

Reevaluation of the effect of Triton X-100 on the assay of superoxide radical by the nitrobluetetrazolium reduction method

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Abstract: Triton X-100 enhances to a marked extent the analytical sensitivity of the nitrobluetetrazolium (NBT) reduction method for the assay of superoxide (O_2^-) production. In the present work, it was attempted to elucidate the physicochemical nature of this Triton X-100 effect, focusing on not only the surfactant-caused stabilization of the water-insoluble formazan colloid but also the kinetic competition between the NBT- O_2^- reaction and the autoproportionation of concomitantly occurring in aqueous media. The measurements of formazan and H_2O_2 formed in a number of reaction systems, as prepared by vortex-mixing potassium superoxide dissolved in an aprotic solvent with aqueous solutions of NBT, revealed that Triton X-100 exerts its effect both through preventing formazan colloid from aggregation and thereby increasing the formazan absorbance and through suppressing the autoproportionation reaction of O_2^- . It also turned out that the relative shares of the colloid stabilization effect and the kinetic effect in the contribution to the sensitivity amplification of the NBT method are dependent upon the reaction conditions, particularly the molar concentration ratio of NBT to O_2^- in the reaction systems (Received August 23, 1993; accepted September 7, 1993).

Introduction

There is a good body of compelling evidence that superoxide radical (O_2^-) is an important cause of oxygen toxicity to living organisms.^{1,2)} Although O_2^- is by itself relatively unreactive³⁾, this activated oxygen species can give rise to the formation of hydroxyl radical (OH) which is one of the most reactive chemical species almost indiscriminately attacking organic compounds of biological importance.^{2,4)}

Because O_2^- undergoes rapid, spontaneous disproportionation in protic media, studies of superoxide chemistry in aqueous solutions require specialized rapid techniques. As to the assay of O_2^- , ESR spectroscopy of spin-trapped superoxide radical is probably the only direct method applicable to aqueous systems. All other

methods indirectly measure O_2^- by optically monitoring the chemical conversion of such indicator compounds^{5,6)} as tetranitromethane, epinephrin, adrenaline, luminol and its derivatives, lucigenine, and nitrobluetetrazolium (NBT) salt: among these the last is used rather widely because the NBT reduction method is relatively sensitive and very simple.

It has long been observed that the inclusion of Triton X-100 in reaction mixtures containing NBT and a certain producing system results in a marked increase in the sensitivity of the method. This fact is quite attractive particularly when O_2^- production at low levels is to be assayed. While this effect of Triton X-100 is generally considered to arise from the surfactant-caused stabilization of the colloidal suspensions of formazan, the reduction product of NBT, by preventing par-

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ticle aggregation, there are several papers from which it can be inferred that the surfactant brings about an increase in the rate of the NBT-O_2^- reaction⁷⁻⁹. Because the kinetic data have been obtained by following changes in absorbance at the maximum absorption wavelength of formazan and because absorbance tends to decrease when light-absorbing colloidal particles aggregate, however, the apparent kinetic effect could rather be the stabilization effect exerted on formazan colloid. In this respect, it may be timely to reevaluate the Triton X-100 effect, aiming to elucidate it based on a rather quantitative basis and to establish experimental conditions under which the advantage of using Triton X-100 or other surface-active agents in the NBT reduction method for the assay of O_2^- production is thoroughly exploited.

Materials and Methods

NBT, xanthine, xanthine oxidase and horseradish peroxidase from Sigma Chemical Co. (St. Louis, MO), Triton X-100 and potassium superoxide from Merck (Darmstadt, Germany), and other chemicals from either Fluka Chemie AG (Buchs, Switzerland) or Wako Pure Chemical (Osaka, Japan) were of the finest grade available and used as supplied.

The stock solution of KO_2 in dimethylsulfoxide (DMSO) was made as described in Rivers *et al.*¹⁰. Crystalline KO_2 (100 mg) was mortar-ground with a few drops of DMSO, transferred to a flask containing 25 ml of DMSO, stirred for 30 min. and then filtered to remove undissolved KO_2 . Being diluted, the final concentration of the stock solution was determined by converting O_2^- into H_2O_2 via the autodisproportionation in aqueous media and spectrofluorometrically measuring H_2O_2 employing the scopoletin-peroxidase method.¹¹ Except for KO_2 , all other solutions of NBT, scopoletin, xanthine, xanthine oxidase, horseradish peroxidase and Triton X-100 respectively were prepared with a potassium phosphate buffer containing 0.1 mM EDTA and 50 mM K-PO_4 (pH 7.0). Glass-distilled and deionized water was used throughout.

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The NBT-O_2^- reaction was initiated by vortex-mixing 0.5 ml of KO_2 solutions with 4 ml of NBT solutions. The xanthine-xanthine oxidase system was also used as an enzymatic source of O_2^- .

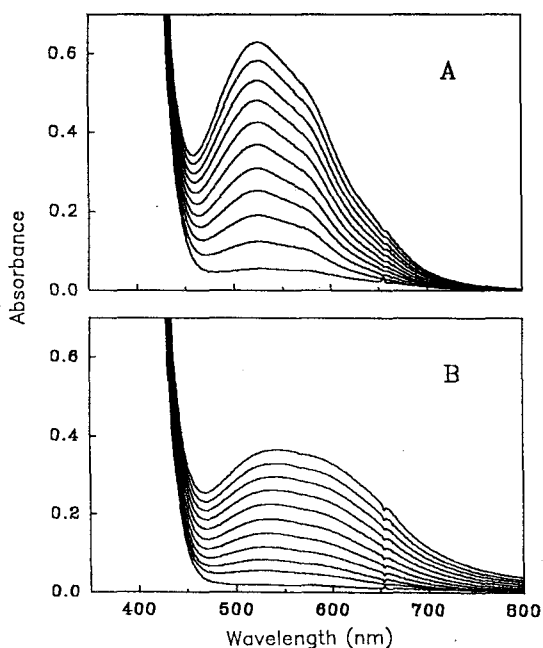


Fig. 1. Time-sequential measurements of the visible absorption spectra of formazan formed through the reduction of NBT by enzymatically generated O_2^- in the presence of 0.25% Triton X-100(A) and in its absence(B).

Reactions were initiated by rapidly mixing xanthine oxidase (0.1 unit; 0.1 mL) with 2 mL of mixtures containing xanthine (0.375 mM) and NBT (0.5 mM) in 50 mM K-PO_4 buffer (pH 7.0). Spectra were recorded in every 30 sec after the mixing.

Throughout the work, absorption and fluorescence spectra were measured by using a HP 8452A diode-array spectrophotometer (Hewlett Packard Co., Palo Alto, CA) and a RF-500 spectrofluorophotometer (Shimadzu, Seisakusho, Japan)

Results and Discussion

Time sequential spectral measurements with reaction mixtures containing NBT and xanthine-xanthine oxidase revealed not only that the presence of Triton X-100 gives rise to a sizable amplification of the sensitivity of the NBT method but also that it causes blue shift of the absorption band of formazan to some extent, as shown in Fig. 1. Because the activity of xanthine oxidase is not affected by Triton X-100 at the concentration (0.25%) used in this experiment⁷⁾ and because the surfactant can easily solubilize formazan aggregate, the amplification effect of Triton X-100 may intuitively be viewed as mainly resulting from the stabilization of formazan colloid, as generally conceived. In order to roughly estimate how much the colloid-stabilization effect indeed contributed to the sensitivity amplification, the surfactant-free mixture was heated to thermally inactivate xanthine oxidase when the maximum absorbance of formazan formed in the mixture reached to *ca.* 0.1 and Triton X-100 was admixed with it. After stirring a few minutes to solubilize the formazan aggregate supposedly formed in the absence of the surfactant, the absorption spectrum was re-measured. It turned out that, while the subsequent addition of Triton X-100 resulted in blue shift of the absorption band as seen from the spectra of the reaction mixture originally containing the surfactant, it increased the absorbance of formazan suspension to somewhat less extent than expected (data not shown), indicating that preventing colloid aggregation is not necessarily the only cause of the Triton X-100 effect.

In this regard, we assumed that both the colloid stabilization effect and the kinetic effect contribute concomitantly to the surfactant-caused enhancement of the analytical sensitivity of the NBT method and that the respective shares of these effects may largely depend on experimental conditions. To ascertain the feasibility of this assumption, the NBT- O_2^- reaction was initiated

in the presence and absence of Triton X-100, using the KO_2 -DMSO solution instead of the xanthine-xanthine oxidase system and a number of aqueous solutions of NBT whose molar concentrations in the final mixtures were of 0.5~6 times as large as that of O_2^- (25 μ M). After measuring the absorption spectra for all reaction mixtures, Triton X-100 was subsequently added to the group of mixtures which had been free of the surfactant and the spectra were recorded again. As can be noted from Fig. 2, when the molar concentration ratio of NBT/ O_2^- was very low, the subsequent addition of Triton X-100 to the originally surfactant-free mixture did not bring about a further increase in the absorbance of formazan suspension to any significant extent even though the insoluble, large formazan particles, if any, suspended in the mixture must be solubilized by the surfactant: this observation implies that the large amplification (*ca.* 3 times when [NBT]/ $[O_2^-]$ is 0.5) is primarily a result of the kinetic effect; that is, Triton X-100 somehow enhances the rate of the NBT reduction by O_2^- . In contrast, when [NBT]/ $[O_2^-]$ was as large as 6, the subsequent addition of Triton X-100 results in a substantial increase in the absorbance up to that of the formazan suspension formed under the influence of the surfactant, clearly indicating that the colloid stabilization is the major cause

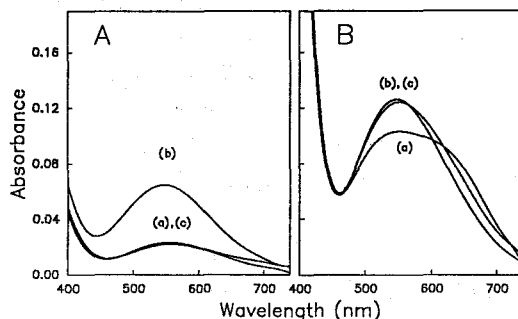
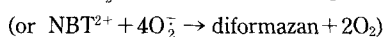
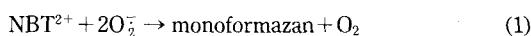


Fig. 2. Absorption spectra of formazan suspensions prepared by vortex-mixing 25 μ M KO_2 -DMSO solution with aqueous solutions of NBT (10 μ M in A and 150 μ M in B respectively).

Traces in (a) were scanned with the suspensions of formazan formed in the surfactant-free reaction mixtures; for traces in (b) 0.25% Triton X-100 was subsequently added to the samples which had been used for traces in (a); and traces in (c) were done with formazan formed in the presence of 0.25% Triton X-100.

of the Triton X-100 effect.

We chose $A_{O+T}-A_0$ and A_T-A_{O+T} as the indices for the colloid stabilization effect and the kinetic effect respectively, where A_0 and A_T are the absorbances of formazan suspensions formed in the absence and presence of 0.25% Triton X-100 respectively, while A_{O+T} is the absorbance of formazan formed in the surfactant-free reaction mixtures and solubilized by Triton X-100 subsequently admixed. Fig. 3 shows changes in A_0 , A_T , A_{O+T} , $A_{O+T}-A_0$, and A_T-A_{O+T} as the functions of NBT concentration, demonstrating that the colloid stabilization effect becomes larger, whereas the kinetic effect becomes smaller, nearly in proportion to the concentration of NBT.



Because these two reactions are competing in aqueous media, there would be a certain point of NBT concentration (*ca.* 150 μM in Fig. 3) at which reaction

(1) completely prevails over reaction (2). In this situation, the kinetic effect of Triton X-100 should be out of operation; instead, the surfactant apparently exerted its effect largely through the stabilization of the formazan colloid. When the concentration of NBT is fairly low as relative to that of O_2^- , reaction (2) could effectively compete with reaction (1), resulting in a decrease in the rate of formazan formation. Under such circumstances, particularly in the extreme case, the resultant formazan concentration is so low that the colloidal particles may have little tendency to aggregate and to settle out from solution; the effect of Triton X-100 should therefore be examined from a kinetic standpoint.

If A_T-A_{O+T} is indeed a proper index for the kinetic effect, the corresponding concentration difference of formazan should be reflected by that of H_2O_2 produced concomitantly through reaction (2). For instance, when 25 μM NBT solution was admixed with 25 μM O_2^- solution, A_T-A_{O+T} was determined to be 0.030 (see Fig. 3): this value corresponds to 2 μM monoformazan (MF) or 1 μM diformazan (DF) because the maximum extinction coefficients of MF and DF in aqueous solution at pH 7.0 are $1.5 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and $3.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ respectively.^{12,13} Either 2 μM MF or 1 μM DF in turn corresponds to 2 μM H_2O_2 . Such conjecture can of course be supported only by the measurements of H_2O_2 formed during the mixing of NBT and solu-

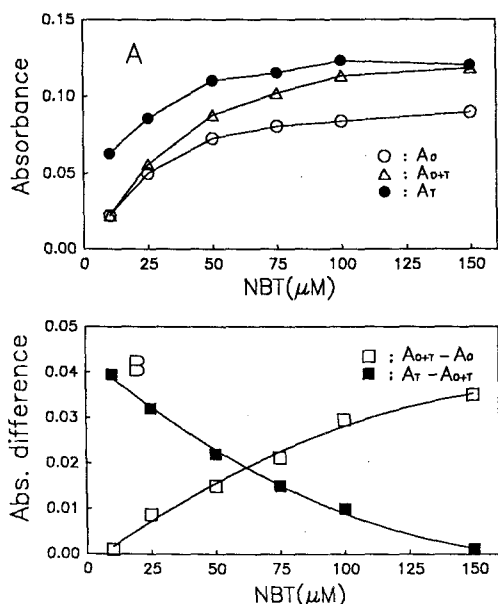


Fig. 3. Changes in the 540 nm absorbance of formazan formed through the reaction between KO_2 (25 μM) and NBT (10~150 μM) as the functions of NBT concentration.

A_0 , A_{O+T} , and A_T correspond to the maximum absorption of traces (a), (b) and (c) in Fig. 2 (also refer to the text).

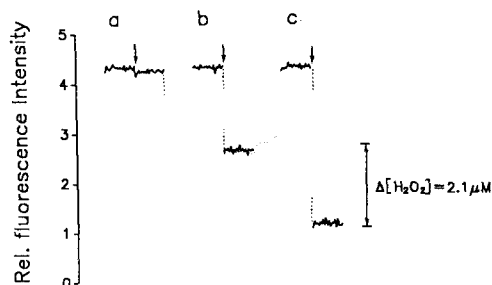


Fig. 4. Decrease in the relative intensity of fluorescence from scooletin (6.5 μM) in samples, as caused by the addition of horseradish peroxidase as indicated by arrows.

In sample (a), scooletin was dissolved in the K-PO_4 buffer containing 0.25% Triton X-100, whereas the fluorescent dye was dissolved in NBT (25 μM)- O_2^- (25 μM) reaction mixtures where formazan had been formed in the presence of 0.25% Triton X-100 (sample b) and in its absence (sample c) respectively.

tions. For these, the scopoletin-peroxidase method was employed. As is well known, scopoletin, an intense fluorescence dye, is enzymatically peroxidized to form a nonfluorescent product, providing the methodological basis for a sensitive fluorometric determination of H_2O_2 .¹¹⁾

Scopoletin fluorescence intensities which were measured with samples containing the NBT- O_2^- reaction mixtures, scopoletin and horseradish peroxidase demonstrated that the concentration of H_2O_2 formed via the autodisproportionation of in the presence of Triton X-100 was lower than that of H_2O_2 formed in the absence of the surface-active agent, as shown in Fig. 4. From the values of $A_T - A_{O+T}$ (see Fig. 3), we estimated the theoretical values of $\Delta[H_2O_2]$, the difference of the concentrations of H_2O_2 formed in the presence of Triton X-100 and in its absence. As can be noted from Table 1, there is a reasonably good agreement between the estimated values of $\Delta[H_2O_2]$ and those measured

by using the scopoletin fluorescence method.

The above results, however, do not necessarily indicate that the rate constant of reaction (1) is increased by the presence of Triton X-100; for a decrease in the rate constant of reaction (2) could give the same result. Any conclusion regarding the kinetic aspect of the Triton X-100 effect should therefore be reserved till further detailed studies are carried out.

Finally, we also checked whether other surfactants stimulate the NBT reduction by O_2^- in aqueous solutions. Just from a comparative standpoint we chose Tween 80, a neutral surfactant with polyethylene glycol chains, C_{16} Tab, a cationic surfactant with a saturated hydrocarbon chain, and deoxycholate, an anionic sterol surfactant. It turned out that, while the effectiveness of Tween 80 and C_{16} Tab as the amplifying agents are certainly comparable to that of Triton X-100, deoxycholate is nearly noneffective, as summarized in Table 2. At the moment, it seems hard to relate the effectiveness of a certain surfactant to its chemical structure. Nonetheless, it can at least be said that there would be a variety of surface-active agents which are commercially available and may be useful as stimulants for the NBT- O_2^- reaction unless they affect the O_2^- -generating biological systems.

In conclusion, the results obtained in the present work suggest that, in spite of skepticism about the use of the NBT reduction method for the quantitative assay of O_2^- production in aqueous media because of the complexity of the redox chemistry of NBT as well as the water-insolubility of the reduction product,¹²⁾ the method appears still useful as one of the simplest techniques if an excess amount of NBT and a certain amount of Triton X-100, which are supposedly to be determined by preliminary experiments, are admixed with the systems investigated. Furthermore, more importantly, this study elucidated, at least in part, the physicochemical nature of the Triton X-100 effect by interpreting it in terms of the kinetic effect as well as the colloid stabilization effect.

Acknowledgement

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Table 1. Decrease in the concentration of H_2O_2 formed during the mixing of the KO_2 (25 μM)-DMSO solution with aqueous solutions of NBT at various concentrations by the presence of 0.25% Triton X-100

NBT (μM)	$A_T - A_{O+T}$	$\Delta[H_2O_2]$ in μM	
		Observed	Estimated
10	0.040	2.8	2.7
25	0.030	2.1	2.0
50	0.022	1.3	1.5
75	0.015	0.6	1.0
100	0.005	0.0	0.3
150	0.000	0.0	0.0

Table 2. Effects of several detergents on the sensitivity amplification of the NBT reduction method for O_2^- assay

Detergents*	Degree of amplification at various [NBT] in μM		
	10	20	40
Triton X-100 (neutral)	2.6	1.9	1.4
Tween 80 (neutral)	3.3	2.3	1.3
C_{16} Tab (cationic)	2.2	1.4	1.2
Deoxycholate (anionic)	1.5	1.0	1.0

* The final concentrations of detergents in the reaction mixtures were 0.25% (w/v).

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NBT 환원방법에 의한 superoxide 라디칼의 검량에 미치는 Triton X-100 효과에 대한 재평가

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초록 : 널리 이용되는 검량방법중의 하나인 NBT 환원방법은 계면활성제인 Triton X-100의 존재하에서 그 측정감도가 크게 증폭된다. 본 연구는 이러한 Triton X-100 효과의 물리화학적 본질을 이해하기 위하여 수행하였으며, 이를 위하여 이 계면활성제가 NBT의 환원생성물인 불용성 formazan 콜로이드를 안정화시키는 효과와 수용액 중에서 동시에 진행되는 두 반응(NBT-O₂⁻ 산화환원 반응과 O₂⁻의 disproportionation 반응) 간의 경쟁관계에 미치는 속도론적 영향에 초점을 맞추었다. Triton X-100 유무 조건하에서 KO₂-DMSO 용액과 K-PO₄ 완충용액에 녹인 여러가지 농도의 NBT 용액을 빠르게 혼합하고, 이때 생성된 formazan과 H₂O₂를 각각 정량한 결과, 이 계면활성제는 formazan 콜로이드를 안정화 시킴으로써 결과적으로 formazan의 흡수도를 상승시키는 효과를 보일뿐만 아니라 O₂⁻의 자발적인 dismutation을 억제하는 결과를 초래함을 확인하였다. 또한 종합적인 Triton X-100의 효과에 대한 콜로이드 안정화 효과와 속도론적 효과의 상대적 기여도는 실험조건에 따라 바뀌는데 특히 반응계의 NBT/O₂⁻ 농도비에 크게 좌우되는 것으로 밝혀졌다.