's conclusion[3] based on ESR studies of SQ formation and decay.

V. Effect of Potential Competitive Inhibitors and EDTA

Organic molecules structurally similar to hydroquinone potentially may inhibit laccase activity by competing for binding sites involved in substrate oxidation. This possibility was checked in several experiments at pH 7.0, 25° with added 1,4-cyclohexanediol and 1,3-dihydroxybenzene (resorcinol). However, with $[H_2Q] = 1.25 \times 10^{-3}$ M and concentrations of added organic molecules up to and over 100-fold in excess, no appreciable variations in rate were found at either 614 nm or 330 nm.

The possibility of trace metal catalysis of protein redox reactions is distinct, especially with a two-electron organic reductant such as hydroquinone. Inhibition with EDTA has pointed to Fe(III), Fe(II)

catalysis of ceruloplasmin redox reactions,[4] but no effect was observed in an analogous experiment with Rhus laccase.[5] This result was confirmed by finding 1×10^{-4} M Na_2H_2EDTA has no influence on 330 nm and 614 nm k_{obs} values with $[H_2Q] = 1.25 \times 10^{-3}$ M at pH 7.0, 25°. Consequently, no special precautions were taken against trace metal contamination of kinetics solutions.

VI. Ionic Strength Dependence

A series of pH 7.0 phosphate buffers in the range $0.024 \leq [\text{phosphate}]_{\text{total}} \leq 0.211$ M was prepared for use in a study of the ionic strength dependence of laccase reduction rates with H_2Q. In view of the sensitivity of laccase to anion binding and the fact that phosphate is by no means a non-complexing anion,[6] these experiments may also be seen as a phosphate concentration dependence study. Substantial stability constants have been reported[6] for the association of mono- and dihydrogen phosphate ions with aqueous copper(II) (log $K_{H_2PO_4^-} = 1.3 \pm 0.1$, $\log_{HPO_4^{2-}} = 3.3 \pm 0.1$; 37°, 0.15 M K_{NO_3}).

Rate data collected over the range $0.05 \leq \mu \leq 0.50$ are tabulated in Table 7 and illustrated in Figure 7. Laccase in ionic strength 0.05 buffer was mixed with hydroquinone solutions containing enough phosphate to give the final concentrations indicated in Table 7. As the ionic strength increases from 0.05 to 0.50, 330 nm and 614 nm observed rate constants for $[H_2Q] = 2.5 \times 10^{-3}$M both are enhanced by approximately 40%.

Table 7

Ionic Strength Dependence of Observed Rate Constants for the Reaction of Hydroquinone with Laccase; 25.5°, pH 7.0, $[H_2Q] = 2.5 \times 10^{-3}M$

$[\text{phosphate}]_{tot}$	μ	$k_{obs}(sec^{-1})$ 614 nm	$k_{obs}(sec^{-1})$ 330 nm
0.0241	0.050	0.796	1.317
		0.803	1.292
0.0836	0.192	0.989	1.606
		1.023	1.607
0.125	0.294	1.054	1.728
		1.054	1.746
0.167	0.399	1.093	1.724
		1.066	1.869
0.211	0.509	1.110	1.830
		1.145	1.801

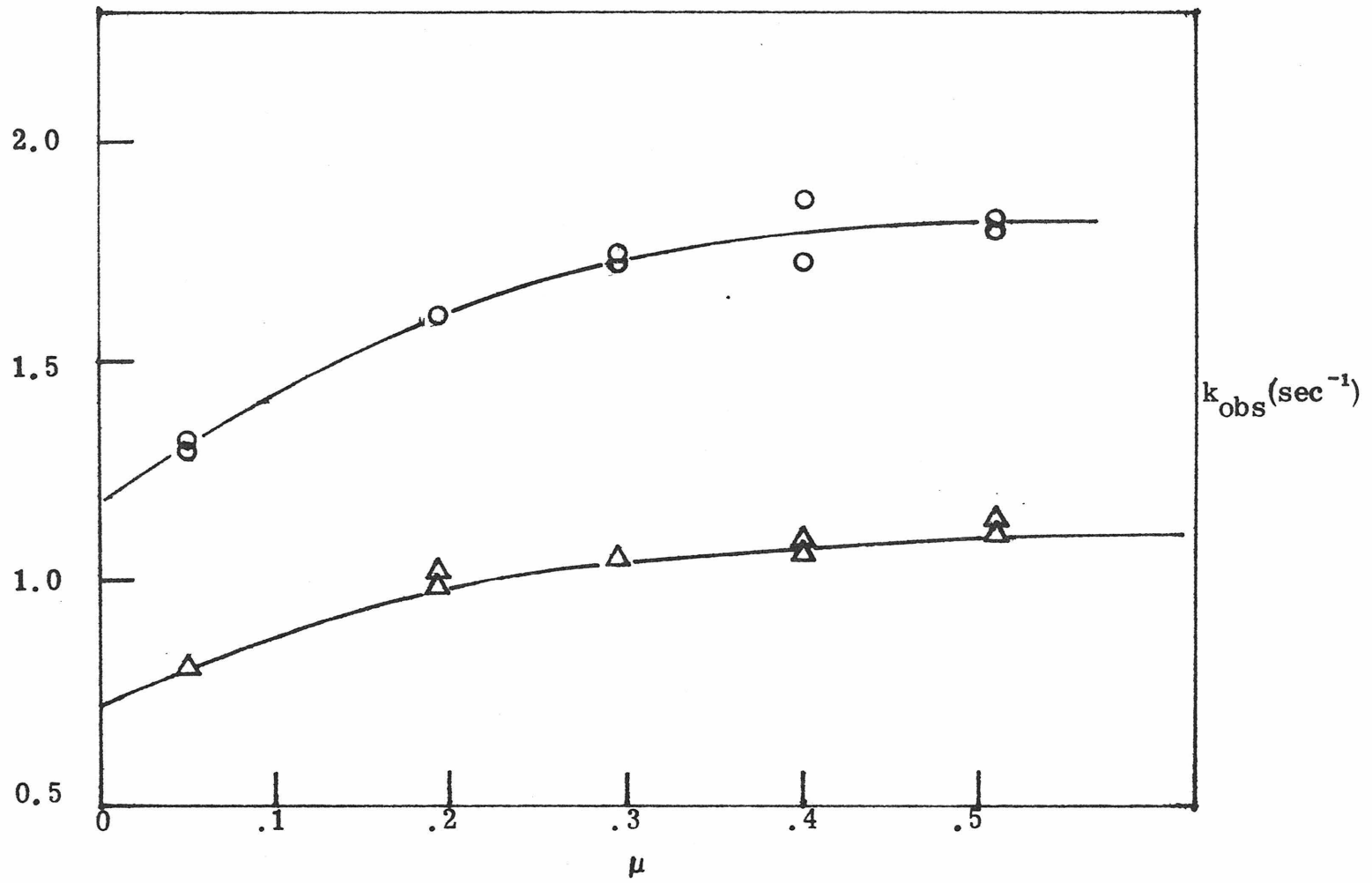


Figure 7. Ionic strength dependence of observed rate constants for the reduction of laccase by hydroquinone; 25.5°, pH 7.0, $[H_2Q] = 2.5 \times 10^{-3}$M; ○, 330 nm, △, 614 nm.

References

1. J. H. Baxendale, H. R. Hardy, and C. H. Sutcliffe, Trans. Farad. Soc., 47, 963 (1951).
2. W. M. Clark, Oxidation-Reduction Potentials of Organic Systems, The Williams and Wilkins Co., Baltimore, 1960.
3. T. Nakamura, in M. S. Blois, Jr., H. W. Brown, R. M. Lemmon, R. O. Lindblom, and M. Weissbluth, eds., Free Radicals in Biological Systems, Academic Press, New York, 1961, p. 169.
4. J. A. McDermott, T. C. Hunter, S. O. Saki, and E. Frieden, Biochim. Biophys. Acta, 151, 541 (1968).
5. J. Peisach and W. G. Levine, J. Biol. Chem., 240, 2284 (1965).
6. C. W. Childs, Inorg. Chem., 9, 2465 (1970).

HYDROQUINONE REDUCTION OF LACCASE: ANION-INHIBITED PROTEIN

I. Spectrum of the Laccase Type 2 Cu(II)-Azide Complex

Addition of NaN_3 to laccase causes a fast color change from blue to green, but no significant change in the protein visible-ultraviolet spectrum results when NaF is added. Difference spectra between azide-complexed and native laccase (Figure 8) reveal an intense new band with maximum at 405 nm. Little absorbance change in the vicinity of 330 nm occurs, but the "blue" absorption becomes appreciably more intense in the presence of azide ion. Formation of the laccase-azide complex is reversible, as the new absorption peak was abolished by dialysis against a large volume of distilled water. High concentrations of azide were also added to stellacyanin in one experiment, but no change in the visible spectrum was observed even for $[N_3^-] = 0.2$ M at pH 6 (0.01 M phosphate).

II. Rate of Formation of the Laccase-Azide Complex

Preliminary kinetic studies of the reaction of laccase with azide at pH 7.0 showed very complicated behavior. For $0.01 \leq [N_3^-] \leq 0.05$ M and [laccase] $\cong 1 \times 10^{-5}$ M, very small absorbance increases occur at 405 nm at an azide-independent rate of 0.26 ± 0.01 sec^{-1}. Both fast and slow rate processes are found at 405 nm for higher azide concentrations, and the total absorbance changes at this

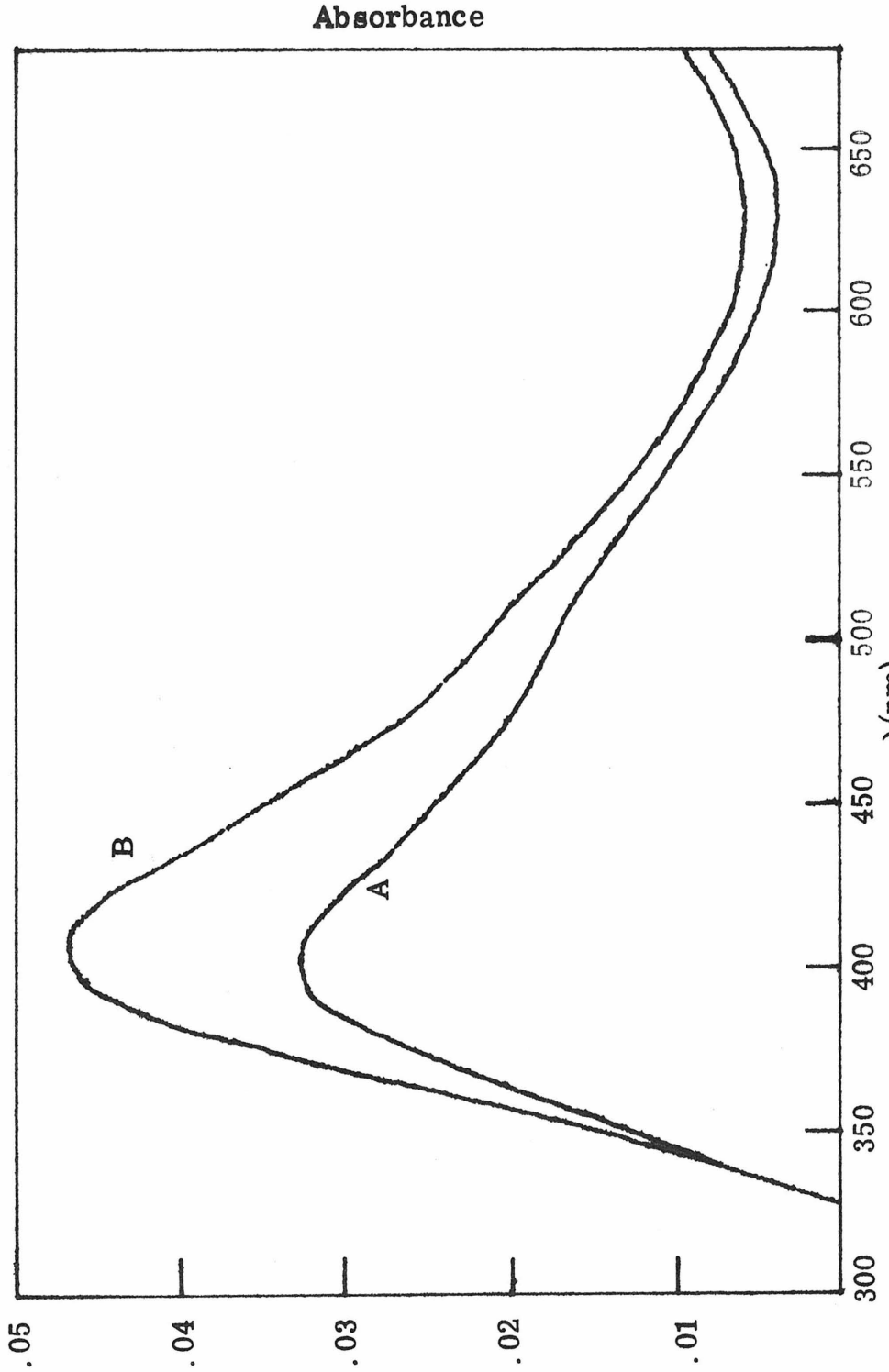


Figure 8. Azide complex minus native laccase difference spectrum; pH 6.1 (3.87×10^{-2} M phosphate), 25°, $[\text{laccase}]_{tot} = 1.83 \times 10^{-5}$ M, 1 cm path-length; A: $[N_3^-] = 0.002$ M, B: $[N_3^-] = 0.10$ M.

wavelength are not consistent with formation of a 1:1 laccase-azide complex.

The kinetics and stoichiometry of azide binding to laccase are much more straightforward at pH 6.1. A single first order relaxation process is observed at 405 nm for all azide concentrations up to at least 0.15 M. Observed rate constants were obtained from the least squares slopes of log $(A_e - A_t)$ vs. time plots where A_e and A_t represent absorbances at equilibrium and at time t, respectively. Total absorbance changes, $A_e - A_o$ (A at time 0), were evaluated from the intercepts of log $(A_e - A_t)$ vs. time plots. All solutions were made up in 0.0387 M phosphate buffer, pH 6.1, and the ionic strength was maintained at 0.2 using sodium nitrate. Azide ion contributes over 96% of the value of $[\text{azide}]_{tot}$ at pH 6.1, ($pK_a = 4.75$ for HN_3[1]), so reaction of laccase with hydrazoic acid need not be considered.

It may be easily shown that a plot of $(A_e - A_o)^{-1}$ vs. $[N_3^-]^{-1}$ is expected to be linear only if laccase and azide form a 1:1 complex exclusively over the entire range of azide concentrations employed. Figure 9 indicates that the pH 6.1 data are consistent with 1:1 stoichiometry over the concentration range of $0.0125 \leq [N_3^-] \leq 0.15$ M used in the kinetic measurements. The formation constant K_f of the complex ($K_f = 45 \pm 2\ M^{-1}$; 25.1°, pH 6.1, $\mu = 0.2$) is given by the ratio of intercept to slope in the $(A_e - A_o)^{-1}$ vs. $[N_3^-]^{-1}$ plot. It was possible to estimate $\Delta\epsilon_{405}$, the azide complex minus native protein molar extinction coefficient at 405 nm ($\Delta\epsilon_{405} = 1.6 \pm 0.1 \times 10^3\ M^{-1}cm^{-1}$), using the relationship: slope $= K_f\, \ell\, \Delta\epsilon_{405}\, [\text{laccase}]_{tot}$ where ℓ is the

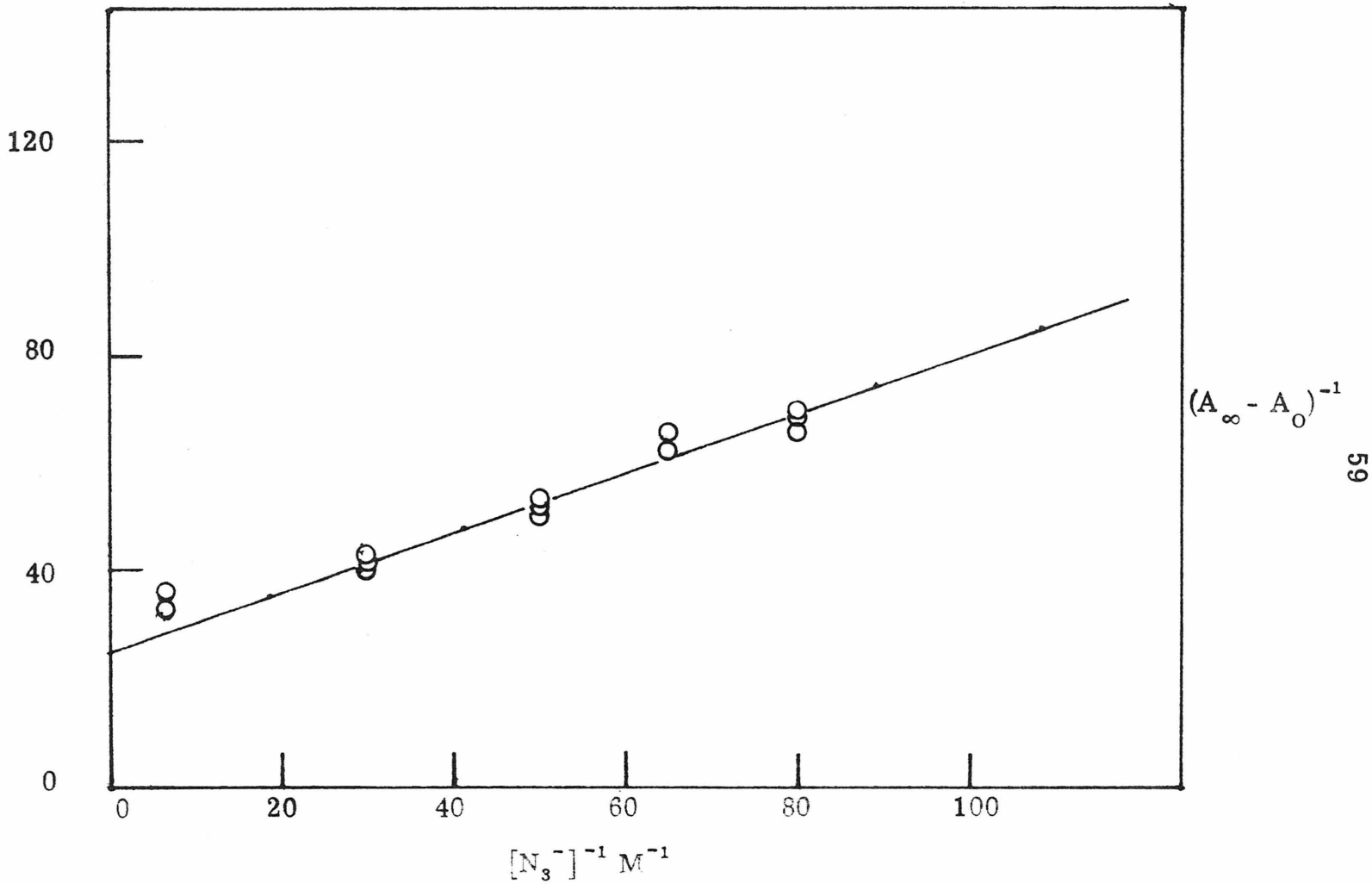


Figure 9. Plot of $(A_\infty - A_O)^{-1}$ vs. $[N_3^-]^{-1}$ for the anation of laccase by azide ion; 405 nm, 25.1°, pH 6.1, $\mu = 0.2$, $[\text{laccase}]_{tot} = 1.3 \times 10^{-5}$ M.

spectrophotometric path length.

A typical log $(A_e - A_t)$ *vs.* time plot for the azide anation of laccase is shown in Figure 10. Observed rate constants are presented in Table 8 and are displayed as a function of azide concentration in Figure 11. For a simple anation mechanism of the form:

$$M^{n+} + L^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ML^{(n-1)+}$$

the expression characterizing observed rate constants is simply[2]:

$$k_{obs} = k_1[L^-] + k_{-1}$$

Figure 11 shows that the anation of laccase by azide ion is not susceptible to this simple analysis. As $[N_3^-]$ increases, k_{obs} values tend towards a saturation value. Sutin and Yandell[3] observed similar behavior for anation reactions of cytochrome *c*, and have pointed out two mechanisms consistent with their data. The first possibility requires an S_N1 protein-dependent step, most likely dissociation of of a ligand, followed by attack of the incoming anion. For laccase this mechanism may be written:

$$\text{Laccase} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Laccase}^*$$

$$\text{Laccase}^* + N_3^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Laccase} - N_3^-$$

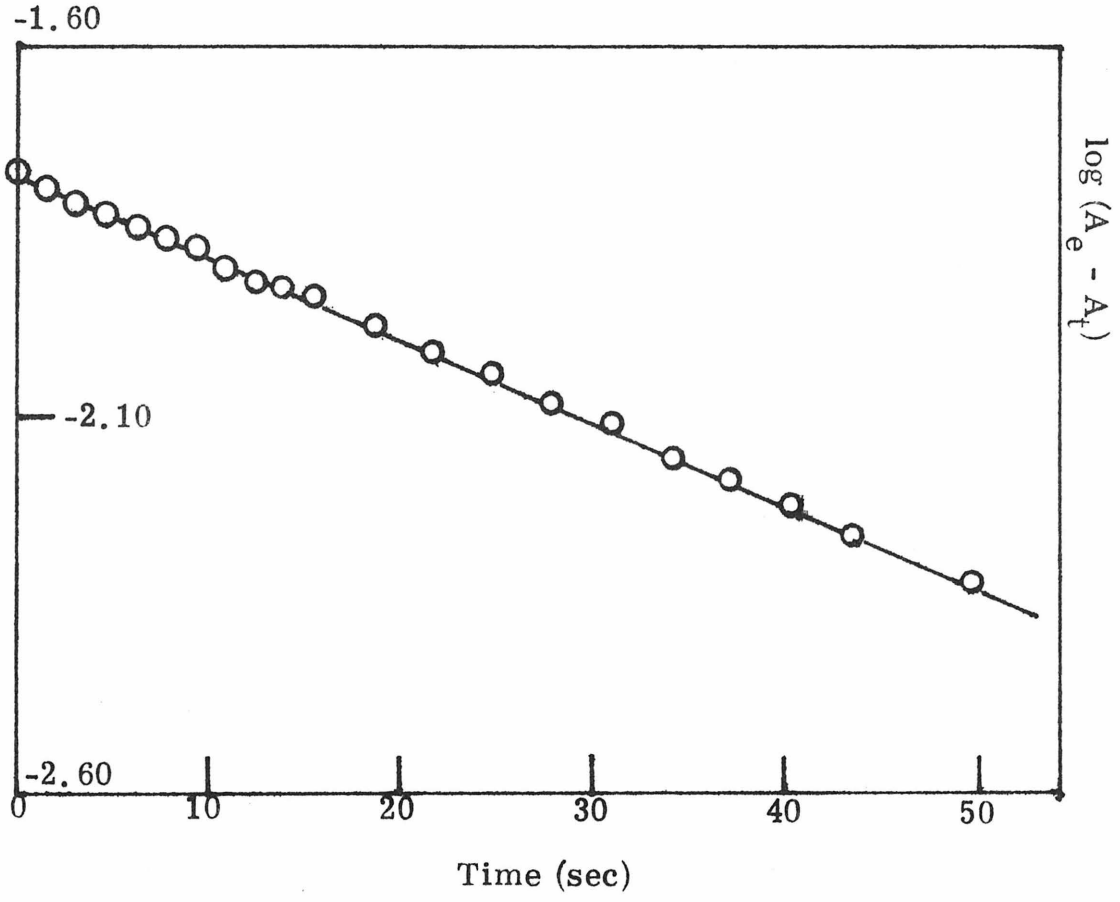


Figure 10. Typical first order plot for the anation of laccase by azide ion; 405 nm, 25.1°, pH 6.1, μ = 0.2, $[N_3^-]$ = 0.02 M.

Table 8

Rate Data for the Anation of Laccase by Azide Ion;

405 nm, 25.1°, pH 6.1, $\mu = 0.2$

$[N_3^-] \times 10^2$(M)	$k_{obs} \times 10^2$(sec^{-1})
1.25	2.06
	1.90
	2.03
	2.19
1.54	2.12
	2.22
2.00	2.55
	2.55
	2.55
	2.45
3.33	3.05
	3.02
	3.06
5.00	3.82
	3.84
	3.77
7.50	4.63
	4.56
	5.27
	4.83
10.00	5.97
	5.34
	5.73
	5.74
12.5	6.08
	6.01

Table 8 (Continued)

$[N_3^-] \times 10^2$(M)	$k_{obs} \times 10^2$(sec^{-1})
15.0	6.28
	6.15

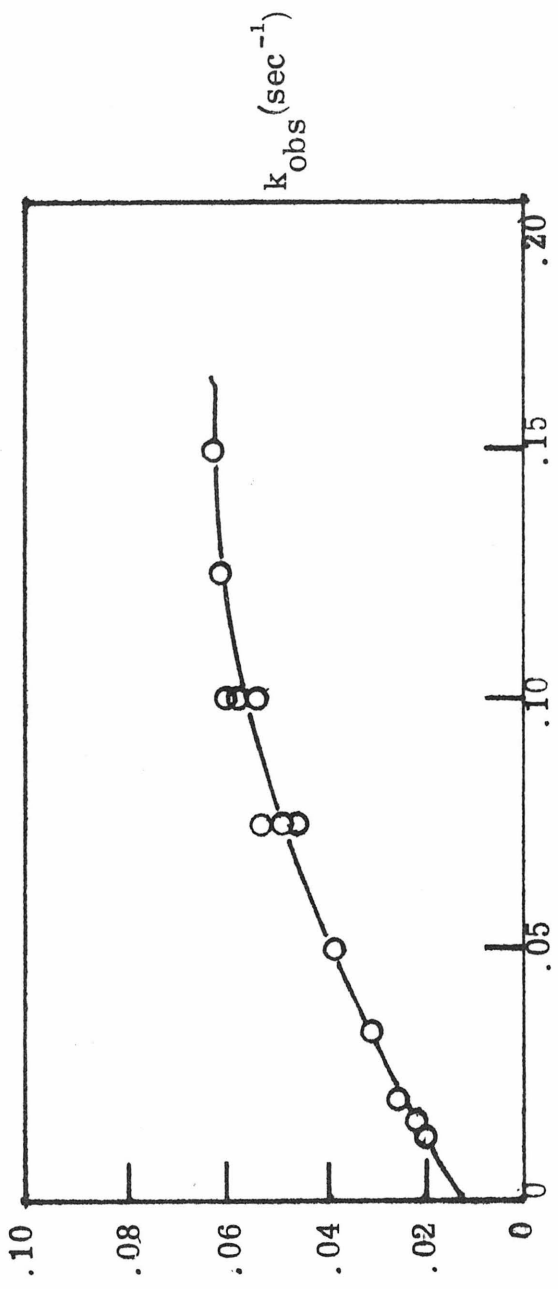


Figure 11. Plot of k_{obs} vs. $[N_3^-]$ for the anation of laccase by azide ion: 405 nm, 25.1°, pH 6.1, $\mu = 0.2$.

where Laccase* represents the S_N1 intermediate and Laccase $-N_3^-$ the species absorbing at 405 nm. The observed rate constant expected for a reaction following this scheme is[3]:

$$k_{obs} = \frac{k_1 k_2[N_3^-] + k_{-1} k_{-2}}{k_1 + k_{-1} + k_2[N_3^-] + k_{-2}}$$

Making the steady state approximation for Laccase* yields the analytically useful expression:

$$\frac{1}{\alpha k_{obs}} = \frac{1}{k_1} + \frac{1}{k_{-2} K_f [N_3^-]}$$

where $\alpha = K_f[N_3^-]/(1 + K_f[N_3^-])$. Plotting (αk_{obs}) vs. $[N_3^-]^{-1}$ then should give a line with slope $(k_{-2}K_f)^{-1}$ and intercept $(k_{-1})^{-1}$.

The other mechanism consistent with saturation in k_{obs} requires fast pre-equilibrium formation of an outer-sphere complex (Laccase · N_3^-) followed by rate-determining dissociative interchange of azide with a native laccase ligand to give the inner-sphere complex Laccase - N_3^-:

$$\text{Laccase} + N_3^- \rightleftharpoons \text{Laccase} \cdot N_3^- \quad K_{os}$$

$$\text{Laccase} \cdot N_3^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Laccase} - N_3^-$$

The apparent first order rate constant implied by this mechanism is easily shown to be:

$$k_{obs} = k_{-2} + \frac{k_2 K_{os}[N_3^-]}{1 + K_{os}[N_3^-]}$$

if a large excess of azide over laccase is present. This expression may

be rearranged to:

$$\frac{1}{\alpha' k_{obs}} = \frac{1}{k_{-2}(K_f + K_{os})[N_3^-]} + \frac{K_f}{(K_f + K_{os})k_2}$$

where $\alpha' = (K_f + K_{os})[N_3^-]/(1 + (K_f + K_{os})[N_3^-])$. If $K_{os} \ll K_f$ this equation reduces to an expression of the same form as that expected for the S_N1 anation mechanism.

Figure 12 is a $(\alpha k_{obs})^{-1}$ vs. $[N_3^-]^{-1}$ plot for the laccase-azide complex formation data. The excellent line obtained indicates that the data are consistent with either one of the proposed mechanisms. Least squares analysis of the linear plot allows estimation of the dissociation rate of the azide complex ($k_{-2} = 1.36 \pm 0.08 \times 10^{-2}$ sec^{-1}) and k_1 (or k_2 for the outer-sphere complex interpretation) ($1.94 \pm 0.10 \times 10^{-1}$ sec^{-1}).

III. Attempts to Measure Thermodynamic and Kinetic Parameters for the Laccase-Fluoride Complex

Complex formation between laccase and fluoride ion gives rise to no new absorptions in the region 300-700 nm which may be used to estimate formation constants and anation rate parameters. One attempt to measure formation constants at pH 7.0 involved a potentiometric fluoride titration using an Orion fluoride sensitive electrode (AgCl/Ag reference electrode). The detection limit of this electrode exceeds 10^{-5} M, but for easily attainable laccase concentrations (10^{-5} - 10^{-4} M) it did not prove to be sensitive enough to accurately measure uptake of F^- by the protein.

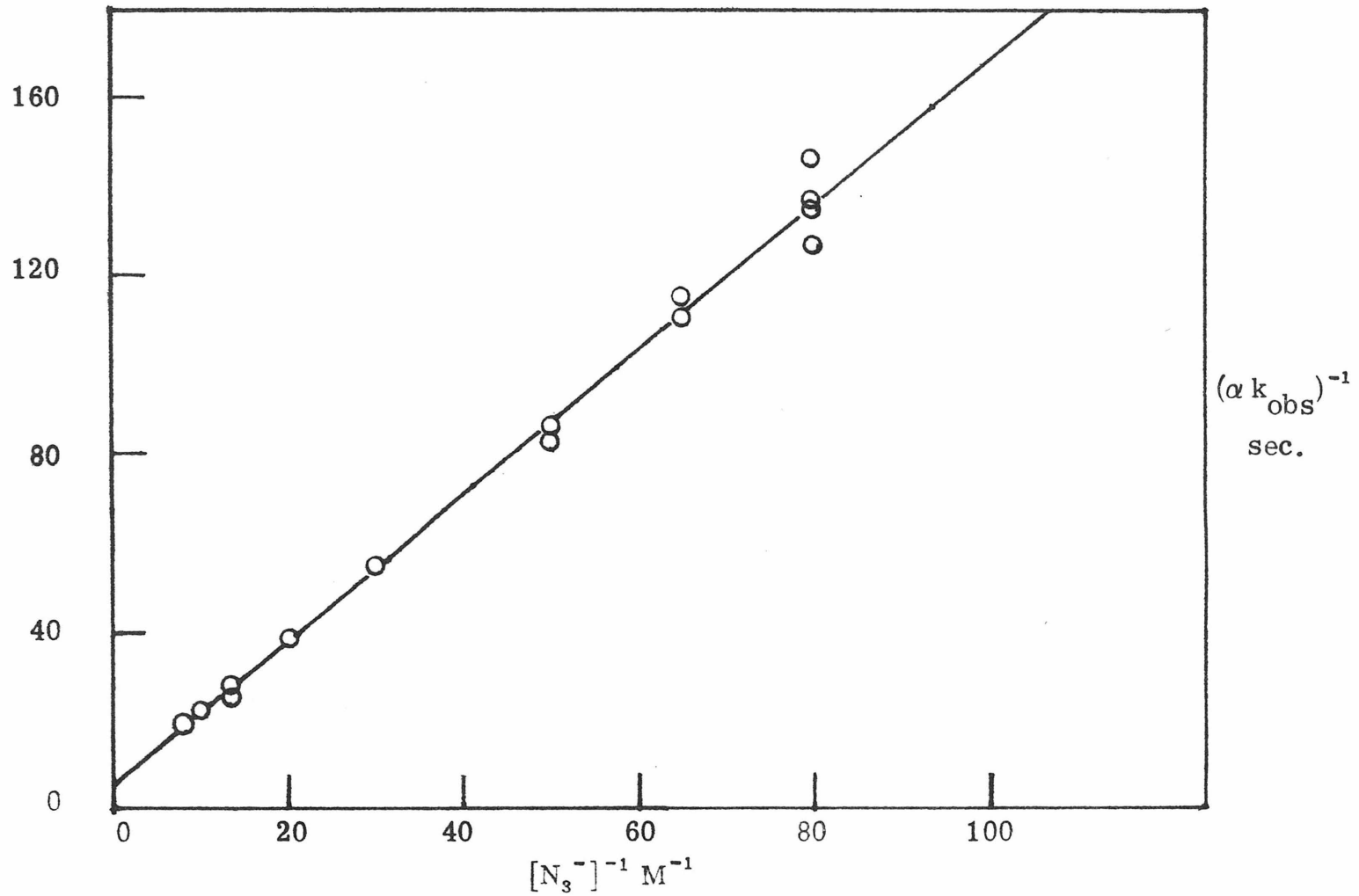


Figure 12. Plot of $(\alpha k_{obs})^{-1}$ vs. $[N_3^-]^{-1}$ for the reaction of azide ion with laccase; 405 nm, 25.1°, pH 6.1, $\mu = 0.2$.

A competition experiment with azide ion was run in the hope of characterizing the reaction between fluoride ion and laccase at pH 6.0. Under virtually the same conditions employed in the azide anation study, NaF solutions were mixed with ca. 1×10^{-5}M laccase pre-equilibrated with 0.02 M NaN_3. Displacement of laccase-bound azide by fluoride was followed through the decay in 405 nm absorbance. A constant first order rate of $1.6 \pm 0.3 \times 10^{-3}$ sec^{-1} (25.2°, pH 6.0, $\mu = 0.2$) was observed over the interval $0.02 \leq [F^-] \leq 0.12$ M, preventing the calculation of any rate parameters pertaining directly to the reaction of laccase with fluoride ion. Fluoride ion appeared to quantitatively displace azide from coordination to laccase for all concentrations employed, but a value of K_f for the laccase-fluoride complex has not yet been obtained. Results of the trial competition experiment between azide and fluoride provide very little information about the reaction of laccase with F^-, but they do demonstrate that the two anions compete for the same laccase binding site.

IV. Reduction of the Laccase-Azide Complex by Hydroquinone, pH 6.1

The reaction of the laccase-azide complex with hydroquinone at pH 6.1 was followed at 330, 405, and 614 nm using the same conditions as were employed in the anation study. Laccase was pre-equilibrated with NaN_3 or NaF in all experiments described in this and subsequent sections. In view of the limited stability of laccase-azide solutions against copper reduction, these were used within several hours after their preparation.

Hydroquinone solutions in the range 2.5×10^{-3} to 5.0×10^{-2}M were used in making kinetics measurements. Native laccase is reduced rapidly using reducing agent concentrations in this range, leaving a slower-reacting component at 614 nm presumably attributable to reduction of the azide-complexed protein. Observed rate constants for "blue" copper reduction are presented in Table 9. The rate is essentially independent of the reducing agent concentration and the azide concentration ($0.02 \leq [N_3^-] \leq 0.15$ M). Actually, k_{obs} reaches a shallow maximum at ca. $[H_2Q] = 2.0 \times 10^{-2}$ M, but deviations from the average value of the observed rate constant over the twenty-fold concentration range (0.13 ± 0.02 sec^{-1}) are not large enough to be considered outside the limits of experimental error. The rate law for reduction of the "blue" copper in the laccase-azide complex thus is:

$$\frac{-d[\text{Cu}(614)]_{tot}}{dt} = k_1[\text{Cu}(614)]_{tot}$$

Following a fast small decrease, most of the 405 nm absorbance decays slowly by a first order process when hydroquinone is mixed with the laccase-azide complex. Rate data are accumulated in Table 10, and a plot of k_{obs} as a function of $[N_3^-]$ is given in Figure 13 for $[H_2Q] = 1.0 \times 10^{-1}$ M. The 405 nm rate is independent of reducing agent concentration over the interval $2.5 \times 10^{-3} \leq [H_2Q] \leq 1.0 \times 10^{-1}$ M ($k_{obs} = 5.6 \pm 0.4$ sec^{-1}; 25.1°, pH 6.1, $\mu = 0.2$, $[N_3^-] = 0.05$ M) but first order in the azide concentration. The rate law for bleaching of the 405 nm absorbance may be written:

Table 9

Observed Rate Constants for the Reduction of the Laccase-Azide Complex; 614 nm, 25.1°, pH 6.1, $\mu = 0.2$

$[H_2Q] \times 10^2$(M)	$[N_3^-] \times 10^2$(M)	k_{obs}(sec^{-1})
0.25	5.0	0.098
		0.095
0.50	5.0	0.122
		0.123
1.0	5.0	0.157
2.5	5.0	0.161
		0.155
5.0	5.0	0.132
		0.140
1.0	2.0	0.160
		0.163
1.0	7.5	0.168
		0.175
1.0	10.0	0.175
		0.165
1.0	15.0	0.153
		0.162

Table 10

Observed Rate Constants for the Reduction of the Laccase-Azide Complex; 405 nm, 25.1°, pH 6.1, $\mu = 0.2$

$[H_2Q] \times 10^2$(M)	$[N_3^-] \times 10^2$M	$k_{obs} \times 10^3$(sec^{-1})
0.25	5.0	5.0
		5.1
0.50	5.0	5.4
		5.3
1.0	5.0	6.1
		6.3
2.5	5.0	5.6
		5.9
5.0	5.0	6.0
		6.1
7.5	5.0	5.3
		5.2
10.0	5.0	5.3
		5.0
10.0	2.0	2.2
		1.9
		1.8
10.0	4.0	4.0
		4.1

Table 10 (Continued)

$[H_2Q] \times 10^2$(M)	$[N_3^-] \times 10^2$M	$k_{obs} \times 10^3$(sec^{-1})
10.0	6.0	6.0
		6.2
		6.4
10.0	8.0	8.8
		8.9
		8.5
		8.8
10.0	10.0	13.0
		10.7
		11.3
		12.3
10.0	15.0	15.5
		15.7
		15.4

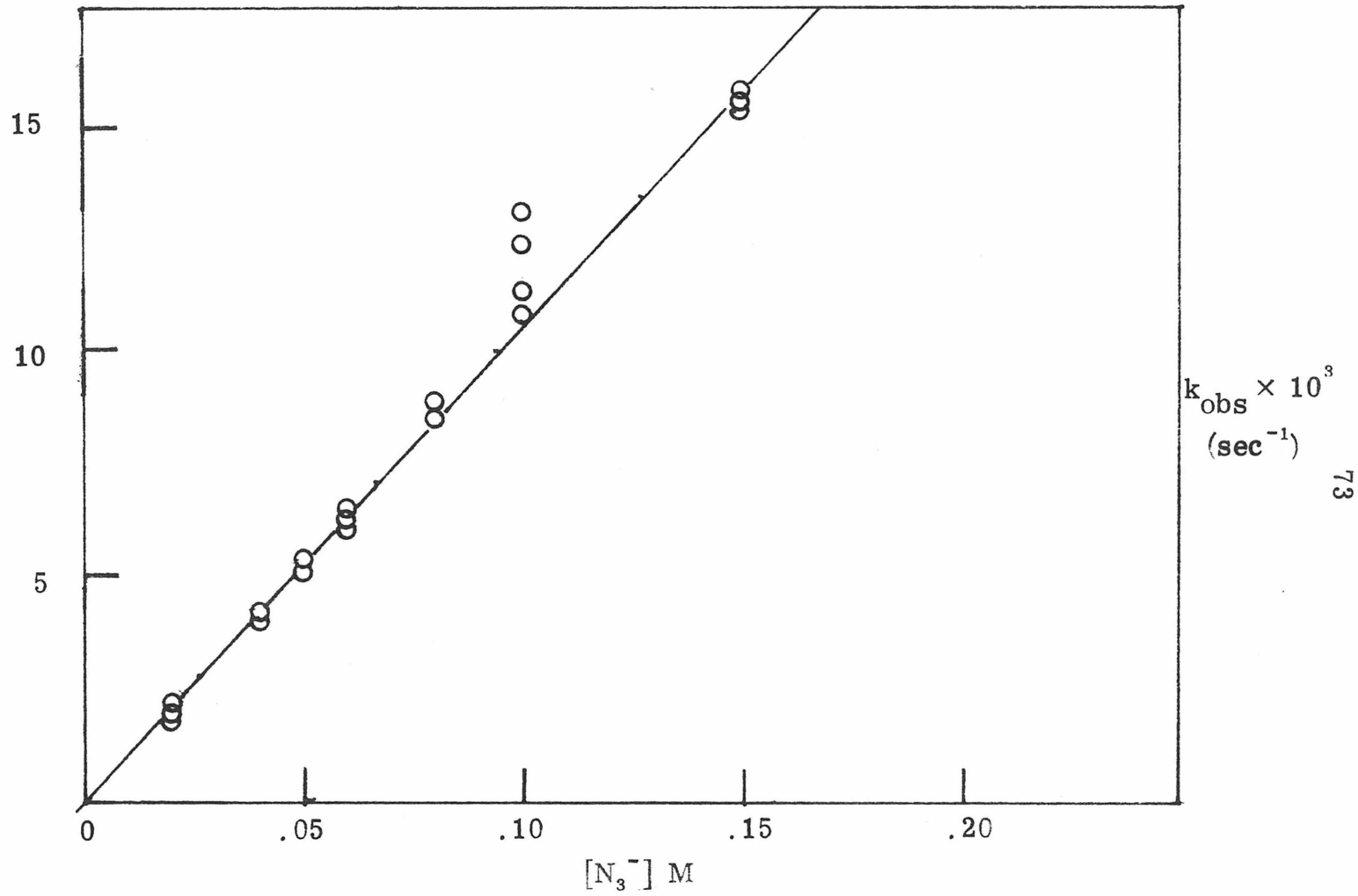


Figure 13. Plot of k_{obs} vs. $[N_3^-]$ for the reduction of the laccase-azide complex by hydroquinone; 405 nm, 25.1°, pH 6.1, $\mu = 0.2$, $[H_2Q] = 0.1$ M.

$$\frac{-d[Cu(405)]}{dt}_{tot} = k_2[N_3^-][Cu(405)]_{tot}$$

where $k_2 = 0.106 \pm 0.006\ M^{-1}sec^{-1}$ (25.1°, pH 6.1, $\mu = 0.2$).

The small initial absorbance decrease at 405 nm takes place on the same time scale as 614 nm absorbance decay. It probably appears because the absorbance changes at the two wavelengths are not totally independent of each other. A small decrease in A_{405} does accompany reduction of native laccase. Absorbance changes at 614 nm were complete before the first data points for the considerably slower 405 nm reaction were taken, so these do not pose a potential interference in the evaluation of 405 nm results.

The 330 nm absorbance actually begins to rise after a small initial decrease when hydroquinone reacts with the laccase-azide complex. Absorbance changes at this wavelength appear to follow those at 405 nm very closely, but slow upward absorbance drifts continue long after the decay in 405 nm absorbance is complete. No attempt was made to quantitatively derive rate parameters from 330 nm absorbance-time curves. An increase in 330 nm absorbance also occurs if azide is added to laccase already fully reduced by hydroquinone.

V. Reduction of the Laccase-Fluoride Complex by Hydroquinone, pH 6.0

Reduction of the laccase-fluoride complex by hydroquinone was followed at 614 and 330 nm, again using the same conditions as were employed in the pH 6 anation experiments. Absorption at both wavelengths decreases normally upon the addition of hydroquinone; Table 11

Table 11

Rate Data for the Reaction of Hydroquinone with the Laccase-Fluoride Complex; 25.1°, pH 6.0, $\mu = 0.2$

$[H_2Q] \times 10^2$(M)	$[F^-] \times 10^2$(M)	$k_{obs} \times 10^2(sec^{-1})$ 614 nm	330 nm
0.25	5.0	3.0	4.1
		2.8	4.2
0.50	5.0	5.1	5.7
		4.8	5.7
1.0	5.0	7.2	7.7
		7.5	7.6
2.5	5.0	8.7	8.7
		8.8	8.7
5.0	5.0	10.5	9.0
		9.2	8.8
		9.5	
		8.0	
10.0	5.0	---	7.4
			7.4
10.0	2.0	---	7.2
			7.5
10.0	7.5	---	7.1
			7.2
10.0	10.0	---	7.1
			7.3
10.0	15.0	---	5.2
			5.2

Table 11 (Continued)

		$k_{obs} \times 10^2 (sec^{-1})$	
$[H_2Q] \times 10^2 (M)$	$[F^-] \times 10^2 (M)$	614 nm	330 nm
1.0	2.0	7.7	---
		7.6	
1.0	10.0	7.4	---
		7.0	
1.0	15.0	7.6	---
		7.6	

contains observed rate constants derived from the absorbance-time data. The absence of any fast reduction component in the absorbance-time profiles suggests that laccase is present only as the fluoride complex for all concentrations of NaF employed.

Figure 14 illustrates the dependence of k_{obs} (330) on $[H_2Q]$ for $[F^-] = 0.05$ M. Rate saturation with increasing hydroquinone concentration occurs, and slight apparent substrate inhibition is evident as $[H_2Q]$ is raised above the minimum level required to saturate the rate. Observed rate constants at 614 nm approach the same saturation rate, but k_{obs} (614) values at lower reducing agent concentrations invariably are somewhat smaller than corresponding 330 nm results. Reduction rates at both wavelengths are independent of the fluoride ion concentration over the interval $0.02 \leq [F^-] \leq 0.15$ M.

Rate saturation with increasing substrate concentration in enzyme systems has been treated in many instances using the Michaelis-Menten formalism.[4] A mechanism based on the Michaelis-Menten approach is proposed here to quantitatively account for the laccase-fluoride complex reduction results:

$$(2,2F^-, Ox) + HQ \rightleftharpoons (2,2F^-, Ox) \cdot HQ \quad K_s'$$

$$(2,2F^-, Ox) \cdot HQ \rightleftharpoons (2,2F^-, Ox) \cdot 2HQ \quad K_s''$$

$$(2,2F^-, Ox) \cdot HQ \xrightarrow{k_1} (2,1, Ox) + SQ + F^-$$

$$(2,2F^-, Ox) \cdot 2HQ \xrightarrow{k_1} (2,1, Ox) \cdot HQ + SQ + F^-$$

$$HQ + (2,1, Ox) \xrightarrow{fast} (2,2, Red) + SQ$$

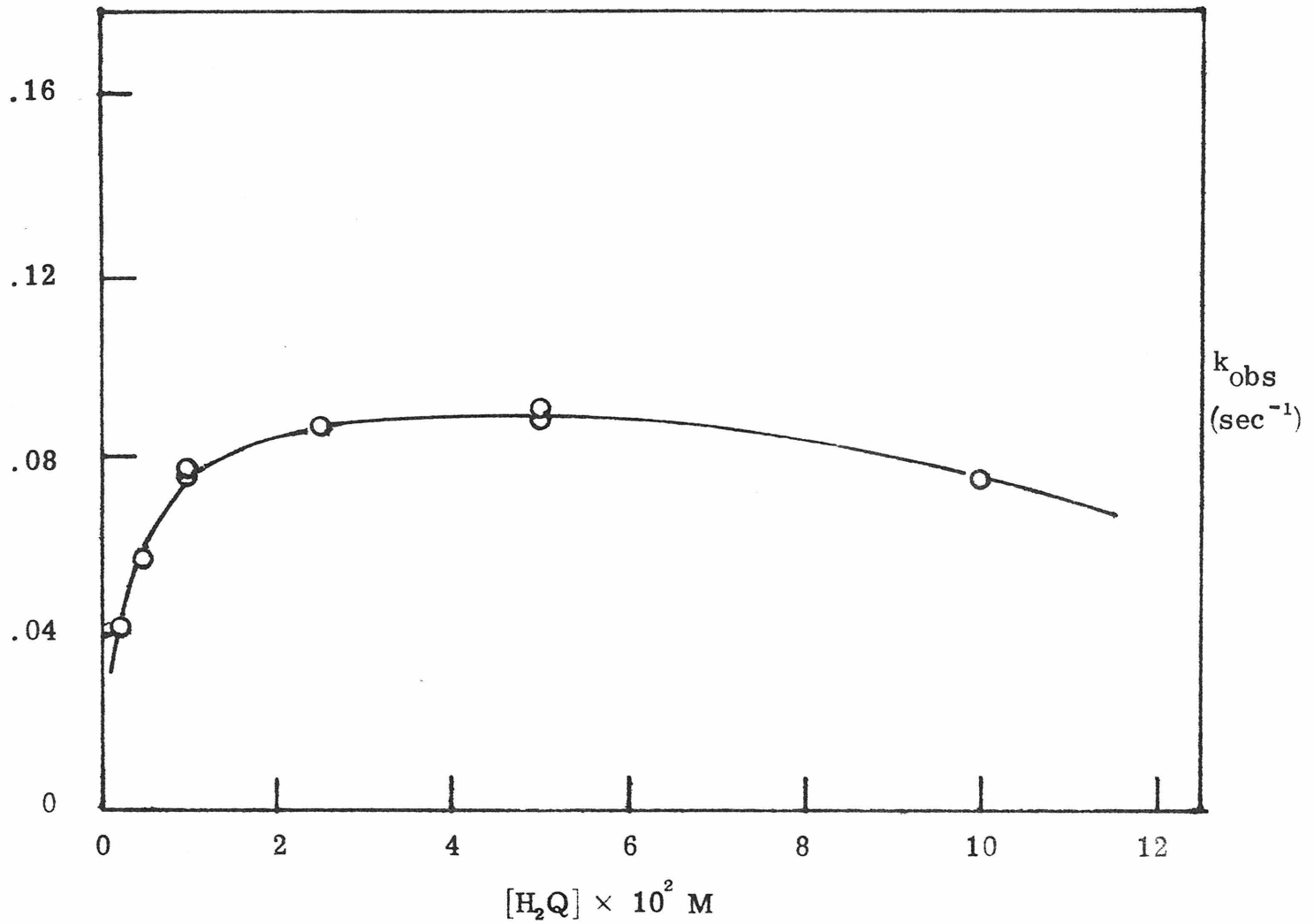


Figure 14. Plot of k_{obs} vs. $[H_2Q]$ for the reduction of the laccase-fluoride complex by hydroquinone; 330 nm, 25.1°, pH 6.0, $\mu = 0.2$, $[F^-] = 0.05$ M.

$$HQ + (2,1,Ox) \cdot HQ \xrightarrow{\text{fast}} (1,2,Red) + 2SQ$$

The species $(2,2F^-,Ox)$ refers to the oxidized laccase Type 2 fluoride complex, and the successive binding constants K_s' and K_s'' pertain to fast pre-equilibria involving enzyme-substrate complex formation at two independent protein sites. The symbol HQ is used to indicate either HQ^- or H_2Q as it is not possible to tell which form of the reductant is participating on the basis of experiments at constant pH.

The rate laws derived from the above mechanism are:

$$\frac{-d[Cu(330)]}{dt}_{tot} = \frac{k_1 K_s'[HQ][Cu(330)]_{tot}}{1 + K_s'[HQ]}$$

$$= k_{obs}(330)[Cu(330)]_{tot}$$

$$\frac{-d[Cu(614)]}{dt} = \frac{k_1 K_s' K_s''[HQ]^2[Cu(614)]_{tot}}{1 + K_s'[HQ] + K_s' K_s''[HQ]^2}$$

$$= k_{obs}(614)[Cu(614)]_{tot}$$

Several assumptions required to reach these rate laws should now be mentioned. First, it is assumed that all redox transformations subsequent to the slow k_1 step are fast. Second, only the hydroquinone molecule associated with K_s' is thought to be involved in fluoride displacement and reduction of the Type 3 copper site. Third, it is suggested that the hydroquinone molecule associated with K_s'' has no

effect on the rate of fluoride displacement and is situated in a position enabling it to attack the "blue" copper site only.

Rearranging the expression for $k_{obs}(330)$ gives:

$$\frac{1}{k_{obs}(330)} = \frac{1}{k_1 K_S' [HQ]} + \frac{1}{k_1}$$

so a plot $k_{obs}(330)^{-1}$ vs. $[H_2Q]^{-1}$ is expected to be linear on the basis of the proposed mechanism for reduction of the laccase-fluoride complex. A linear plot is obtained with the experimental data (Figure 15), and the parameters derived from the least squares slope and intercept are: $k_1 = 9.8 \pm 0.2 \times 10^{-2}$ sec^{-1}, $K_S' = 293$ M^{-1} (25.1°, pH 6.0, $\mu = 0.2$). Rearranging the expression for $k_{obs}(614)$ gives:

$$\frac{1}{k_{obs}(614)} - \frac{1}{k_1} = \frac{1}{k_1 K_S''} \left(\frac{[HQ] + K_S' [HQ]^2}{K_S' [HQ]^3} \right)$$

Using the k_1 and K_S' values evaluated in the 330 nm experiments, the quantity on the right hand side of the equation may be plotted against $(k_{obs}(614)^{-1} - k_1^{-1})$ (Figure 16). As expected from the equation, a reasonably good line with intercept essentially zero (0.26 sec) is obtained. Taking the slope to be $(k_1 K_S'')^{-1}$, K_S'' is estimated at 396 M^{-1}.

VI. Reduction of the Laccase-Fluoride Complex by Hydroquinone, pH 7.0

The reactivity of laccase-anion complexes with hydroquinone changes dramatically as the pH is altered only one unit from 6 to 7. A series of runs at $[F^-] = 0.02$ M, $\mu = 0.1$, pH 7.0 are discussed in this section; phosphate buffer and NaF alone maintained the ionic strength.

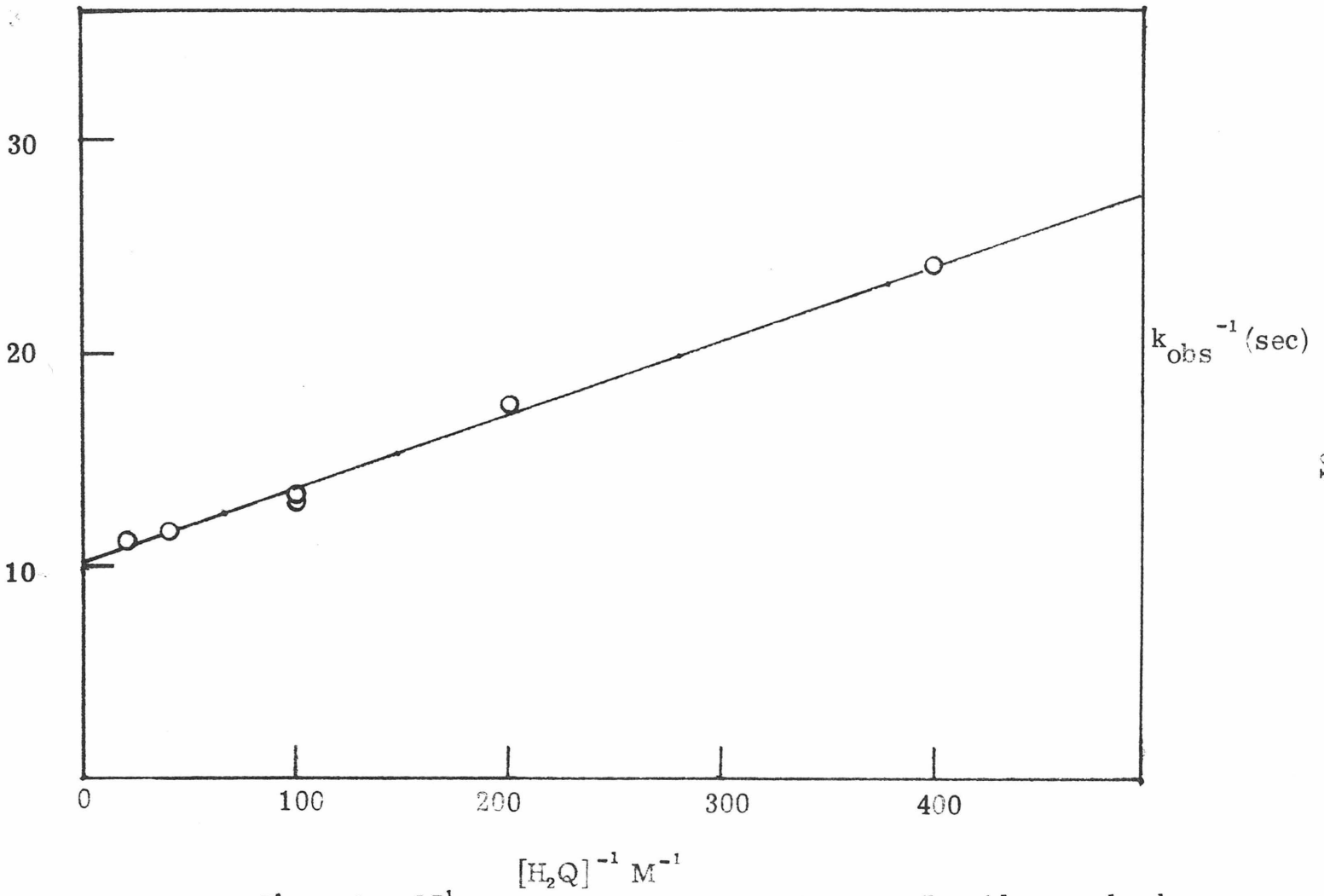


Figure 15. Plot of k_{obs}^{-1} vs. $[H_2Q]^{-1}$ for the reduction of the laccase-fluoride complex by hydroquinone; 330 nm, 25.1°, pH 6.0, $\mu = 0.2$, $[F^-] = 0.05$ M.

Figure 16. Plot of $(k_{obs}^{-1} - k_1^{-1})$ vs. $([H_2Q] + K_S'[H_2Q]^2)/K_S'[H_2Q]^3$ for the reaction of hydroquinone with the laccase-fluoride complex; 614 nm, 25.1°, pH 6.0, $\mu = 0.2$, $[F^-] = 0.05$ M.

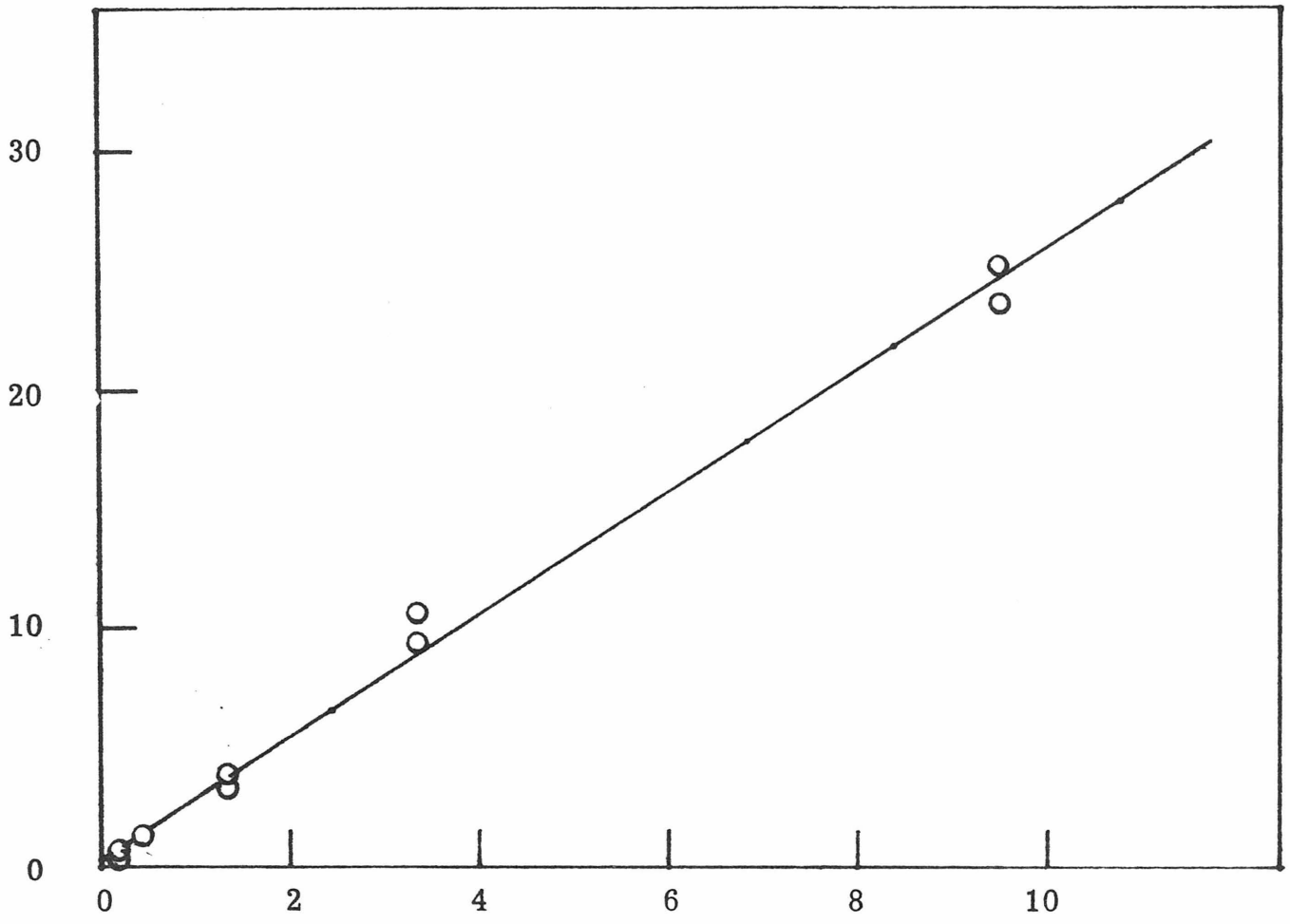

30
20
10
0
0
2
4
6
8
10
$(k_{obs}^{-1} - k_1^{-1})$ sec.
$([H_2Q] + K_S'[H_2Q]^2)/K_S'[H_2Q]^3 \times 10^{-2}$ M^{-1}

Figure 17 illustrates a typical oscilloscope trace found for the hydroquinone reduction of laccase "blue" copper. The absorbance initially decays rapidly to a first baseline (1). Following a short period, the absorbance again begins to drop and ultimately reaches the final baseline (2). For the purpose of clarity, the second slower A_{614} decrease is not included in the illustrated example. We suggest that the initial absorbance drop corresponds to reduction of "blue" copper in a laccase-fluoride complex which does not dissociate rapidly to give the native enzyme; the second component is assigned to reduction of native laccase. This interpretation implies that the reduced laccase-fluoride complex is not rapidly reoxidized by oxygen, permitting reduction of the oxidized laccase-fluoride derivative to be observed while the native enzyme is participating in steady-state oxygen consumption.

Laccase absorbance typically drops to a steady-state level in the presence of oxygen and a reducing agent, and then falls to the value characteristic of fully reduced protein when oxygen is fully consumed. Absorbance decay towards the steady-state value is exponential, so it is conceivable that this phenomenon might be mistaken for reduction of a non-oxidizable laccase species. It is easily shown that the steady-state 614 nm absorbance value will decrease as the hydroquinone concentration increases if the initial oxygen concentration is held constant from one experiment to the next. No variation in the absorbance level of baseline 1 was observed as the hydroquinone concentration was

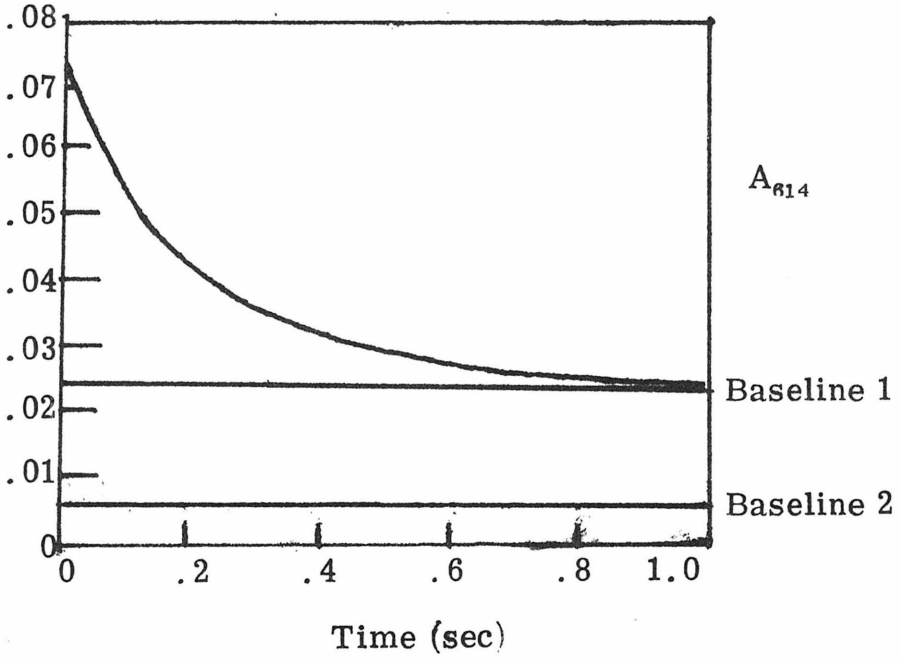


Figure 17. Typical oscilloscope trace for the reaction of hydroquinone with the laccase-fluoride complex; 614 nm, 26.3°, pH 7.0, $\mu = 0.1$, $[H_2Q] = 2.5 \times 10^{-3}$ M, $[F^-] =$ 0.02 M.

varied over a ten-fold range in making stopped flow kinetic measurements of the reduction of laccase in the presence of added fluoride at pH 7.0. The initial absorbance decrease always accounts for 73 ± 3% of the total 614 nm absorbance change at room temperature, ruling out the possibility that relaxation to the steady-state condition accounts for the observed two-step reduction pattern.

Accurate rate parameters for reduction of the slower-reacting laccase component could not be obtained in view of the small extent of the second A_{614} decrease. Approximate observed rate constants are in reasonable agreement with values expected for native laccase at the same temperature, ionic strength, and pH.

Analytical plots based on the fast absorbance change yield the observed rate constants given in Table 12 and plotted as a function of hydroquinone concentration in Figure 18. An Eyring plot constructed as described previously is shown in Figure 19. The reaction is first order in the hydroquinone concentration, and the room temperature second order rate constant k_1 is approximately five times larger than that found for the native protein under the same conditions-

$$k_{obs}(614) = 0.394 + 1.62 \times 10^{3}[H_2Q] \quad (26.3°, \text{pH } 7.0, \mu = 0.1)$$

Plots of $k_{obs}(614)$ vs. $[H_2Q]$ for data taken at several temperatures show small positive intercepts. These are large enough to be considered outside the limits of experimental error from zero, but too small to justify firmly assigning them to a parallel reduction pathway zeroth order in $[H_2Q]$. Even if the intercepts do correspond to an

Table 12

Rate Data for the Reduction

of the Laccase-Fluoride Complex;

614 nm, pH 7.0, $\mu = 0.1$, $[F^-] = 0.02$ M

	$k_{obs}(sec^{-1})$			
$[H_2Q] \times 10^3$(M)	10.7°	18.3°	26.3°	37.3°
1.25	0.496	1.21	2.39	6.3
	0.483	1.18	2.52	6.2
2.50	0.845	1.91	4.44	12.1
	0.783	2.11	4.52	13.8
				12.5
5.00	1.56		8.24	26.3
	1.53		8.42	28.2
				27.8
10.00	2.86		16.66	
	2.86			

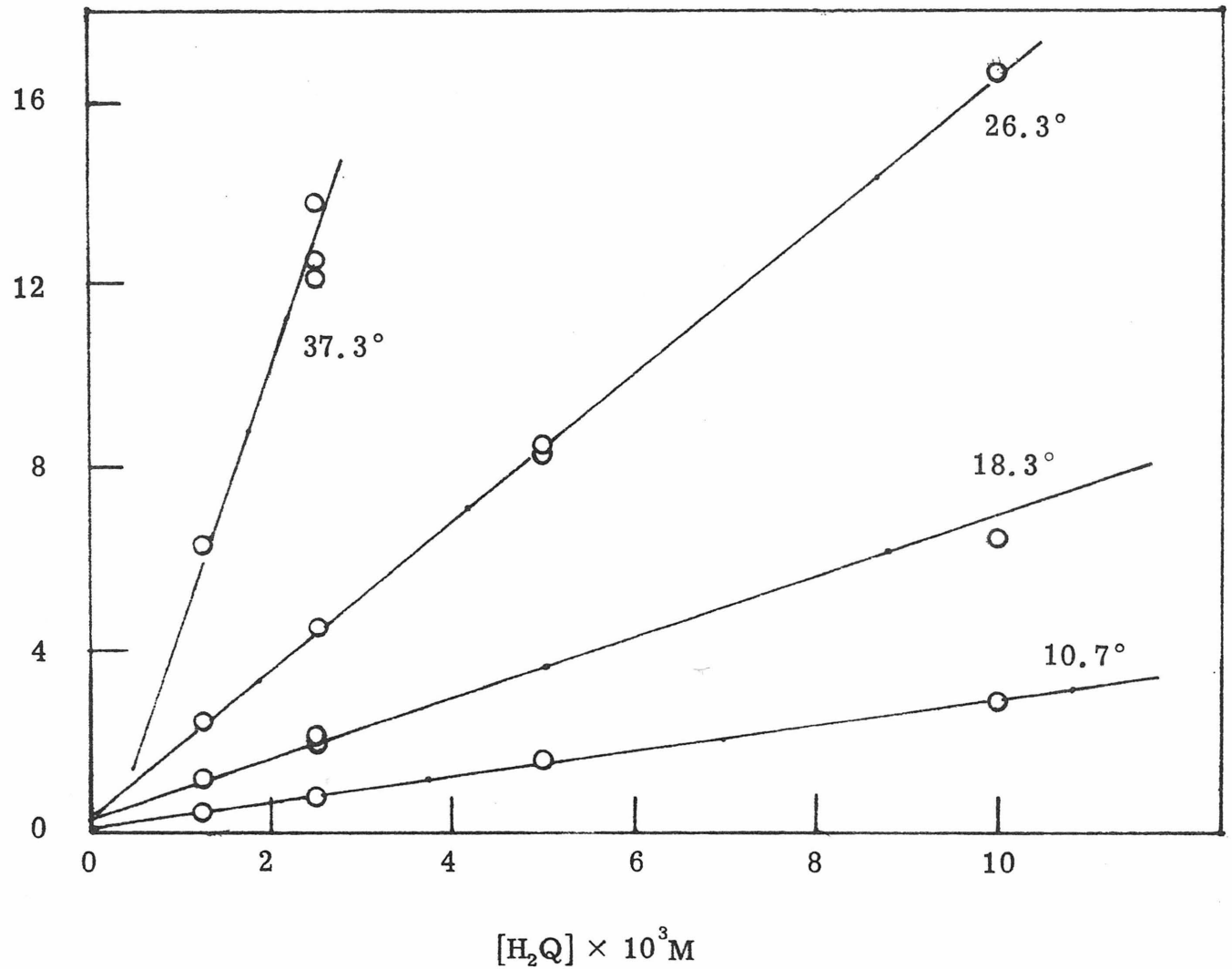


Figure 18. Plot of k_{obs} vs. $[H_2Q]$ for the reaction of hydroquinone with the laccase -fluoride complex; fast phase-614 nm, pH 7.0, $\mu = 0.1$, $[F^-] = 0.02$ M.

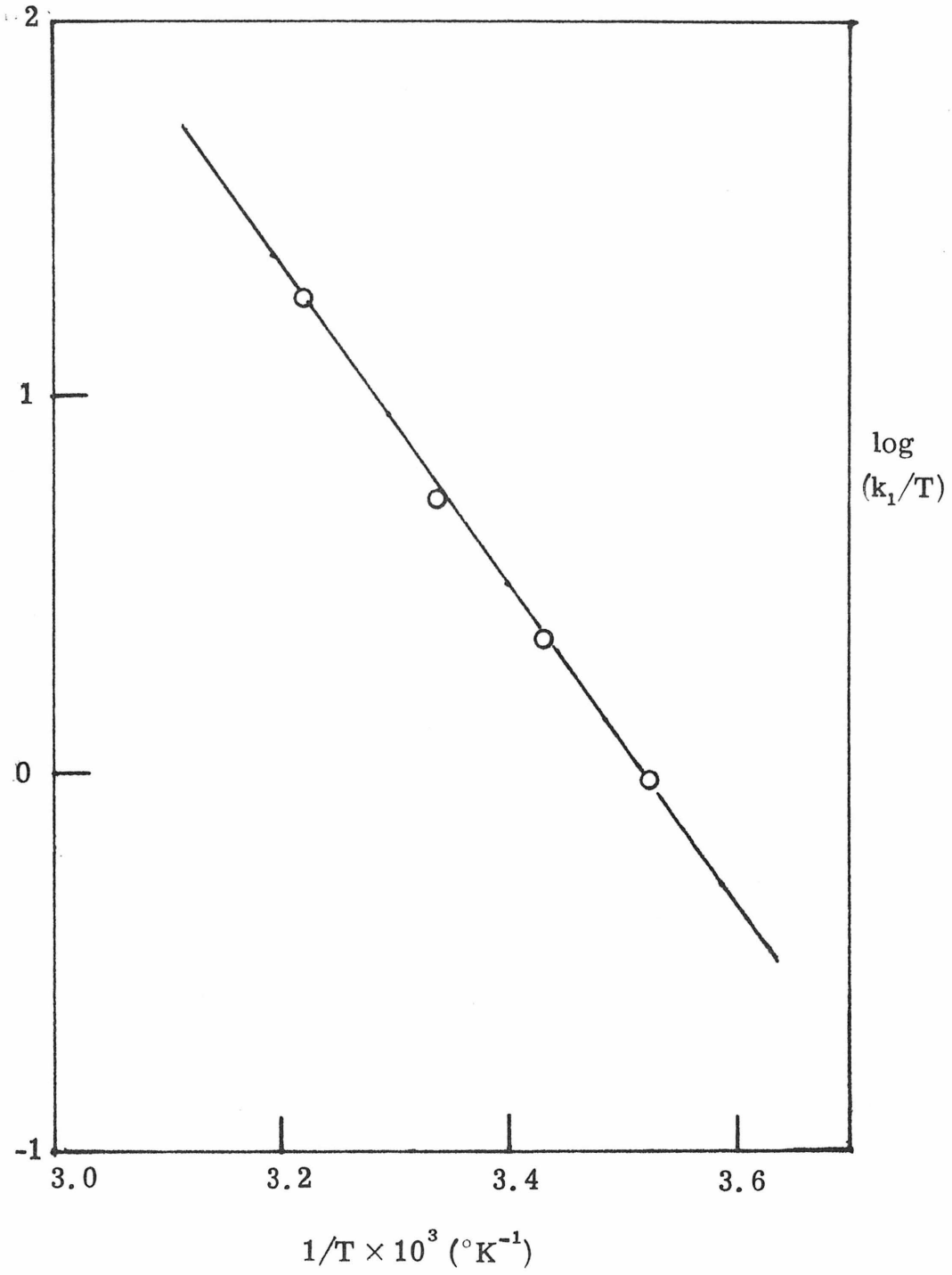


Figure 19. Eyring plot of the rate data for the fast phase of the 614 nm absorbance change in the reaction of hydroquinone with the laccase-fluoride complex; pH 7.0, $\mu = 0.1$, $[F^-] = 0.02$ M.

alternative reduction pathway, its contribution to the overall rate is so small as to be neglected at all but the lowest hydroquinone concentrations.

The activation enthalpy for reduction of the pH 7.0 laccase-fluoride complex "blue" copper site is 19.5 ± 0.5 kcal/mole (referred to $[H_2Q] = 1M$), only about 1 kcal/mole smaller than the value recorded earlier for the native protein. If the dependence of k_{obs} on $[H^+]$ is not changed by association of F^- with laccase at pH 7 (a reasonable expectation based on the proposed origin of the kinetic $[H^+]$ dependence), then $\Delta S^{\ddagger}$ for reduction of the fluoride complex is essentially identical with the native protein activation entropy.

The reactivity of laccase "blue" copper is almost unchanged in the presence of fluoride ion at pH 7. In contrast, reduction of the Type 3 site is severely inhibited and the rate law for reduction is considerably altered. Following the usual steady-state period, a fast absorbance decrease accounting for ca. 25% of the total 330 nm absorbance change occurs. Considering observations at 614 nm, this fast change most logically corresponds to reduction of native laccase. The remaining 330 nm absorbance change occurs by a very slow first order process, attributed to reduction of the ESR-nondetectable copper site in fluoride-inhibited laccase. The kinetic results based on the slow process (Table 13) are consistent with an essentially zeroth order variation of $k_{obs}(330)$ with $[H_2Q]$ at several temperatures. Observed rate constants actually fall off significantly with increasing hydroquinone concentration. The 25.5° reduction rate is $1.0 \pm 0.2 \times 10^{-2}$ sec^{-1} for $1.25 \times 10^{-3} \leq [H_2Q] \leq 7.5 \times 10^{-3}$ M. An Eyring plot

Table 13

Rate Data for the Reaction of

Hydroquinone with the Laccase-Fluoride Complex;

330 nm, pH 7.0, $\mu = 0.1$, $[F^-] = 0.02$ M

	$k_{obs} \times 10^3 (sec^{-1})$			
$[H_2Q] \times 10^3 (M)$	12.0°	18.9°	25.5°	35.9°
1.25	6.6		10.7	19.7
	7.2		11.5	20.3
2.50	5.8	7.9	9.2	18.4
	6.2	8.3	9.4	18.5
5.00	5.0	7.8	10.0	17.8
	5.4	7.6	9.6	17.9
7.50	5.7		8.5	17.1
	5.5		8.4	17.3

constructed from the 330 nm data is non-linear, and no attempt was made to extract activation parameters from it.

VII. Reduction of the Laccase-Azide Complex by Hydroquinone, pH 7.0

In view of the apparent complexity of laccase-azide equilibria at pH 7.0, only a few hydroquinone reduction experiments were attempted with azide-inhibited protein at this pH value. Qualitative comparisons are stressed in this section rather than quantitative results.

One set of kinetic runs with $[N_3^-] = 0.02$, $\mu = 0.1$ was performed for comparison with the fluoride results at the same anion concentration. Reduction of 614 nm absorbance again takes place in two steps, but the baseline for the initial fast process is not well defined. Observed rate constants were estimated by the Guggenheim method,[5] and an order of magnitude value for the room temperature second order rate constant was obtained ($k_1 \cong 1.0 \times 10^3\ M^{-1}\ sec^{-1}$).

The similarity between results of kinetic experiments with added NaF and NaN_3 at pH 7 extends to observations at 330 nm. Although an absorbance increase develops after hydroquinone is mixed with dilute laccase containing 0.02 M NaN_3 at pH 6.1, normal reduction of 330 nm absorbance occurs at pH 7.0 using the same azide concentration. The rate of absorbance decay at 330 nm is independent of $[H_2Q]$ over the interval $1.25 \times 10^{-3} \leq [H_2Q] \leq 1.0 \times 10^{-2}$ M (Table 14) with $k_{obs} = 9.1 \pm 0.2 \times 10^{-2}\ sec^{-1}$ (25.1°).

Table 14

Observed Rate Constants for the Reaction of Hydroquinone with the Laccase-Azide Complex;

330 nm, 25.1°, pH 7.0, $\mu = 0.1$, $[N_3^-] = 0.02$ M

$[H_2Q] \times 10^3$ M	$k_{obs} \times 10^2 (sec^{-1})$
1.25	8.0
	8.4
2.50	9.0
	8.8
5.00	9.1
	9.1
7.50	9.3
	9.1
10.00	9.3
	9.2

Finally, a few points were taken at 25°, $\mu = 0.2$, pH 7.0 for azide concentrations higher than 0.02 M. Under conditions where the concentration of the species absorbing at 405 nm is appreciable, pH 7.0 results for the reaction of hydroquinone with azide-inhibited laccase strongly resemble the pH 6.1 observations presented earlier. Increases in 330 nm absorbance are again noted, and 405 nm absorbance decay takes place even slower than at pH 6.1. For example, with $[H_2Q] = [N_3^-] = 1.0 \times 10^{-1}$ M, $k_{obs}(405)$ is 3.1×10^{-3} sec^{-1} at pH 7.0 compared with 1.2×10^{-2} sec^{-1} at pH 6.1. Observed 405 nm rate constants increase with increasing azide concentration, but the dependence on $[N_3^-]$ is greater than first order.

References

1. D. Yost and H. Russell, Jr., Systematic Inorganic Chemistry, New York, Prentice-Hall, Inc., 1946.
2. A. A. Frost and R. G. Pearson, Kinetics and Mechanism, 2nd ed., Wiley, New York, 1961, p. 186.
3. N. Sutin and J. K. Yandell, J. Biol. Chem., 247, 6932 (1972).
4. H. R. Mahler and E. H. Cordes, Biological Chemistry, Harper and Row, New York, 1966, Chapter 6.
5. Reference 2, p. 49.

DISCUSSION

The fact that first order absorbance decreases occur at both 614 and 330 nm when hydroquinone is mixed with Rhus laccase implies that the Type 1 and Type 3 reduction pathways are parallel rather than consecutive.[1] Reduction of the Type 1 site is somewhat slower than Type 3 reduction for the native enzyme, but this does not necessarily indicate that the faster process is a prerequisite to the slower one. If "blue" and ESR-nondetectable copper reduction pathways were consecutive then two cases would be possible: (1) reduction of Type 1 occurs rapidly subsequent to attack on Type 3, or vice-versa, and the observed first order rate constants at 614 and 330 nm are identical, or (2) Type 1 reduction is governed by two slow steps and decay in A_{614} is not first order. Neither one of these cases is consistent with the experimental observations. Indeed, it is obvious from several experiments reported above that rate effects observed at 330 nm need not be reflected in 614 nm results. For example, drastic inhibition in the Type 3 but not the Type 1 reduction rate is observed in pH 7.0 experiments with added NaF and NaN_3. Similarly, high concentrations of benzoquinone produce rate inhibition at 330 nm, but the reactivity of the Type 1 site appears unaffected.

All of the experimental evidence clearly points to the involvement of a common intermediate in the reduction mechanisms of laccase Type 1 and Type 3 copper sites. Trends in observed rate constants for the native protein found at one wavelength invariably are reflected in

results at the other, and the absolute values of 614 and 330 nm rate constants are always comparable. Only small differences are evident in the activation parameters for Type 1 and Type 3 reduction, strongly suggesting that the two reduction pathways depend on a shared slow step.

The inverse hydrogen ion dependences of 614 and 330 nm rates are consistent with Omura's observation[2] that Rhus laccase activity rises with increasing pH up to pH 7.5. Variations in the oxidizing strengths of laccase copper sites are not a likely source of the kinetic pH-dependence since the standard reduction potential of "blue" copper actually decreases as solutions become more basic.[3] Furthermore, the reduction rate laws are not of the form expected if ionization of laccase amino acid side chains were responsible for the rate increase with increasing pH. We conclude that the inverse hydrogen ion dependences reflect participation of the hydroquinone monoanion HQ^- in the shared slow step governing Type 1 and Type 3 reduction.

Several experimental observations support this hypothesis. It is expected that the phenolate anion HQ^- and the free radical SQ ought to have similar reactivities with laccase. Rate constants calculated assuming HQ^- to be the reactive species in solution are indeed in close agreement with those found for semiquinone as reductant in experiments with added benzoquinone. The ionic strength dependence of k_{obs} (614, 330) at pH 7.0 is consistent with a mechanism requiring approach of the hydroquinone anion to a negatively charged protein site in the rate-determining step. However, a more attractive rationale for the ionic strength dependence results is that rate changes are linked to modifica-

tions in the hydroquinone acid ionization constant induced via the secondary salt effect. Since k_1 is postulated to have the form $k_1' K_{H_2Q}/[H^+]$, variations in K_{H_2Q} as well as in k_1' at constant $[H^+]$ should be reflected in observed rate constants. Baxendale and Hardy[4] have reported $K_{H_2Q} = 1.41 \times 10^{-10}$ M at 25°, $\mu = 0.65$. Comparing this value with the one given above for $\mu = 0.04$ reveals a 42% increase over the interval $0.04 \leq \mu \leq 0.65$. This correlates very well with the 40% enhancement in k_{obs} (614, 330) found over approximately the same range in ionic strength.

Preferential binding of azide and fluoride to Rhus laccase Type 2 copper has been suggested on the basis of perturbations in the ESR spectrum of this component induced by the anions.[5] That high concentrations of fluoride promote Type 2 coordination seems clear from experiments in which ^{19}F superhyperfine splitting was detected in the fungal laccase Type 2 ESR signal.[6] This assignment has also been made for ceruloplasmin.[7] Alterations in the ceruloplasmin Type 2 spectrum induced by azide, however, are thought to reflect anion binding at a Type 3 site closely linked to a Type 2 copper atom.[8] There seems to be disagreement on whether or not fluoride and azide compete for the same coordination position in ceruloplasmin. Byers et al.[8] claim they do not, and suggest that the new visible spectral features accompanying azide coordination have their origin in drastic structural perturbations in the Type 3 site. Andréasson and Vänngård,[7] on the other hand, state that the addition of large excesses of fluoride to the

ceruloplasmin-azide complex yields the ESR spectrum characteristic of the fluoride derivative. A correlation was found between the appearance of the new absorption band around 390 nm and changes in the Type 2 Cu(II) ESR spectrum, and this is taken as strong evidence for Type 2 Cu(II)-azide complex formation. Recently completed experiments in Rome[9] have demonstrated that a similar correlation exists for Rhus laccase when the azide concentration is higher than stoichiometric; Type 2 Cu(II) again is thought to be involved in the appearance of the new band. Correct assignment of the laccase-azide complex absorption with maximum at 405 nm is clearly essential to the interpretation of kinetic results reported here; we feel that the available evidence favors the Type 2 Cu(II)-N_3^- formulation.

The pH 6.1 study of laccase anation by azide shows that absorbance changes at 405 nm are consistent with formation of a 1:1 complex over the entire range of azide concentrations employed. This finding, coupled with the observation that fluoride and azide compete for the same binding site, indicates that the pH 6 results for hydroquinone reduction of anion-inhibited laccase may be safely interpreted in terms of fluoride and azide blocking the Type 2 site. As will be seen later, this is apparently no longer true when the pH is changed to 7.0 and low concentrations of anions are used.

The fluoride inhibition study at pH 6.0 makes it even more clear that the Type 1 and Type 3 reduction mechanisms are closely related and provides an important clue to the nature of the proposed intermediate. Type 1 and Type 3 reduction rates are still similar when

hydroquinone attacks the Type 2 Cu(II)-F^- laccase complex, but saturation in observed rate constants at 614 and 330 nm to a common value indicates that a new protein-dependent slow step is operating. In view of the demonstrated specificity of laccase for HQ^-, we propose that fluoride ion inhibits laccase reduction under these circumstances by occupying a labile inner-sphere coordination position of the Type 2 copper ordinarily competed for rapidly by the phenolate anion. The slow step in the presence of fluoride then probably involves intramolecular dissociative interchange, releasing fluoride back into the solvent. It has already been seen that the experimental results are consistent with rate laws calculated on the basis of a mechanism requiring slow breakdown of an enzyme-substrate complex between the Type 2 Cu(II)-F^- derivative and hydroquinone.

A requirement for inner-sphere penetration of HQ^- at the Type 2 copper site is strongly suggested by the pH 6.0 fluoride inhibition results. The logical inference to make is that the shared slow step governing reduction of the Type 1 and Type 3 sites in native laccase involves inner-sphere electron transfer from HQ^- to Type 2 Cu(II), producing the intermediate (2,1,Ox). Type 2 reduction is not required for the derivation of rate laws consistent with all of the experimental results, however, and a substrate-induced protein conformational change may also be the feature which activates the intermediate for subsequent electron transfer steps. This possibility will be explored further later in the discussion. The following mechanism is one of several closely related possibilities for reduction of laccase Type 1

and Type 3 copper sites by hydroquinone:

$$H_2Q \rightleftharpoons HQ^- + H^+ \qquad K_{H_2Q}$$

$$HQ^- + (2,2,Ox) \xrightarrow{k_1} (2,1,Ox) + SQ$$

$$HQ + (2,1,Ox) \xrightarrow{k_2} (2,2,Red) + SQ$$

$$HQ + (2,1,Ox) \xrightarrow{k_3} (1,1,Ox) + SQ$$

As before, HQ may refer either to H_2Q or HQ^-. If the k_2 and k_3 steps are fast compared with k_1, then the steady-state approximation may be made for (2, 1, Ox) and the calculated rate laws for 614 nm and 330 nm absorbance decay are:

$$\frac{-d[Cu(614)]}{dt}_{tot} = \frac{k_1 k_3 K_{H_2Q}[H_2Q][(2,2,Ox)]}{[H^+](k_2 + k_3)}$$

$$\frac{-d[Cu(330)]}{dt}_{tot} = \frac{k_1 k_2 K_{H_2Q}[H_2Q][(2,2,Ox)]}{[H^+](k_2 + k_3)}$$

The simplified mechanism given above is intended to imply that Type 1 and Type 3 reduction steps subsequent to formation of the intermediate are fast and totally independent of each other, regardless of possible oxidation state changes in the Type 2 copper after completion of the slow step.

The mechanism suggested above accounts for many of the experimental findings for the hydroquinone reduction of laccase: first order oxidizing and reducing agent dependences, inverse hydrogen ion

dependence, and similarity of 614 and 330 nm observed rate constants. If k_2 and k_3 are very large compared with k_1, then it is clear that the observed activation parameters for both Type 1 and Type 3 reduction will effectively be measures of contributions to $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the k_1 step. The apparent agreement between calculated and observed rate laws for this one mechanism does not rule out the consideration of other possibilities. In particular, it is important to determine whether or not the experimental observations for reduction of laccase by hydroquinone are compatible with formation of some kind of enzyme-substrate complex. The scheme proposed above may be modified to include a rapid pre-equilibrium enzyme-substrate complex formation step as follows:

$$H_2Q \rightleftharpoons HQ^- + H^+ \qquad K_{H_2Q}$$

$$HQ^- + (2,2,Ox) \rightleftharpoons (2,2,Ox) \cdot HQ^- \qquad K_S$$

$$(2,2,Ox) \cdot HQ^- \xrightarrow{k_1} (2,1,Ox) + SQ$$

$$(2,1,Ox) + HQ \xrightarrow{k_2} (2,2,Red) + SQ$$

$$(2,1,Ox) + HQ \xrightarrow{k_3} (1,1,Ox) + SQ$$

where $(2,2,Ox) \cdot HQ^-$ now refers to an enzyme-substrate complex with HQ^- presumably bound somewhere in the vicinity of the Type 2 copper atom. This mechanism leads to the rate laws:

$$\frac{-d[\,Cu(614)]_{tot}}{dt} = \frac{k_1 k_3 K_S K_{H_2Q}[H_2Q][(2,2,Ox)]_{tot}}{([H^+] + K_S K_{H_2Q}[H_2Q]](k_2 + k_3)}$$

$$\frac{-d[Cu(330)]_{tot}}{dt} = \frac{k_1 k_2 K_S K_{H_2Q}[H_2Q][(2,2,Ox)]_{tot}}{([H^+] + K_S K_{H_2Q}[H_2Q])(k_2 + k_3)}$$

where $[(2,2,Ox)]_{tot}$ refers to the total concentration of oxidized protein in solution, complexed by substrate or not. Although saturation in observed rate constants with increasing hydroquinone concentration is predicted from these rate laws, it is easily seen that this will not be observed if $K_S K_{H_2Q}[H_2Q] \ll [H^+]$. For $[H^+] = 1 \times 10^{-7}$ M, a typical hydroquinone concentration (1×10^{-3} M), and using $K_{H_2Q} \cong 1 \times 10^{-10}$ M, K_S would have to be ca. 1×10^{6} M^{-1} for the terms $[H^+]$ and $K_S K_{H_2Q}[H_2Q]$ to be comparable in value. For a K_S value even as large as 10^{3} - 10^{4} M^{-1}, the $[H_2Q]$ term in the denominator of the rate expressions may be neglected compared with $[H^+]$, and simple bimolecular kinetics will be found. Considering the consistency of the results of the pH 6.0 fluoride inhibition experiment with formation of some type of enzyme-substrate complex, it is clear that a mechanism requiring a similar pre-equilibrium may not be ruled out for the native enzyme.

The absence of any appreciable effect of cyclohexanediol and resorcinol on the reduction rates of laccase Type 1 and Type 3 copper sites is unsurprising if native laccase has no tendency to form an enzyme-substrate complex. Even if enzyme-substrate complex

formation occurs, the specificity of laccase for HQ^- may rule out interferences from any potential competitive inhibitor which is not anionic. The mild benzoquinone inhibition of Type 3 reduction observed at pH 7 most likely reflects specific interaction of the organic molecule with the ESR-nondetectable copper pair. It has been suggested that laccase substrates may participate in π-complex formation with enzymatic copper atoms.[10]

The ESR properties of Rhus laccase Type 2 Cu(II)[5] suggest that its coordination geometry is essentially axial. If this is the case, the unpaired electron most likely resides in a metal e_g (σ symmetry) orbital, probably 3 $d_{x^2-y^2}$. Bennett[11] has pointed out that reactants transferring electrons from or accepting electrons into orbitals of σ symmetry favor inner-sphere mechanisms more than do complexes with redox orbitals of π symmetry. Few kinetic studies of redox reactions involving low molecular weight copper complexes are available, but existent results are compatible with our hypothesis that Type 2 Cu(II) oxidizes HQ^- by an inner-sphere process. An inverse hydrogen ion path is dominant in the chromium(II) reduction of copper(II) in aqueous solution, and it is suggested that an inner-sphere mechanism with hydroxide ion serving as a bridging ligand between oxidant and reductant may be operating.[12] Chloride bridging may be responsible for the extremely rapid electron exchange reaction between cuprous and cupric ions in concentrated HCl.[13] A mechanism similar to that proposed here for laccase reduction has been suggested to account for cupric and cupric poly-L-hystidine complex catalysis of the reduction of O_2 to H_2O_2 by

hydroquinone.[14] For cupric ion the mechanism is:

$$Cu^{2+} + H_2Q \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Cu(HQ)^+ + H^+$$

$$Cu(HQ)^+ \xrightarrow{k_2} Cu^+ + SQ$$

$$Cu^+ + O_2 \xrightarrow{\text{fast}} Cu^{2+} + O_2^-$$

and an analogous scheme is outlined for the polyhistidine complex. The existence of a catalyst-substrate complex is clearly indicated in the case of polyhistidine-Cu^{2+} catalysis as Michaelis-Menten type substrate saturation was observed.

There is no information available on the ligand environment of laccase Type 2 copper, but Rotilio and co-workers[15] have pointed out the possible structural and functional correspondence between this site and the copper atoms in superoxide dismutase. Evidence has been given for the presence of three to four nitrogen donor ligands as well as a water molecule in the first coordination sphere of bovine superoxide dismutase copper.[16] Both fluoride and azide coordinate to copper in this enzyme, changing the symmetry of the sites from rhombic to axial; an intense new peak with maximum at 370 nm was observed for the azide complex.[15] The anion binding position is suspected to be the one favored by the superoxide anion radical.

Reduction of Rhus laccase by hydroquinone evidently takes place by a mechanism much different than that preferred by the fungal enzyme. Type 1 Cu(II) in fungal laccase A rapidly oxidizes H_2Q with $k = 7.7 \times 10^6\ M^{-1}\ sec^{-1}$ at 2.5°, pH 5.4 (0.1 M acetate buffer).[17] The reduction rate of the fungal laccase B 330 nm chromophore is independent of substrate concentration at high concentrations; k(330) = $1.0\ sec^{-1}$ at 25°, pH 5.5.[18] At least one of the electrons necessary for reduction of the Type 3 site is thought to be transferred intramolecularly from Type 1 Cu(I).[18] Formation of the Type 2 Cu(II)-F^- complex does not affect the Type 1 reduction rate in either chromatographic form of fungal laccase,[17,18] but the rate of reduction of the 330 nm chromophore is slowed by about two orders of magnitude.[18]

Intramolecular electron transfer reactions may be involved in reduction of the ESR-nondetectable copper site in both fungal and tree laccases. We suggest a Type 2 to Type 3 electron transfer step as part of our proposed mechanism for Rhus laccase reduction, but no compelling evidence for the existence of such a reaction is available. If it does exist, its rate must be fast compared with that of the slow step involving Type 2 Cu(II). In contrast, intramolecular electron transfer itself appears to be rate-determining in reduction of the fungal laccase 330 nm chromophore.[18]

Fungal laccase "blue" copper apparently is susceptible to direct attack by hydroquinone whereas the Rhus enzyme Type 1 site clearly is not the first to be reduced. This observation accounts for

the difference in fluoride ion effects on Type 1 reduction rates for the two forms of laccase. That Type 1 Cu(II) is well suited to be the initial electron acceptor in fungal laccase is indicated by its extraordinarily high reduction potential ($E_o' = +785$ mv, pH 5.5, $\mu = 0.2$).[19]

Association of HQ^- with Type 2 Cu(II) may serve a dual purpose: (1) reduction of Cu(II) to Cu(I) and (2) initiation of a facile conformational change permitting subsequent redox steps involving the other two copper sites to occur. A requirement for such a structural change would account for the inability of the Type 1 and Type 3 copper sites to react directly with substrate in the absence of the slow step involving Type 2 Cu(II). The necessity for initial attack at Type 2 copper is remarkable considering the fact[19] that the other two sites are much more effective oxidants thermodynamically. Enzyme-substrate interactions may not only modify the relative thermodynamic stabilities of different protein conformations, but also may lower the free energy barrier to their interconversion.[20] Substrate-induced protein structural changes are an integral part of the induced fit theory of enzyme action initially proposed by Koshland,[21] and crystallographic, chemical, and kinetic evidence has been found for these conformational transitions in a number of instances.[20, 22]

Available evidence strongly suggests that the Type 1 copper site is strongly distorted.[23] Bennett[11] has discussed some of the possible implications of this distortion for redox reactions involving the "blue" copper site. Among these are: (1) adjustment of the reduction

potential to an optimum value, (2) coupling of the oxidation state change at the metal center to important conformational movement remote from the metal, and (3) modification of atomic positions towards those of the activated complex, reducing $\Delta G^{\ddagger}$. Williams and Vallee have stressed the latter point.[24,25] The coordination environments preferred by Cu(II) and Cu(I) are very different, Cu(II) opting for tetragonal complexes and Cu(I) favoring tetrahedral symmetry. The Franck-Condon inner-sphere rearrangement barrier for reduction of Cu(II) to Cu(I) thus is expected to be high, as distortion to a compromise geometry must occur. Nuclear positional rearrangement contributions to $\Delta G^{\ddagger}$ for the "blue" site should be much smaller than for normal square-planar Cu(II) as the ground state geometry presumably is similar to that of the activated complex for electron transfer. This expectation has been realized in a recent study of reactions between inorganic reductants and metalloproteins containing only the "blue" copper structural unit.[26] Chromous reduces spinach plastocyanin with $k = 3.3 \times 10^{4}\ M^{-1}\ sec^{-1}$ at 25°, pH 4.2, $\mu = 0.1$ (NaCl) and $\Delta H^{\ddagger}$ is only 1.3 kcal/mole. Large second order rate constants and very small activation enthalpies are also reported for the reaction of bean plastocyanin and stellacyanin with Cr^{2+} (aq.).[26]

The ability of the Type 1 copper site to participate in extremely rapid redox reactions does not appear to be its most important functional characteristic for laccase enzymatic activity. The reduction mechanism of Rhus laccase is dominated by a slow step not involving the "blue"

copper, preventing the enzyme from taking advantage of its high reactivity in producing high substrate turnover rates. The activation enthalpy of 15.0 kcal/mole observed for reduction of laccase Type 1 Cu(II) by hydroquinone differs considerably from $\Delta H^{\ddagger}$ values for reduction of other "blue" proteins, but this is not surprising considering our assignment of the slow step governing "blue" copper reduction in the enzyme. We propose that the observed value probably refers essentially to the reduction of Type 2 Cu(II) by HQ^{-}. The dominant pathway for reduction of Cu^{2+} (aq.) by Cr^{2+} (aq.) is characterized by: $\Delta H^{\ddagger} = 17.1$ kcal/mole,[12] so the $\Delta H^{\ddagger}$ values pertaining to laccase Type 1 and Type 3 copper sites are compatible with expectations for reduction of a nearly axial Cu(II) species in the rate-limiting step.

Large positive activation entropies have signalled extensive conformational movement in kinetic studies of protein denaturation,[20] but the comparatively small positive $\Delta S^{\ddagger}$ values observed for reduction of laccase Type 1 and Type 3 sites by hydroquinone need not be interpreted in terms of a special conformational effect. The driving force for the association of small molecules with proteins often lies in a favorable entropy change of 10-20 cal/mol-deg linked to solvation effects.[27] Contributions to $\Delta S^{\ddagger}$ from pre-equilibria not directly related to activation of the metal center reasonably could account for the difference between activation entropies for redox reactions involving simple Cu(II) complexes and those reported here. Only a difference of

the order of 20 cal/mol-deg exists between the laccase results and $\Delta S^{\ddagger}$ for the dominant pathway in the Cr^{2+} (aq.) + Cu^{2+}(aq.) reaction (-2.1 ± 1.2 cal/mol-deg).[12]

Our finding of a new absorption band with maximum at 405 nm when NaN_3 is added to laccase is in good agreement with other recent results for Rhus laccase,[9] and similar absorptions have been reported for fungal laccase,[28] and ceruplasmin.[7,8,29] Analogous absorptions in low molecular weight pseudo-halogen complexes have been assigned as ligand to metal charge transfer,[30] and this assignment seems reasonable for the laccase Type 2 Cu(II)-N_3^- complex as well considering the high band intensity and requirement for cupric copper. Our value for the formation constant of the Type 2 Cu(II)-N_3^- complex at pH 6.1, $\mu = 0.2$ is considerably smaller than Morpurgo et al.'s result (4×10^2 M^{-1}; pH 5.5 (acetate)),[9] but the apparent discrepancy probably may be satisfactorily accounted for considering the pH and medium sensitivity of K_f documented by the Italian workers.[9] The K_f value reported here also is about an order of magnitude smaller than stability constants for simple azido copper(II) complexes.[31]

The results for reduction of the Type 2 laccase-azide complex at pH 6.1 offer a marked contrast with findings for the fluoride derivative under virtually the same conditions. In addition, they are not consistent with expectations based on our proposed mechanism for laccase reduction. Displacement of fluoride ion from Type 2 coordination is thought to be a prerequisite to reduction of the Type 1 and Type 3 copper sites, but this clearly is not the case when azide is substituted

for fluoride. Type 1 copper in the Type 2 Cu(II)-N_3^- derivative is fully reduced before significant loss of 405 nm absorbance occurs.

The kinetic results for reduction of azide-inhibited laccase at pH 6.1 are consistent with the following mechanism:

$$(2, 2N_3^-, Ox) + HQ \rightleftharpoons (2, 2N_3^-, Ox) \cdot HQ \qquad K_S$$

$$(2, 2N_3^-, Ox) \cdot HQ \xrightarrow{k_1} (1, 2N_3^-, Ox) + SQ$$

$$(1, 2N_3^-, Ox) + N_3^- \xrightarrow{k_2} \left(1\begin{smallmatrix} \diagup N_3^- \\ \diagdown N_3^- \end{smallmatrix}2, Ox\right)$$

$$\left(1\begin{smallmatrix} \diagup N_3^- \\ \diagdown N_3^- \end{smallmatrix}2, Ox\right) + HQ \xrightarrow{\text{fast}} \left(1\begin{smallmatrix} \diagup N_3^- \\ \diagdown N_3^- \end{smallmatrix}1, Ox\right) + SQ$$

The species $(2, 2, N_3^-, Ox)$, $(2, 2N_3^-, Ox) \cdot HQ$, and $\left(1\begin{smallmatrix} \diagup N_3^- \\ \diagdown N_3^- \end{smallmatrix}2, Ox\right)$ refer, respectively, to the fully oxidized Type 2 azide complex, an enzyme-substrate complex, and a Type 1 to Type 2 copper diazido bridged intermediate. The zeroth order $[H_2Q]$ and $[N_3^-]$ dependences of observed rate constants for Type 1 reduction are consistent with rate-determining electron transfer within an enzyme-substrate complex, but many strictly protein-dependent slow steps are also possible. The first order azide dependence and zeroth order hydroquinone dependence of k_{obs} for bleaching of 405 nm absorbance strongly suggests an intramolecular electron transfer reaction is taking place. The azide dependence rules out a mechanism requiring dissociation of N_3^- from Type 2 coordination, and is most likely explained considering

azide to be a bridging group for electron transfer from Type 1 Cu(I) to Type 2 Cu(II). Since one azide ion is already bound to laccase before introduction of the reducing agent, the data indicate that two azides are present in the activated complex for the electron transfer step which results in decolorization of 405 nm absorbance. The data do not demand that both azides be involved in bridging roles, but the requirement for a second azide entering in the slow k_2 step seems difficult to understand if only one N_3^- ion is required to accomplish electron transfer.

Conversion of the Type 1 site from cupric to cuprous should make the ligands to copper more susceptible to displacement by azide ion. The substitutional lability of laccase copper in the cuprous form is indicated by its fast exchange with radioactive Cu(I) in the medium.[32] Introduction of a second azide ion bridging Type 1 Cu(I) and Type 2 Cu(II) may explain the observation[9] that the affinity of Type 2 Cu(II) for N_3^- appears to increase as the "blue" copper is reduced.

Several studies have demonstrated that the μ-diazido bridged configuration is particularly effective in promoting electron transfer. A single fluoride ion bridge is utilized in the reaction of cis-$(H_2O)_4CrF_2^+$ with Cr^{2+},[33] but transfer of both azides accompanies electron transfer in the analogous reaction with cis-$(H_2O)_4Cr(N_3)_2^+$.[34] In addition, the rate of electron transfer from Cr^{2+} is 45 times faster for cis-$(H_2O)_4Cr(N_3)_2^+$ than it is for $(H_2O)_5CrN_3^{2+}$ at 0°.[35] Evidence for double azide bridging has also been obtained in studies of the reduction of cis-$(NH_3)_4Co(N_3)_2^+$ by Cr^{2+},[36] and for the reaction of $Au(N_3)_4^-$ with

$Pt(PPh_3)_n^0$ (n = 3, 4) in THF/benzene solution, yielding $Au(N_3)_2^-$ and $Pt(PPh_3)_2(N_3)_2^0$.[37]

Susceptibility of cuprous ion to oxidation by an inner-sphere mechanism is implied by our formulation of a Type 1 Cu(I) to Type 2 Cu(II) electron transfer step. The reasonableness of this hypothesis is indicated by the results of a kinetic study of the oxidation of Cu^+ by Fe^{3+} in aqueous acidic solution.[38] Hydroxide ion bridging in the activated complex for electron transfer was suggested to account for the variation of the rate with $1/[H^+]$. This formulation was supported by the observation that introduction of the good bridging ligands F^- and N_3^- causes the rate of oxidation of Cu^+ by Fe^{3-} to be immeasurably fast.

The stoichiometry of the reaction of hydroquinone with the Type 2 Cu(II) laccase-azide complex is still uncertain, but our spectroscopic observations at 330, 405, and 614 nm are compatible with reduction of the anated derivative by less than four electron equivalents. The hydroquinone-induced absorbance rise at 330 nm in particular casts considerable doubt on the reduction of the Type 3 site. An attractive possibility is that the azide bridged structure between the Type 1 and Type 2 copper sites might remain after both copper atoms are reduced, stabilizing Type 2 Cu(I) against intramolecular oxidation by the two-electron acceptor. Indeed, formation of a stable azide-bridged cuprous species might provide the driving force necessary to accomplish electron transfer from Type 1 Cu(I) to Type 2 Cu(II). This reaction is predicted to take place only to a limited extent based on reduction potentials[19] for these sites in the native enzyme. A well-characterized diazido

bridged binuclear cuprous complex , μ-diazido-tetrakis (triphenylphosphine)dicopper(I),[39] is known.

The difference in reactivity of the Type 2 fluoride and azide complexes may be related to the degree to which the two anions perturb the protein structure in the vicinity of the Type 1 copper atom, opening it to direct attack by hydroquinone prior to reduction of the Type 2 site. Although azide binding to the "blue" oxidases is usually associated with Type 2 copper, interactions with the Type 1 site have been documented as well. Half of the "blue" copper absorption intensity for ceruloplasmin disappears in the presence of large excesses of azide,[8,40] but the effect is not attributable to azide reduction of "blue" copper.[40] It is suggested that the coordination environments of one or both ceruloplasmin Type 1 copper atoms may be disrupted by N_3^-.[8,40]

Azide binding at the Type 3 copper site has been suggested to account for spectroscopic observations on laccase solutions containing low concentrations of azide.[9] Our pH 7.0 fluoride and azide inhibition results are compatible with this hypothesis. Azide ion dramatically affects the reactivity of the Type 3 site under conditions where the extent of formation of the species absorbing at 405 nm is minimal and activation requirements for reduction of the Type 1 site are essentially unchanged from those for the native enzyme. Considering our proposed mechanism for reduction of native laccase, the latter observation is compatible with the hypothesis that Type 2 Cu(II)-anion binding does not take place for low anion concentrations at pH 7.0.

Inhibition of electron transfer to the Type 3 site at high pH follows the same pattern as rate effects induced by low concentrations of F^- and N_3^- at pH 7. A laccase species reacting by a hydroquinone-independent Type 3 reduction pathway is present at pH 7.8, but observations at 614 nm indicate that its "blue" copper reduction rate must not be very different from that of the native enzyme. The high pH form of laccase most likely is just the hydroxide analog of the Type 3 complex favored by fluoride and azide ions. The presence of this species in appreciable concentrations above pH 7.5 perhaps explains why Rhus laccase activity reaches a maximum at this pH value and declines appreciably in more basic solutions.[1]

At least two mechanistic possibilities may be considered for reduction of the Type 3 azide, fluoride, and hydroxide complexes by hydroquinone. The zeroth order reducing agent dependencies observed at 330 nm are compatible with a rate-determining intramolecular electron transfer step, perhaps Type 2 Cu(I) to Type 3 Cu(II)-Cu(II). It has been suggested that these two sites are closely conjugated in ceruloplasmin,[8] and anion binding at the Type 3 site might perturb the protein structure enough to considerably change the characteristics of an intramolecular electron transfer step in laccase which ordinarily is fast. Another possibility is that the zeroth order dependences on $[H_2Q]$ reflect rate-determining displacement of the anions from Type 3 coordination, permitting direct attack at the 330 nm chromophore by hydroquinone.

References

1. A. A. Frost and R. G. Pearson, Kinetics and Mechanism, 2nd. ed., Wiley, New York, 1961, p. 166.
2. T. Omura, J. Biochem. (Tokyo), 50, 264 (1961).
3. T. Nakamura, Biochim. Biophys. Acta, 30, 44 (1958).
4. J. D. Baxendale and H. R. Hardy, Trans. Faraday Soc., 49, 1140 (1953).
5. B. G. Malmström, B. Reinhammar, and T. Vänngård, Biochim. Biophys. Acta, 205, 48 (1970).
6. R. Malkin, B. G. Malmström, and T. Vänngård, FEBS Lett., 1, 50 (1968).
7. L.-E. Andréasson and T. Vänngård, Biochim. Biophys. Acta, 200, 247 (1970).
8. W. Byers, G. Curzon, K. Garbett, B. E. Speyer, S. N. Young, and R. J. P. Williams, Biochim. Biophys. Acta, 310, 38 (1973).
9. L. Morpurgo, G. Rotilio, A. Finazzi-Agrò, and B. Mondovi, submitted for publication.
10. L. Broman, B. G. Malmström, R. Aaasa, and T. Vänngård, Biochim. Biophys. Acta, 75, 365 (1963).
11. L. E. Bennett, in Progress in Inorganic Chemistry, S. J. Lippard, ed., Vol. 18, Wiley, New York, 1973, p. 1.
12. K. Shaw and J. H. Espenson, Inorg. Chem., 7, 1619 (1968).
13. H. M. McConnell and H. E. Weaver, Jr., J. Chem. Phys., 25, 307 (1957).

14. I. Pecht, A. Levitzki, and M. Anbar, J. Amer. Chem. Soc., 89, 1587 (1967).

15. G. Rotilio, L. Morpurgo, C. Giovagnoli, L. Calabrese, and B. Mondovi, Biochemistry, 11, 2187 (1972).

16. G. Rotilio, A. Finazzi-Agrò, L. Calabrese, F. Bossa, P. Guerrieri, and B. Mondovi, Biochemistry, 10, 616 (1971).

17. B. G. Malmström, A. Finazzi-Agrò, and E. Antonini, Eur. J. Biochem., 9, 383 (1969).

18. L.-E. Andréasson, B. G. Malmström, C. Strömberg, and T. Vänngård, Eur. J. Biochem., 34, 434 (1973).

19. B. Reinhammar, Biochim. Biophys. Acta, 275, 245 (1972).

20. H. Morawetz, Adv. Prot. Chem., 26, 243 (1972).

21. D. E. Koshland, Jr., in The Enzymes, P. D. Boyer, H. Lardy, and K. Myrbäck, eds., 2nd. ed., Vol. 1, Academic Press, New York, 1959, p. 305.

22. W. P. Jencks, Catalysis in Chemistry and Enzymology, McGraw-Hill, New York, 1969, Chapter 5.

23. R. Malkin and B. G. Malmström, Advan. Enzymol., 33, 177 (1970).

24. B. L. Vallee and R. J. P. Williams, Proc. Nat. Acad. Sci. (U.S.), 59, 498 (1968).

25. R. J. P. Williams, Inorg. Chim. Acta Rev., 5, 137 (1971).

26. J. W. Dawson, H. B. Gray, R. A. Holwerda, and E. W. Westhead, Proc. Natl. Acad. Sci. (U.S.), 69, 30 (1972).

27. W. Kauzmann, Adv. Prot. Chem., 14, 1 (1959).

28. B. G. Malmström, B. Reinhammar, and T. Vänngård, Biochim. Biophys. Acta, 156, 67 (1968).

29. C. B. Kasper, J. Biol. Chem., 243, 3218 (1968).

30. P. Day, Inorg. Chem., 5, 1619 (1966).

31. L. G. Sillen and A. E. Martell, Stability Constants of Metal-Ion Complexes, The Chemical Society (London) Special Publication 17, 1964).

32. T. Omura, J. Biochem. (Tokyo), 50, 305 (1961).

33. Y. T. Chia and E. L. King, Disc. Farad. Soc., 29, 109 (1960).

34. R. Snellgrove and E. L. King, J. Amer. Chem. Soc., 84, 4609 (1962).

35. R. Snellgrove and E. L. King, Inorg. Chem., 3, 288 (1964).

36. A. Haim, J. Amer. Chem. Soc., 88, 2324 (1966).

37. W. Beck and K. Schorpp, Angew. Chem. Int. Ed. Engl., 9, 735 (1970).

38. O. J. Parker and J. H. Espenson, Inorg. Chem., 8, 1523 (1969).

39. R. F. Ziolo, A. P. Gaughan, Z. Dori, C. G. Pierpont, and R. Eisenberg, Inorg. Chem., 10, 1289 (1971).

40. K. E. Falk and B. Reinhammar, Biochim. Biophys. Acta, 285, 84 (1972).

FERROCYANIDE REDUCTION OF LACCASE

I. Stoichiometry

To check the stoichiometry of the reaction between ferrocyanide and Rhus laccase, the 420 nm $Fe(CN)_6^{3-}$ absorption was used to quantitate product formation under rigorously anaerobic conditions at pH 7.0. Ferrocyanide (5.0×10^{-3} M) and laccase (1.69×10^{-5} M) solutions in dilute phosphate buffer were made rigorously anaerobic following normal deoxygenation procedures by the addition of the glucose-glucose oxidase oxygen scavenging system. They were then mixed in the stopped flow apparatus, and absorbance changes at 420 nm and 614 nm were recorded. The concentration of reduced Type 1 copper was obtained from ΔA_{614} using a previously determined value for the oxidized minus reduced extinction coefficient ($\Delta\epsilon_{614} = 4.5 \times 10^3\ M^{-1}cm^{-1}$, ϵ_{614} (ox) $= 4.7 \times 10^3\ M^{-1}\ cm^{-1}$, pH 7.0, 0.0463 M phosphate buffer). Ferricyanide production was evaluated using $1.01 \times 10^3\ M^{-1}\ cm^{-1}$ as $\Delta\epsilon_{420}$ for $Fe(CN)_6^{3-}$. Comparison of the two absorbance changes shows that 2.9 ± 0.2 moles of ferricyanide are produced for every mole of Type 1 copper reduced.

II. Ferrocyanide Concentration and Temperature Dependences of Laccase Reduction Rates; pH 7.0, $\mu = 0.1$

Although only a very narrow ferrocyanide concentration range is available at ionic strength 0.1, it was judged important to evaluate rates and activation parameters for all reducing agents under standard

conditions. Phosphate buffer (0.016 M, pH 7.0) and sodium nitrate maintained the ionic strength. Experiments at three different temperatures were performed using ferrocyanide concentrations in the range $1.5 \times 10^{-3} = [Fe(CN)_6^{4-}] \leq 4.0 \times 10^{-3}$ M. In addition to runs at 614 nm and 330 nm, room temperature measurements of the total rate of ferricyanide production were performed at 420 nm.

Absorbance decreases at 330 nm and 614 nm as well as absorbance increases at 420 nm yield linear first order analytical plots. Observed rate constants for all three wavelengths are assembled in Table 15. It is evident that 614 and 330 nm reduction rates increase linearly with ferrocyanide concentration over the small interval considered, but the least squares lines through the data points have smaller than zero intercepts. It appears that a higher than first order $[Fe(CN)_6^{4-}]$ dependence is obeyed at low reducing agent concentrations, but no data could be obtained to directly verify this.

Second order rate constants for Type 1 and Type 3 reduction obtained as the least squares slopes of k_{obs} vs. $[Fe(CN)_6^{4-}]$ plots are virtually identical at all temperatures considered. For example, $k_1(614) = 42.4 \pm 2.1\ M^{-1}\ sec^{-1}$, 25.7°; $k_1(330) = 42.7 \pm 2.2\ M^{-1}\ sec^{-1}$, 25.3°. The close agreement between 614 and 330 nm rates is further emphasized in an Eyring plot (Figure 20) based on experimental k_1 values. All of the data may be fit by a single line, indicating that the same rate-determining step governs reduction of both optically observable sites. The activation parameters derived from the log (k_1/T) vs. 1/T plot are: $\Delta H^{\ddagger} = 18.4 \pm 0.5$ kcal/mole; $\Delta S^{\ddagger} = +10 \pm 2$ cal/mol-deg.

Table 15

Rate Data for the Reduction of Laccase by Ferrocyanide Ion;

pH 7.0, $\mu = 0.1$

	$k_{obs}(sec^{-1})$		
		330 nm	
$[Fe(CN)_6^{4-}] \times 10^3 M$	12.8°	25.3°	36.0°
1.5	0.010	0.039	
	0.010	0.042	
2.0		0.058	0.222
		0.063	0.194
			0.216
2.5	0.018	0.081	
	0.019	0.081	
3.0		0.103	0.332
			0.332
4.0	0.037	0.147	0.463
	0.037	0.147	0.483
		614 nm	
	12.8°	25.7°	35.3°
1.5		0.048	
		0.047	
2.0	0.016	0.067	0.188
	0.015	0.070	0.180
2.5		0.086	0.258
		0.085	0.262
3.0	0.027	0.109	0.298
			0.310
4.0	0.037	0.155	
	0.036		

Table 15 (Continued)

	420 nm
	25.7°
1.5	0.048
	0.048
2.0	0.081
	0.082
	0.080
2.5	0.107
	0.103
3.0	0.132
	0.130
	0.130
4.0	0.169
	0.170

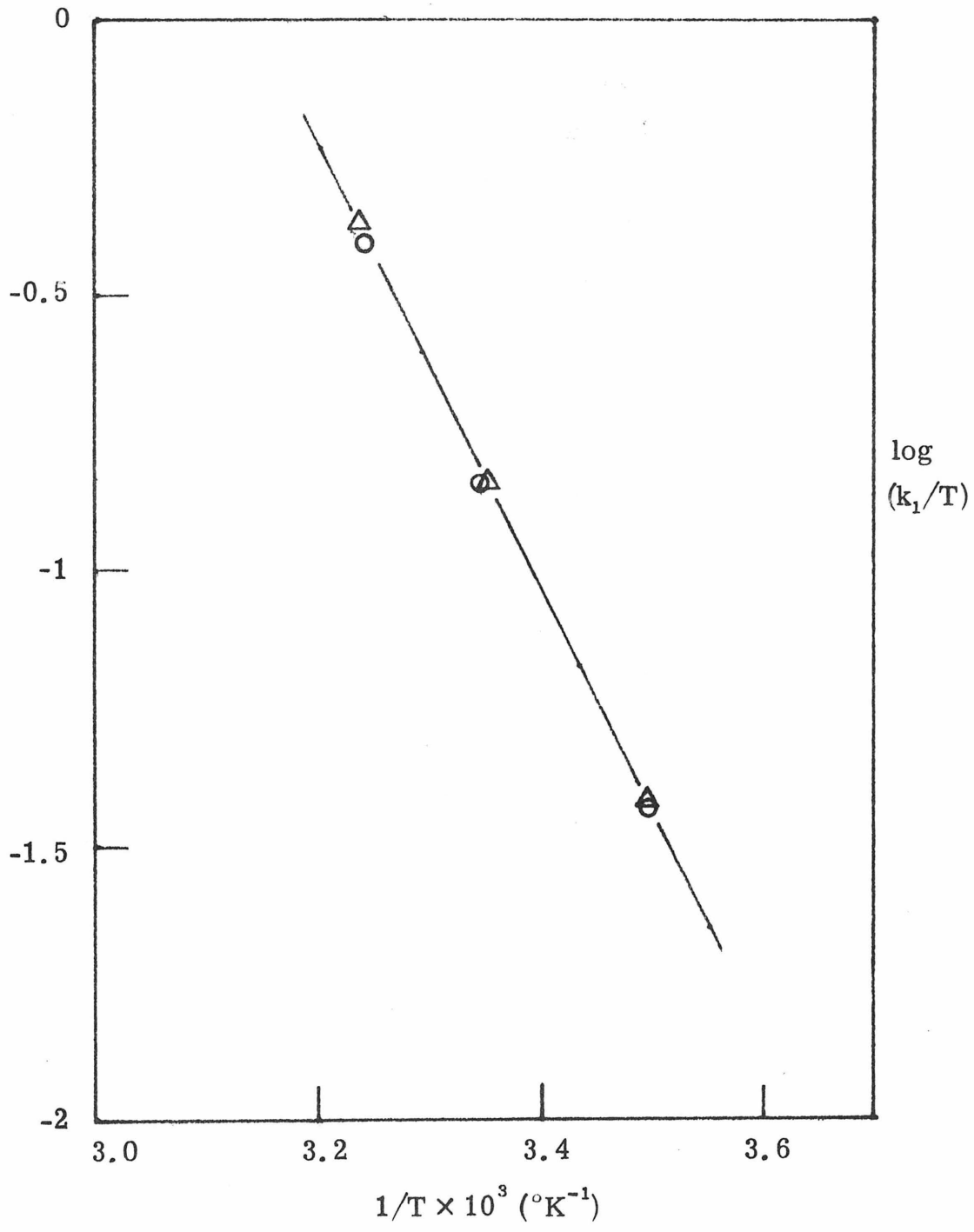


Figure 20. Eyring plot of the rate data for the reaction of ferrocyanide with laccase; pH 7.0, $\mu = 0.1$; ○, 614; Δ, 330 nm.

It was initially feared that the apparent agreement between Type 1 and Type 3 reduction rates might be an artifact caused by the ferricyanide-induced coupling of 614 nm absorbance changes to those at 330 nm. This suspicion was ruled out, however, after considering the 420 nm results. If the rates of reduction of Type 1 and Type 3 copper sites are indeed equal, then the following relationships should hold:

$$\frac{-d[Cu(614)]_{tot}}{dt} = \frac{1}{3}\frac{d[Fe(CN)_6^{3-}]}{dt}$$

$$= k_1[Fe(CN)_6^{4-}][(2,2,Ox)]$$

$$= k_1[Fe(CN)_6^{4-}]([laccase]_{tot} - \frac{1}{3}[Fe(CN)_6^{3-}])$$

where $[laccase]_{tot}$ refers to the total concentration of protein in solution, oxidized and reduced. From the last equation it is seen that the specific rate constant for ferricyanide production should be identical with k_1, the value already evaluated for reduction of "blue" copper. The k_{obs} (420) values vary linearly with $[Fe(CN)_6^{4-}]$, as expected, and a second order rate constant of $44.7 \pm 2.5\ M^{-1}\ sec^{-1}$ (25.7°) was calculated for $Fe(CN)_6^{3-}$ production. The agreement between this value and the corresponding room temperature results at the other two wavelengths is good evidence that there actually is only one rate characterizing Type 1 reduction, Type 3 reduction, and ferricyanide generation. The linearity of 330 nm log $(A_t - A_\infty)$ *vs.* time plots is fortuitous, the result of exact agreement between Type 1 and Type 3 reduction rates.

III. Ferrocyanide Concentration Dependence; pH 6.9, $\mu = 0.5$

The ionic strength 0.1 experiments did not allow a very wide range of ferrocyanide concentrations to be covered for kinetic measurements. Consequently, a room temperature 614 nm experiment was designed to allow ferrocyanide concentrations in the range 1.0×10^{-3} M $\leq [Fe(CN)_6^{4-}] \leq 1.5 \times 10^{-2}$ M to be used. All solutions were prepared in 0.133 M phosphate buffer, pH 6.9, and sodium nitrate again was used to maintain the ionic strength.

Kinetic results are given in Table 16 and plotted in Figure 21. Observed rate constants at 614 nm vary linearly with $[Fe(CN)_6^{4-}]$ only over a small concentration range: 1.0×10^{-3} M $\leq [Fe(CN)_6^{4-}] \leq 7.5 \times 10^{-3}$ M. Data for higher ferrocyanide concentrations deviate considerably from the best line drawn through the lower concentration points, and a smaller than zero intercept again is evident. The least squares line gives a value for k_1 of 16.1 ± 0.8 M^{-1} sec^{-1} at 24.9°. An attempt was made to fit all of the data to a rate law containing terms both first and second order in reducing agent concentration, but this was not successful.

IV. Correlation Between Anaerobic Reduction Rates and Ferrocyanide Turnover Rates in the Presence of Oxygen at $\mu = 0.5$

Although ferrocyanide only partially reduces laccase anaerobically, the enzyme is still capable of catalyzing the oxidation of $Fe(CN)_6^{4-}$ by oxygen. Solutions of ferrocyanide and laccase left open to the air rapidly turn bright yellow, and observations at 420 nm show continuing

Table 16

Rate Data for the Reduction of

Laccase by $Fe(CN)_6^{4-}$;

614 nm, 24.9°, pH 6.9, $\mu = 0.5$

$[Fe(CN)_6^{4-}] \times 10^3$(M)	k_{obs}(sec^{-1})
1.0	0.011
	0.011
2.5	0.030
	0.029
5.0	0.072
	0.070
7.5	0.110
	0.116
10.0	0.171
	0.172
15.0	0.382
	0.395
	0.384

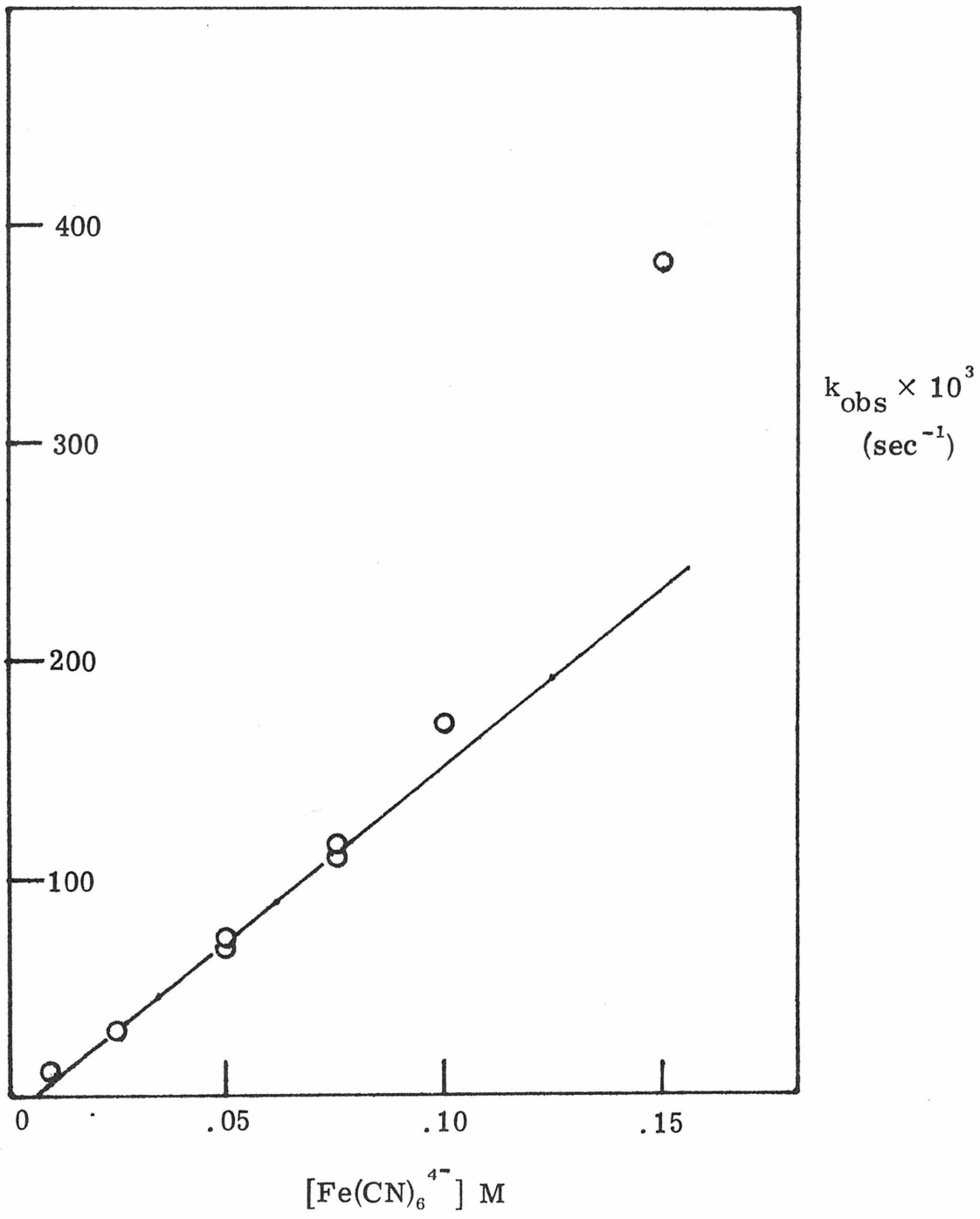


Figure 21. Plot of k_{obs} vs. $[Fe(CN)_6^{4-}]$ for the reaction of ferrocyanide with laccase; 614 nm, 24.9°, pH 6.9, $\mu = 0.5$.

increases in absorbance. The solutions prepared for the ionic strength 0.5 concentration dependence study were also used for measurements of aerobic ferrocyanide turnover rates at 420 nm. No care was taken to exclude oxygen in these runs, and turnover rates were evaluated from the slopes of A_{420} vs. time traces shortly after mixing. A_{420} vs. time traces were linear initially before oxygen consumption was significant, indicating a constant rate of ferricyanide generation.

Table 17 lists turnover rates, values of the observed turnover constant $k_{obs}(420, O_2)$ = (turnover rate)/$[\text{laccase}]_{tot}$, and compares $k_{obs}(420, O_2)$ with average $k_{obs}(614)$ values found under the same conditions. For each of the five ferrocyanide concentrations considered, the ratio $k_{obs}(420, O_2)/k_{obs}(614)$ is very nearly equal to 4. The average value of this ratio for all concentrations is 4.1 ± 0.2.

V. Ionic Strength Dependence

A series of pH 7.0 phosphate buffers was used in a study of the ionic strength dependence of laccase reduction rates with ferrocyanide. As for the hydroquinone experiments, laccase was initially prepared in an ionic strength 0.05 buffer. The ferrocyanide concentration was 2.5×10^{-3} M throughout, and phosphate buffer and ferrocyanide alone contributed to the ionic strength.

Observed rate constants may be found in Table 18, and Figure 22 contains a plot of $k_{obs}(614, 330)$ against ionic strength. As expected, the 330 nm and 614 nm data are indistinguishable to within experimental error over the entire range of ionic strength from 0.075 to 0.536.

Table 17

Turnover Rates for the Laccase-Catalyzed Oxidation of $Fe(CN)_6^{4-}$ by O_2; 420 nm, 24.9°, pH 6.9, $\mu = 0.5$

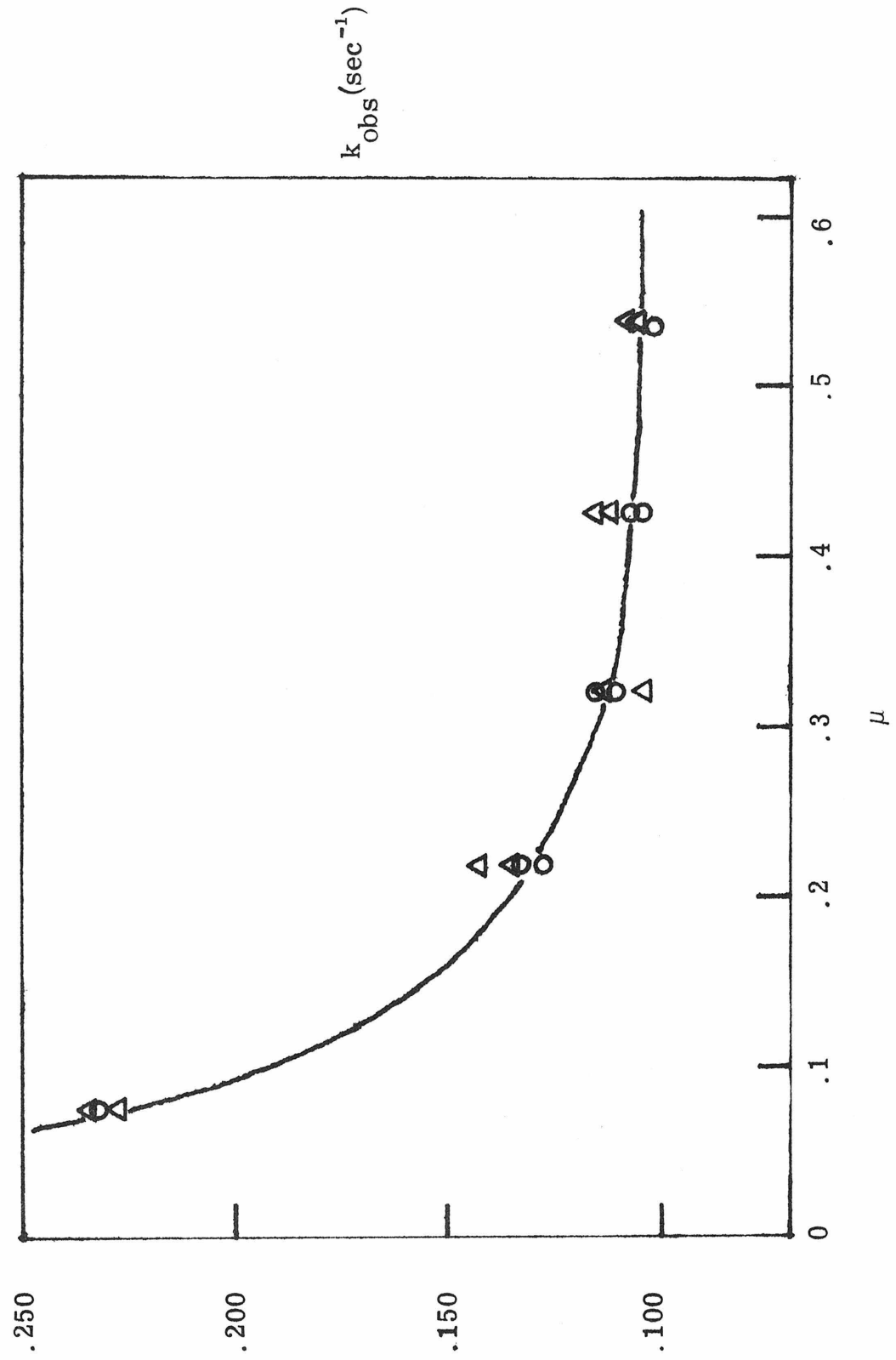
$[Fe(CN)_6^{4-}] \times 10^3$(M)	Turnover Rate $\times 10^6$(M sec^{-1})	$k_{obs}(420, O_2)(sec^{-1})$	$\frac{k_{obs}(420, O_2)}{k_{obs}(614)}$
2.5	1.15	0.138	4.6
	1.17	0.140	4.7
5.0	2.44	0.292	4.1
	2.45	0.293	4.1
7.5	3.90	0.466	4.1
	3.96	0.474	4.2
10.0	5.85	0.700	4.1
	5.66	0.677	4.0
15.0	11.81	1.413	3.6
	12.36	1.480	3.8
	11.64	1.393	3.7

Table 18

Ionic Strength Dependence of Observed Rate Constants for the Reaction of Ferrocyanide Ion with Laccase; 25.5°, pH 7.0, $[Fe(CN)_6^{4-}] = 2.5 \times 10^{-3}$ M

$[\text{phosphate}]_{tot}$	μ	k_{obs} (sec^{-1}) 614 nm	k_{obs} (sec^{-1}) 330 nm
0.0241	0.075	0.231	0.234
		0.232	0.227
0.0836	0.218	0.132	0.134
		0.127	0.142
0.125	0.320	0.110	0.104
		0.115	0.113
0.167	0.425	0.107	0.112
		0.104	0.115
0.211	0.536	0.100	0.108
		0.102	0.106

Figure 22. Ionic strength dependence of observed rate constants for the reduction of laccase by ferrocyanide; 25.5°, pH 7.0, $[Fe(CN)_6^{4-}]$ $= 2.5 \times 10^{-3}$ M; ○, 614 nm; Δ, 330 nm.


$k_{obs}(sec^{-1})$
.250
.200
.150
.100
0
.1
.2
.3
.4
.5
.6
μ

The observed rate constants drop by ca. 56% over this same interval.

VI. pH Dependence

The effect of $[H^+]$ on the rate of laccase reduction by ferrocyanide was evaluated at 614 nm, 24.9°, using ionic strength 0.3 phosphate buffers in the range pH 5-8. A constant ferrocyanide concentration of 0.02 M made up the total ionic strength to 0.5 in each case. Separate protein solutions were made up in the appropriate buffer for each pH considered.

Observed rate constants as a function of pH are tabulated in Table 19 and illustrated in Figure 23. The variation of rate with hydrogen ion concentration for $Fe(CN)_6^{4-}$ as reductant clearly is much different than it is with H_2Q as reductant. The rate now increases with decreasing pH, and its variation with $[H^+]$ is not as simple as the inverse hydrogen ion dependence found earlier for hydroquinone. The plot in Figure 23 resembles a titration curve, and it is suspected that ionization of a particular amino acid side chain is responsible for the observed trend in laccase reduction rates with pH. Assuming this to be the case, the following mechanism was used to interpret the data.

$$Fe(CN)_6^{4-} + (2,2,Ox) \cdot H^+ \xrightarrow{k_p} \text{products}$$

$$Fe(CN)_6^{4-} + (2,2,Ox) \xrightarrow{k_u} \text{products}$$

$$(2,2,Ox) \cdot H^+ \overset{K_H}{\rightleftharpoons} (2,2,Ox) + H^+$$

Table 19

pH Dependence of Observed Rate Constants for the Reaction of Laccase with Ferrocyanide Ion; 614 nm, 24.9°, $\mu = 0.5$, $[Fe(CN)_6^{4-}] = 0.02$ M

pH	k_{obs} (sec^{-1})
5.20	3.26
	3.78
5.66	3.58
	3.57
6.17	2.59
	2.63
6.55	1.82
	1.85
6.98	1.01
	1.03
7.53	0.405
	0.399
8.00	0.310
	0.303

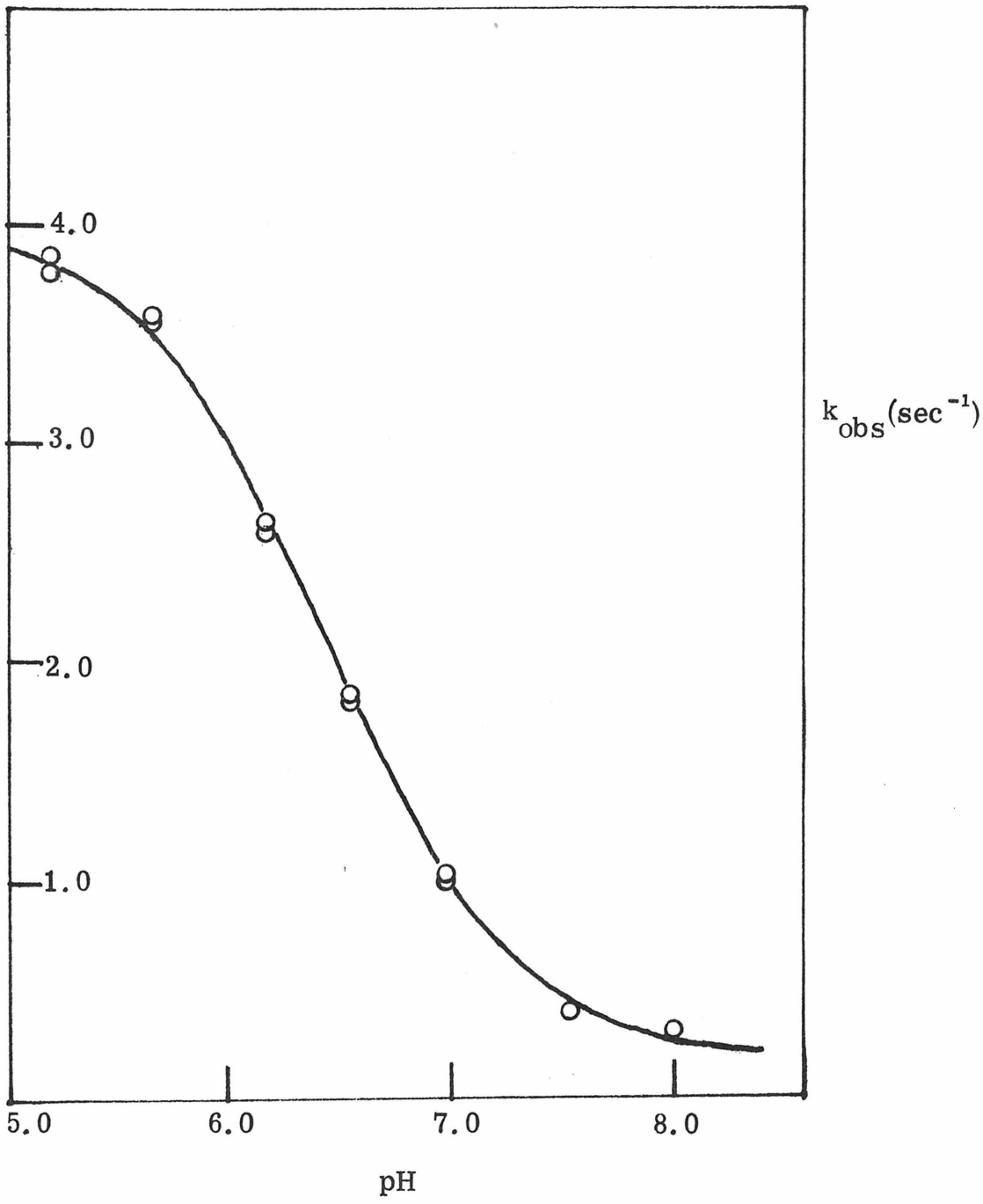


Figure 23. Plot of k_{obs} vs. pH for the reaction of ferrocyanide with laccase; 614 nm, 24.9°, $\mu = 0.5$, $[Fe(CN)_6^{4-}] = 0.02$ M.

The species (2, 2, Ox) and (2, 2, Ox) · H^+ represent unprotonated and protonated forms of the metalloprotein, respectively, with k_p and k_u the associated specific reduction rate constants. The equilibrium between unprotonated and protonated forms, governed by the acid ionization constant K_H, is assumed to be rapid.

The rate law derived from this mechanism is:

$$\frac{-d[Cu(614)]}{dt}_{tot} =$$

$$[Fe(CN)_6^{4-}](k_p[2,2,Ox) \cdot H^+] + k_u[(2,2,Ox)])$$

$$= \left(\frac{k_p[H^+] + k_u K_H}{[H^+] + K_H}\right)[Fe(CN)_6^{4-}][Cu(614)]_{tot}$$

$$= k_{obs}(614)\,[Cu(614)]_{tot}$$

The experimental data were fit to the theoretical expression as follows. The relationship for $k_{obs}(614)$ was rearranged to:

$$K_H = [H^+]\left(\frac{k_p[Fe(CN)_6^{4-}] - k_{obs}(614)}{k_{obs}(614) - k_u[Fe(CN)_6^{4-}]}\right)$$

and trial values for $k_p[Fe(CN)_6^{4-}]$ and $k_u[Fe(CN)_6^{4-}]$ were chosen by inspecting the data. Using these values in the expression on the right hand side of the equation, apparent K_H values were obtained for each $k_{obs}(614)$, $[H^+]$ pair and the standard deviation of the mean for K_H was calculated. New trial values were then chosen, and the calculation was iterated until a minimum in the standard deviation of K_H was reached. The parameters yielding a minimum in $\sigma(K_H)$ are: $k_p[Fe(CN)_6^{4-}]$ =

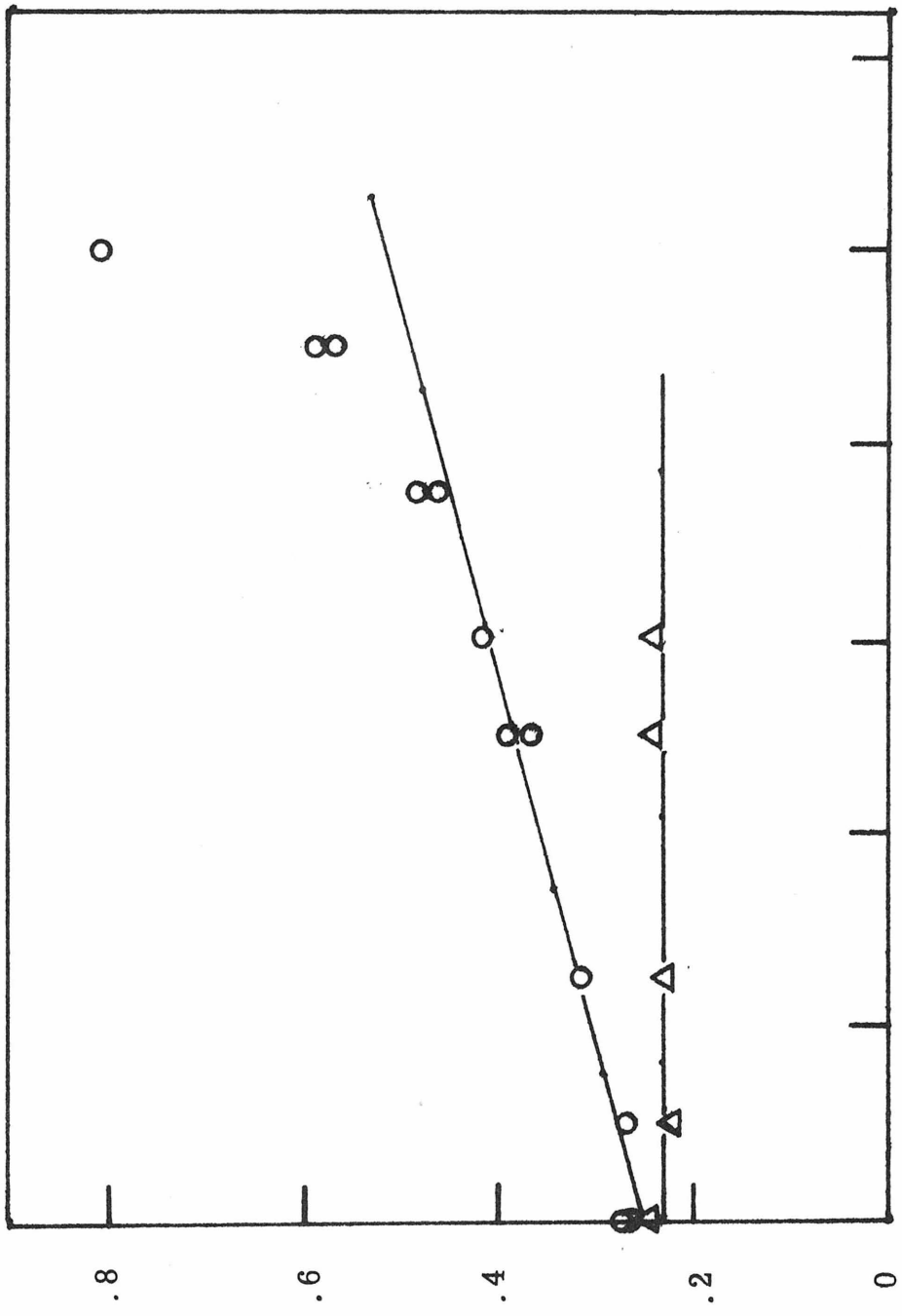
4.03 sec^{-1}, $k_u[Fe(CN)_6^{4-}] = 0.16\ sec^{-1}$, and $K_H = 3.53 \pm 0.63 \times 10^{-7}$ M ($pK_H = 6.45 \pm 0.09$). The curve drawn through the data points in Figure 23 is the calculated dependence of k_{obs} on pH using these parameters, and clearly a good fit of the experimental observations is achieved.

VII. Effect of Ferricyanide

The standard reduction potentials of laccase copper sites are so close to that of ferricyanide that it ought to be possible to reoxidize reduced laccase with $Fe(CN)_6^{3-}$ under appropriate concentration conditions. Comparable concentrations of ferro- and ferricyanide were added to oxidized laccase in experiments described in this section, and relaxation to redox equilibrium was followed at 614 nm. All solutions were prepared in 1.85×10^{-2} M phosphate buffer, pH 6.9, the concentration of ferrocyanide was 1.0×10^{-2} M throughout, and sodium nitrate was again used to maintain the ionic strength. The ferricyanide concentration was varied from zero to 1.0×10^{-2} M.

The extent of 614 nm absorbance changes decreases markedly with increasing ferricyanide concentration, consistent with the reaction between $Fe(CN)_6^{4-}$ and laccase coming to equilbrium short of full reduction of available "blue" copper sites. Decay of 614 nm absorbance to its equilibrium value (A_e) is first order, since plots of log ($A_t - A_e$) vs. time were found to be linear. Observed rate constants based on these plots are given in Table 20 and are plotted against ferricyanide concentration in Figure 24. As a check that any

Figure 24. Effect of hexacyanometallate ions on k_{obs} for the reaction of ferrocyanide with laccase; 614 nm, 25.3°, pH 7.0, $\mu = 0.2$, $[Fe(CN)_6^{4-}] = 0.01$ M; ○, $Fe(CN)_6^{3-}$; Δ, $Cr(CN)_6^{3-}$.


$k_{obs}(sec^{-1})$
.8
.6
.4
.2
0
2
4
6
8
10
$[M(CN)_6^{3-}] \times 10^3 M$

rate effects seen with ferricyanide are related to redox processes, some blank runs were performed keeping the conditions constant but substituting the redox-inert hexacyanochromate(III) ion for ferricyanide. These results also appear in Table 20 and in Figure 24.

The variation of k_{obs} with $[Fe(CN)_6^{3-}]$ at constant $[Fe(CN)_6^{4-}]$ is linear up to about 7.5×10^{-3} M where observed rate constants begin to deviate high from the best line drawn through the low concentration points. Observed rate constants measured in the absence of added ferricyanide are in good agreement with the intercept of the least squares line. The data may be understood in terms of the rate law expected for the approach of $Fe(CN)_6^{4-}$ and Cu(II) to redox equilibrium.

$$Fe(CN)_6^{4-} + Cu(II) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe(CN)_6^{3-} + Cu(I)$$

$$-\frac{d[Cu(II)]}{dt} = (k_1[Fe(CN)_6^{4-}] + k_{-1}[Fe(CN)_6^{3-}])([Cu(II)] - [Cu(II)]_{eq})$$

$$= k_{obs}([Cu(II)] - [Cu(II)]_{eq})$$

The rate law has been derived for the present conditions where both ferro- and ferricyanide concentrations are effectively constant throughout the reaction. The slope of the ferricyanide dependence plot ($26.8\ M^{-1}\ sec^{-1}$) is thus assigned to k_{-1}, and the intercept is, as expected, $k_1[Fe(CN)_6^{4-}]$. The value of k_1 is estimated to be $24.9\ M^{-1}\ sec^{-1}$ under these conditions (pH 6.9, $\mu = 0.2$, 25.1°).

Table 20

Effect of Hexacyanometallate(III) Ions on Observed Rate Constants for the Reaction of Ferrocyanide Ion with Laccase; 614 nm, 25.3°, pH 7.0 ± 0.1, $\mu = 0.2$, $[Fe(CN)_6^{4-}] = 0.1$ M

$[M(CN)_6^{3-}] \times 10^3$(M)	$k_{obs}(sec^{-1})$	
	$Fe(CN)_6^{3-}$	$Cr(CN)_6^{3-}$
0.0	0.245	0.247
	0.270	0.240
	0.257	
1.0	0.269	0.225
	0.267	0.217
2.5	0.312	0.223
	0.316	0.232
5.0	0.364	0.238
	0.391	0.253
	0.387	
6.0	0.419	0.241
	0.408	0.239
7.5	0.463	
	0.483	
9.0	0.569	
	0.588	
10.0	0.804	
	0.811	

Full reduction of laccase 614 nm absorbance was still achieved in the presence of added $K_3Cr(CN)_6$ and no variation in k_{obs} with $[Cr(CN)_6^{3-}]$ was found over the same concentration range used in the ferricyanide experiments. It seems safe to conclude then that the increases in k_{obs} with added $Fe(CN)_6^{3-}$ are redox-related and not attributable to interaction between the anion and laccase.

Knowing the forward and reverse rate constants, the equilibrium constant for the reaction between ferrocyanide and the initial copper site attacked by this reducing agent may be evaluated:

$$K_{eq} = \frac{[Cu(I)][Fe(CN)_6^{3-}]}{[Cu(II)][Fe(CN)_6^{4-}]} = \frac{k_1}{k_{-1}} = 0.93$$

From K_{eq} and the known standard reduction potential for ferricyanide under the present experimental conditions[1] ($E^0_{Fe(CN)_6^{3-}} = +0.42$ volt, 25°, $\mu = 0.2$, sodium phosphate media), the E^0 value of the reactive laccase copper site may be calculated:

$$\log_{10} K_{eq} = \frac{(E^0_{Cu(II)} - E^0_{Fe(CN)_6^{3-}})}{0.0592}$$

$$= -0.0315$$

$$E^0_{Cu(II)} = E^0_{Fe(CN)_6^{3-}} - 0.002 \text{ volt}$$

$$\cong +0.42 \pm 0.02 \text{ volt}$$

VII. Reduction of the Laccase-Fluoride Complex; pH 7.0

Several room temperature kinetic runs at varying $[Fe(CN)_6^{4-}]$ were performed for pH 7.0, $\mu = 0.1$ pre-equilibrating laccase with 0.02 M NaF. The observations at 614 and 330 nm were complicated,

however, and not readily susceptible to analysis. As for the ferricyanide experiments, the extent of reduction varies considerably with $[Fe(CN)_6^{4-}]$. In retrospect, this is not surprising in light of decreases in laccase reduction potentials known to accompany fluoride binding.[2] Observed rate constants were calculated for $1.5 \times 10^{-3} \leq [Fe(CN)_6^{4-}] \leq 5.0 \times 10^{-3}$ M, but their quantitative significance is doubtful considering they do not correspond to a reaction going all the way to completion. The numbers will not be listed here, but several qualitative observations are important to note. Unlike the hydroquinone reaction with fluoride-inhibited protein, no dramatic decrease in the rate of 330 nm absorbance decay was observed. The 614 and 330 nm rate constants are still equal to within experimental error, and their values are comparable to those found for the native enzyme.

FERROUS(ETHYLENEDIAMINETETRAACETATE) REDUCTION OF LACCASE AND STELLACYANIN

I. Comparison of Laccase and Stellacyanin "Blue" Copper Reactivities with $Fe(EDTA)^{2-}$

Experiments with $Fe(EDTA)^{2-}$ provide an opportunity to compare the reactivities of laccase and stellacyanin "blue" copper sites with a common reducing agent; $Fe(EDTA)^{2-}$ is a strong enough reductant (E^{0} = +120 mv, pH 7.0)[3] to fully reduce both proteins under physiological conditions. The reduction rates of laccase and stellacyanin "blue" sites by $Fe(EDTA)^{2-}$ were evaluated under conditions almost exactly duplicating those for the $Fe(CN)_6^{4-}$ concentration dependence study at $\mu = 0.5$ (0.133 M phosphate buffer, pH 6.9, 25.1°). Stellacyanin reduction was followed at its "blue" absorption maximum, 604 nm. Since nitrate ion is attacked by strong Fe(II) reductants, ammonium sulfate was substituted in these experiments to maintain the ionic strength.

Laccase and stellacyanin "blue" sites are both rapidly attacked by $Fe(EDTA)^{2-}$, and first order plots of log $(A_t - A_\infty)$ vs. time are nicely linear for both proteins. Observed rate constants are collected in Table 21, and Figure 25 illustrates the first order $[Fe(EDTA)^{2-}]$ variation obeyed by the laccase results. Stellacyanin reduction was so fast that the reaction could only be detected for the two smallest reducing agent concentrations employed. The stellacyanin results are

Table 21

Rate Data for the Reduction of Laccase and Stellacyanin by $Fe(EDTA)^{2-}$; 25.1°, pH 6.9, $\mu = 0.5$

	Laccase	Stellacyanin
$[Fe(EDTA)^{2-}] \times 10^{3}$(M)	$k_{obs}(614)(sec^{-1})$	$k_{obs}(604)(sec^{-1})$
0.5	0.068	202.2
	0.074	206.5
1.0	0.212	419.5
	0.216	416.3
	0.211	
	0.219	
2.5	0.615	
	0.615	
5.0	1.176	
	1.290	
10.0	2.284	
	2.218	

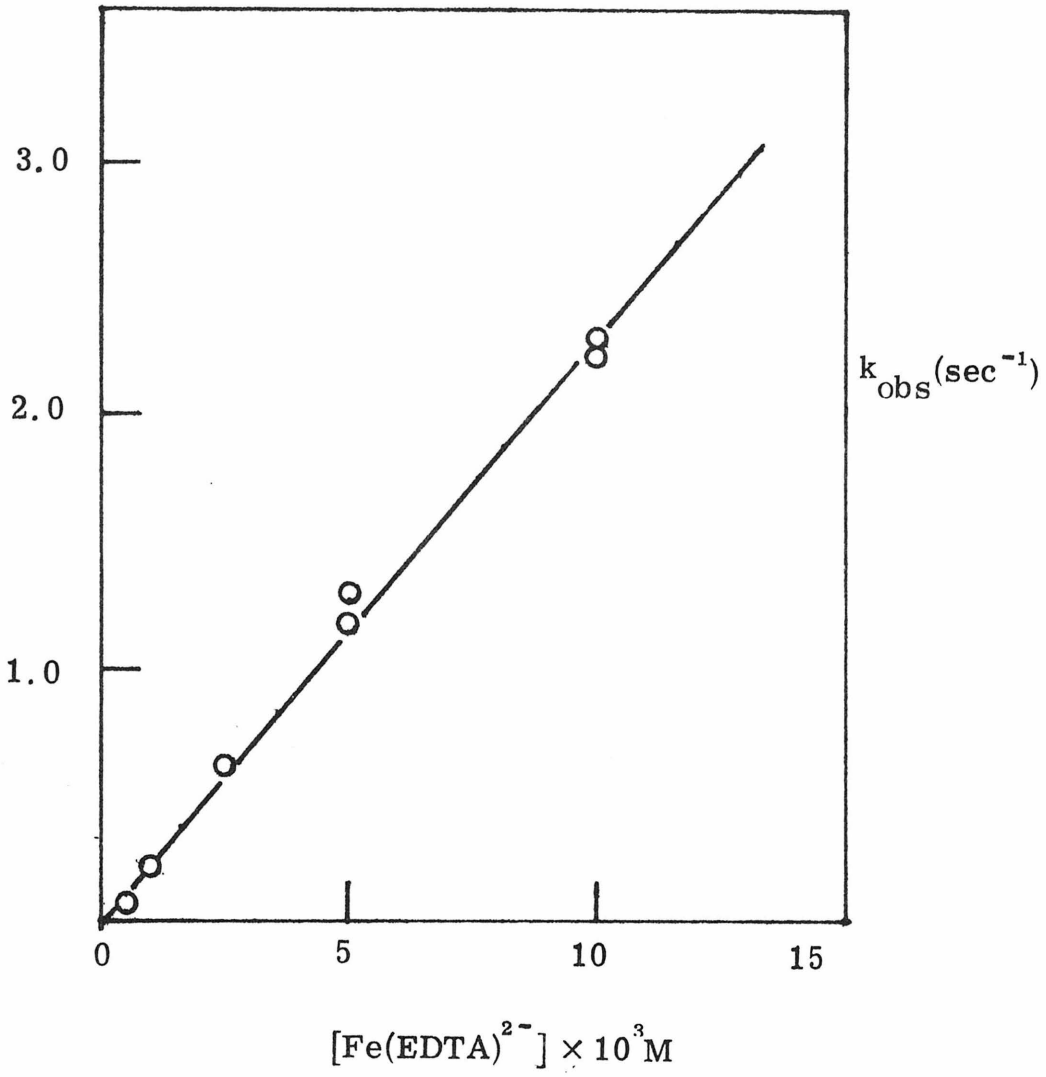


Figure 25. Plot of k_{obs} vs. $[Fe(EDTA)^{2-}]$ for the reduction of laccase by $Fe(EDTA)^{2-}$; 614 nm, 25.1°, pH 6.9, $\mu = 0.5$.

consistent with a bimolecular rate law characterized by a second order rate constant of $4.2 \pm 0.1 \times 10^5\ M^{-1}\ sec^{-1}$.

Unlike the analogous ferrocyanide reaction, $Fe(EDTA)^{2-}$ reduction of laccase "blue" copper follows a second order rate law:

$$\frac{-d[Cu(614)]}{dt}_{tot} = k_1[Fe(EDTA)^{2-}][Cu(614)]_{tot}$$

The value of k_1 ($231 \pm 12\ M^{-1}\ sec^{-1}$) is over 1000 times smaller than the rate constant for stellacyanin reduction, but is about 14 times larger than k_1 for $Fe(CN)_6^{4-}$ reduction of laccase "blue" copper under the same conditions.

II. Temperature Dependence of the $Fe(EDTA)^{2-}$ Reduction Rate of Laccase "Blue" Copper

The temperature dependence of the laccase "blue" copper reduction rate with $Fe(EDTA)^{2-}$ was measured at $\mu = 0.1$, pH 7.0 (0.0185 M phosphate). Only two reducing agent concentrations were used at each temperature, assuming the first order $[Fe(EDTA)^{2-}]$ dependence found at $\mu = 0.5$ would still hold. Observed rate constants as a function of temperature are given in Table 22, and Figure 26 shows an Eyring plot of the data. The activation parameters calculated from this plot are: $\Delta H^{\ddagger} = 13.0 \pm 0.5$ kcal/mol, $\Delta S^{\ddagger} = -4 \pm 2$ cal/mol-deg. These numbers should be regarded as tentative pending the completion of hydrogen ion dependence experiments for this system.

Table 22

Temperature Dependence of Observed Rate Constants for the Reaction of $Fe(EDTA)^{2-}$ with Laccase; 614 nm, pH 7.0, $\mu = 0.1$ *

Temperature	$k_{obs}(sec^{-1})$ A	$k_{obs}(sec^{-1})$ B
8.3°	0.065	0.175
	0.067	0.176
16.7°	0.142	0.373
	0.138	0.387
25.7°	0.251	0.759
	0.259	0.763
31.6°	0.395	1.23
	0.403	1.19
38.2°	0.675	2.02
	0.658	1.89

*$[Fe(EDTA)^{2-}] = 1.09 \times 10^{-3}$ M (A), 2.73×10^{-3} M (B)

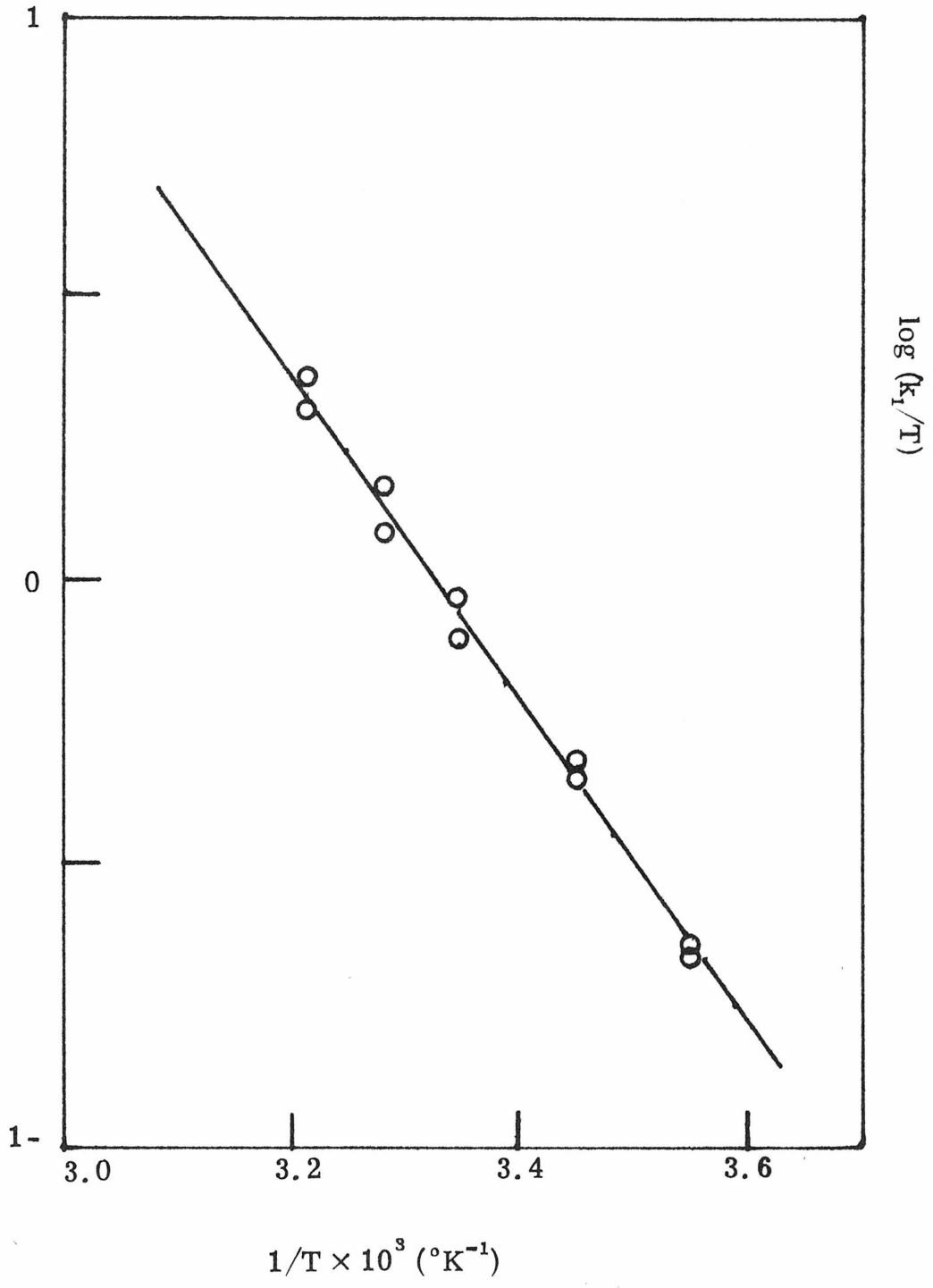


Figure 26. Eyring plot of the rate data for reduction of laccase by $Fe(EDTA)^{2-}$; 614 nm, pH 7.0, $\mu = 0.1$.

The ionic strength effect on k_1 for the $Fe(EDTA)^{2-}$ reduction of Cu(614) is quite different than for the analogous reaction with ferrocyanide. The second order rate constant extrapolated from the Eyring plot for 25.1° (249 M^{-1} sec^{-1}) is in essential agreement with the ionic strength 0.5 result at this temperature.

References

1. I. M. Kolthoff and W. J. Tomsicek, J. Phys. Chem., 39, 945 (1935).

2. B. Reinhammar, Biochim. Biophys. Acta, 275, 245 (1972).

3. R. Belcher, D. Gibbons, and T. S. West, Anal. Chim. Acta, 12, 107 (1955).

DISCUSSION

The kinetic results for the reaction of Rhus laccase with ferrocyanide are complicated in a number of ways. The variation of observed rate constants with the reducing agent concentration deviates from first order at both low and high concentrations, and the metalloprotein is reduced by only three electrons anaerobically under conditions where thermodynamic considerations suggest that it ought to be fully reduced at equilibrium. Ferrocyanide also reduces fungal laccase A by only three electrons anaerobically,[1] but fungal laccase B is reported to be fully reduced by this redox agent.[2] Since full reduction of the Type 1 and Type 3 copper sites is evident from observations at 614 and 330 nm, it seems likely that Type 2 Cu(II) is not reduced by $Fe(CN)_6^{4-}$ on the time scale of our experiments.

Nevertheless, interaction between hexacyanoferrate(II) and Type 1 Cu(II) is evident from changes in the metalloprotein ESR spectrum induced even by stoichiometric concentrations of the reducing agent.[3] We propose that $Fe(CN)_6^{4-}$ associates with laccase at a substrate-binding position in the vicinity of Type 2 Cu(II), but prefers, for reasons related to its high negative charge or low reducing strength, a reduction mechanism involving initial attack at an alternative copper site. This proposal is outlined in the scheme given below:

$$Fe(CN)_6^{4-} + (2,2,Ox) \underset{}{\overset{K_d}{\rightleftharpoons}} (2,2,Ox) \cdot Fe(CN)_6^{4-}$$

$$Fe(CN)_6^{4-} + (2,2,Ox) \cdot Fe(CN)_6^{4-} \xrightarrow{k_1} \text{products}$$

where $(2,2,Ox) \cdot Fe(CN)_6^{4-}$ refers to the laccase-ferrocyanide complex. The rate law expected from this reaction sequence is:

$$\frac{-d[(2,2,Ox)]_{tot}}{dt} = \frac{k_1 K_c [Fe(CN)_6^{4-}]^{2-} [(2,2,Ox)]_{tot}}{1 + K_c [Fe(CN)_6^{4-}]}$$

The quantity $[(2,2,Ox)]_{tot}$ indicates the total quantity of fully oxidized laccase in solution. This mechanism predicts, as we have observed, a greater than first order dependence of observed rate constants on $[Fe(CN)_6^{4-}]$ at low concentrations. For $K_c[Fe(CN)_6^{4-}] >> 1$, the rate is expected to follow a first order reducing agent dependence with $k_{obs} = k_1[Fe(CN)_6^{4-}]$. It is important to note that second order rate constants reported here for the reaction between ferrocyanide and laccase probably pertain to reduction of a ferrocyanide-complexed species and not the native enzyme.

Deviations from a first order $[Fe(CN)_6^{4-}]$ dependence at high reducing agent concentrations do not appear to correspond to authentic terms in the rate law higher than first order in the hexacyanoferrate(II) concentration. Rather, it is suspected that the anomalous high concentration rates reflect a breakdown in this case of the standard kinetic technique of keeping the ionic strength constant to minimize rate effects induced by changes in the medium. This technique is very effective when one 1:1 electrolyte is replaced by another; proportions of $HClO_4$ and $LiClO_4$ are often varied in the course of kinetic acid dependence studies, for example. Changes in the concentration of a 1:4 electrolyte ($K_4Fe(CN)_6^{4-}$) were balanced by the addition or

subtraction of a 1:1 electrolyte ($NaNO_3$) in this study, and it is not clear that the effective ionic strength will remain constant over large variations in the relative concentrations of the two salts.

The inability of partially reduced fungal laccase to rapidly reduce oxygen[1] leads to the plausible expectation that the Rhus enzyme should not be an effective catalyst for the oxidation of $Fe(CN)_6^{4-}$ by O_2. Nevertheless, our observations demonstrate that laccase catalysis of this reaction does occur even though the metalloprotein only accepts three electrons from $Fe(CN)_6^{4-}$ anaerobically. A rationale for this seemingly contradictory phenomenon is presented below.

$$3Fe(CN)_6^{4-} + (2,2,Ox) \xrightarrow{k_1} 3Fe(CN)_6^{3-} + (1,2,Red)$$

$$Fe(CN)_6^{4-} + (1,2,Ox) + O_2 + 4H^+ \xrightarrow{fast} Fe(CN)_6^{3-} + (2,2,Ox) + 2H_2O$$

This scheme is not intended to serve as a mechanism, but rather emphasizes what probably are the essential features of ferrocyanide turnover. The second step implies that oxygen rapidly induces the oxidation of a fourth ferrocyanide ion, either by causing the enzyme to accept four electron equivalents or by reacting with $Fe(CN)_6^{4-}$ directly.

Making the steady-state approximation for (1,2,Red), the aerobic ferrocyanide turnover rate expected from the proposed scheme is:

$$\frac{d[Fe(CN)_6^{3-}]}{dt} = 4k_1[Fe(CN)_6^{4-}][(2,2,Ox)] = k_{obs}(420,O_2)[(2,2,Ox)]$$

This expression is consistent with our experimental findings, as a constant turnover rate is expected when the ferrocyanide concentration is large enough to be effectively constant. In addition, our results confirm that four electrons are turned over to oxygen at a rate determined by that of the initial reduction step; the experimental ratio of $k_{obs}(420,O_2)/k_{obs}(614)$ is in excellent agreement with the predicted value of 4.

It is pleasing to note that quantitative results of anaerobic laccase reduction experiments are applicable to the understanding of aerobic ferrocyanide turnover. However, the correlation between observed rate constants for reduction and aerobic turnover only partially justifies the hypothesis that the overall mechanism of laccase action may be treated as the sum of separable reduction and reoxidation processes. On the one hand, the observed turnover rates suggest that oxygen does not influence the rate of the initial laccase reduction step. On the other hand, it is clear that oxygen does induce substrate oxidation beyond what occurs anaerobically.

The ionic strength and pH dependences of observed rate constants for the reaction between hydroquinone and laccase were instrumental pieces of evidence leading to the proposal that the phenolate monoanion attacks Type 2 Cu(II) in the slow step for this reaction. The analogous rate dependences for $Fe(CN)_6^{4-}$ as reductant support our suggestion that the rate-determining step governing reduction of both the Type 1 and

Type 3 copper sites involves reducing agent attack at a position other than Type 2 Cu(II). The hydroquinone results could be rationalized considering factors influencing the extent of ionization of hydroquinone to the monoanion. No evidence was obtained suggesting that the ionization state of laccase amino acid side chains is an important influence on reduction rates in the range $5 \leq pH \leq 8$. For ferrocyanide, this clearly is not the case. The pH dependence results show that a 25-fold rate decrease accompanies ionization of a group (or groups) with pK = 6.45. Acid-base titrations of a number of proteins have indicated that the histidine imidazole group typically ionizes with $6.4 \leq pK \leq 6.9$, and no other side chain is expected to have a pK value falling between 6 and 7.[4] Rhus laccase contains about 17 histidine residues per mole.[5]

The influence of the ionization state of histidine on the rate of the reaction between laccase and ferrocyanide strongly suggests that at least one imidazolium cation has an important function in attracting the highly anionic reductant to its attack site. The fact that the rate decreases markedly with increasing ionic strength is compatible with this hypothesis. Bennett[6] has distinguished between normal outer-sphere reduction and outer-sphere reduction involving pre-equilibrium ion-pair formation in considering the mechanistic alternatives for reactions between metalloproteins and inorganic redox agents.[6] Rate-determining electron transfer within an ion-pair intermediate has been documented for the reaction between $Fe(CN)_6^{4-}$ and $Co(NH_3)_5OH_2^{3+}$.[7]

Rate saturation with increasing reducing agent concentration is expected under circumstances where the ion-pair formation constant is large, but the complicated rate behavior at high $[Fe(CN)_6^{4-}]$ prevents the detection of such an effect, if it exists, for the reaction between ferrocyanide and laccase.

Our value of 0.42 ± 0.02 volt for the reduction potential of the copper site initially attacked by ferrocyanide is similar to that obtained for Type 1 Cu(II) under conditions similar to ours (0.432 volt; pH 6.8, $\mu = 0.35$, [hexacyanoferrate]/[laccase] = 100).[8] Although Type 1 copper appears to be the best choice, the reduction potentials of all laccase copper sites fall within the range 0.35 - 0.50 volt,[3] and a well-defined assignment is not possible in view of the reported[3] dependence of E^0 values on the hexacyanoferrate concentration.

If Type 1 Cu(II) is the initial attack site, then the subsequent rate of reduction of the 330 nm chromophore must be very fast for $k_{obs}(614)$ and $k_{obs}(330)$ to be equal. Low concentrations of fluoride ion inhibit reduction of the Type 3 site by hydroquinone at pH 7.0, but no inhibition is evident with ferrocyanide as reductant. Specific interaction of F^- with the Type 3 unit has been suggested to account for the hydroquinone results; one possible explanation is that the rate of a Type 2 Cu(I) to Type 3 Cu(II)-Cu(II) intramolecular electron transfer step is drastically decreased by structural rearrangements accompanying fluoride binding. Fluoride probably does not inhibit the reaction of ferrocyanide with laccase at pH 7 because an intramolecular redox reaction is not part of the reduction mechanism.

Kinetic results for the reaction of hydroquinone with laccase indicate that the Type 1 copper is susceptible to reduction only after a slow step involving attack at the Type 2 copper atom. The highly unfavorable activation enthalpy (18.4 kcal/mole) for the ferrocyanide reduction of Type 1 Cu(II) supports the hypothesis that this site is not positioned properly for facile reduction through direct attack by an external reducing agent. This is in contrast to observations[1,2] for fungal laccase A and B, whose Type 1 copper atoms react with $Fe(CN)_6^{4-}$ at specific rates of the order of 10^6 M^{-1} sec^{-1}. Rate parameters for the reaction of ferrocyanide with spinach (k (25°) = 2.11×10^4 $M^{-1}sec^{-1}$, $\Delta H^{\ddagger}$ = 7.6 kcal/mole, $\Delta S^{\ddagger}$ = -13 cal/mol-deg)[9] and bean (k (25°) = 1.56×10^4 M^{-1} sec^{-1}, $\Delta H^{\ddagger}$ = 8.6 kcal/mole, $\Delta S^{\ddagger}$ = -10 cal/mol-deg)[9] plastocyanins at μ = 0.2, pH 6.0 (acetate) are much different than those for the ferrocyanide reduction of Rhus laccase even though the driving force for reduction of laccase and plastocyanin Type 1 sites is about the same (for spinach plastocyanin: E° = 0.37 volt, $5.4 \leq pH \leq 9.9$).[10] Part of the difference between $\Delta S^{\ddagger}$ values for the laccase and plastocyanin systems probably represents contributions to the overall activation entropy from the ion-pairing pre-equilibrium thought to exist in the ferrocyanide-laccase reaction.

Our preliminary kinetic results for laccase reduction with $Fe(EDTA)^{2-}$ indicate that this redox agent probably interacts much differently with laccase than does $Fe(CN)_6^{4-}$. The rate of laccase "blue" copper reduction by $Fe(EDTA)^{2-}$ is first order in the reducing

agent concentration over the entire concentration range employed, and its lack of sensitivity to the ionic strength suggests that ion-pairing of the type proposed for the $Fe(CN)_6^{4-}$ reaction is not a major influence on the reactivity of $Fe(EDTA)^{2-}$ with the protein.

Considering arguments advanced previously, it is not too surprising that the reactivity of stellacyanin and laccase Type 1 sites with $Fe(EDTA)^{2-}$ is so different. As is the case for HQ^- as reductant, the $Fe(EDTA)^{2-}$ activation parameters are compatible with electron transfer to Type 2 Cu(II) in the rate-determining step for Type 1 reduction. It is interesting to note that the rate enhancement for laccase reduction by HQ^- relative to $Fe(EDTA)^{2-}$ has its origin almost exclusively in a difference of about 20 cal/mol-deg in $\Delta S^{\ddagger}$.

An interesting final point concerns the susceptibility of laccase and ceruloplasmin copper sites to attack by outer-sphere reductants. Both $Fe(CN)_6^{4-}$ and $Fe(EDTA)^{2-}$ almost certainly favor outer-sphere electron transfer mechanisms in reducing cytochrome c,[11] and this assignment probably holds for the laccase systems as well. Although $Fe(CN)_6^{4-}$ and $Fe(EDTA)^{2-}$ both react readily with Rhus laccase, neither one of these reductants evidently is capable of reducing ceruloplasmin Type 1 Cu(II) in the absence of the mediator Fe(II).[12,13] Indeed, only a few substrates, inorganic or organic, react directly with the serum "blue" protein.[12] One possible explanation for this behavior is that hydrogen atom transfer is required to accomplish reduction of ceruloplasmin; further experiments are required to thoroughly test

this hypothesis and to establish the basis for the difference in susceptibility of laccase and ceruloplasmin to direct outer-sphere attack.

References

1. B. G. Malmström, A. Finazzi-Agrò, and E. Antonini, Eur. J. Biochem., 9, 383 (1969).
2. L. E. Andréasson, B. G. Malmström, C. Strömberg, and T. Vänngård, Eur. J. Biochem., 34, 434 (1973).
3. B. R. M. Reinhammar, Biochim. Biophys. Acta, 275, 245 (1972).
4. C. Tanford, Adv. Prot. Chem., 17, 69 (1962).
5. B. Reinhammar, Biochim. Biophys. Acta, 205, 35 (1970).
6. L. E. Bennett, in Progress in Inorganic Chemistry, S. J. Lippard, ed., Vol. 18, Wiley, New York, 1973, p. 1.
7. D. Gaswick and A. Haim, J. Amer. Chem. Soc., 93, 7348 (1971).
8. B. Reinhammar and T. Vänngård, Eur. J. Biochem., 18, 463 (1971).
9. D. Fensom, unpublished results.
10. S. Katoh, I. Shiratori, and A. Takamiya, J. Biochem. (Tokyo), 51, 32 (1962).
11. H. L. Hodges, R. A. Holwerda and H. B. Gray, manuscript in preparation.
12. J. A. McDermott, T. C. Hunter, S. O. Saki, and E. Frieden, Biochim. Biophys. Acta, 151, 541 (1968).
13. G. Curzon and S. N. Young, Biochim. Biophys. Acta, 268, 41 (1972).

PROPOSITION I - Study of the Binoxalatochromium(III) Ion

Chelate complexes of chromium(III) and oxalate ion have been studied extensively in the past twenty years. A review[1] published in 1961 demonstrates that even by that time many structural, equilibrium, and kinetic studies had been performed, particularly on the trioxalatochromate(III) ion. Although monodentate oxalatochromium(III) complexes have been suggested as intermediates in aquation,[2] anation,[3,4] oxalate exchange,[6] and racemization[7] studies, there are no known examples of stable monodentate oxalatochromium(III) ions with aquo ligands. Although the chelated forms of oxalato metal complexes certainly are favored at equilibrium, there is some question about the kinetic stability of the monodentate form. Mechanisms have been given both supporting and discounting the hypothesis that formation of the oxalate chelate ring is so fast that monodentate oxalatochromium(III) complexes will never exist in larger than trace concentrations. We propose to settle this question by preparing the binoxalatopentaaquochromium(III) ion and measuring its rate of intramolecular ring closure.

The question of the kinetic stability of $[Cr(H_2O)_5OxH]^{2+}$ is perhaps best focused in the work of Taube and Price[8] on the reduction of $[(NH_3)_5Co(III)OxH]^{2+}$ by chromous ion in aqueous acidic solution. These workers disagree with Fraser's mechanism,[9] but confirm his observation that almost all of the chromium(III) product is the monovalent oxalatotetraaquochromium(III) ion. This product is thought

to reflect chelation of Cr(II) in the activated complex,[8] but chelation subsequent to electron transfer cannot be ruled out. Observations for the malonatopentaaquochromium(III) ion[10] suggest that chelation subsequent to Cr(III) formation is unlikely since closing of the six-membered malonate chelate ring is very slow: $k(25°) = 4.0 \times 10^{-6} + 2.2 \times 10^{-7}\,[H^+]\ sec^{-1}$.

In general it is not difficult to prepare complexes of the form: $Cr(H_2O)_5X^{2+}$, but there are complications in the case X = OxH. Taube and co-workers have prepared these ions in many instances by reducing $Co(NH_3)_5X^{2+}$ with Cr^{2+} in aqueous acid solution, but the product analysis mentioned earlier clearly makes this approach useless for X = OxH. Huchital and Taube[10] prepared $[Cr(H_2O)_5OOCCH_2COOH]^{2+}$ by reacting Cr^{2+} with Fe^{3+} in the presence of malonic acid (0.1 F $HClO_4$ medium), but oxalic acid is easily reduced by Cr^{2+}, yielding glycolic acid rapidly and quantitatively.[11] This second commonly effective technique must therefore also be ruled out in attempting to synthesize $Cr(H_2O)_5OxH^{2+}$.

A third synthetic possibility is based on recent results[12] concerning the mechanism of oxidation of the hydrated glyoxalato-pentaamminecobalt(III) ion by dichromate. In one experiment, Fe(II) was added slowly to a solution containing excess Cr(VI) and the cobalt(III) complex at $[H^+] = 0.05$ F. At this low acid concentration direct reaction of Cr(VI) with the coordinated organic acid is negligible, and the principal initial reaction is the one-electron reduction of Cr(VI) by Fe(II). Yields of binuclear products containing

both cobalt and chromium are maximized under these conditions. These may result when Cr(V) is reduced by two electrons in an inner-sphere process[12]:

$$[(NH_3)_5Co(III)OOCCH(OH_2)]^{2+} + Cr(V)$$

$$\rightarrow [(NH_3)_5Co(III)-O-\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{C}}-\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{C}}-O-Cr(III)(H_2O)_5]^{4+}$$

$$+ 3H^+$$

The oxalate-bridged binuclear product was eluted from a Dowex X-2 cation exchange column with a 4 M $LiClO_4/HClO_4$ solution, and the 1:1 cobalt:chromium stoichiometry was verified.

It is evident that the binoxalatopentaaquochromium(III) ion might be prepared from this oxalate-bridged complex by reacting it with a reagent which would not coordinate to oxalate in the process of reducing Co(III) to Co(II):

$$[(NH_3)_5Co(III)-O-\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{C}}-\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{C}}-O-Cr(III)(H_2O)_5]^{4+}$$

$$+ 6H^+ + Red. \rightarrow 5\,NH_4^+ + Co^{2+} + Oxid.$$

$$+ [(H_2O)_5Cr(III)-O-\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{C}}-\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{C}}-OH]^{2+}$$

Cobalt(II) conveniently is much more substitution labile than Co(III), so hydrogen ion should complete effectively for the oxalate oxygen formerly coordinated to Co(III). Ferrous and vanadous ions are two possibilities for the reducing agent (Red.). Both are known to reduce $[(NH_3)_5Co(III)OxH]^{2+}$ at reasonable rates.[8,13] Choosing a

reducing agent with appropriate visible spectral qualities will permit study of $Cr(H_2O)_5OxH^{2+}$ without having to separate it from the reaction mixture in which it was generated. If the binoxalato ion is as slow to chelate as is the malonato analog, it should be possible to isolate it in pure form by ion exchange chromatography or at least observe its reactions after the generation step is complete. Otherwise, the observed chromium product of the reaction between the oxalate-bridged precursor and a reducing agent probably will be exclusively $Cr(H_2O)_4(Ox)^+$.

Ring closing in the binoxalatopentaaquochromium(III) ion may be followed at 418 nm where the molar extinction coefficient of the oxalatotetraaquochromium(III) ion is 40.1 M^{-1} cm^{-1}.[8] At this wavelength the extinction coefficient for a monodentate carboxylatopentaaquochromium(III) ion is expected to be only about half as large.[14] Both aquation and chelation reactions will contribute to the observed absorbance changes. Huchital found that the chelation reaction is predominant over short time intervals in his study of the malonatopentaaquochromium(III) ion.[10]

References

1. K. V. Krishnamurty and G. M. Harris, Chem. Rev., 61, 213 (1961).
2. K. V. Krishnamurty and G. M. Harris, J. Phys. Chem., 64, 346 (1960).
3. R. E. Hamm and R. H. Perkins, J. Amer. Chem. Soc., 77, 2083 (1955).
4. R. E. Hamm and R. E. Davis, J. Amer. Chem. Soc., 75, 3085 (1953).
5. D. Banerjea and S. D. Chaudhuri, J. Inorg. Nucl. Chem., 32, 1617 (1970).
6. F. D. Graziano and G. M. Harris, J. Phys. Chem., 63, 330 (1959).
7. F. A. Long, J. Amer. Chem. Soc., 63, 1353 (1941); 61, 570 (1939).
8. H. J. Price and H. Taube, Inorg. Chem., 7, 1 (1968).
9. R. T. M. Fraser, J. Amer. Chem. Soc., 85, 1747 (1963).
10. D. H. Huchital and H. Taube, Inorg. Chem., 4, 1660 (1965).
11. R. M. Milburn and H. Taube, J. Phys. Chem., 64, 1776 (1960).
12. A. L. Shugarman, J. C. Sullivan, and H. Taube, to be published, Stanford University.
13. J. H. Espenson, Inorg. Chem., 4, 121 (1965).
14. E. Deutsch and H. Taube, Inorg. Chem., 7, 1532 (1968).

PROPOSITION II - Chemical Modification Studies of Rhus Laccase

Kinetic studies of Rhus vernicifera laccase have suggested that the optically invisible, EPR-detectable Type 2 copper atom fulfills an important role in the enzymatic oxidation of hydroquinone.[1] Experiments with Type 2-binding anions have provided a background for discussion, but kinetic ambiquities and structural unknowns prevent the elucidation of some of the most interesting mechanistic points. A three-point program is proposed here involving chemical modifications of laccase which will be useful in determining structural and functional features of the enzyme essential to its oxidase activity.

A key concept in protein chemical modification studies is that of "essential groups." These are involved in or in some way required for a particular enzymatic property.[2] In the case of laccase, "essential groups" would be those whose elimination or modification directly affects oxidase activity. As Means and Feeney have pointed out,[2] one of the most difficult tasks in modification studies is to distinguish between essential groups and those whose alteration leads to secondary effects (i.e., changes in the charge distribution on the surface of the protein) which ultimately result in activity-destroying general conformational changes.

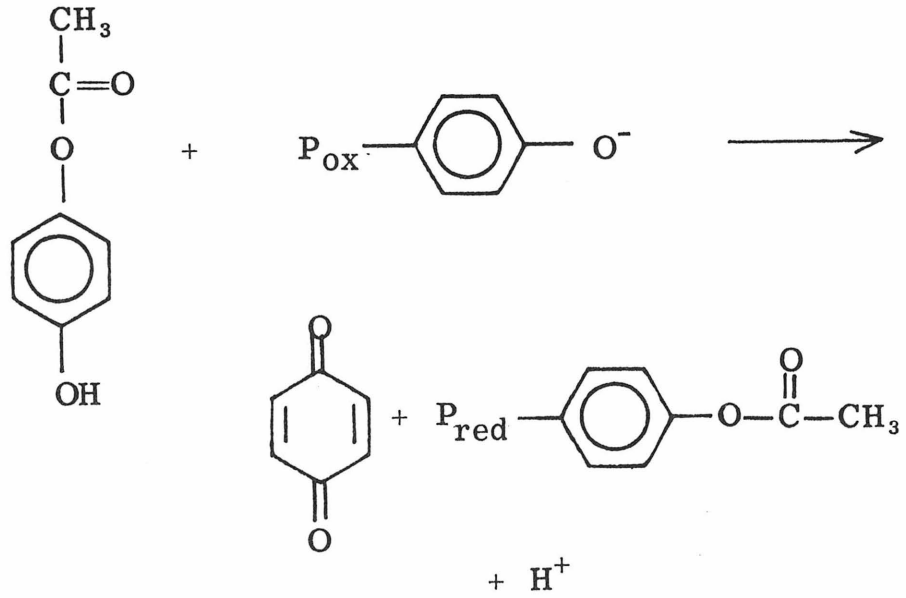
Although many protein modification schemes have been devised,[3] few are specific enough or mild enough to be applied to the understanding of laccase activity. The ideal modification reagent would react only in the vicinity of the active site, and then only with

amino acid residues conbributing to enzymatic activity. Such "site-specific reagents" are currently the subject of much interest, and have been used successfully in a number of instances.[4] A particularly interesting application of the site-specific concept for the inorganic chemist is the use of metal ions which bind near the attack site after participating in an electron transfer reaction with a metalloprotein. For example, Fleischer and co-workers currently are attempting to assign the attack site for Cr(II) in its reaction with cytochrome c(III) by identifying the amino acid side chains which are coordinated to Cr in the substitution-inert chromium(III) product.

Affinity labeling, one form of the site-specific modification technique, depends on the existence of normal enzyme-substrate interactions to produce a large local concentration of reagent at the active site. An affinity labeled reactive group is constrained to attack side chains in the immediate vicinity of the active site, and is likely to modify essential residues. Subsequent protein hydrolysis and identification of modified residues allows correlation of activity losses with specific protein structural changes. For example, irradiation of chymotrypsin-bound p-nitrophenyldiazoacetate results in alkylation of serine, histidine, and tyrosine residues and loss of peptide hydrolysis activity.[5]

To selectively alkylate essential laccase residues it may suffice to introduce an aryl halide whose overall structure is similar to that of hydroquinone. An alternative scheme which potentially is more

specific is suggested by some interesting work on quinone phosphates. When oxidized by bromine, napthoquinol phosphate transfers a phosphoryl group to adenosine-5′ phosphate (AMP), producing adenosine-5′ diphosphate (ADP) non-enzymatically in good yield.[6] Similarly, ATP may be prepared by the bromine oxidation of the pyrophosphate diester of napthoquinol and adenosine in the presence of phosphate ions.[7] Several other oxidizing agents have been shown to be effective in this oxidative phosphorylation procedure.[8] Replacing the phosphoryl group in hydroquinone phosphate with an acyl or alkyl function may result in a reductant with acylating or alkylating ability directly linked to the oxidation process. For example, labeling of an essential tyrosine might proceed as follows:



where P_{ox} refers to fully oxidized laccase and P_{red} refers to the half-reduced enzyme. Thus, in addition to being affinity-labeled, these hydroquinone derivatives would be activated only when participating in the enzymatic reaction of interest. Acylated or alkylated hydroquinones undoubtedly will not be as easily oxidized as hydroquinone itself, but the inherent reducing strength of these compounds fortunately may be increased if necessary by placing electron-donating substituents on the aromatic ring.

Although many studies of laccase have appeared in recent years, no information about the coordination environments of the enzymatic copper atoms is available. Following partial copper removal from the enzyme it may be possible to identify the now-exposed ligands to copper by comparing the reactivities of native and copper-depleted samples with functional group-specific reagents. The fungal laccase Type 2 copper atom may be specifically removed using a cuprous chelating agent, bathocuproine disulfonate (BCS), under mildly denaturing conditions.[9] Preliminary experiments with Rhus laccase[1] have shown that at least three of four copper atoms may be removed using similar conditions. More importantly, removal rates are slow enough that it should be possible to quench the process after as many coppers as desired have been extracted. Copper removal is easily quantitated using the large absorbance of the cuprous bathocuproine complex at 480 nm, and uv, visible, and ESR spectral measurements on copper-depleted protein samples will indicate which copper atoms are absent.

Since metalloprotein ligand identification techniques are well documented,[10] only a brief introduction to some of the procedures which may be applicable to laccase will be given here. The most likely ligands to laccase copper atoms are the nitrogen donor side chains of histidine, arginine, tryptophan and lysine, and the sulfur atom of cysteine. Cysteine and tryptophan have already been identified as part of the stellacyanin coordination environment.[11] The dye rose bengal acts specifically as a photo-oxidation sensitizer for histidine, and has been used to identify essential residues in cytochrome c[12] and in cytochrome c peroxidase.[13] 2-hydroxy-5-nitrobenzyl bromide (HNBB) condenses with tryptophan at neutral pH producing a product which absorbs at 410 nm.[14] Spectrophotometric titrations with p-mercuri benzoate at 255 nm[15] are widely employed to determine the number of reactive protein sulfhydryl groups.

In addition to their usefulness in ligand determination procedures, copper-depleted laccase samples will be valuable in testing hypotheses concerning the mechanism of the hydroquinone reduction of laccase. It has been suggested that attack of the hydroquinone mono-anion at the Type 2 site serves as a common rate-determining step for reduction of the Type 1 and Type 3 sites. Removal of the Type 2 copper atom then should "uncouple" the redox processes leading to reduction of the "blue" and ESR-nondetectable sites. If a Type 2 to Type 3 electron transfer step is involved in the reduction mechanism for the native enzyme, as is suspected, electron transfer to the 330 nm chromophore may not occur at all in the Type 2 Cu-depleted enzyme.

Finally, several spectrophotometric experiments may be suggested for Type 2-depleted laccase. First, direct confirmation that the 405 nm azide-complex band arises from Type 2 coordination will now be possible. Second, since laccase is only rapidly reoxidized when four electrons are available to oxygen,[16] it may be possible to isolate and characterize a laccase-oxygen complex using the reduced 3-copper enzyme. If the laccase-oxygen complex indeed involves cuprous interaction with O_2 at the Type 3 site, a near ultraviolet absorption spectrum similar to that of oxyhemocyanin ($\epsilon_{347} = 8.9 \times 10^3$)[17] may be observed.

References

1. R. Holwerda, unpublished results.
2. G. E. Means and R. E. Feeney, Chemical Modification of Proteins, Holden-Day, San Francisco, 1971, p. 21.
3. See ref. 2 for examples.
4. Reference 2, p. 25-30.
5. J. P. Schafer, P. Baronowsky, R. Laursen, F. Finn, and F. H. Westheimer, J. Biol. Chem., 241, 421 (1966).
6. V. M. Clark, D. W. Hutchinson, and A. Todd, J. Chem. Soc., 722 (1961).
7. A. Todd, Proc. Chem. Soc., 199 (1962).
8. V. M. Clark, D. W. Hutchinson, G. W. Kirby, and A. Todd, J. Chem. Soc., 715 (1961).
9. R. Malkin, B. G. Malmström, and T. Vänngård, Eur. J. Biochem., 7, 253 (1969).
10. B. L. Vallee and E. W. Wacker, in The Proteins, H. Neurath, ed., Academic Press, New York, Vol. 5, 2nd, ed., 1970, p. 61-87.
11. L. Morpurgo, A. Finazzi-Agro, G. Rotilio, and B. Mondovi, Biochim. Biophys. Acta, 271, 292 (1972).
12. M. Nakatani, J. Biochem. (Tokyo), 48, 633 (1960).
13. A. F. W. Coulson and Y. Yonetani, J. Biol. Chem., 247, 2278 (1972).

14. T. E. Barman and P. E. Koshland, J. Biol. Chem., 242, 5771 (1967).

15. P. D. Boyer, J. Amer. Chem. Soc., 26, 4331 (1954).

16. B. G. Malmström, A. Finazzi-Agro, and E. Antonini, Eur. J. Biochem., 9, 383 (1969).

17. K. E. van Holde, Biochemistry, 6, 93 (1967).

PROPOSITION III - Detection of Cytochrome c Conformational Changes Using Fluorescent Organic Probes

In considering the implications of conformational changes accompanying the reduction of cytochrome c, Dickerson et al.[1] have brought up some interesting questions. Are the conformational transitions a cause or an effect of the change in oxidation state of the heme iron? Does the reduced structure reflect relaxation of the polypeptide chain after electron transfer is complete, or are the conformational changes intimately related to the reduction mechanism? These important questions are far from being answered, even in the presence of well-defined X-ray structural data for oxidized horse-heart[2] and reduced tuna-heart[1] cytochrome c. Experimentally, measurements directly related to changes in protein structure are difficult to come by, especially when considering fast redox reactions which must be studied using stopped flow or temperature-jump techniques. It is proposed here that some environmentally sensitive fluorescent molecules may bind specifically to the hydrophobic right channel of ferricytochrome c, allowing direct evaluation of the rates of reduction-linked conformational changes in this region.

Pulsed radiolysis experiments[4,5] have shown that it is possible to separate electron transfer and conformational contributions to spectral changes accompanying reduction of cytochrome c. Heme reduction occurs with about 20 μs after a 200 rad pulse is delivered

to cytochrome c in neutral solution ($k = 1.3 \times 10^{11}$ $M^{-1}sec^{-1}$, 20°, pH 6.0), but the 20 μs reduced minus oxidized difference spectrum is not in agreement with literature results.[4] The 20 μs spectrum is significantly blue-shifted relative to the normal difference spectrum, especially in the Soret region; relaxation of the transient to the normal spectrum occurs at a rate of 8.5 ± 0.3 sec^{-1}. Pecht and Faraggi[4] attribute the transient spectrum to ferrocytochrome c being produced in the protein environment of ferricytochrome c; the first order relaxation process subsequent to heme reduction is assigned to conformational rearrangement of the intermediate to the equilibrium ferrocytochrome c structure.

Unfortunately, results with the hydrated electron as reductant allow no conclusions to be drawn concerning relationships between electron transfer and conformational processes when less potent reductants are used. It has been pointed out that conformational and redox transformations are likely to be concerted when cytochrome c functions *in vivo*.[1] The use of absorbance changes in the Soret region to characterize conformational movement probably is a technique applicable only to the limiting case represented by the hydrated electron experiments.

In experiments with model reductants such as $Fe(EDTA)^{2-}$, what is needed to characterize fast conformational transitions is a physical measurement other than absorbance which is sensitive to changes in the three-dimensional structure of cyt c. Stryer[5] recently

has summarized the biochemical applications of fluorescent probes, and discusses a number of examples. Distinction is made between intrinsic chromophores, such as the fluorescent aromatic side chains of tyrosine and tryptophan, and extrinsic chromophores, such as the dye 1-anilino-8-naphthalene sulfonate (ANS) which binds non-covalently to protein hydrophobic pockets. Criteria are also listed[5] for evaluating the usefulness of potential extrinsic chromophores:

1. The chromophore must be bound or covalently attached to the protein at a unique location.
2. The fluorescent properties of the probe must be sensitive to the structure and dynamics of its environment, and in ways that are amenable to definitive interpretation.
3. Insertion of the probe should not appreciably disturb those features of the protein that are being investigated.

One of the applications of protein-bound fluorescent groups is as polarity probes.[5] Since the positions of emission maxima and magnitudes of corresponding quantum yields are strongly dependent on the environment of the excited state, the fluorescence characteristics of some protein-bound molecules are drastically altered when they are displaced from a hydrophobic into an aqueous environment as a consequence of a conformational change. Low emission quantum yields, poor resolution between absorption and emission spectra, and, most importantly, poor placement in the protein relative to the conformation change of interest are factors limiting the usefulness of tryptophan and tyrosine as native conformational probes.[5,6] On the other hand, a

number of fluorescent organic probes have been developed which offer all of the desirable properties suggested by Stryer. Several applications of ANS and of TNS (2-p-toluidinylnaphthalene-6-sulfonate) will be briefly discussed here in way of introduction to the field.

ANS emits maximally at 515 nm in water with a quantum yield of only 0.004 (excitation wavelength 365 nm), but upon binding to the hydrophobic heme crevice of apomyoglobin the quantum yield (Q) increases to 0.98 and the emission maximum (λ_{max}) is blue-shifted to 454 nm.[7] This behavior is paralleled by trends observed in alcohol solvents of varying polarity.[7] As the dielectric constant of the medium decreases, λ_{max} values tend towards the ultraviolet and Q values increase. The stoichiometry of ANS binding to apomyoglobin was shown to be 1:1 and a dissociation constant of ca. 10^{-5} M was calculated for the dye-protein complex.[7]

Identical trends in λ_{max} and Q with solvent dielectric constant have been observed for TNS, and binding of this dye to a number of proteins occurs.[8] TNS was used to characterize the binding of competive inhibitors to chymotrypsin,[9] and also was employed in a kinetic study of the conformational transition accompanying activation of chymotrypsinogen by trypsin.[10]

It seems likely that the right channel of ferricytochrome c offers a unique binding site for fluorescent probes. The only regions in the oxidized protein where enough space is available to accommodate extrinsic molecules or ions are the heme pocket and the right channel.[2]

Furthermore, the dimensions of the pocket are such that a molecule as large as ANS is not likely to penetrate very deeply. Conversely, the right channel offers ample space for introduction of a hydrophobic molecule; it has been suggested that this structural feature may be important in allowing recognition and binding of cytochrome oxidase to cytochrome c.[2]

Amino acid residues 19 to 25 form the bottom part of the right channel in ferricytochrome c, but shift up to block the channel in the reduced molecule.[1] Ferrocytochrome c is much more compact than the oxidized molecule, and contains no hydrophobic pockets which would be accessible to a fluorescent dye. It is expected then that reduction of cytochrome c will bring about displacement of any non-covalently bound species residing in the right channel; fluorescence changes induced by the return of ANS or similar dyes to an aqueous environment may be used to estimate the rate of the redox-linked conformational transition in this region. Binding of ANS in the ferricytochrome c right channel should not alter reduction of the enzyme from its normal course, as the most likely attack sites for reductants all are remote from the right channel. Thus, association of fluorescent molecules with ferricytochrome c should fulfill all three criteria for acceptable conformational probes: a unique binding site, a large change in fluorescent properties upon binding to the site, and little interference with the reactions of interest.

Finally, it should be noted that experimental techniques for following fast fluorescence changes have been documented.[11,12] Stopped flow instruments designed for both absorbance and fluorescence measurements are commercially available. Conveniently, instrumental sensitivity to small concentrations of fluorescing substances is often superior to that achieved in absorption measurements.[13]

References

1. T. Takano, R. Swanson, O. B. Kallai, and R. E. Dickerson, Cold Spring Harbor Symp. Quant. Biol., 36, 397 (1971).
2. R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, and E. Margoliash, J. Biol. Chem., 246, 1511 (1971).
3. E. J. Land and A. J. Swallow, Arch. Biochem. Biophys., 145, 365 (1971).
4. I. Pecht and M. Faraggi, Proc. Nat. Acad. Sci. (U.S.), 69, 902 (1972).
5. L. Brand and B. Witholt, in Methods in Enzymology, C. H. W. Hirs, ed., Vol. 11, Academic Press, New York, 1967, p. 776.
6. L. Stryer, Science, 162, 526 (1968).
7. L. Stryer, J. Mol. Biol., 13, 482 (1965).
8. W. O. McClure and G. M. Edelman, Biochemistry, 5, 1908 (1966).
9. W. O. McClure and G. M. Edelman, Biochemistry, 6, 559 (1967).
10. W. O. McClure and G. M. Edelman, Biochemistry, 6, 567 (1967).
11. Q. H. Gibson, in Methods in Enzymology, K. Kustin, ed., Vol. 16, Academic Press, New York, 1969, p. 187.
12. A. N. Schechter, Science, 170, 273 (1970).
13. J. E. Stewart, Durrum Applications Notes, Number 7, Durrum Instrument Corp., Palo Alto(November 1971).

PROPOSITION IV - Spectroscopic and Kinetic Studies of Mixed Valence Copper Complexes

Interest in mixed-valence transition metal complexes currently is very high. Robin and Day[1] have reviewed the literature, and offer a useful classification system for mixed-valence compounds. A number of mixed-valence Cu(II)-Cu(I) complexes have been reported, many of which are intensely colored because of charge transfer bands associated with this electronic configuration. Quantitative understanding of these bands is limited in view of the lack of structural information for most known examples of Cu(II)-Cu(I) compounds. We propose that a number of well-characterized binuclear Cu(II)-Cu(II) complexes may be partially reduced to yield Cu(II)-Cu(I) species whose structures may be readily predicted and whose ligands may be systematically varied. It is suggested that these complexes will be useful not only in spectroscopic studies, but also in NMR experiments probing factors affecting the cupric-cuprous electron exchange rate.

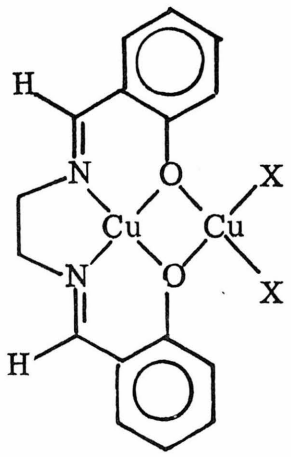
Chlorocuprate(I,II) complexes are perhaps the best studied mixed-valence copper system. Crystals containing varying proportions of Cu(II) and Cu(I) have been obtained from chloride media employing $Co(NH_3)_6^{3+}$ as the cation,[2] and a new absorption band at 17,000 cm^{-1}[3] has an intensity proportional to the product of the mole fractions of the two valence states.[4,5] Single crystal spectra of hexaamminecobalt(III) chlorocuprates (I,II) have been measured, and some of the factors

possibly contributing to the low intensity and broadness of the mixed-valence transition have been discussed.[6] A peak near 18,000 cm^{-1} appears for the chlorocuprate (I, II) system in 10 M HCl,[7] and again it has been shown that the band has its origin in a 1:1 Cu(I)-Cu(II) interaction.[8] Although McConnell and Davidson[9] established the formula of the chlorocuprate (I, II) complex to be Cu_2Cl_3, Robin and Day[1] have pointed out that it is not possible to distinguish between bridged and outer-sphere interaction in this species on the basis of available evidence.

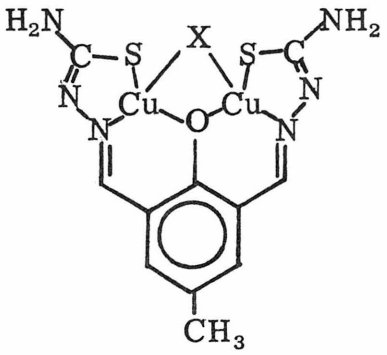
Structural unknowns have also prevented the full understanding of several other interesting mixed-valence copper systems. An intense violet color develops when thiomalic acid is mixed with Cu(II), and the stoichiometry of optical titrations at 520 nm suggests the formulation of a species: $Cu(II)[RS\text{-}Cu(I)]_4$.[10] Hemmerich, et al. have suggested[11] that valence tautomerism of the form:

may need to be considered in discussing the origin of the 520 nm band, but the structure of the thiomalic acid complex has never been determined. A deep violet 1:1 Cu(II)-Cu(I) complex containing equimolar copper and acetate has been synthesized in methanol solution[12]; the presence of bridging acetates in the mixed-valence species is suggested.

A systematic study of the spectroscopic properties of mixed-valence copper complexes as a function of the coordination environment has never been made. A reasonable approach to this problem may be to partially reduce some of the many well-characterized binuclear copper(II) complexes chemically or electrochemically in the hope that dramatic structural perturbations will not occur. Since Cu(II) and Cu(I) ordinarily prefer very different coordination environments, best results will probably be obtained with binucleating ligands which impose a specific ligand field on both metal atoms. Tetradentate Schiff base ligands appear to be ideal in this respect.[13] A study of non-bridging ligand effects on mixed-valence spectra could be based compounds of the form:



where X is virtually any monodentate ligand. Variations in a bridging ligand are possible with compounds like:



where X may be RO^-, RS^-, N_3^-, Cl^-, and many other anionic species.[14] Several variations on this theme are possible starting with a wide variety of binuclear cupric complexes listed in two recent reviews.[13,15]

One major concern in attempting the reduction of binuclear cupric complexes is that binucleating ligands will promote square-planar coordination to the extent that reduction of Cu(II) to Cu(I) will be extremely unfavorable. Still, it has been observed that if one of the metal ions in a binuclear complex is in a planar environment, the adjacent metal ion will probably favor a ligand field distorted away from planarity.[13] Stabilization of cuprous copper may be accomplished by the use of ligand configurations explicitly designed to bind metal ions in a compromise geometry between square planar and tetrahedral.[13,15]

Another application of well-characterized mixed-valence copper complexes is in the study of factors affecting the cuprous-cupric electron exchange rate. McConnell and Weaver[16] used broadening of the $^{63}Cu(I)$ NMR signal induced by cupric ion to measure the Cu(I)-Cu(II) electron exchange rate in concentrated HCl ($k = 5 \times 10^7\ M^{-1}\ sec^{-1}$). This interesting pioneering study has never been followed up, presumably because of the difficulties involved in maintaining cuprous

ion in solution. Evaluation of intramolecular electron transfer rates in mixed-valence copper complexes by the ^{63}Cu line-broadening method offers several advantages over classical solution kinetic methods. No rapid mixing or perturbation techniques are required, and faster rate processes may be observed. More importantly, even though some ligands may still exchange rapidly, the geometry and composition of the activated complex for electron exchange should be largely predetermined by the ground structure of the mixed-valence species. Thus, it may be possible to understand for the first time some of the structural factors (i.e., bridging group effects) influencing the rates of copper redox processes.

Several practical points should be emphasized in proposing to study intramolecular electron transfer rates by the ^{63}NMR line-broadening method. First, observed rates may be so fast for many of the species considered that it will be difficult to quantitatively measure and compare them. Second, in order to estimate electron exchange rates by the published method,[16] it will be necessary to obtain a reference value for the transverse relaxation time of cuprous ion in the absence of electron exchange. This quantity probably may be reliably estimated using model compounds containing cuprous ion in a coordination environment similar to that in the binuclear complexes.

References

1. M. B. Robin and P. Day, Advan. Inorg. Chem. Radiochem., 10, 247 (1967).
2. M. Mori, Bull. Chem. Soc. Japan, 33, 985 (1960).
3. M. Mori, Bull. Chem. Soc. Japan, 34, 1249 (1961).
4. D. Culpin, P. Day, P. R. Edwards, and R. J. P. Williams, Chem. Comm., 450 (1965).
5. R. J. P. Williams, in The Biochemistry of Copper, J. Peisach, P. Aisen, and W. E. Blumberg, eds., Academic Press, New York, 1966, p. 131.
6. P. Day and D. W. Smith, J. Chem. Soc., A, 1045 (1967).
7. E. Doehlemann and H. Fromherz, Z. Physik.Chem., A171, 353 (1934).
8. H. Diehl, P. A. Carlson, D. Christian, E. H. Dewel, M. R. Emerson, F. K. Hermann, and H. W. Standage, Proc. Iowa Acad. Sci., 55, 241 (1948).
9. H. M. McConnell and N. Davidson, J. Amer. Chem. Soc., 72, 3168 (1959).
10. I. M. Klotz, G. H. Czerlinski, and H. A. Fiess, J. Amer. Chem. Soc., 80, 2920 (1958).
11. P. Hemmerich, H. Beinert, and T. Vänngård, Angew. Chem., 78, 449 (1966).
12. C. Sigwart, P. Hemmerich, and J. T. Spence, Inorg. Chem., 7, 2545 (1968).

13. E. Sinn and C. M. Harris, Coord. Chem. Rev., 4, 391 (1969).

14. W. D. McFadyen, R. Robson, and H. Schaap, Inorg. Chem., 11, 1777 (1972).

15. D. St. C. Black and A. J. Hartshorn, Coord. Chem. Rev., 9, 219 (1972).

16. H. M. McConnell and H. E. Weaver, Jr., J. Chem. Phys., 25, 307 (1956).

PROPOSITION V - Metallochemotherapy of Cancer

Cancer has been defined[1] as "a disease of multicellular organisms which is characterized by the seemingly uncontrolled multiplication and spread within the organism of apparently abnormal forms of the organism's own cells." A great many compounds are known to induce this disease, many of which are used and should be respected by the laboratory chemist.[2] Cancer research certainly is not a new phenomenon, but participation of inorganic chemists in this field definitely is. In the absence of detailed information concerning the molecular mechanism of carcinogenesis, it remains essentially impossible to prescribe anticancer drugs which attack only malignant cells. Nevertheless, the demonstrated success of metallotherapeuticals discovered in the past five years has led to the empirical correlation of stereochemical, electronic, and thermodynamic properties of metal complexes effective in treating cancer. In addition, several research workers have realized that the application of presently used anti-cancer drugs in the form of metal complexes may enhance their specificity of action. Several such applications are proposed here.

Furst suggested the development of transition metal chelate complexes as anti-cancer agents in 1960,[3] and went so far as to propose that the effectiveness of some carcinogens may lie in their ability to compete for and abnormally transport physiological metal ions. Schubert provides a number of examples confirming the feasibility of this hypothesis in his interesting discussion of chelation in medicine.[4]

Rosenberg and co-workers recent finding[5,6,7] that a number of Pt(II) compounds have potent anti-tumor activity inspired the first major surge of bioinorganic cancer research. D. R. Williams' timely review[8] serves as an introduction to the biology of cancer for the inorganic chemist, and discusses many of the pioneering studies of cancer metallochemotherapy.

A major problem with drugs employed in the chemotherapy of cancer is their lack of specificity for cancerous cells.[8,9] Stock has emphasized that anti-cancer drugs essentially are anti-growth agents.[9] Kirschner and co-workers were among the first researchers to realize some of the potential advantages in therapeutic specificity available when metal complexes are used as drugs.[10] They introduced the concept of "appropriateness" in evaluating the properties a complex should possess in order to inhibit malevolent viruses by coordinating to specific protein or nucleic acid ligands. To be "appropriate", a complex ion must not be so labile that it is readily complexed by non-viral species such as enzymes and free amino acids; still, it must be capable of interacting with specific ligands offered by malevolent viruses. Williams[8] has emphasized the usefulness of hard and soft acid-base concepts in designing complexes which will favor coordination to some donor atoms more than to others.

It has been proposed that transition metal complexes of ligands with chemotherapeutic activity might release the toxic chemicals upon binding to malevolent viruses, effectively channeling the

medication to where it is needed.[10] Several group VIII complexes of 6-mercaptopurine were shown to be somewhat more effective as anti-cancer drugs than the free ligand,[10] but the "carrier" hypothesis has never been fully tested. Many factors contributing to the remarkable chemotherapeutic activity of cis-$Pt(NH_3)_2Cl_2$ and related compounds are understood,[11,12] but the effectiveness of these complexes probably is linked to formation of stable chelates with DNA.[13,14] Platinum(II) complexes appear to be ideal as drugs which function by forming specific cancer-inhibiting complexes with nucleic acids, but they are not particularly good choices as carrier complexes for anti-cancer ligands such as 6-mercaptopurine (6-mp). The thermodynamic and kinetic stability of Pt(II) complexes with nitrogen and sulfur donors is such that no physiological ligands are expected to displace 6-mp, especially if it is chelated. This point is illustrated by some kinetic results for the reaction:

$$Pt(dien)X^+ + py \rightarrow Pt(dien)py + X^-$$

in water. Observed rate constants at 25° for $[py] = 5.9 \times 10^{-3}$ M are 35, 0.30, and 0.83×10^{-6} sec^{-1} with $X = Cl^-$, SCN^-, and N_3^-, respectively.[15]

Livingstone and co-workers have prepared some potential carrier complexes with nickel(II),[16,17] including the square planar compounds $Ni(dtp)_2$ (dtp = dialkyldithiophosphate).[16] The effectiveness of these complexes as carriers for the fungicidal ligands is probably limited by the tendency of square planar Ni(II) to add two ligands to

form high-spin octahedral complexes instead of substituting ligands in the square plane.[18] It was shown that $Ni(dtp)_2$ (R = CH_3) is susceptible to attack by pyridine to give the octahedral complex trans-$[Ni(dtp)_2(py)_2]$.[16]

We propose that palladium(II) complexes of the form $[Pd(dien)X]^+$ and $Pd[(terpy)X]^+$ will be good carriers for X = 6-mp and other anti-cancer ligands. Substitution of the X ligand in diethylenetriaminepalladium(II) complexes is known to be much more facile than for the Pt(II) analogs, in accord with weaker Pd-ligand bond strengths.[19] Furthermore, Pd(II) does favor the low-spin d^8 square-planar configuration over octahedral coordination. The tridentate dien and terpy ligands are suggested in order that only 6-mp will be substituted for, and modification of these ligands (i.e., replacement of dien with Et_4dien) allows variation in steric and charge factors which may be crucial to the medicinal activity of the Pd complexes. The influence of ionic charge is especially important in determining the physiological attack sites available to complex ions. Distribution studies of platinum complexes in whole bacteria have supported this hypothesis.[20] The bacteriocidal complex $PtCl_6^{2-}$ is not transported across membranes and reacts principally with cytoplasmic protein. The neutral species $[Pt(NH_3)_4Cl_2]$ presumably may cross membranes and associates extensively with nucleic acids and metabolic intermediates such as amino acids and peptides.

The study of palladium(II) compounds as carriers for anti-cancer ligands should include several aspects. Synthetic work with a

variety of carcinostatic ligands listed elsewhere[9] will be important. Equilibrium and kinetic studies of displacement of the drugs from palladium coordination by biologically important species will also be essential. Finally and most important, assays of effectiveness against cancers in laboratory animals should be run.

References

1. E. J. Ambrose and F. J. C. Roe, eds., The Biology of Cancer, Van Nostrand, London, 1966.
2. C. E. Searle, Chem. Brit., 6, 5 (1970).
3. A. Furst, in M. J. Seven and L. A. Johnson, eds., Metal Binding in Medicine, Lippincott Co., Philadelphia, 1960, Chap. 46.
4. J. Schubert, Sci. Amer., 214 (5), 40 (1966).
5. B. Rosenberg, L. VanCamp, J. E. Trosko, and V. H. Mansour, Nature, 222, 385 (1969).
6. B. Rosenberg and L. VanCamp, Cancer Res., 30, 1799 (1970).
7. B. Rosenberg, Plat. Met. Rev., 15(2), 42 (1971).
8. D. R. Williams, Chem. Rev., 72, 203 (1972).
9. J. A. Stock, Chem. Brit., 6, 11 (1970).
10. S. Kirschner, Y. K. Wei, D. Francis, and J. Bergman, J. Med. Chem., 9, 369 (1966).
11. A. J. Thomson, R. J. P. Williams, and S. Reslova, Struct. Bonding (Berlin), 11, 1 (1972).
12. M. J. Cleare and J. D. Hoeschele, Bioinorganic Chemistry, 2, 187 (1973).
13. H. C. Harden and B. Rosenberg, Int. J. Cancer, 6, 207 (1970).
14. J. A. Howle and G. R. Gale, Biochem. Pharmacol., 19, 2757 (1970).
15. F. Basolo, H. B. Gray, and R. G. Pearson, J. Amer. Chem. Soc., 82, 4200 (1960).

16. S. E. Livingstone and A. E. Mihkelson, Inorg. Chem., 9, 2545 (1970).

17. S. E. Livingstone and J. P. Nolan, ibid., 7, 1447 (1968).

18. F. A. Cotton and G. Wilkinson, Advanced Inorganic Chemistry, 3rd. ed., Interscience, 1972, p. 897.

19. F. Basolo and R. G. Pearson, Mechanisms of Inorganic Reactions, 2nd. ed., Wiley, 1967, p. 415.

20. E. Renshaw and A. J. Thomson, J. Bacteriol., 94, 1915 (1967).