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The Micellar Acceleration of the Reduction of Nitroblue Tetrazolium by Superoxide Anion Radicals during the Autoxidation of Polyphenols

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Synopsis. The rates of the reduction of Nitroblue Tetrazolium by O_2^- during the autoxidation of pyrogallol, epinephrine, and catechol were increased 110, 55, and 37-fold respectively in the presence of hexadecyltrimethylammonium bromide. A nonionic micelle of polyoxyethylene dodecyl ether also accelerated these reactions. The microenvironment effects on the electron-transfer from O_2^- to Nitroblue Tetrazolium were discussed.

The superoxide anion radical (O_2^-) is known to be produced by a number of biologically significant oxidations and is probably generated, to some degree, in all oxygen-metabolizing cells.[1] Recently, O_2^- has also been shown to be generated during the autoxidation of some phenols[2,3] and thiols.[4] Many other autoxidizable compounds may be found to generate O_2^- during autoxidation. Therefore, it is useful to develop easy and sensitive methods to detect O_2^-. The detection of O_2^- has been carried out by using direct kinetics techniques, such as EPR, or pulse radiolysis, and using inhibition by superoxide dismutase (SOD).[1] Nitroblue Tetrazolium (NBT) has been used for the detection of small amounts of such reducing agents as O_2^-, S^{2-}, and $S_2O_4^{2-}$.[5] Since it has been reported that the nucleophilic reactivity of the hydroxide ion and thiol are affected by the cationic micellar environment,[6] one may anticipate that the reaction rate between O_2^- and NBT is also accelerated by the micellar systems. In this study, we will report that the rate of electron transfer from O_2^- to NBT is accelerated by the surface-active cation hexadecyltrimethylammonium bromide (CTAB) and by nonionic micelles of polyoxyethylene dodecyl ether (Brij 35), and that NBT in micellar systems may be used in an easy and sensitive method to detect O_2^- during autoxidation.

Experimental

The pyrogallol was purified by sublimation. The epinephrine (Merck A. G.) and catechol (Katayama Chem. Co.) were used without further purification. Sodium dodecyl sulfate (NaLS) and CTAB were recrystallized from methanol and water respectively. Brij 35 (Katayama Chem. Co.), NBT (Sigma Chem. Co.), and bovin Cu-Zn SOD (Sigma Chem. Co.) were used without further purification. The water was deionized and distilled.

The spectroscopic measurements were made with a Shimadzu UV-200S recording spectrometer at 25 °C. The rates of the autoxidation of polyphenols were taken from the increase in the absorbance at 420 nm for pyrogallol[2] and at 480 nm for epinephrine[3] and catechol. The reduction of NBT was measured from the increase in the absorbance at 560 nm due to the accumulation of blue formazan. In all cases, reactions were started by adding an aliquot of an acidic (pH 2.0) stock solution of polyphenol to the buffered reaction mixture.

Results and Discussion

Since it has been shown that the autoxidations of pyrogallol, epinephrine, and catechol all involve O_2^-,[2,3] these polyphenols can transfer electrons to NBT *via* oxygen under aerobic conditions. Therefore, we investigated the reduction of NBT by O_2^- in micellar systems. As the mixing of NaLS and NBT resulted in precipitation, we used CTAB and Brij 35 for the experiment. The initial rates of the aerobic reduction of NBT during the autoxidation of pyrogallol were linear functions of the NBT concentration in either the absence or presence of a detergent, as is shown in Fig. 1. This result indicates that the rates of the reduction of NBT were accelerated by both CTAB and Brij 35. We show in Fig. 2 the effects of the concentrations of CTAB and Brij 35 on the reduction of NBT by O_2^- during the autoxidation of pyrogallol at pH 8.0. The reduction rate of NBT was markedly accelerated above 1×10^{-3} M CTAB (the critical micelle concentration is 0.92×10^{-3} M[6]) and tended to be saturated beyond 7×10^{-3} M CTAB. The rate was also accelerated by Brij 35 and saturated above the detergent concentration of 2×10^{-3} M. 5×10^{-3} M of CTAB and Brij 35 increased the rates by factors of 110 and 13-fold respectively. Similar results were obtained for the autoxidations of epinephrine and catechol, as is shown in Table 1. We can see that 5×10^{-3} M CTAB accelerated the reduction of NBT during the autoxidations of epinephrine and catechol by factors 55 and 37-fold respectively. 5×10^{-3} M Brij 35 also accelerated these reactions by factors of 7 and 5-fold respectively. The drastic acceleration in micellar systems described above raises the question whether these micelles accelerate the rate of autoxidation or electron-transfer from O_2^- to NBT. In order to clarify this point, we examined the micellar effects on the rates of the autoxidation of polyphenols by the methods described in the experimental section. We found that CTAB, Brij 35, and NaLS had almost no effect on the rates of autoxidation of these polyphenols with the

TABLE 1. MICELLAR EFFECT ON THE REDUCTION OF NBT DURING THE AUTOXIDATION OF EPINEPHRINE AND CATECHOL

The reaction mixture contained 2.5×10^{-4} M of polyphenol, 8.3×10^{-5} M of NBT, 5×10^{-3} M of the surfactant, and 1×10^{-4} M of EDTA in 0.1 M Tris-HCl at pH 9.0.

	Δ Absorbance at 560 nm/min		
	Non-surfactant	CTAB	Brij 35
Epinephrine	0.011	0.590	0.079
Catechol	0.007	0.260	0.033

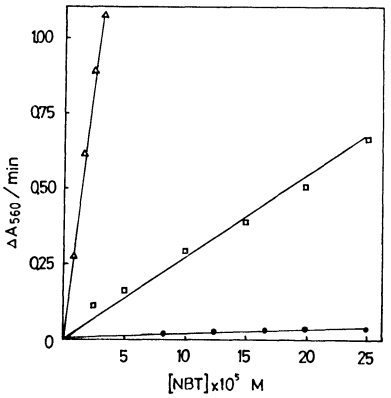

Fig. 1. The initial reduction rate of NBT as a function of NBT concentration. 2.5×10^{-4} M pyrogallol was added to the reaction mixture containing indicated concentration of NBT, 5×10^{-3} M surfactant, 1×10^{-4} M EDTA, and 0.1 M Tris-HCl at pH 8.0. ●; No surfactant, △; CTAB, □; Brij 35.

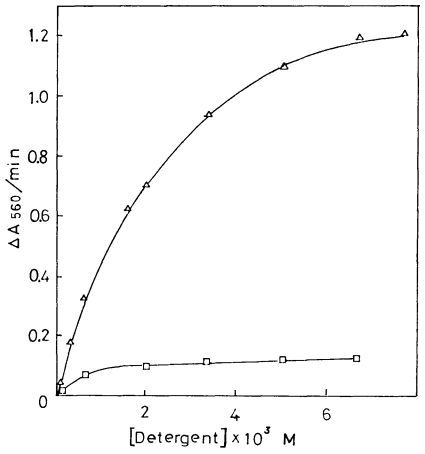

Fig. 2. Plots of the observed pseudo-first-order rate constant against the surfactant concentration for the reduction of NBT during the autoxidation of pyrogallol. Reaction-mixture contained 2.5×10^{-4} M pyrogallol, 3.3×10^{-5} M NBT, 0.1 M Tris-HCl at pH 8.0, and the indicated concentration of CTAB (△) and Brij 35 (□).

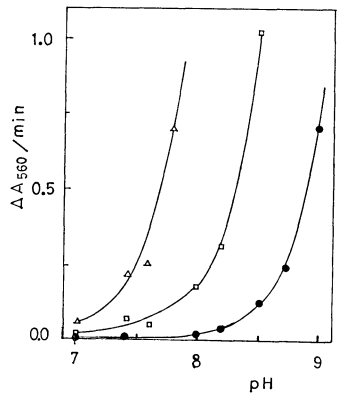

Fig. 3. Effect of pH on the reduction rate of NBT during the autoxidation of pyrogallol. Reaction mixture contanied 2.5×10^{-4} M pyrogallol, 8.3×10^{-5} M NBT, 5×10^{-3} M detergent, and 1×10^{-4} M EDTA in the following buffer systems; pH 7.0 and 7.4, 0.1 M potassium phosphate; other pH, 0.1 M Tris-HCl. ●; No surfactant, △; CTAB, □; Brij 35.

exception of the autoxidation of pyrogallol in the presence of CTAB. 5×10^{-3} M CTAB accelerated the autoxidation of pyrogallol 11-fold. However, its value is quite small in comparison with that for the reduction of NBT (110-fold). Therefore, these results indicate that CTAB and Brij 35 accelerated the rate of electron-transfer from O_2^- to NBT.

Since it has been shown that polyphenols are autoxidized rapidly in an aqueous alkaline solution,[2,3] we investigated the pH effects on the reduction of NBT. As is shown in Fig. 3, the reduction of NBT was accelerated by both CTAB and Brij 35 over the whole pH range of 7.0—9.0. This indicates that even a small amount of O_2^- generated during the autoxidation at a neutral pH is detectable by NBT in the micellar systems. From these result, it seems likely that the catalytic functions of both CTAB and Brij 35 are due to binding organic substrates to the micellar phase, giving more favorable environment for the O_2^- attack on NBT (the orientation effect[6]). Both Fig. 3 and Table 1 indicate that CTAB accelerates the reduction of NBT about 8 times more than does that Brij 35. Therefore, for the rate-enhancement effect by CTAB, besides the orientation effect, electrostatic interactions between the cationic micelle and O_2^- might be important. When KO_2 was directly added to the NBT solution, the rate of the reduction of NBT was accelerated only 30 percent by CTAB and Brij 35 compared with that in the non-micellar system. Therefore, the generation of O_2^- in the micellar phase may be important for the rapid reduction of NBT.

In order to determine whether or not O_2^-, which is generated during the autoxidation of polyphenols, practically reduces NBT in these micellar systems, we examined the effect of SOD on the reduction of NBT. As is shown in Fig. 4, the reduction of NBT during the autoxidation of pyrogallol was inhibited by SOD in either the absence or the presence of a surfactant. The

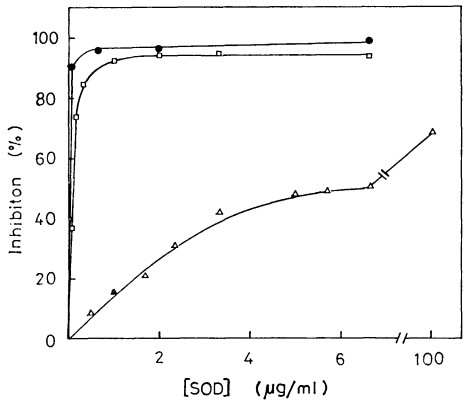

Fig. 4. Inhibition of the reduction of NBT during the autoxidation of pyrogallol by SOD. Cuvets contained 2.5×10^{-5} M NBT, 5×10^{-5} M pyrogallol, 5×10^{-3} M surfactant, 1×10^{-4} M EDTA, and 0.1 M Tris-HCl at pH 8.0. ●; No surfactant, △: CTAB, □; Brij 35.

reductions of NBT during the autoxidation of epinephrine and catechol were also inhibited by SOD in a manner similar to that is the case of pyrogallol. Quite similar micellar effects were also observed in the cases of the autoxidations of anthrone and such thiols as dithiothreitol, 2-mercaptoethanol, glutathione, and *l*-cysteine, all involving O_2^-. Therefore, NBT in the micellar systems might be useful as an easy and sensitive method to detect O_2^- during autoxidation.

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