The Oxidation of Catechols by Reduced Flavins and Dehydrogenases

AN ELECTRON SPIN RESONANCE STUDY OF THE KINETICS AND INITIAL PRODUCTS OF OXIDATION*

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SUMMARY

1,2-Dihydroxybenzene-3,5-disulfonic acid (Tiron) has been investigated with regard to oxidation to the o-semiquinone form by both photochemical and enzymatic systems. The formation of the o-semiquinone radical can be recorded at pH 6.8 in the electron spin resonance spectrometer at room temperature. The kinetics of formation and decay of the radical has been determined, since it is relatively stable in solution. The parent catechol is not oxidized to a significant extent below pH 7. The utility of Tiron as a model compound for catechol oxidation reactions lies in the stability of the o-semiquinone at lower pH values and the relative absence of side reactions or formation of highly oxidized pigments. The reaction between oxygen-reductant complexes and this catechol may be diagnostic of the formation of such intermediates where superoxide dismutase fails to inhibit effectively oxygen-dependent electron transfer. It has been previously reported (Massey, V., Palmer, G., and Ballo, D. (1971) in Flavins and Flavoproteins (Kamin, H., ed) p. 349, University Park Press, Baltimore) that in the photochemical system, reduced flavin mononucleotide reacts with oxygen to form a compound which then may dissociate to yield the flavin semiquinone and free superoxide anion. The equilibrium position for dissociation of the reduced flavin-oxygen compound may be shifted toward free superoxide anion at higher pH values. The flavin radical is shown to be unreactive with Tiron at pH 6.8. The disulfonated catechol which was used in the present work is chemically similar to catecholamines and other catechols of biological origin in that metal-catalyzed autoxidation to the o-semiquinone takes place readily above pH 8.5. Evidence is presented indicating that catechols and ferrocyanochrome c can react directly with the reduced flavin-oxygen compound by a pathway which is not susceptible to inhibition by superoxide dismutase. In contrast, the oxidation of Tiron by iron-flavoproteins is completely inhibited by superoxide dismutase. Thus, in the latter case, superoxide anion must be released from the enzyme active site before reacting with catechols or cytochrome c.

Previous investigations into the role of o-semiquinone forms of biologically important catechols have been hampered by the relative instability of these free radicals in the physiological pH range. Although many catechols form stable semiquinones at pH 8.5 and above (1, 2), the observation of the electron spin resonance spectra of these compounds at neutral pH is difficult because of the short lifetime of the radicals in aqueous media. For this reason direct determinations of the kinetics of formation and decay of o-semiquinones in photochemical or enzymatic systems has been limited to flow mixing systems constructed within the cavity of an ESR spectrometer (3, 4).

Two different approaches may overcome some of the difficulties in the direct observation of the formation of unstable o-semiquinones. Copper protein such as ceruloplasmin (5) and superoxide dismutase (6) may oxidize catechols and under some conditions stabilize the semiquinones. The reaction of nor epinephrine or caffeic acid with superoxide dismutase isolated from Neurospora crassa yields appreciable concentrations of the o-semiquinone at pH 7.5 (6). Enzyme-bound copper is reduced concomitantly with catechol oxidation. However, in these cases the spectrum of the free radical product appears only at 77° K. The oxidation product of 1,2-dihydroxybenzene-3,5-disulfonate (Tiron) is much more stable than the products of oxidation of the naturally occurring catechols. In the presence of the copper protein or other oxidizing agents such as silver oxide (Ag2O), Tiron yields a room temperature-observable o-semiquinone. Sulfonation of the aromatic nucleus of pyrocatechol renders the free radical relatively stable near pH 7.

Because of indirect evidence for the oxidation of catechols, particularly of 1,2-dihydroxybenzene-3,5-disulfonate, by superoxide anion (7-9), we have employed Tiron as a chemical trapping agent for univalently reduced oxygen in photochemical and enzymatic systems. Tiron o-semiquinone was proposed to be a 1-electron oxidant for ferrocyanochrome c (7, 8). Direct evidence for the selective reactivity of the catechol radical with ferrocyanochrome c is reported. As shown below, the formation and dissipation of Tiron o-semiquinone can be followed by ESR spectroscopy at 25° in the physiological pH range. Moreover, since the kinetics of appearance of the free radical can be recorded directly, it can be determined whether the o-semiquinone is formed initially or whether it is the product of a reaction between the original catechol and the corresponding o-quinone.

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The exact role of superoxide anion in the oxidation and autoxidation of catechols at neutral pH is not completely known. Above pH 8.5 it is clear that superoxide anion plays a major role in the metal-catalyzed autoxidation process because the reaction is severely inhibited by superoxide dismutase (9). However, at lower pH, superoxide dismutase fails to inhibit the reaction at all (9). This finding indicates that free superoxide anion is not responsible for autoxidation below pH 8.5. Oxygen in the singlet excited state conceivably might be involved in the oxidation of Tiron. However, recent reports suggest that this species also may react with superoxide dismutase (10, 11).

Although superoxide anion can be observed directly by trapping it at low temperature in the ESR spectrometer (12), the formation of this species at higher temperatures cannot be followed readily in photochemical or enzyme-catalyzed systems at neutral pH. Tiron can act as an indicator for superoxide generation in these systems because it apparently reacts rapidly with superoxide anion to give the o-semiquinone. Autoxidation of the catechol is eliminated as a complicating side reaction because at pH 6.8 the o-semiquinone is not formed in the absence of a superoxide-generating system. Fundamental differences between the enzymatic and photochemical processes as determined by this method are reported below.

The primary site of formation of superoxide anion by metalloflavoproteins is not entirely clear (13, 14). The catechol probe might be expected to give some information as to the mode of the univalent reduction of oxygen by the flavin or iron-sulfur components of enzymes such as xanthine oxidase and dihydroorotate dehydrogenase.

MATERIALS AND METHODS

Dihydroorotate dehydrogenase was purified from cells of Zymobacterium aroticum. The enzyme was preactivated by dialysis against 2 mM cysteine as part of the purification procedure and was crystallized by the method of Aleman and Handler (15). Reaction of this activated enzyme preparation with dihydroorotate is possible in the absence of ascorbic acid-containing reagents (16). Xanthine oxidase purified from buttermilk was obtained as an active ammonium sulfate suspension from Sigma Chemical Co. Superoxide dismutase was prepared from bovine erythrocytes by the method of McCord and Fridovich (17). The electrophoretically homogeneous enzyme had an activity of 1300 enzyme units per mg of protein when assayed by a continuous recording photochemical procedure described previously (6). An enzyme unit defined by this assay method is equivalent to 2.5 enzyme units in the assay method of McCord and Fridovich (17). Dithionite-free ferrocyanochrome c was prepared by a previously published method (8). FMN, catecholamines, and other biochemicals were obtained from Sigma. Tiron was supplied by Mann Research Laboratories, Inc. Double distilled water and inorganic reagents having low heavy metal analyses were used in all enzyme preparations and experiments.

Oxygen was reduced univalently in two ways, photochemically (13, 18) and enzymatically. Cuvettes or ESR cells containing the photochemical reaction mixture (see legends to figures) were irradiated with a 60-watt tungsten lamp which produced a total radiant energy flux of about 2 \times 10^7 ergs per cm²-s at the outer surface of the cell. Photoexcited FMN reacted with EDTA to give fully reduced FMN (FMNH₂) and oxidation products of EDTA (18). The complex course of reoxidation of reduced FMN by molecular oxygen has been determined by Massey et al. (13). It is well established that the end products of this reaction are superoxide anion and hydrogen peroxide. However, a compound consisting of reduced FMN and oxygen is formed initially followed by the production of FMN semiquinone and free superoxide anion (19). These reactions are summarized under "Discussion."

ESR spectra and kinetic data were obtained with the Varian E-3 ESR spectrometer. A quartz flat cell having an internal depth of 0.25 mm was fixed in place in the cavity and filled by means of a plastic capillary tube attached to the bottom. A sealing, disposable 1-ml syringe attached to the top of the cell was used to introduce the sample solutions into the cell or expel them from it. The spectra were recorded as a plot of the first derivative of microwave absorption against the magnetic field. For kinetic studies, the magnetic field and the microwave frequency were held constant while the signal height was monitored.

Photochemical reactions were carried out in an ESR cavity which had the front cover removed for irradiation. When the reaction was to be carried out in the absence of oxygen, the mixture was purged with purified helium or nitrogen before being placed in an oxygen-free cell. This procedure gave a final oxygen concentration of less than 2 \muM, as determined with a Clark oxygen electrode. The reactions were started by switching on the 60-watt tungsten lamp, which was located 3 cm from the cell between the poles of the spectrometer magnet.

For enzyme-catalyzed reactions a mixture lacking only the enzyme was placed in the flat cell and the ESR spectrometer was tured. The reaction was initiated by pumping the buffer-substrate mixture out of the flat cell with the sealed syringe and into an aliquot of the enzyme which was held in a test tube (10 cm \times 9 mm). The solution was then re-introduced into the flat cell. If air bubbles were avoided, the spectrometer immediately reassumed the tuned conditions. In this way the rate of change of the signal height could be measured continuously from about 4 s after the actual initiation of the reaction. At low spin concentrations encountered in this work, interactions between paramagnetic centers would be expected to be negligible in solution. The kinetic course of semiquinone formation may be assessed from plots of signal height against time (e.g. Reference 19), since relative signal widths should remain constant throughout a kinetic experiment. Although catechol-derivative radicals exhibit hyperfine splitting patterns characteristic of interactions of the unpaired electron with protons of the aromatic ring, overmodulation may be used to broaden the signal and hence simplify the setting of the magnetic field at the exact position of maximal signal intensity.

RESULTS

Photochemical Oxidation of Tiron—Fig. 1 shows the time course of the appearance of the ESR signal of the Tiron semiquinone at two modulation amplitudes. The recordings were made under similar conditions at pH 6.8. Overmodulation of the semiquinone signal produced Spectrum A, whereas a 10-fold lower modulation amplitude gave Spectrum B, in which the hyperfine splitting is well resolved. Tiron semiquinone yields four identical resonances because the free electron interacts with the 2 non-equivalent ring protons (1). Each proton splits the resonance into a pair of signals because of the 2 protons’ differing spatial positions. Although the hyperfine interactions are not resolved in the overmodulated signal, the kinetics of the formation and decay of the Tiron semiquinone is not significantly altered.

The vertical arrows of Fig. 1 indicate initiation and reversal of the reaction by alternately starting and stopping irradiation of the FMN-EDTA-Tiron mixture in the cavity of the spec-
trometer. The traces appeared to be simple exponentials when log \((S_{\text{max}}/s)\) was plotted against time, where \(S_{\text{max}}\) represents maximum first derivative amplitude and \(s\) is the difference between \(S_{\text{max}}\) and the amplitude at a given time. Pseudo-first order rate constants for rise and fall of the signal are given in Table I. The modulation amplitude did not noticeably affect \(k_1\), as can be seen from an inspection of Curves 1 and 3.

Curve 2 of Fig. 1 also establishes the absolute requirement for molecular oxygen for the oxidation of Tiron to the \(\alpha\)-semiquinone. The initial oxygen concentration was less than 2 \(\mu\text{M}\). On illumination (first vertical arrow, Curve 2) residual oxygen was consumed and the small amount of \(\alpha\)-semiquinone which was formed disappeared after a few minutes. Cessation of illumination produced no significant change. When the cell contents were mixed with air followed by re-illumination (third vertical arrow, Curve 2), the reaction proceeded normally as expected.

The kinetic course of the formation and the decay of the Tiron radical indicated that the \(\alpha\)-semiquinone is the primary product of the oxidation of the catechol. If the \(\alpha\)-quinone were required to be formed first, a lag in the appearance of the semiquinone after illumination might be expected. This was never observed, indicating that a side reaction between the catechol and the quinone was probably not responsible for initial \(\alpha\)-semiquinone formation. Moreover, cessation of illumination caused a first order return of the radical concentration to a very low level. If accumulated \(\alpha\)-quinone reacted with Tiron to maintain a relatively high concentration of semiquinone, the \(\alpha\)-semiquinone signal would be expected to remain for a finite period of time after the exciting lamp had been turned off. Since no lag was observed in the decay of the Tiron radical, it must be assumed that this was a pseudo-first order process which involved a reaction between the \(\alpha\)-semiquinone and other components of the reaction medium which were in excess.

Although the data of Fig. 1 showed that oxygen is required for the oxidation of Tiron, it remained to be determined whether the FMN-semiquinone was also a direct oxidant for the catechol. It was necessary to eliminate this possibility in order to rigorously implicate some reduced form of oxygen in the oxidation of Tiron. Spectrum 2 of Fig. 2 was obtained when a mixture containing FMN, Tiron, and EDTA was irradiated under anaerobic conditions. The FMN concentration was increased in this experiment in order to make possible the direct observation of the FMN radical which is formed in the oxygen-free system under these conditions. The identity of Spectrum 2 with the FMN radical is established by the broad line width (20 G), by the requirement for large modulation amplitudes for observation of the signal, and by the lack of hyperfine splitting at any modulation amplitude. At a much lower modulation amplitude (Spectrum 3) there is virtually no signal. The FMN radical did not decay if the illuminating lamp was turned off, showing that the system was in equilibrium after 30 min. These results show that there is no Tiron radical in the illuminated anaerobic reaction mixture even though the FMN radical is present. On equilibration of the mixture with oxygen the FMN radical completely disappeared. On re-illumination a totally different signal appeared which was recorded as Spectrum 4 at a 10 fold lower gain setting.

This spectrum exhibits the expected hyperfine pattern of the Tiron radical. Even at the higher modulation amplitude, the catechol radical is distinguishable from the FMN semiquinone because the signal from the former is narrower (7.3 G) and the peak-to-peak amplitude does not increase with increasing modulation amplitude, indicating saturation of the signal. From Fig. 2 it may be concluded that neither the FMN radical nor \(\text{FMNH}_2\) is an oxidant for Tiron, that no appreciable concentration of FMN radical exists in the aerobic mixture, and that oxidation of Tiron requires mediation by some species derived from molecular oxygen.

Modification of Photochemical System—Three categories of reagents were added to the photochemical FMN-EDTA-Tiron reaction mixture.

1. Reactants which completely eliminate \(\alpha\)-semiquinone accumulation by trapping free superoxide anion or its precursor, the photochemically induced \(\text{FMNH}_2\)-oxygen compound (13). Rapid reaction between an electron acceptor and the catechol radical would produce a similar result.

2. Reactants which partially inhibit the accumulation of \(\alpha\)-semiquinone.
Fig. 2. Lack of reactivity of FMN semiquinone with Tiron. FMN and Tiron solutions were made anaerobic separately and then combined in the dark to give final concentrations as follows: FMN, 4.2 x 10^{-5} M; EDTA, 6.7 x 10^{-3} M; potassium phosphate buffer, 0.04 M (pH 6.80); and Tiron, 0.083 M. The residual oxygen concentration was less than 2 μM. The reaction mixture was introduced anaerobically into a nitrogen-filled ESR cell which was kept dark until after Spectrum 1 had been recorded. Spectrum 2 was obtained after the cuvette had been illuminated in the spectrophotometer with light from a tungsten source at a total radiant flux of 10^9 ergs per cm²-s for 30 min. The broad signal of Spectrum 2 remained stable on termination of illumination unless oxygen was introduced. Spectrum 3 was recorded under the same conditions as 1 and 2 except that the 100 kHz modulation amplitude was decreased from 20 G to 0.8 G. The reaction mixture was returned to an opaque tube, below the spectrometer cavity, and O₂ gas was introduced through a capillary. Bubbling of O₂ was continued for 60 s before the mixture was returned to the darkened spectrometer cell. No signals were observed until the illuminating lamp was activated. Spectrum 4 was recorded 5 min after illumination began. Spectrum 5 was obtained 10 min later at a much higher modulation amplitude. Spectrometer parameters are listed below for the five spectra, which were all recorded with 50 milliwatts of microwave power at 970 MHz. The g values of the Tiron and FMN radicals are represented by g₁ and g₂, respectively.

| Spectrum No. | Gain | Modulation amplitude | Time constant | Scan time | Illumination time |
|--------------|------|----------------------|---------------|----------|------------------|
| 1            | 2.5 x 10^4 | 20                  | 3             | 12.5     | 0                |
| 2            | 2.5 x 10^4 | 20                  | 10            | 6.25     | 30               |
| 3            | 2.5 x 10^4 | 0.8                 | 1             | 3.33     | 38               |
| 4            | 3.2 x 10^4 | 0.8                 | 0.3           | 3.33     | 5                |
| 5            | 2.5 x 10^4 | 20                  | 0.3           | 12.5     | 15               |

3. Reagents which have no effect on the course of the reactions.

Ferrocytochrome c falls into the first category. In the concentration range 1 to 5 x 10^{-5} M, reduced cytochrome c completely suppressed the results described in Fig. 1 by reacting with the Tiron radical (see below). Ferricytochrome c also eliminated o-semiquinone accumulation, probably by competing for a form of univalently reduced oxygen. The resulting conversion to ferrocytochrome c compounded this observed effect. When l-epinephrine or norepinephrine (10^{-3} M) was added to the Tiron-FMN reaction mixture, Tiron semiquinone formation was not observed. This result indicates a direct reaction between these hormones and either the immediate oxidant of Tiron or the Tiron radical itself. In reaction mixtures which contained the catecholamines, no free radicals could be detected at pH 6.8 because of conversion of the hormones to nonradical, oxidized forms as evidenced by the formation of light-absorbing pigments.

In the partially inhibitory category were organic compounds that are known scavengers of activated oxygen intermediates, such as indole-3 acetate (10^{-4} M), which caused 50% inhibition of the photochemical reaction as followed by ESR. Surprisingly, superoxide dismutase was relatively ineffective in inhibiting the oxidation of Tiron in the photochemical system. Very high concentrations of the dismutase could not be used because of the oxidation of the catechol by the copper-protein. At or below a concentration of 3 x 10^{-5} M enzyme copper, inhibition of the initial rate of o-semiquinone production was never greater than 8% at pH 6.8. This lack of dismutase effect in spite of the absolute requirement for oxygen is considered further below.

The initial rate of oxidation of Tiron was not affected by catalytic concentrations of catalase or by 5 x 10^{-4} M horse-radish peroxidase. Hydrogen peroxide at a concentration of 5 x 10^{-2} M had no effect on the complete photochemical reaction or on Tiron alone at pH 6.8.

Oxidation of Ferrocytochrome c—The rapid oxidation of ferrocytochrome c was proportional to the concentration of the catechol semiquinone which was present in a reaction mixture. In this experiment, a phosphate buffer solution containing 0.018 M Tiron at pH 6.8 was allowed to react with 10 mg of Ag₂O for 5 min at 25°. The solid oxidant then was removed by rapid filtration, which effectively stopped the formation of semiquinone. Aliquots of the mixture were added to 3.8 x 10^{-5} M ferrocytochrome c in a spectrophotometer which was set to record absorbance at 550 nm. On addition of the oxidized Tiron an immediate oxidation of the cytochrome occurred in the presence of the Tiron radical. This initial process was followed by a much slower oxidation reaction, which probably was due to a reaction between the yellow Tiron o-semiquinone and ferrocytochrome c at pH 6.8. The data correlating the magnitude of the immediate phase in the oxidation of ferrocytochrome c with the height of the ESR signal at specific times are presented in Table II. It is apparent that the rapid reaction phase requires appreciable concentrations of the Tiron radical. When the semiquinone concentration has dropped to a minimum level, virtually no fast reoxidation of ferrocytochrome c is observed. The Tiron semiquinone must be regarded as the oxidant of ferrocytochrome c in both photochemical (7) and enzymatic (8) systems.

Either Tiron or pyrocatechol may mediate the oxygen-dependent reoxidation of ferrocytochrome c (8). This reaction and the effect of superoxide dismutase on it are shown in Fig. 3. Although the figure indicates that the reaction requires FMN and Tiron, superoxide dismutase inhibits only about 50%. Previous data established the absolute requirement for oxygen (7).

Photochemical Oxidation of l-Epinephrine—Although catecholamines produced no observable free radicals in the FMN-EDTA photochemical system, the oxygen-dependent formation of adrenochrome was followed spectrophotometrically at 480 nm (9). Irradiated reaction cuvettes were removed from the light beam periodically and the absorbance was determined. Table III shows that the initial rate of increase in absorbance at 480 nm increased rapidly with pH between pH 6.5 and pH 8.3. However, superoxide dismutase inhibited 80% of the reaction at the higher pH but only 50% at pH 6.5. Again the over-all oxidation requires molecular oxygen (14).
The photochemical reduction of ferricytochrome c exhibited a rate, 4 min.

The height of the signal is proportional to the amount of semiquinone added to cytochrome c at the indicated times. ESR parameters were modulation amplitude, 0.8 G; microwave power, 50 milliwatts; receiver gain, 3.2 $\times$ 10$^4$; time constant, 0.1 s; scan rate, 4 min.

The first column lists times after stopping the Ag$_2$O oxidation of Tiron by filtration. The absorbance of ferri-cytochrome c at 550 nm was determined after addition of aliquots of the mixture of Tiron, Tiron semiquinone, and Tiron o-quinone. The magnitude of the ESR first derivative signal as a function of time was followed in a parallel experiment in the absence of cytochrome c.

The metalloflavoproteins also oxidize epinephrine (17, 22). Although the formation of the Tiron o-semiquinone would be expected to be complex, plots of time against the log of the ratio of maximum signal amplitude to the difference between this maximum signal and the signal amplitude at a given time yielded straight lines, indicating pseudo-first order behavior. The rate constants are given in Table I and were related to enzyme concentration.

The photochemical reduction of ferricytochrome c exhibited a similar pattern of inhibition by superoxide dismutase. This reaction is known to require univalently reduced oxygen. At pH 8.5, maximal inhibition by the dismutase was 95%, while at pH 6.8 this effect decreased markedly to 80%.

Enzymatic Oxidation of Tiron—In view of the seemingly anomalous behavior of the photochemical system toward superoxide dismutase, two non-heme iron-containing flavoproteins were used to replace photoreduced FMN. Substrate-reduced dihydroorototate dehydrogenase and xanthine oxidase are known to form superoxide anion (8, 20), and this univalently reduced species is considered to be the reducer for ferri-cytochrome c (21). Indirect evidence also indicated that dihydroorototate dehydrogenase could oxidize Tiron to the o-semiquinone under aerobic conditions (7, 8). This free radical in turn could oxidize ferri-cytochrome c. The upper section of Fig. 4 shows the kinetic course of the enzyme-dependent formation of the o-semiquinone. The reaction was started by mixing the enzyme with a solution of the reducing substrate, dihydroorotatc, and Tiron at pH 7.0. Mixing was complete in 3 to 4 s. The traces show that Tiron semiquinone rapidly attained a steady state level on introduction of the enzyme. The rate and extent of oxidation of Tiron were dependent on enzyme concentration, as shown by the difference in Curves 1 and 2. For Curve 1, 5 times as much enzyme was used. As expected, the rate of decay of Tiron o-semiquinone was not dependent on enzyme concentration (Curves 2 and 4). The lower portion of Fig. 4 shows the nonlinear relationship between the amount of enzyme and the initial rate of increase in the o-semiquinone signal. During the steady state period (0.5 to 2 min) the rate of formation of the radical due to the reaction of univalently reduced oxygen and Tiron balanced the rate of dismutation of the o-semiquinone or its further oxidation.

When the source of reducing equivalents (dihydroorotate) neared exhaustion, the latter reactions predominated and the strength of the o-semiquinone signal decreased very slowly. A xanthine-xanthine oxidase system produced similar results, as shown by the solid square data points near Curves 3 and 4 of Fig. 4.

The enzymatic oxidation of Tiron was completely eliminated by inclusion of 50 $\mu$g of bovine superoxide dismutase in the reaction mixture. Therefore, in the enzymatic reaction all of the electron flux takes place through freely diffusing superoxide anion even at pH 7.

Although the formation of the Tiron o-semiquinone would be expected to be complex, plots of time against the log of the ratio of maximum signal amplitude to the difference between this maximum signal and the signal amplitude at a given time yielded straight lines, indicating pseudo-first order behavior. The rate constants are given in Table I and were related to enzyme concentration.

The metalloflavoproteins also oxidize epinephrine (17, 22).

### Table II

| Time | Height of ESR signal | Ferrocytochrome c oxidized, immediate phase | Rate of slow oxidation of cytochrome c | Value |
|------|---------------------|-------------------------------------------|-------------------------------------|-------|
| min  | × 10$^{-6}$ M       | × 10$^{-4}$ M/min                        |                                     |       |
| 2.1  | 55                  | 1.7                                       | 1.2                                 |       |
| 4.5  | 20                  | 0.56                                      | 2.4                                 |       |
| 6.0  | 18                  | 0.41                                      |                                     |       |
| 0.3  | 16                  | 0.25                                      | 0.5                                 |       |
| 12.0 | 14                  | 0.12                                      | 15.7                                |       |
| 15.5 | 13                  | 0.09                                      | 20.4                                |       |
| 30.0 | 4                   | 0                                         | 22.0                                |       |

* Arbitrary units.

### Table III

| pH | Dismutase | O$_2$ concentration | Initial rate | Inhibition |
|----|-----------|---------------------|--------------|------------|
| 6.5| +         | 240                 | 0.065        | 52         |
| 6.5| +         | 240                 | 0.065        | 52         |
| 7.2| +         | 240                 | 0.068        | 52         |
| 7.2| +         | 240                 | 0.024        | 65         |
| 8.3| +         | 240                 | 0.100        | 80         |
| 8.3| +         | 240                 | 0.020        | 80         |
| 6.5| +         | 3                   | 0.0001       | 98         |
| 6.5| +         | 3                   | 0.0001       | 98         |

* Where indicated 100 $\mu$g of bovine erythrocytoperoxidase were added to otherwise complete reaction mixtures prior to irradiation.
FIG. 4. Aerobic, substrate-dependent conversion of Tiron to the o-semiquinone form by reduced metalloflavoproteins. A mixture consisting of 20 mM sodium phosphate buffer, pH 7.2, 3 mM Tiron, and 15 mM sodium dihydroorotate was placed in flat ESR cells (0.5 mm inside width). The spectrometer was tuned with the parameters detailed below. The dihydroorotate dehydrogenase (DHOD) (1.2 nmoles of flavin per ml, Curves 1 and 2, or 0.24 nmole of flavin per ml, Curves 3 and 4) was then added. For similar experiments with xanthine oxidase, 0.67 mM xanthine replaced dihydroorotate in the substrate mixture and 0.2 mg of xanthine oxidase replaced dihydroorotate dehydrogenase. II, xanthine oxidase data. Spectrometer conditions were modulation amplitude, 16 G; microwave frequency, 9.449 GHz; microwave power, 100 milliwatts; receiver gain, $6.2 \times 10^6$; time constant, 1 s; and temperature, 25°.

This reaction can be completely inhibited by superoxide dismutase. For example, under the conditions of Fig. 4, α-epinephrine was oxidized to adrenochrome at an initial rate of 0.11 absorbance unit per min at 480 nm in the presence of dihydroorotate dehydrogenase when the catecholamine replaced Tiron in the reaction mixture. Although no free radical could be observed at pH 7, the visible spectrum showed that the reaction was completely inhibited by 100 μg of superoxide dismutase. In contrast to the reaction with photochemically oxidized catechols, the enzymatic reaction displayed far greater sensitivity to inhibition by superoxide dismutase.

**DISCUSSION**

The utility of considering the reactions of a disulfonated catechol as models for the oxidation of biologically occurring catechols and catecholamines becomes apparent when the close similarity in chemical properties among Tiron, pyrocatechol, 1,2-dihydroxybenzoic acid, and caffeic acid are considered. All of the compounds autoxidize rapidly above pH 8.5 and give stabilized free radicals at 77° K with N. crassa superoxide dismutase at lower pH values (6). Tiron is aerobically oxidized by reduced FMN and by reduced iron-sulfur-containing flavoproteins, in analogy with the oxidation of epinephrine and norepinephrine by these systems. In all cases the reaction shows an absolute requirement for molecular oxygen.

Massey et al. have reported evidence for a series of reactions to account for the complex reoxidation of photooxidized flavins (13). An essential feature of this mechanism is the formation of a covalent compound between reduced flavin and oxygen which can dissociate to yield free superoxide anion and flavin semiquinone, according to Reactions 1 and 2 (13).

\[
\text{FH}_2 + O_2 \rightarrow \text{FH}_2 \cdot - O_2 \quad (1)
\]

\[
\text{FH}_2 \cdot + O_2^{-} \rightarrow \text{FH}^+ + O_2 \quad (2)
\]

The flavin semiquinone may also reduce oxygen univalently, according to Reactions 3 and 4 (13). Reactions 3 and 4 are apparently fast, since no flavin radical can be observed under aerobic conditions.

\[
\text{FH}^+ + O_2^{-} \rightarrow \text{F} + O_2 + H^+ \quad (3)
\]

\[
H^+ + \text{FH}^+ + O_2^{-} \rightarrow \text{F} + \text{H}_2\text{O}_2 \quad (4)
\]

We propose that in our photochemical system the reduced forms which contain the oxygen molecule may react with a catechol molecule (Reactions 5 and 6) or may disproportionate (Reaction 7). FMN radicals were shown to be unreactive with Tiron.

\[
\text{R} \quad \text{R}
\]

\[
\text{OH} + O_2 \rightarrow \text{HO}^+ + \text{R} \quad (5)
\]

\[
\text{R} \quad \text{R}
\]

\[
\text{OH} + \text{FH}_2 \cdot \rightarrow \text{FH}^+ \quad (6)
\]

\[
20_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + 0_2 \quad (7)
\]

While Reaction 5 would be completely inhibited by superoxide dismutase, Reaction 6 should not be, since the reduced flavin–oxygen compound would not be expected to fit the active site of the dismutase. The catechol molecule, however, could readily attack the compound in a bimolecular reaction. The fact that superoxide dismutase is ineffective in blocking the oxygen-dependent transfer of electrons from reduced flavin to Tiron supports Reaction 6 as the major mechanism in the photochemical oxidation of Tiron in the physiological pH range. The only other explanation for this phenomenon would be an inhibition of superoxide dismutase by Tiron. No evidence for this was found, although it is a difficult point to test because a high concentration of catechols (> 1 mM) would competitively remove the substrate of the enzyme and also would reduce enzyme-bound copper (6).

Da Silva Araujo et al. (23) have pointed out that superoxide anion is usually reversibly generated within a pre-existing charge transfer complex between molecular oxygen and an electron donor. α-Diphenols may play a dual role by reducing superoxide anion and transferring a proton to the resulting peroxy ion according to Reactions 5 and 6. Hence catechols could shift an unfavorable equilibrium (Reaction 2) between the electron transfer to oxygen and the back reaction. Hydrogen ion-dependent shifts in this type of equilibrium could account for the effect of pH on the sensitivity of catechol oxidation to inhibition by superoxide dismutase.

At pH 6.8 Tiron is oxidized almost exclusively by the reduced...
FMN-oxygen compound, and only a very small percentage of free superoxide anion is released into the reaction medium. L-Epinephrine and norepinephrine, in contrast, must react less rapidly with the oxygen compound and thus allow about one-half of the reducing equivalents to be released as freely diffusing superoxide anion. This species also oxidizes these electron donors, but in this case superoxide dismutase may intervene in the electron transfer. Since the catecholamines completely eliminate the accumulation of the Tiron radical it may be assumed that they also react directly with the Tiron radical.

The reactions of oxidized and reduced cytochrome c (cyt) which are of importance in the present work are as follows.

\[
\begin{align*}
\text{Cyt-Fe}^{2+} + O_2^{-} & \rightarrow \text{cyt-Fe}^{2+} + O_2 \\
\text{Cyt-Fe}^{2+} + F\text{H}_2 & \rightarrow \text{cyt-Fe}^{2+} + F\text{H}^+ + H^+ \\
\text{Cyt-Fe}^{2+} + F\text{H}_2O_2 & \rightarrow \text{cyt-Fe}^{2+} + F\text{H}^+ + O_2 + H^+ \\
\text{Cyt-Fe}^{2+} + T^2 & \rightarrow \text{cyt-Fe}^{2+} + T 
\end{align*}
\]

Ferricytochrome effectively traps reducing equivalents from oxygen radicals and reduced flavin according to Reactions 9 and 10. Under aerobic conditions Reaction 11 is of some importance, as illustrated by the failure of superoxide dismutase to completely inhibit the photochemical aerobic reduction of cytochrome c. Evidence for Reaction 10 was presented previously (7). Tiron (T) fails to inhibit cytochrome c reduction by photo-reduced flavin under anaerobic conditions, but in the presence of oxygen inhibition is nearly complete. Therefore, under aerobic conditions all reducing equivalents pass through oxygen according to Reactions 9 and 11. Tiron radical, which is formed in the reaction with the F\text{H}_2O_2 compound, can react in turn with ferricytochrome c (Reaction 12). This process prevents observation of the catechol semiquinone when ferricytochrome is present in the reaction mixture. The versatile reactions of cytochrome c with both oxygen and catechol radicals suggest that this mobile electron carrier may have a protective function in living cells which to a degree supplements the protective action of superoxide dismutase (24).

t-Epinephrine and to a lesser extent ferricytochrome c must be able to react directly with the reduced flavin-oxygen compound, because at pH 6.8 aerobic oxidation is only partially inhibited by excess superoxide dismutase. The transfer of electrons from univalently reduced oxygen to cytochrome c or from catechols to univalently reduced oxygen is distributed between two pathways, only one of which involves free superoxide anion. However, the failure of superoxide dismutase to inhibit a reaction does not rule out the involvement of activated oxygen complexes which are not accessible to the catalytic centers of the enzyme. The enzyme gives no information on univalent electron flux through compounds containing the reductant and oxygen as shown in Reaction 6.

In the case of metalloflavoprotein-catalyzed generation of superoxide anion it is apparent that the anion must be released into the solvent before catechols can be oxidized or cytochrome c reduced. If a complex or compound between univalently reduced oxygen and a prosthetic group of the enzyme is formed it is inaccessible to these reagents. The fact that metalloflavoprotein dehydrogenases co-oxidize catechols through free superoxide anion does not necessarily indicate that superoxide is generated specifically at the iron sites of these enzymes. In the case of flavins and metal-free flavoproteins the reactive site may be structured so that univalent electron acceptors or donors may react either with the reduced flavin-oxygen complex or with free superoxide anion. This difference might explain the reported differences in behavior of univalent electron flow between the two types of flavoproteins (14).

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