0.9998–0.9954 (15–20 points). The dependence of pseudo-first-order rate constants on the β-cyclodextrin concentration was analyzed by the use of eq 6. Data processing was carried out using a nonlinear least-square regression program on a Union System 77 computer.

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$$\begin{array}{ccc} a(1-x)\mathrm{CD}\cdot\mathrm{AH} & \overset{pK_a}{\rightleftharpoons} & ax\mathrm{CD}\cdot\mathrm{A} \\ K_{ass}=490\ \mathrm{M}^{-1} \uparrow\downarrow & & \uparrow\downarrow K_{ass}' \\ b(1-y)\mathrm{AH} & \overset{pK_a'=9.45}{\rightleftharpoons} & by\mathrm{A} \end{array}$$

respectively. At the titration point (pH 8.93), the amount of CD·AH plus AH should be equal to the amount of CD·A plus A. The corrected pK_a value of 8.90 and K_{ass}' value of 1737 were obtained based on the above assumptions.

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(20) 9,10-Anthraquinone, which is ca. 11 Å in width and, therefore, is too big to be inserted sideways into the cavity of β-cyclodextrin, exhibited no significant blue shift of the λ_{max} upon inclusion (332 nm in water, ca. 331 nm in the inclusion complex). Meanwhile, the inclusion of 2-methyl-1,4-naphthoquinone (**8**) by β-cyclodextrin showed a blue shift of the λ_{max} from 339 nm (in water or in 30% MeOH–water) to 336 nm, strongly suggesting that the mode of inclusion differs from that of anthraquinone. The different modes of inclusion of **8** and 9,10-anthraquinone are also reflected in the differences in the K_{ass} values: 310 M^{-1} for **8**; 45 ± 20 M^{-1} for 9,10-anthraquinone, pH 9, borate buffer (30% MeOH). These results strongly support the postulate that **8** is included sideways by β-cyclodextrin, as shown in Scheme I, in marked contrast to anthraquinone. The protection of **8** from attack by hydrogen peroxide also strongly supports the postulated mode of interaction of the naphthalene ring with β-cyclodextrin.

Superoxide Dismutase Activities of an Iron Porphyrin and Other Iron Complexes

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Abstract: The superoxide dismutase (SOD) activities of a number of metal complexes have been measured and a mathematical model is proposed which allows their rate constants to be calculated. $Fe(CN)_6^{4-}$ showed no activity whereas $Fe(CN)_6^{3-}$ appeared to react stoichiometrically with superoxide. True catalytic SOD activity appeared to be shown by chelates of iron with EDTA [$Fe(EDTA)^-$ and $Fe(EDTA)^{2-}$], with diethylenetriaminepentaacetic acid [$Fe(DTPA)^{2-}$ and $Fe(DTPA)^{3-}$], and with *o*-phenanthroline [$Fe(phen)_3^{2+}$]. The most efficient superoxide dismutase catalyst of the compounds tested here was found to be tetrakis(4-*N*-methylpyridyl)porphineiron(III) [$Fe^{III}TMpyP$], with a rate constant of 3×10^7 M^{-1} s^{-1} at pH 10.1. Exposure of this compound to H_2O_2 produced in a superoxide-generating system caused its degradation to a product with lower SOD activity, but it could be protected by catalase. H_2TMpyP itself showed no SOD activity, nor did its complexes with Zn(II) or Cu(II). $Co^{III}TMpyP$ showed much lower SOD activity than the Fe(III) complex.

That molecular oxygen is indispensable for most life forms has been known since the time of Priestly and Lavoisier. More recently, investigations have demonstrated that, as oxygen is utilized by organisms, highly toxic intermediates are produced on the pathway to the formation of water. Respiring organisms have evolved a system of defenses against these dangerous metabolites which ailows them to enjoy the benefits of living in an oxygen-rich environment. As part of this protective arsenal, superoxide dismutases are present in aerobes to catalyze the conversion of superoxide ion into H_2O_2 and O_2, while catalase and the peroxidases function to scavenge peroxides. Not only are H_2O_2 and O_2^- potentially deleterious in their own right, but evidence exists that under appropriate conditions they can react together to form the even more lethal OH· radical.[2-5]

Peroxide eliminating enzymes as well as superoxide dis-

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mutases, which catalyze the reaction

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

all contain metals. Furthermore, it is now known that certain metal-containing model compounds show catalase and/or superoxide dismutase activity. Reports have appeared on the catalase-like activity of the cobalt(III) derivative of hematoporphyrin IX,[6,7] deuteroferriheme, and protoferriheme.[8,9] The dismutation of superoxide ion has been shown[10-14] to be catalyzed by low molecular weight copper(II) complexes and by $Fe(EDTA)^{2-}$ and $Fe(EDTA)^-$. The present report deals with the influence of a number of metal derivatives of the water-soluble porphyrin tetrakis(4-*N*-methylpyridyl)porphine ($MeTMpyP^{n+}$) on the rate of dismutation of superoxide ion. These results are compared with those obtained for a number of simpler iron complexes. It is our purpose in investigating the reactions of superoxide ion with a series of model compounds to determine the features of substances which make them effective in catalyzing the dismutation reaction or in otherwise scavenging superoxide ion.

Experimental Section

Superoxide dismutase (copper–zinc enzyme), nitroblue tetrazolium, xanthine, diethylenetriaminepentaacetic acid (DTPA), ferric chloride, and ferrous sulfate were purchased from the Sigma Chemical Corp. Analar ethylenediaminetetraacetic acid (EDTA), potassium ferricyanide, potassium ferrocyanide, mannitol, and dimethyl sulfoxide were obtained from BDH Chemicals Ltd. Fisons sodium bicarbonate, Fluka dicyclohexyl 18-crown-6, and ICN potassium superoxide were used. Catalase (20 mg/mL) and xanthine oxidase (10 mg/mL) were purchased from Boehringer Corp. The catalase exhibited no SOD activity.[15] The tetraiodide salt of $\alpha,\beta,\gamma,\delta$-tetrakis(4-*N*-methylpyridyl)porphine ($H_2TMpyP I_4$) was purchased from Strem Chemicals, Inc.; the iron, cobalt, copper, and zinc derivatives of this porphyrin were prepared by published methods.[16-18]

Kinetic experiments were conducted on a Unicam SP600 UV spectrophotometer and spectral experiments on a Unicam SP800 UV recording spectrophotometer. The superoxide dismutase activity of a test substance was assayed by its ability to inhibit the reduction of nitroblue tetrazolium by a xanthine–xanthine oxidase system at pH 10.1 (0.05 M carbonate buffer) and ambient temperature.[19] The course of the reaction was followed by the increase in absorbance at 560 nm as nitroblue tetrazolium was converted to formazan. When a compound being tested showed apparent superoxide dismutase activity as manifested by an inhibition of the rate of production of formazan, a separate test was made to ensure that the rate of superoxide production was unaffected by the added substance.

Superoxide is produced as xanthine is converted to uric acid; the rate of uric acid production was monitored at 290 nm in the presence of the maximum concentration of the compound tested for SOD activity. Only those compounds showing 10% or less inhibition of uric acid formation are included in this report.

Results and Discussion

The experimental basis for the present study is the comparison of rates of reaction of superoxide ion with nitroblue tetrazolium (NBT) and a substance being tested for superoxide dismutase activity. Superoxide ion is generated continuously during the enzymatically catalyzed conversion of xanthine to uric acid at pH 10.1 (0.05 M carbonate buffer). NBT, which is yellow in aqueous solution, reacts with O_2^- to form the blue-colored formazan whose production is monitored at 560 nm.[19] A comparison is made between the rate of formation of the 560-nm absorbing product in the presence and absence of the tested substance. Figure 1 shows a typical experimental result; a linear kinetic profile of absorbance vs. time is obtained until one of the reactants (either xanthine or NBT, depending on reaction conditions) nears depletion.

The following model is proposed to account for and to interpret the kinetic results. It is reasonable to assume that during the linear portion of the kinetic profile, superoxide is produced

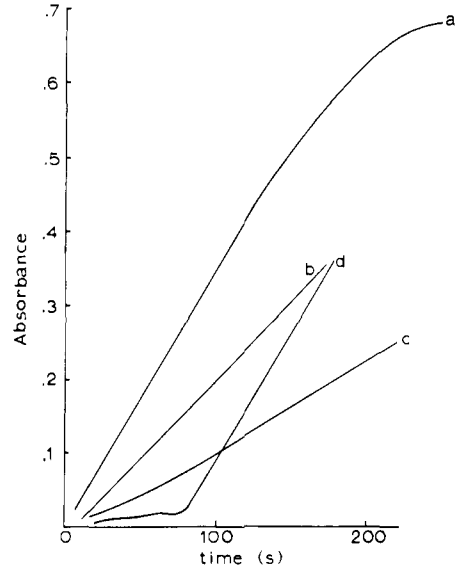

Figure 1. Effect of iron complexes on reduction of nitroblue tetrazolium by O_2^- at pH 10.1. (a) No added compound, Q. This experiment was run for a period of time sufficient to show the curvature which results as either NBT or xanthine (depending upon reaction conditions) approaches depletion. An identical kinetic profile was obtained when 3.3×10^{-5} M $Fe(CN)_6^{4-}$ was added to the reaction mixture. (b) Assay mixture to which 6.7×10^{-6} M $Fe(EDTA)^{2-}$ has been added. (c) Assay mixture to which 3.3×10^{-5} M $Fe(EDTA)^-$ has been added. (d) Assay mixture to which 3.3×10^{-5} M $Fe(CN)_6^{3-}$ has been added. See text for further description and interpretation of these kinetic results.

at a constant rate.[20] Superoxide ion disappears in any of three parallel pathways: (a) spontaneous dismutation, (b) reaction with NBT, and/or (c) reaction with an added substance, Q.

$$HO_2 + O_2^- + H_2O \xrightarrow{k_{SD}} H_2O_2 + O_2 + OH^- \quad (2a)$$

$$O_2^- + NBT \xrightarrow{k_N} F \quad (2b)$$

$$O_2^- + Q \xrightarrow{k_Q} P \quad (2c)$$

Although the pK_a of HO_2 is 4.75, the spontaneous dismutation is shown as in eq 2a because it has been found that the direct reaction of two O_2^- anions

$$2O_2^- + 2H_2O \rightarrow H_2O_2 + O_2 + 2OH^- \quad (3)$$

proceeds at a negligible rate.[21,22]

The rate of production of formazan is given by

$$\text{rate} = \frac{d[F]}{dt} = k_N[O_2^-][NBT] \quad (4)$$

If we apply the steady-state approximation to superoxide ion, we obtain

$$\frac{d[O_2^-]}{dt} = 0 = v - k_{SD}\frac{[H^+]}{K_a}[O_2^-]^2 - k_N[NBT][O_2^-] - k_Q[O_2^-][Q] \quad (5)$$

The quantity v in eq 5 is the rate of superoxide production, which in turn depends on the amount of xanthine oxidase added,[20] since the xanthine is in large excess during the linear portion of the kinetic profile. Therefore, the concentration of O_2^- is given as

$$[O_2^-] = \frac{v}{k_{SD}\frac{[H^+]}{K_a}[O_2^-] + k_N[NBT] + k_Q[Q]} \quad (6)$$

Table I. Experimental Testing of Kinetic Model

	conditions					
	[xanthine] × 10^4	[NBT] × 10^5	xanthine oxidase, μg	[Q] ($\times 10^7$)	ρ	rate ($\times 10^3$), s^{-1} / k_Q ($\times 10^{-6}$), M^{-1} s^{-1}
		I. No Q				
a	1	2.5	200			3.0
b	2	2.5	200			3.3
c	1	5.0	200			3.2
d	1	2.5	100			1.6
	II. In Presence of Q					
e	1	5.4	200	9.2	0.62	2
f	1	2.7	200	9.2	0.45	2
g	1	5.4	200	18	0.34	3
h	1	5.4	200	37	0.26	2

The value of the spontaneous dismutation rate constant for eq 2a has been determined[21] as $k_{SD} = 8.9 \times 10^7$ M^{-1} s^{-1}. At pH 10.1, k_{SD} ($[H^+]/K_a$) equals 4×10^2 M^{-1} s^{-1}. Furthermore, the reaction rate of O_2^- with NBT has been measured under similar conditions[23] as $k_N = 6 \times 10^4$ M^{-1} s^{-1}. Therefore, eq 6 can be rewritten to give

$$[O_2^-] = \frac{v}{400[O_2^-] + 6 \times 10^4[NBT] + k_Q[Q]} \quad (7)$$

However, under the conditions of these experiments [NBT] $\gg$ $[O_2^-]$[20] and so we may neglect the spontaneous dismutation step and eq 7 simplifies to give

$$[O_2^-] = \frac{v}{k_N[NBT] + k_Q[Q]} \quad (8)$$

If we substitute the terms of eq 8 where $[O_2^-]$ appears into eq 4 we obtain the final expression

$$\text{rate} = \frac{vk_N[NBT]}{k_N[NBT] + k_Q[Q]} \quad (9)$$

If xanthine and NBT are present in large excess and if Q is either present in large excess or acts catalytically the rate becomes constant (k_{obsd}) and the reaction is zero order, leading to a linear dependence of absorbance upon time for a portion of the reaction.

When several reactions are run at constant enzyme concentration even with variable but excess substrate (xanthine), v is a constant for each reaction, and

$$\frac{k_{obsd}}{v} = \rho = \frac{k_N[NBT]}{k_N[NBT] + k_Q[Q]} \quad (10)$$

The value of v is obtained by conducting an experiment in which no Q is added. Then

$$k_Q = \frac{k_N[NBT]}{[Q]}\left(\frac{1}{\rho} - 1\right) \quad (11)$$

This model must be modified to take into account the occurrence of a very slow direct reduction of NBT by xanthine oxidase.[19] The rate of this process is easily determined by conducting experiments in the presence of large excess of superoxide dismutase (SOD). Although under these conditions NBT cannot compete with SOD for O_2^-, the formazan blue color is slowly generated. This small rate of direct reduction has been subtracted from observed rates to obtain corrected rates, which are used throughout.

The above model has been tested in a number of ways as summarized in Table I. The model predicts that the rate, R, is independent of xanthine concentration (as long as xanthine is present in excess) and of NBT concentration if there is no added Q. Both these predictions are borne out (Table I, experiments a–c). On the other hand, the rate should depend on the amount of xanthine oxidase added (experiments a and d). In the presence of a fixed concentration of some superoxide scavenger, Q, the rate should depend on [NBT], to give the same value for k_Q regardless of experimental conditions. The result of such an experiment is shown in Table I for Q = Fe^{III}TMpyP (phase II (vide infra); experiments e and f). Finally, if the reaction is first order with respect to Q, then k_Q calculated from eq 11 will be independent of [Q]. This result is realized for Q = Fe^{III}TMpyP (phase II) as shown by experiments e, g, and h of Table I.

The total absorbance change during the reaction was the same in experiments a and b shown in Table I; i.e., doubling the xanthine concentration had no effect on the $A_{t=\infty}$ at 560 nm, indicating that, under the conditions of these experiments, the limiting reagent is NBT. This permits a calculation of the molar absorptivity of formazan at 560 nm in this reaction medium: $\epsilon_F = 3 \times 10^4$ M^{-1} cm^{-1}. Doubling the NBT concentration in experiment c in Table I does not lead to a doubling of the final absorbance at 560 nm, suggesting that, under these conditions, xanthine is the limiting reagent.

A number of iron complexes were considered as potential superoxide scavengers including $Fe(EDTA)^{2-}$, $Fe(EDTA)^-$, $Fe(DTPA)^{3-}$, $Fe(DTPA)^{2-}$, $Fe(phen)_3^{2+}$, $Fe(CN)_6^{3-}$, and $Fe(CN)_6^{4-}$. Halliwell had demonstrated earlier that $Fe(EDTA)^{2-}$ shows superoxide dismutase activity[12] and consistent with that result we obtain a value of $k_Q = 3 \times 10^5$ M^{-1} s^{-1} for this complex, a value some five times greater than that for the reaction of NBT with superoxide ion (cf. Table II). We estimate the number of superoxide ions which react with a given metal complex ion from the formula[24]

$$\text{minimum superoxide turnover} = (1 - \rho)(0.6) \times [\text{xanthine}]/[Q] \quad (12)$$

Thus, as claimed earlier,[12] the reaction of $Fe(EDTA)^{2-}$ with O_2^- is shown to be catalytic. The reactions of $Fe(DTPA)^{3-}$ and $Fe(phen)_3^{2+}$ with O_2^- proceed somewhat more slowly than the $Fe(EDTA)^{2-}$ reaction, thus requiring higher concentrations of the metal complex to substantially influence the rate of formazan production. Thus it is not certain for these latter cases whether the process is catalytic (Table II). The iron(III) complexes of EDTA and DTPA were also tested and both $Fe(EDTA)^-$ and $Fe(DTPA)^{2-}$ show a lag period at initial stages of the reaction (Figure 1) but then formazan blue is produced at a rate comparable to but somewhat faster than when the iron(II) complexes are added.

The rate of production of formazan from NBT is not influenced by the presence of ferrocyanide, [Q] = 3.3×10^{-5} M.

Table II. Results for Metal Complexes as Superoxide Scavengers

substance	comments	min superoxide turnover
$Fe(EDTA)^{2-}$	$k_Q = 3 \times 10^5$	5.3
$Fe(EDTA)^{-}$	incubation period followed by $k_Q = 2 \times 10^5$	1.4
$Fe(DTPA)^{3-}$	$k_Q = 1 \times 10^5$	2.6
$Fe(DTPA)^{2-}$	incubation period followed by $k_Q = 0.8 \times 10^5$	0.9
$Fe(phen)_3^{2+}$	$k_Q = 1 \times 10^5$	1.2
$Fe(CN)_6^{4-}$	$\rho \sim 1$, $[Q] = 3.3 \times 10^{-5}$ M	
$Fe(CN)_6^{3-}$	incubation period followed by rapid formazan production (see text)	
H_2TMpyP^{4+}	$\rho \sim 1$, $[Q] = 1.0 \times 10^{-5}$ M	
$Cu^{II}TMpyP$	$\rho \sim 1$, $[Q] = 1.3 \times 10^{-5}$ M	
$Zn^{II}TMpyP$	$\rho \sim 1$, $[Q] = 1.0 \times 10^{-5}$ M	
$Co^{III}TMpyP$	some inhibition of formazan production at $[Q] = 2.3 \times 10^{-5}$ M (see text for details)	
$Fe^{III}TMpyP$	biphasic when catalase not added	
	k_Q (phase I) $= 3 \times 10^7$	21
	k_Q (phase II) $= 2 \times 10^6$	135

However, when the reaction mixture is made 3.3×10^{-5} M ($\sim 10^{-7}$ mol) in ferricyanide, a lag period results during which essentially none of the O_2^- produced reacts with NBT. The rate of formazan production then accelerates to $\rho \sim 1$ (Figure 1). We suggest that, in this early stage of the reaction, O_2^- reduces $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ so rapidly that NBT cannot compete for the produced O_2^-. The formed $Fe(CN)_6^{4-}$ does not interfere with formazan production. A similar reaction of $Fe(CN)_6^{3-}$ with O_2^- was demonstrated by previous workers[25] but these experiments have been included here in order to emphasize the different behavior, in our system, of substances that react stoichiometrically and catalytically with O_2^-.

The reactions with superoxide of the water-soluble porphyrin tetrakis(4-*N*-methylpyridyl)porphine and a number of its metal derivatives have also been investigated. We find that none of the species H_2TMpyP^{4+}, $CuTMpyP^{4+}$, nor $ZnTMpyP^{4+}$ significantly affects the rate of formazan production at $[Q] \sim 10^{-5}$ M. $Co^{III}TMpyP$ shows superoxide dismutase activity at concentrations above 2×10^{-5} M, but the kinetic profile is biphasic with the two processes having similar rates. Experiments with $Fe^{III}TMpyP$ also lead to biphasic kinetic profiles but (1) the two steps proceed at rates which are sufficiently different to allow a resolution of the profile (cf. Figure 2) and (2) this substance appeared to be considerably more efficient at preventing formazan blue production than is the cobalt derivative.

Phase I of the reaction proceeds at a rate characteristic of the slow direct reduction of NBT to formazan at all $Fe^{III}TMpyP$ concentrations above 9×10^{-7} M. Although the rate of formazan production during phase I is independent of FeTMpyP concentration ($9 \times 10^{-7} \leq [FeTMpyP] \leq 2 \times 10^{-5}$), the duration of phase I depends on metalloporphyrin concentration. In typical experiments in which superoxide is produced at $\sim 10^{-7}$ M/s for $[FeTMpyP] = 9 \times 10^{-7}$ M, the phase I time is about 55 s; for $[FeTMpyP] = 2 \times 10^{-6}$ M, phase I time ~100 s, and for $[FeTMpyP] = 4 \times 10^{-6}$ M, phase I time ~220 s. The number of moles of superoxide produced during this period exceeds the number of moles of iron porphyrin by a factor of about 6 (Table II).

Phase II of the reaction shows a linear portion like that of the other iron complexes studied, with the slope dependent on starting FeTMpyP concentration. As discussed earlier, $k_Q = 2 \times 10^6$ M^{-1} s^{-1} (cf. Table I) for this portion of the reaction and the action of the superoxide scavenger is catalytic.

In an effort to determine the chemical basis for the biphasic nature of the reaction, we conducted experiments in which the Soret band of FeTMpyP was observed; NBT was not added to the reaction mixture. For the solution to absorb sufficient light, we had to work at $Fe^{III}TMpyP$ concentrations at which no phase II is evident. We found that the effect of producing superoxide in the reaction mixture is to decrease the intensity of the Soret band at 420 nm irreversibly. Once superoxide was no longer produced because the xanthine supply had been exhausted, the absorbance of the Soret band remained constant (Figure 3).

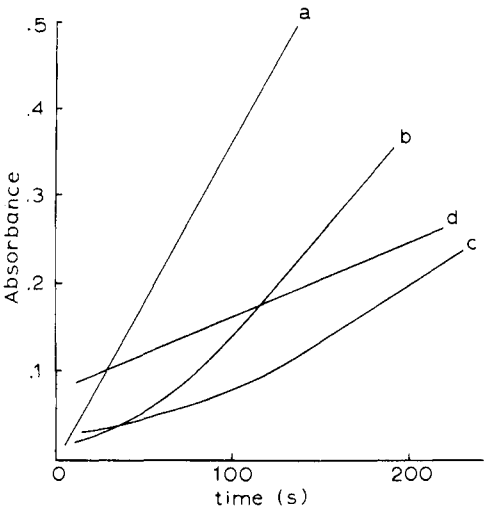

Figure 2. Effect of presence of FeTMpyP on reduction of nitroblue tetrazolium by O_2^- at pH 10.1. (a) No metalloporphyrin added; (b) $[FeTMpyP] = 1.8 \times 10^{-6}$ M; (c) $[FeTMpyP] = 3.7 \times 10^{-6}$ M. Kinetic curves (b) and (c) cross because the metalloporphyrin absorbs weakly (relative to other wavelengths) at 560 nm. (d) $[FeTMpyP] = 1.4 \times 10^{-6}$ M, $\sim 10^4$ units of catalase added.

These spectral experiments suggest that $Fe^{III}TMpyP$ is somehow irreversibly modified during phase I of the reaction and that it is this modified form (referred to as B for convenience) which then reacts with superoxide in phase II.

$$FeTMpyP + ? \rightarrow B \text{ (phase I)}$$

$$B + O_2^- \rightarrow \text{products (phase II)}$$

The form B does not appear to be porphyrin in nature because of the absence of a band of Soret intensity but it is quite efficient at catalyzing the dismutation of superoxide as is evidenced by its rate constant, $k_Q = 2 \times 10^6$ M^{-1} s^{-1}.

In the dismutation process, superoxide is converted to H_2O_2 and, in the presence of metal ions, H_2O_2 can react with O_2^- to form hydroxyl radicals (Haber-Weiss reaction).[2-5] In an effort to determine which, if any, of these species—O_2^-, H_2O_2,

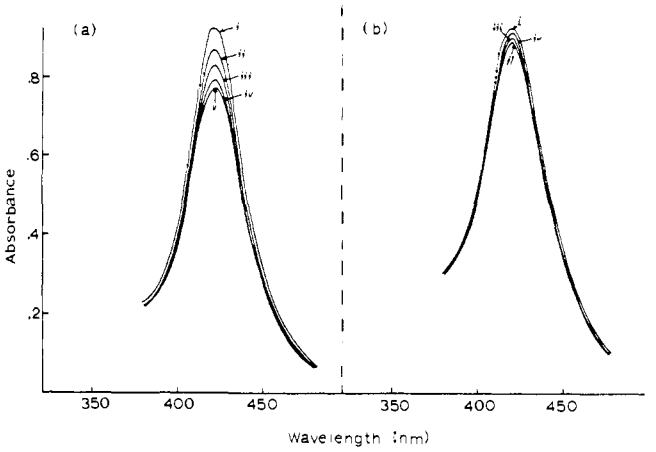

Figure 3. Spectrum of reaction mixture in the Soret region of FeTMpyP as a function of time. (a) No catalase added, [FeTMpyP] = 9.7×10^{-6} M, (i) spectrum before addition of xanthine oxidase corrected for xanthine oxidase medium, (ii) 35 s after addition of xanthine oxidase, (iii) 1.5 min after addition, (iv) 2.5 min after addition, (v) 4.5 min after addition. (b) $\sim 10^4$ units of catalase added, [FeTMpyP] = 9.7×10^{-6}, (i) spectrum before addition of xanthine oxidase corrected for xanthine oxidase medium, (ii) 35 s after addition of xanthine oxidase, (iii) 1.5 min after addition, (iv) 2.5 min after addition. From 4.5 min after addition onwards, the spectrum returns to (i).

and/or OH·—are involved in converting FeTMpyP into B, we conducted experiments in which either (1) superoxide dismutase, (2) catalase, or (3) mannitol, a potent hydroxyl radical scavenger,[5] was added to the reaction mixture. Both competition kinetic and spectral experiments were conducted for each medium. Mannitol had no effect on either the kinetics or the spectral experiments. In contrast, the presence of SOD markedly changed the kinetic profile to one in which formazan is produced via the slow direct reduction step only. SOD had no effect on the spectral experiment; the intensity of the Soret band still decreased irreversibly with time. However, in the presence of as little as 100 μg ($\sim 10^4$ enzyme units) of catalase, the kinetics show only one phase and the Soret band decreases and broadens slightly at first, remains stable for a period, and then reattains its original intensity (Figure 3). Thus, it is the hydrogen peroxide produced by the xanthine–xanthine oxidase system which, unless destroyed by catalase, attacks the metalloporphyrin and converts it into B. Previous work on the catalase activity of deuteroferriheme and protoferriheme has shown that hydrogen peroxide attacks and splits open the porphyrin ring in a fairly rapid step to form bile pigments and CO.[26,27] Meso-substituted metalloporphyrins appear to be even more susceptible to ring opening than are the metal derivatives of deuteroporphyrin or protoporphyrin.[28]

These findings were further tested by using as a superoxide source KO_2 dissolved in Me_2SO to which some dicyclohexyl 18-crown-6 had been added. Addition of 20 μL of this solution ($\sim$0.06 M KO_2) to 3 mL of 10^{-5} M FeTMpyP led to a bleaching of the spectrum so that some 90% of the Soret intensity was lost. The presence of SOD or mannitol in the reaction mixture had no effect. However, when O_2^- from this KO_2 source was added to a FeTMpyP solution to which $\sim 10^4$ enzyme units of catalase had been added, less than 4% of the Soret intensity was lost.

The decrease and broadening of the Soret band during the initial stages of reaction between superoxide generated enzymatically and FeTMpyP is likely to be due to the formation of some $Fe^{II}TMpyP$ which has its Soret band at $\sim$440 nm.[29] Thus a mechanism for the catalytic action of FeTMpyP is suggested which parallels that proposed for $Fe(EDTA)^{2-}$ and similar compounds:[12]

$$Fe^{III}TMpyP + O_2^- \rightarrow Fe^{II}TMpyP + O_2$$

$$Fe^{II}TMpyP + O_2^- + 2H_2O \rightarrow Fe^{III}TMpyP + H_2O_2 + 2OH^-$$

Kinetic experiments conducted at FeTMpyP concentrations below 4×10^{-7} M in the presence of catalase (which shows no SOD activity) lead to a rate constant for the dismutation of O_2^- of $k_Q = 3 \times 10^7$ M^{-1} s^{-1}. At these concentration levels at pH 10.1, the stable form of the porphyrin is the dihydroxo monomer, $FeP(OH)_2^{3+}$, the equilibrium constant for dimer formation being about 10^6:[15]

$$K_D = [O-(FeP(OH))_2]/[FeP(OH)_2]^2 = 10^6\ M^{-1}$$

It is thus quite likely that the reduction of $FeP(OH)_2^{3+}$ by O_2^- proceeds via an outer-sphere mechanism. Although the rate constant (k_f) for reactions of the type

$$FeP(H_2O)^{5+} + 2L \xrightarrow{k_f} FePL_2^{5+} + H_2O \qquad (13)$$

has been determined for $Fe^{III}TMpyP$[30] as $\sim 3 \times 10^8$ M^{-1} s^{-1}, a direct extrapolation to the rate of ligand substitution of $FeP(OH)_2^{3+}$ is not possible. The reaction 13 involves a change in coordination number and a spin-state change with ligand addition/substitution. It is probably more to the point to recognize that the substitution reactions of $CoP(OH)_2^{3+}$ are orders of magnitude slower than those of $CoP(H_2O)_2^{5+}$.[27,31-33] The large value of the rate constant for catalytic action of FeTMpyP, $k_Q = 3 \times 10^7$ M^{-1} s^{-1}, argues against ligand substitution preceding electron transfer. Thus, the reduction of the dihydroxo form of the iron porphyrin by superoxide is likely to be an outer-sphere process.

Conclusions

1. A number of iron complexes catalyze the dismutation of superoxide ion. Of the various complexes studied, the most efficient is an iron porphyrin, FeTMpyP, which has a catalytic rate constant about 1–3% of the value for copper–zinc superoxide dismutase at pH 10.1[34]

2. In the absence of a protective agent, FeTMpyP is attacked by H_2O_2 formed in the O_2^- generating system. The resulting product has no Soret absorbance and previous work[26,27] suggests that it may be a bile-pigment type of compound. This product also catalyzes the dismutation of superoxide, but with only about 10% of the efficiency of FeTMpyP. Addition of traces of catalase protects the FeTMpyP from undergoing degradation by H_2O_2.

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Communications to the Editor

Caution in Using ^{15}N–^{13}C Spin–Spin Coupling for Determining (Bio)synthetic Pathways

Sir:

A recent communication by Suzuki et al.[1] purported a successful application of $^{15}N,^{13}C$ double label technique for determining the synthetic pathway to adenine from hydrogen cyanide and formamide.[1] The idea was the same as the well-established ^{13}C double label technique which depends upon the fairly large coupling constants between directly bonded nuclei.[2] It was assumed that the doubly labeled precursors could be detected unambiguously by the coupling constants $J(^{13}C-^{15}N)$, whereas the recombined C–N bonds in the products would lack these couplings. This is a reasonable approach, but unfortunately the magnitude of directly bonded ^{15}N–^{13}C coupling constants is not fully understood.[3] In particular, in the course of the structural investigation using ^{15}N-enriched nucleic acid derivatives, we have found numerous unusually small ^{15}N–^{13}C coupling constants for directly bonded pairs,[4] and therefore the application of $^{15}N,^{13}C$ double label technique used to determine synthetic pathways may be unreliable.

About 100 mg of the fully ^{15}N-labeled adenosine[5] (95% ^{15}N enrichment based on a mass spectrometric analysis) dissolved in 1.5 mL of deuteriodimethyl sulfoxide showed a complex ^{13}C NMR spectrum under proton wide-band irradiation (Figure 1A). The fine structure of the spectrum, which does not exist in the case of normal adenosine (Figure 1B), should be due to ^{15}N coupling constants. The tentative assignment of these coupling constants, which is shown in Figure 1A, was made by comparing the data for the other nucleic acid derivatives[4] and also by the spectral data for [^{15}N]adenine derived from [$^{15}N,^{13}C$]hydrogen cyanide.[1] The directly bonded coupling constants obtained are shown below with the tentative assignment: $J(C_6-N_6)$, 20.5 (20.5); $J(C_4-N_3)$, 4.4 (9.5); $J(C_4-N_9)$, 19.3; $J(C_8-N_7)$, 10.4; $J(C_5-N_7)$, 8.5[6] (7.3); and $J(C_1-N_9)$, 11.1 Hz.[6] The values in the parentheses represent the coupling constants in the [^{15}N]adenine,[1] and the uncertainty of the values in the [^{15}N]adenosine is 0.5 Hz (600-Hz spectral width and 4K real data points). Once again note that the assignments may not all be correct without doing selective ^{15}N decoupling while observing ^{13}C NMR or comparing these with spectra of selectively ^{15}N-enriched adenosines.

In any case, C-8 of the fully ^{15}N-enriched adenosine showed only one measurable spin–spin coupling constant, either with N-7 or N-9, and C-2 showed a rather broad singlet with a line width of ~7 Hz but did not show any resolved coupling to ^{15}N, even though the latter carbon also has two adjacent ^{15}N nuclei. This fact itself is an interesting subject, which we are currently investigating with respect to the sensitivity of ^{15}N–^{13}C coupling constants to the electronic structure of intervening bonds, but at the same time it introduces more complicated situations in using $^{15}N,^{13}C$ double label technique to ascertain the intact ^{15}N–^{13}C fragments in the products. For instance, we now know that "the presence of enhanced peaks instead of ^{13}C–^{15}N coupled peaks can *not necessarily* be explained by the thermal fission and re-formation of the C–N bond in formamide during prolonged heating procedure".[1]

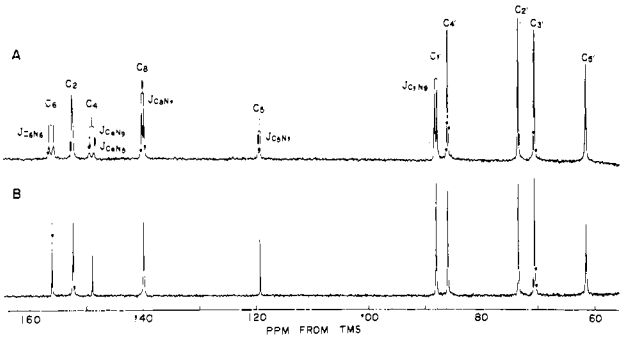

Figure 1.

In conclusion, this method can be used unambiguously only for systems in which the relevant coupling constants are large enough.[8]

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(5) ^{15}N-Enriched adenosine was produced by the microbial fermentation using [^{15}N]ammonium sulfate as the sole nitrogen source, and the details of the procedure will be given in a separate paper.
(6) These values were obtained from the spectrum shown in Figure 1A and may

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