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THE INFLUENCE OF FATTY ACID MICELLES ON THE ASSAYS FOR SOD ACTIVITY

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The influence of fatty acid (FA) micelles on cytochrome c(cyt.c) reduction and nitroblue tetrazolium (NBT) reduction assays for SOD activity, which continue to be widely used, has been studied. In the presence of FA micelles, the use of cyt.c reduction assay was found to overestimate the real activity of SOD. This effect is attributed to the following reasons. 1. The FA micelles lead to the denaturation of cyt.c, which gives rise to suppression of the reactivity of ferricyt.c (cyt.c(ox)) towards O_2^-. Furthermore, this denaturation increases the reoxidation rate of ferrocyt.c, and consequently the reoxidation causes a decreased rate of cyt.c(ox) reduction. 2. Positively charged cyt.c(ox) interacts with negatively charged FA micelles, and so cyt.c(ox) on the surface of FA micelles reacts less with negatively charged O_2^- because of electrostatic repulsion. Also in NBT reduction assay using a positively charged probe molecule, FA micelles cause the appearance of enhancement of SOD activity, due to suppression of the reactivity of NBT towards O_2^- by electrostatic repulsion. However, in both chemiluminescence assay using the uncharged probe molecule and LDH-NADH assay using the negatively charged probe molecule, FA micelles cannot influence the assays of the SOD activity, because the micelles do not interact electrostatically with probe molecules.

KEY WORDS: SOD, supereoxide, linoleic acid micelles, cytochrome c, NBT, MCLA.

INTRODUCTION

Evidence that suggests the involvement of free radicals, especially superoxide (O_2^-), in a variety of pathological events has accumulated[1,2] since the discovery of superoxide dismutase (SOD) by McCord and Fridovich.[3] Many investigators have attempted to use the SOD as medicine for oxygen free radical-dependent diseases.[4,5]

With a view to using SOD in therapeutic practice, correct determination of the SOD activity in the tissues is necessary. There are a number of assays for SOD activity, of which cytochrome c(cyt.c) reduction and nitroblue tetrazolium (NBT) reduction assays have been widely used.[3,6] The former is the assay used in the first isolation of SOD.[3] In practice, one unit of commercial SOD is defined as that amount causing a 50% decrease in the rate of cyt.c reduction under the assay conditions of McCord and Fridovich.[3] The latter has the advantage of high sensitivity and has been used to measure SOD activity in crude homogenates of tumor tissue, which have very low SOD activity.[7]

Some of the various compounds in crude tissue extract might interfere with assays of tissue extracts for SOD activity either by directly reacting with O_2^-,[8] or by deactivation of xathine oxidase (XOD),[9] or by oxidizing or reducing the probe molecules such as cyt.c and NBT.[9] It has been reported that care should be taken in using cyt.c

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reduction[10] and NBT reduction assays[11] for SOD activity.[12] However, to our knowledge, there is no report on the influence of fatty acid (FA) micelles on the two major assays for SOD activity.

In the course of our study on the biological role of naturally occurring furan fatty acids (F acids),[13–15] we have found that FA micelles affect the cytochrome c reduction and the NBT reduction assays for SOD activity.

MATERIALS AND METHODS

Reagents

Linoleic acid (Ln, 99.5% pure), XOD (grade 1), catalase (bovine liver), cyt.c (horse heart, type III) and SOD (bovine erythrocytes) were obtained from Sigma Chemical Co. (St. Louis, MO). Ln was found to be free of hydroperoxides by thin-layer chromatography and by potassium iodide spray. Xanthine (XO), hypoxanthine (HXO) and NBT were purchased from Kanto Chemical Co. (Tokyo, Japan). 2-Methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]pyradin-3-one (MCLA) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Sodium deoxycholate (SDC) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Ferrocyt.c (cyt.c(red)) was prepared by the method of Baba *et al.*.[16] All other reagents were of analytical grade.

Preparation of FA Micelles

FA micelles were prepared by the method of Gebicki *et al.*.[17] Briefly, stock solutions of FA micelles (10^{-2}M) were prepared by dissolving FA in dilute potassium hydroxide solution (pH 10–11) and were then stored at −20°C. These aliquots were subsequently diluted to the appropriate concentration in water. The micelles in reaction mixtures were confirmed using the dyes pinacyanol and Rhodamine 6G.[18]

Assay Methods for SOD Activity

1) XOD-cyt.c assay. XOD-cyt.c assay followed the original method of McCord and Fridovich.[3] The reaction mixture contained, in a final volume of 3 ml, 50 mM potassium phosphate buffer with 0.1 mM EDTA, pH 7.8, 10 μg/ml catalase, 50 μM XO, 10 μM cyt.c, 10 nM XOD and, when necessary, the appropriate concentrations of additives such as SOD and potassium salt of FAs prepared as described above. Reaction was started by adding 0.2 ml of 0.15 μM XOD (in a final concentration of 10 nM) after equilibration at 25°C, and the initial rate of ferricyt.c (cyt.c(ox)) reduction was determined by measuring the increase in absorption at 550 nm with a Hitachi 557 spectrophotometer equipped with a thermostatted cell block.

2) XOD-NBT assay method. This assay was carried out according to the method of Beauchamp and Fridovich.[6] The reaction mixture contained, in a final volume of 3 ml, 50 mM sodium carbonate buffer with 0.1 mM EDTA, pH 10.2, 10 μg/ml catalase, 0.1 mM XO, 25 μM NBT, 10 nM XOD and, when necessary, the appropriate concentrations of additives such as SOD and potassium salt of FAs. Reaction was started by 0.2 ml of 0.15 μM XOD after equilibration at 25°C, and the rate of NBT reduction was measured at 560 nm.

3) Chemiluminescence method. The chemiluminescence method was performed using the method of Nakano.[19] The mixture contained, in a final volume of 3 ml, 50 mM

Tris-HCl buffer with 0.1 mM EDTA at pH 7.8, 10 μg/ml catalase, 50 μM HXO, 0.2 μM MCLA, 50 nM XOD and, when necessary, appropriate concentrations of additives such as SOD and potassium linoleate prepared as described above. The chemiluminescence measurement was started by the addition of MCLA to the mixture excluding XOD and was continued for an additional 16 min after the addition of XOD. Chemiluminescence was measured with a Luminescence Reader (Aloka, BLR301). During the measurement, the mixture was agitated by rotation at 25°C in the Luminescence Reader.

4) LDH-NADH method. This assay was carried out according to the method previously reported.[20]

RESULTS AND DISCUSSION

Inhibition of Cyt.c(ox) Reduction by FA Micelles

The influence of FA micelles on cyt.c reduction assay for SOD activity, which continues to be widely used, has been studied. This quantitative method for SOD activity is based on the ability of O_2^- to reduce cyt.c(ox) to cyt.c(red), measured as an increase in absorbance at 550 nm. SOD competing with cyt.c(ox) for O_2^- inhibits its reduction. As shown in Figure 1, when SOD at 0.15 μg/ml in a final concentration was added to the control system containing 50 mM potassium phosphate buffer with 0.1 mM EDTA, 10 μg/ml catalase, 50 μM XO, 10 μM cyt.c and 10 nM XOD, the rate of cyt.c(ox) reduction was inhibited by 40% compared to that in the control system. Additions of both SOD and Ln inhibited the rate of cyt.c(ox) reduction more efficiently than did SOD alone. For example, when both SOD at 0.15 μg/ml and Ln at 0.2 mM in final concentrations were added, the rate of cyt.c(ox) reduction was inhibited by 32% compared to SOD alone. Thus Ln was found to suppress the rate of cyt.c(ox) reduction by O_2^- and so to affect the estimate of SOD activity by the cyt.c reduction assay. Substantially the same results were obtained when oleic, γ-linolenic and arachidonic acids were added in place of Ln. On the other hand, common saturated FAs, palmitic and stearic acids did not reveal such an effect under these assay conditions, because they have high Kraft points (70–80°C).

We examined the mechanism of inhibition of the cyt.c(ox) reduction by unsaturated FAs. As shown in Figure 2A, also in the absence of SOD, additions of Ln to the control system inhibited the reduction of cyt.c(ox) by O_2^-, indicating that this inhibition is not attributed to the enhancement of SOD activity by FAs. A direct inhibitory action of Ln on XOD can be excluded as Ln does not modify the rate of urate formation from XO (data not shown).[8] In addition, the decrease in the rate of cyt.c(ox) reduction by the reaction of Ln with O_2^- was also ruled out, because unsaturated FAs are not capable of reacting with O_2^- as shown previously by Bielski,[20] and this was confirmed in our assay conditions. It is clear that FAs do not act as dismutases which induce dismutation of O_2^- into O_2 and H_2O_2, because, as described below, the intensities of MCLA-dependent chemiluminescence by O_2^- generated by XO-XOD in the presence of FAs were essentially equal to those in their absence.

Participation of Micelle Form of FAs in Inhibition of Cyt.c(ox) Reduction

As shown in Figure 2B, Ln began to inhibit the cyt.c reduction at 0.05 mM, which was almost identical with the critical micelle concentration (CMC) of Ln under these assay

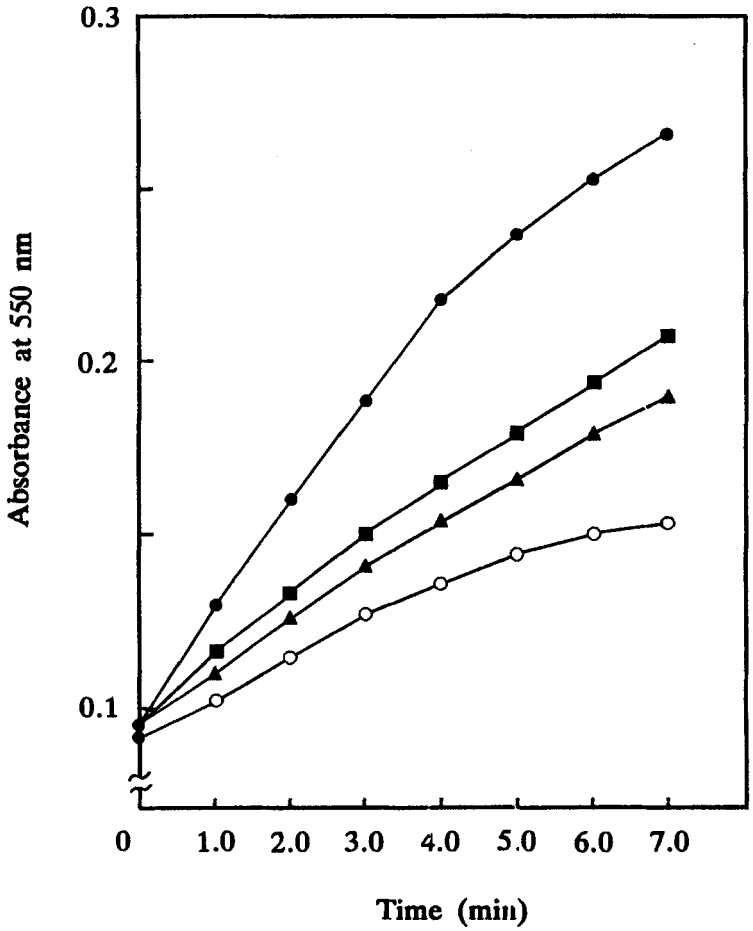

FIGURE 1 Effects of linoleate addition on the reduction of cyt.c(ox) by O_2^- generated by a XO-XOD system in the presence of SOD. A mixture of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 10μg/ml catalase, 50μM XO, 10μM cyt.c(ox), and 10 nM XOD, in a total volume of 3.0 ml, was incubated at 25°C without any addition (●) or with 0.15μg/ml SOD (■), 0.15μg/ml SOD and 0.1 mM linoleate (▲), or 0.15μg/ml SOD and 0.2 mM linoleate (○).

conditions. Furthermore, additions of a saturated FA, lauric acid, above its CMC (6 mM) were capable of inhibiting the cyt.c(ox) reduction above its Kraft point (40°C), but not when below its Kraft point. These results suggest that the micelle form of FA participates in the inhibition of the cyt.c(ox) reduction. Also SDC, which possesses a carboxylic group and is a well-known anionic detergent, was found to inhibit the cyt.c(ox) reduction above its CMC (2 mM), supporting the participation of the micelle form of FAs in inhibiting the cyt.c(ox) reduction.

As reported by Letellier and Shechter,[21] additions of lipids, methanol and uric acid to cyt.c(ox) lead to the disruption of the heme-Met 80 bond of cyt.c(ox). The presence of the heme-Met 80 bond of cyt.c(ox) has been shown to give rise to an absorption band at 695 nm, and this absorption can be used as a measure of the integrity of this bond. Therefore, absence of this absorption indicates the disruption of this bond,[22] i.e., the opening of the heme crevice. In order to examine whether or not FA micelles can disrupt

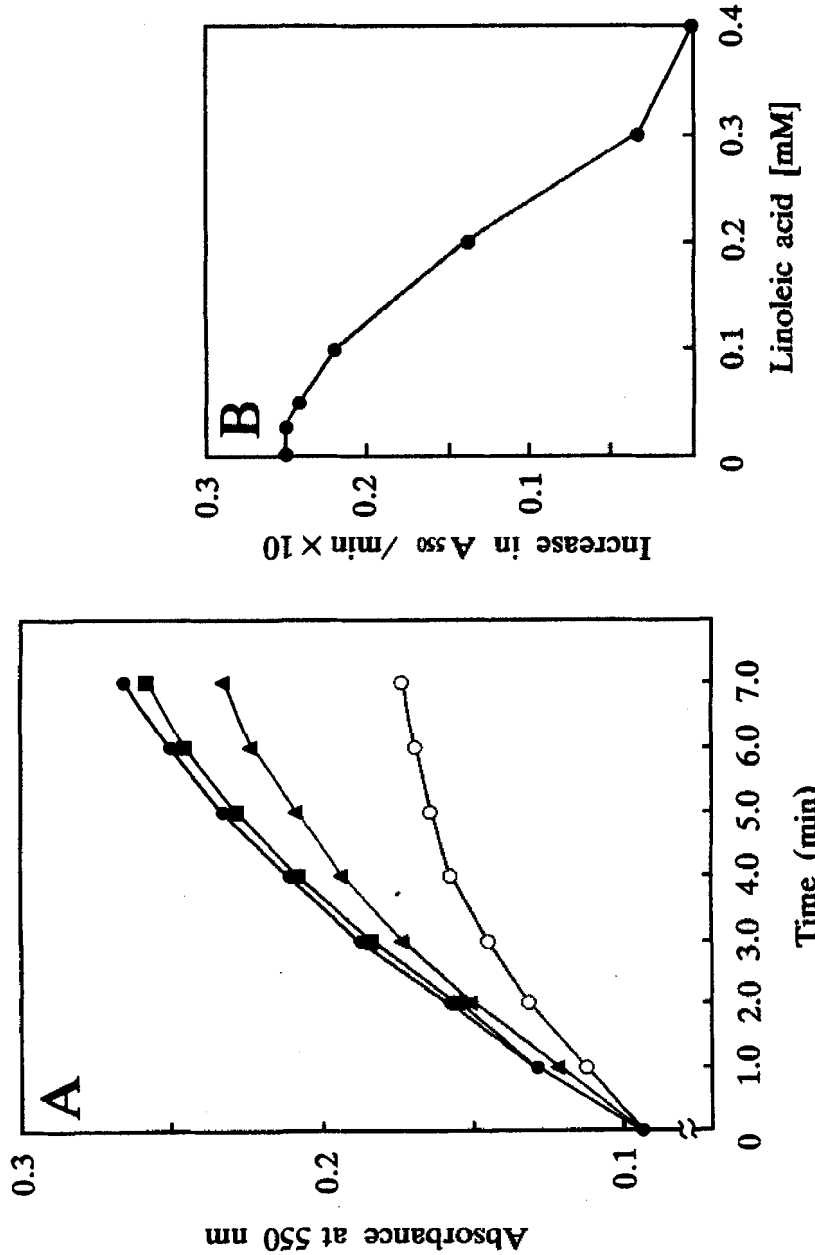

FIGURE 2 (A) Effects of linoleate addition on the reduction of cyt.c(ox) by O_2^- generated by a XO-XOD system in the absence of SOD. A mixture of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 10μg/ml catalase, 50μM XO, 10μM cyt.c(ox), and 10 nM XOD, in a total volume of 3.0 ml, was incubated at 25°C without any addition (●) or with 0.05 (■), 0.1(▲) or 0.3 mM linoleate (○). (B) Effects of concentrations of linoleate added on the rate of cyt.c(ox) reduction by O_2^- generated by a XO-XOD system in the absence of SOD. The same mixture as shown in the legend to Figure 2(A), in a total volume of 3.0 ml, was incubated with various concentrations of linoleate at 25°C for 3.0 min. The rate of cyt.c(ox) reduction was measured as an increase in absorbance at 550 nm per minute.

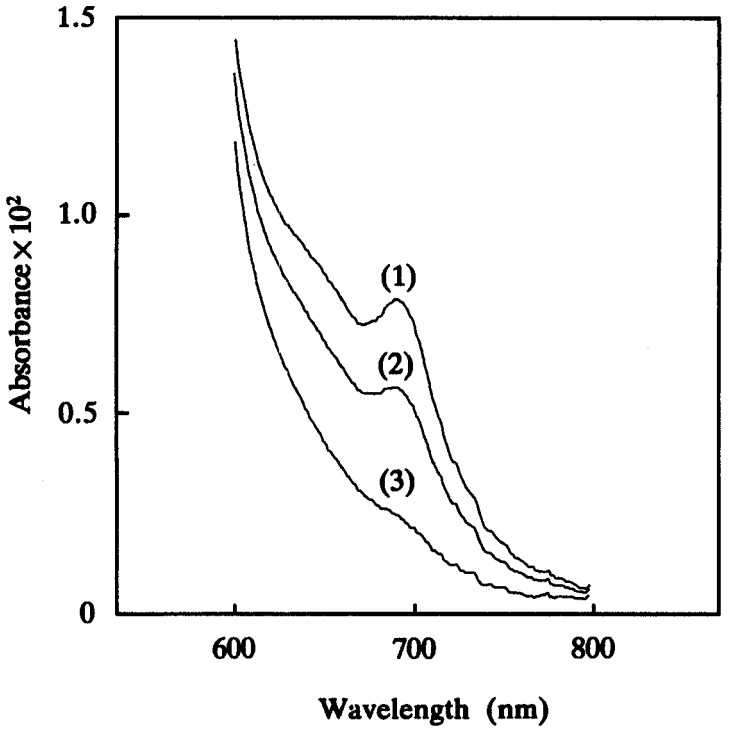

FIGURE 3 Decrease in absorbance at 695 nm indicating the presence of the heme-Met80 bond of cyt.c(ox) with the addition of linoleate. A mixture of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, and 10μM cyt. c(ox), in a total volume of 3.0 ml, was incubated at 25°C for 5.0 min without any addition (1) or with 0.1(2) or 0.3 mM linoleate (3).

the heme-Met 80 bond, we measured the absorption spectra of cyt.c(ox) in the presence of Ln micelles. As shown in Figure 3, the absorption at 695 nm which indicates the integrity of the heme-Met 80 bond was found to decrease with increasing concentrations of Ln. The addition of Ln at 0.4 mM in a final concentration produced a complete quenching of the absorbance at 695 nm, and simultaneously the reduction of cyt.c(ox) by O_2^- was almost completely inhibited (Figure 2B). These results indicate that the reactivity of this structurally changed cyt.c(ox) against O_2^- was reduced, compared to that of the native one. Sodium dodecylsulfate (SDS), a commonly used anionic surfactant, is reported to induce a disruption of the heme-Met 80 bond of cyt.c(ox).[23] As shown in Figure 4, cyt.c(red) in the presence of Ln micelles was found to be more susceptible to reoxidation than in their absence. This finding suggests that the structurally changed cyt.c(red) is much more susceptible to reoxidation than the native one.[24] Therefore, this reoxidation is also responsible for the decreased rate of the cyt.c(ox) reduction by O_2^-.

Electrostatic Interaction of Cyt.c(ox) with Ln Micelles

Cyt.c(ox) is known to interact electrostatically with negatively charged liposomes.[25] To examine the possibility of the electrostatic interaction of cyt.c(ox) with Ln micelles and the contribution of its interaction to the inhibition of cyt.c(ox) reduction by O_2^-, we

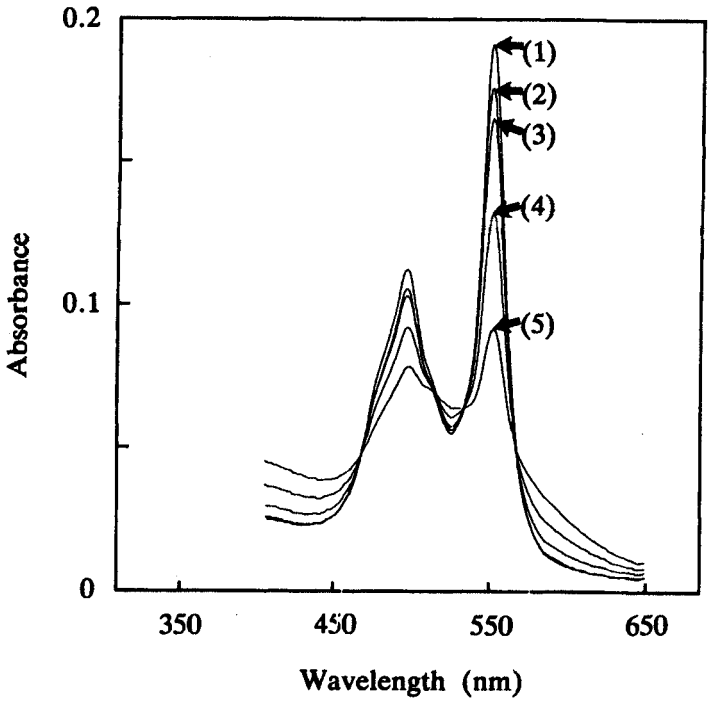

FIGURE 4 Changes in absorption spectra of cyt.c(red) with the addition of linoleate. A mixture of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, and 10μM cyt.c(red), in a total volume of 3.0 ml, was incubated at 25°C for 5.0 min without any addition(1) or with 0.05(2), 0.1(3), 0.2(4) or 0.3 mM linoleate (5).

first measured the absorption at 406 nm (Soret band) and the fluorescence emission at 330 nm caused by Trp-59 of cyt.c(ox) in the presence of Ln micelles. As shown in Figure 5A, the absorption at 406 nm (Soret band) underwent an intensity increase and a small blue shift compared to that in the absence of Ln micelles. Then a very weak fluorescence at 330 nm from Trp-59 in the absence of Ln micelles was observed (Figure 5B), because of the quenching of excited Trp by the heme[26] (the peak at 305 nm is attributed to a Raman spectrum of H_2O). However, in the presence of Ln micelles, the Trp fluorescence emission underwent a large increase (Figure 5B). This observation is characteristic of proteins with Trp in a highly hydrophobic environment. It was reported that, in reverse micelles, the Soret band (406 nm) in the absorption spectrum of cyt.c underwent an intensity increase and a small blue shift, and the Trp fluorescence emission at 330 nm displayed a large increase.[27] Thus, the findings in the absorption and the fluorescence spectra of cyt.c(ox) in the presence of Ln micelles suggest that cyt.c(ox) can interact electrostatically with Ln micelles.

Fukuzawa *et al.*[28] reported that tocopherol held in positively charged micelles (tetradecyltrimethylammonium bromide (TTAB) micelles) was more susceptible to oxidation by O_2^- than that in negatively charged micelles (SDS micelles), because positively charged micelles allowed relatively easy access to O_2^-, but the negative charge on SDS kept it effectively away from the substance by electrostatic repulsion. Similarly, the inhibition of cyt.c(ox) reduction by O_2^- may be explained as follows. Positively charged cyt.c(ox) interacts with negatively charged Ln micelles, and so negatively

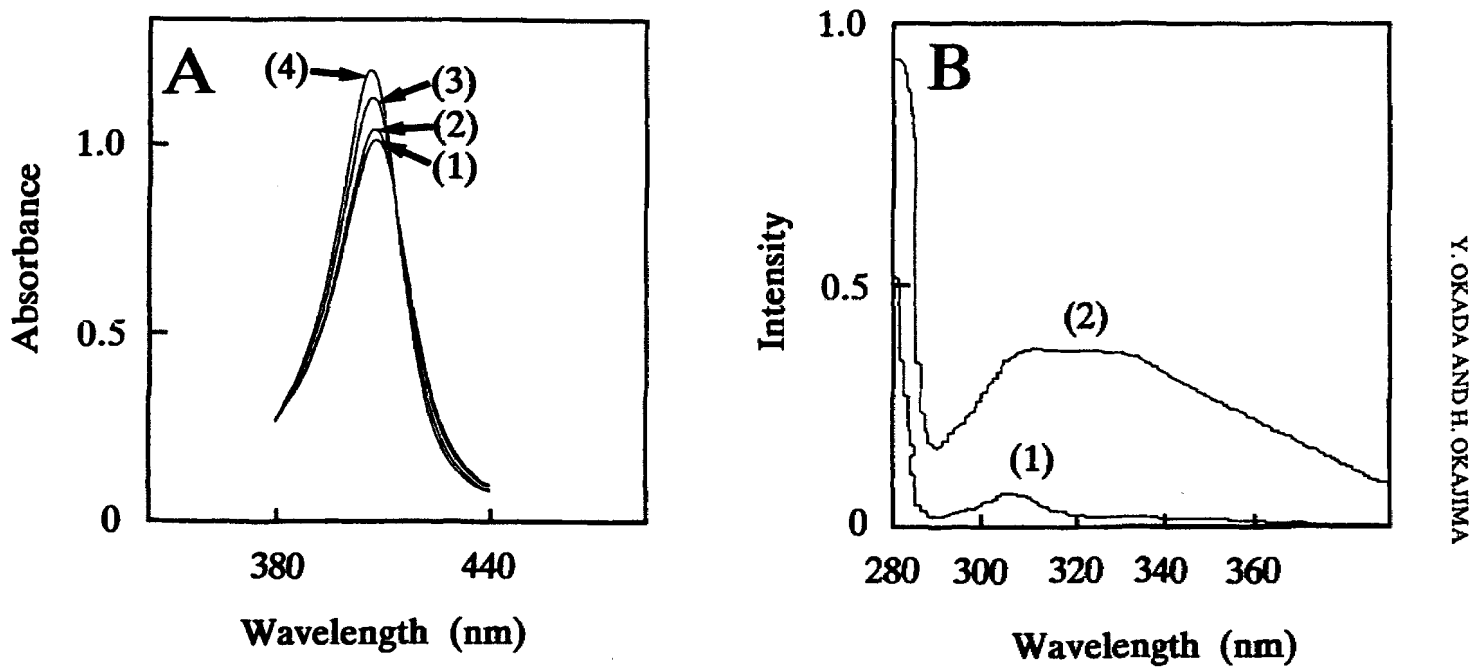

FIGURE 5 (A) Changes in absorption spectra of cyt.c(ox) with the addition of linoleate. A mixture of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, and 10μM cyt.c(ox), in a total volume of 3.0 ml, was incubated at 25°C for 5.0 min without any addition(1) or with 0.1(2), 0.2(3) or 0.3 mM linoleate(4). (B) Changes in fluorescence spectra of cyt.c(ox) with the addition of linoleate. A mixture of 50 mM potassium phosphate buffer, pH 7.8, and 0.1 mM EDTA, in a total volume of 3.0 ml, was incubated at 25°C for 5.0 min with 10μM cyt.c(ox) (1) and 10μM cyt.c(ox) and 0.2 mM linoleate(2). The excitation wavelength was 280 nm. The peak at 305 nm is attributed to a Raman spectrum of H_2O.

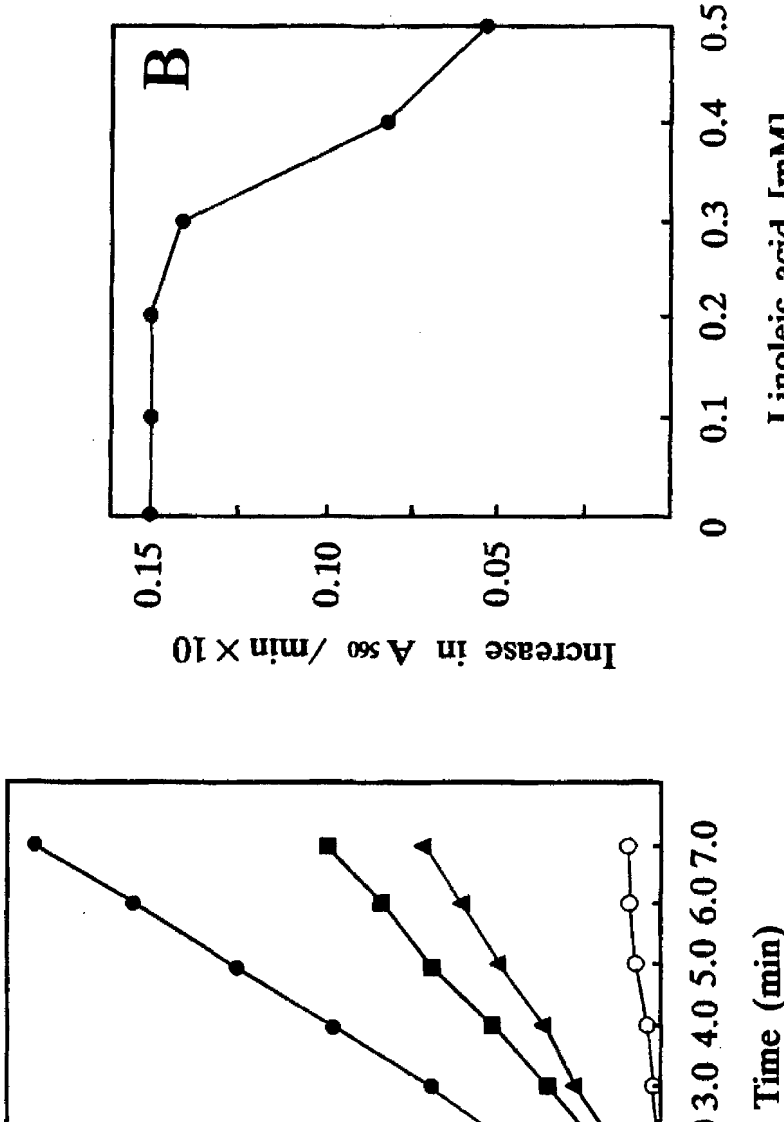

FIGURE 6 (A) Effects of linoleate addition on the reduction of NBT by O_2^- generated by a XO-XOD system in the presence of SOD. A mixture of 50 mM sodium carbonate buffer, pH 10.2, 0.1 mM EDTA, 10μg/ml catalase, 0.1 mM XO, 25μM NBT, and 10 nM XOD, in a total volume of 3.0 ml, was incubated at 25°C without any addition (●) or with 0.4 mM linoleate (■), 0.02μg/ml SOD (▲) or 0.02μg/ml SOD and 0.4 mM linoleate (○). (B) Effects of concentrations of linoleate added on the rate of NBT reduction by O_2^- generated by a XO-XOD system in the absence of SOD. The same mixture as shown in the legend to Figure 6(A), in a total volume of 3.0 ml, was incubated with various concentrations of linoleate at 25°C for 3.0 min.

charged O_2^- reacts less with cyt.c(ox) on the surface of the micelles because of electrostatic repulsion.

From the results described above, it is concluded that in addition to the structural changes in cyt.c(ox), the electrostatic interaction with Ln micelles is also a contributor to the inhibition of cyt.c(ox) reduction by O_2^-.

Influence of FA Micelles on NBT Reduction Method

Also in NBT reduction assay, Ln micelles gave the appearance of enhancement of SOD activity as in the cyt.c(ox) reduction assay (Figure 6A), though a structural change in NBT was not observed. Also other unsaturated FAs, such as oleic and arachidonic acids, produced essentially the same results as Ln. Even a saturated FA, lauric acid, exhibited an effect similar to that of the unsaturated FAs described above when the measurement was carried out above the Kraft point of the acid. Moreover, Ln began to inhibit the reduction of NBT at about 0.2 mM (Figure 6B), which is almost equal to the CMC of Ln under the assay conditions, and also SDC began to affect the rate of reduction at about its CMC. These findings suggest that Ln micelles, not monomer, participate in the inhibition of NBT reduction as well as in that of cyt.c(ox) reduction. To examine the electrostatic interaction of Ln micelles with NBT, we measured the absorption spectrum of NBT. As shown in Figure 7, the maximum absorption

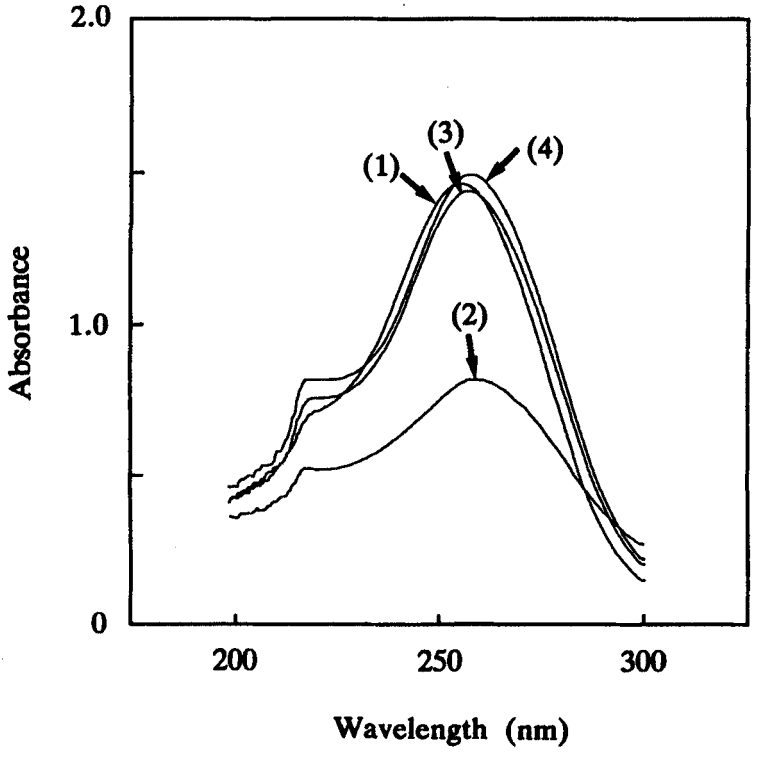

FIGURE 7 Changes in absorption spectra of NBT with the addition of linoleate. A mixture of 50 mM sodium carbonate buffer, pH 10.2, 0.1 mM EDTA, 10μg/ml catalase, and 25μM NBT, in a total volume of 3.0 ml, was incubated at 25°C for 5.0 min without any addition(1) or with 0.1(2), 0.3(3) or 0.5 mM linoleate(4).

underwent a large decrease in the absorbance when Ln was below the CMC. On the other hand, when Ln was above the CMC, the intensity of the maximum absorption returned to that in the absence of Ln and the wavelength of maximum absorption underwent a small red shift. Similar phenomena were observed in the determination of the CMC using the dyes pinacyanol and Rhodamine 6G.[18] These findings indicate that positively charged NBT, like cyt.c(ox), can interact electrostatically with negatively charged Ln micelles, and consequently NBT on the surface of negatively charged Ln micelles reacts less with negatively charged O_2^- because of electrostatic repulsion. Therefore, this interaction interferes with easy access of O_2^- to NBT and so inhibits the rate of NBT reduction by O_2^-.

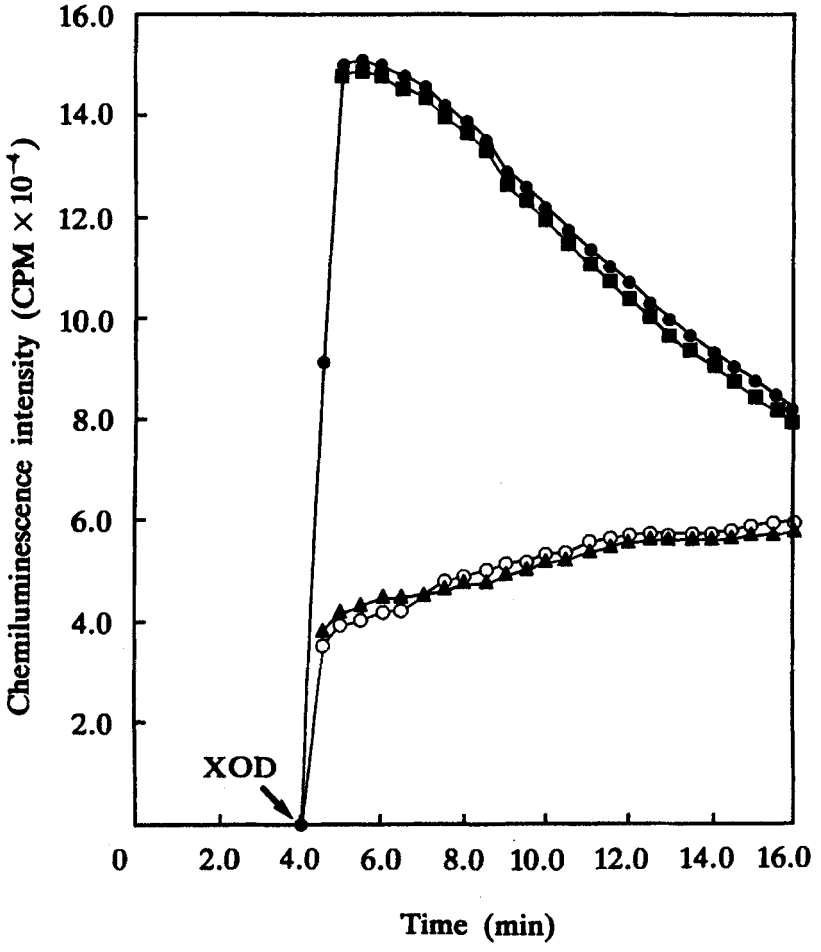

FIGURE 8 Effects of linoleate addition on MCLA-dependent chemiluminescence by O_2^- generated by a HXO-XOD system in the presence of SOD. A mixture of 50 mM Tris-HCl buffer, pH 7.8, 0.1 mM EDTA, 10μg/ml catalase, 50μM HXO, 0.2μM MCLA, and 50 nM XOD, in a total volume of 3.0 ml, was incubated at 25°C without any addition (●) or with 0.2 mM linoleate (■), 0.01μg/ml SOD and 0.2 mM linoleate (▲) or 0.01μg/ml SOD (○).

Influence of FA Micelles on Chemiluminescence and LDH-NADH Assays

We examined the influence of Ln micelles on a chemiluminescence assay for SOD activity using MCLA as a chemiluminescence probe. It has been found that MCLA emits light through reactions with O_2^- and $^1O_2$. [19] Unlike cyt.c reduction and NBT reduction assays in the presence of Ln micelles, a chemiluminescence assay did not affect the SOD activity (Figure 8). The absorption spectrum of MCLA in the presence of Ln micelles was almost in agreement with that in their absence, suggesting that MCLA cannot interact electrostatically with Ln micelles. Because the pKa of dihydropyrazine ring of MCLA is 8.3,[29-31] under these assay conditions (pH 7.8) it exists mostly as uncharged molecules. Therefore, negatively charged Ln micelles cannot interact electrostatically with uncharged MCLA[32] and so cannot reduce the reactivity of MCLA toward O_2^-. In LDH-NADH assay[20] using a negatively charged probe molecule, LDH-NADH complex, Ln micelles cannot influence the asaay of SOD activity, because Ln micelles cannot approach with the probe molecule because of electrostatic repulsion.

The results in the present paper show that, for the measurement of SOD activity in the presence of negatively charged micelles such as FA micelles, the assays using uncharged or negatively charged probe molecules are especially suitable.

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