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FREE RADICALS IN NADPH–MICROSOMES–TRIPHENYLTETRAZOLIUM CHLORIDE SYSTEM AS EVIDENCED BY INITIATION OF SULFITE OXIDATION

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SUMMARY

1. Triphenyltetrazolium chloride (TTC) was found to stimulate aerobic oxidation of sulfite in the presence of NADPH and microsomes from rat liver and Ehrlich ascites tumor cells, indicating its involvement in the reaction in the form of a reactive free-radical intermediate.

2. TTC was found also to initiate sulfite oxidation non-enzymatically in the presence of a low concentration of EDTA.

3. Results are presented which suggest that EDTA acts as a weak chain-breaker.

INTRODUCTION

In a previous paper[1], evidence was presented that triphenyltetrazolium chloride (TTC) stimulates the aerobic oxidation of NADPH in the presence of microsomes, probably by acting as an electron carrier from NADPH to molecular O_2 in some way. Since, however, two-electron-reduced TTC (triphenylformazan) is not readily autoxidizable, an alternate reduced form must be sought. The possibility that this form might be a one-electron-reduced intermediate (TTC$^{\cdot}$) which may be reactive with molecular O_2 was considered. Stepwise reduction of organic molecules in overall two-electron-reduction reactions is not uncommon[2–6]. It has been shown that enzymatically formed free radicals can reduce molecular O_2 (ref. 7). Electron transfers effecting the transformation of catalyst intermediates in the half reduced form to either the fully oxidized[8,9] or the fully reduced state[10,11] are also known to occur in enzymatic systems.

The aerobic oxidation of sulfite proceeds by a free-radical reaction[12–14] which may be initiated by any process that effects either the univalent oxidation of sulfite or the univalent reduction of O_2 (refs. 15, 16). FRIDOVICH AND HANDLER[16–18] demonstrated the utility of this aerobic oxidation of sulfite as a method of detecting free-radical chain reactions in several oxidative enzyme systems. These results prompted the present study to see whether the NADPH–microsome–TTC system can initiate sulfite oxidation. This paper reports data which indicate that TTC acts by a free-radical mechanism. Sensitivity of the system to variations of components of the

Abbreviation: TTC, 2,3,5-triphenyltetrazolium chloride.

reaction mixture, the effects of inhibitors (particularly of EDTA) and non-enzymatic initiation of sulfite oxidation by TTC will also be reported.

METHODS

Microsomes were prepared as previously described[1]. The preparations were stored at −15° and used within 10 days.

O_2 uptake was measured by a conventional Warburg apparatus. All experiments, unless stated otherwise, were performed in a total volume of 3 ml at 30° under an air gas phase after temperature equilibration for 7 min. "Basic solution" consisted of 40 mM potassium phosphate (pH 7.4), 80 mM KCl and 40 mM sodium sulfite, the final pH being about 7.8. Further additions and modifications will be specified in the legends to the figures. Concentrations expressed are the final ones. Sulfite and TTC solutions were prepared immediately prior to use. The TTC solution was kept in the dark and added last. All solutions were prepared using glass re-distilled water.

Inorganic iron and copper were determined colorimetrically by the method described by FISTER[19] and manganese and zinc according to MALMSTRÖM[20]. Formazan and nitrogen were determined as previously described[1].

TTC was obtained from the Sigma Chemical Co. (St. Louis), sodium sulfite and potassium phosphate, monobasic, from Wako Pure Chemical Industries, Ltd. (Osaka), and EDTA, disodium salt, from Daiichi Pure Chemicals Co., Ltd. (Tokyo). Nicotinamide nucleotide coenzymes and milk xanthine oxidase (EC 1.2.3.2) were the products of Boehringer and Soehne (Mannheim). All other chemicals used were of analytical-reagent grade.

RESULTS

Preliminary experiments

Since the autoxidation of sulfite ions in aqueous solutions is known to be very sensitive to both positive and negative catalysts[12–14] some preliminary experiments, especially with TTC, were carried out using the basic solution (see METHODS) in which microsomal activity can be tested. In the absence of added EDTA, the autoxidation of sulfite in the basic solution was high, O_2 uptake amounting to more than 30 μmoles during 60 min incubation. Addition of 3 mM TTC reduced this by about 20%. In the presence of EDTA, as would be expected, the autoxidation was strongly inhibited, the inhibition being more than 90% at 0.133 mM and complete at 2 mM. The strongly suppressed O_2 uptake in the presence of 0.133 mM EDTA was progressively reduced with increasing addition of TTC (Fig. 1a). The unexpected increase in O_2 uptake observed at 3 mM TTC was, in turn, progressively reduced by raising the concentration of EDTA to 2 mM, where O_2 uptake was again slight (Fig. 1b).

The last two observations might lead one to suspect that the TTC contained heavy metals such as Cu^{2+} as impurities. This suspicion was eliminated, from the following observations. (a) The stimulation by Cu^{2+} added in slight excess of EDTA is much higher than that by TTC. The former was very sensitive to the further addition of EDTA, a typical chelating agent, and was completely abolished in the presence of 2–4 mM EDTA. In contrast, the latter was not so susceptible to the reagent and even in the presence of 4 mM EDTA a residual, but definite, O_2 uptake was always observed.

The results clearly indicate that the action of TTC differs from that of Cu^{2+}. (b) TTC used contained no heavy metals such as copper, iron, zinc or manganese as determined colorimetrically[19,20]. It should be added that of the constituents of the reaction

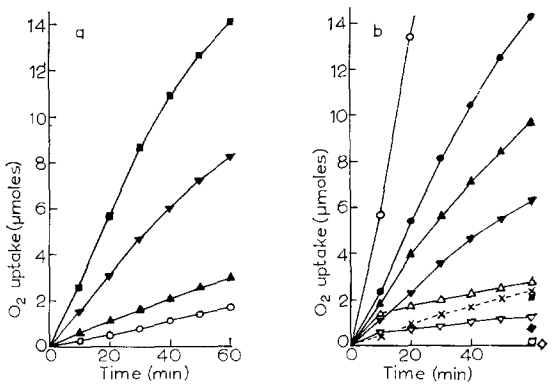

Fig. 1. a. Release by TTC of EDTA-suppressed autoxidation of sulfite. Reaction mixture consisted of basic solution (40 mM potassium phosphate buffer (pH 7.4), 80 mM KCl and 40 mM sulfite, final pH being 7.8) and 0.133 mM EDTA (○). Further addition of TTC: ▲, 0.03 mM; ▼, 0.3 mM; and ■, 3 mM. b. Comparison of effect of EDTA on TTC- and Cu^{2+}-stimulated sulfite oxidations. Reaction mixture consisting of basic solution *plus* 0.133 mM EDTA (×) also contained either 3 mM TTC (filled symbols) or 0.16 mM $CuSO_4$ (open symbols). Further addition of EDTA: ○, ●, None; △, ▲, 0.2 mM; ▽, ▼, 0.4 mM; □, ■, 2 mM; and ◇, ◆, 4 mM. Reactions were started by tipping in sulfite *plus* EDTA from side arms in both cases.

mixture, only 0.2 M stock phosphate buffer solution, but not 40 mM, showed a positive test for heavy metals when 1 vol. was vigorously shaken with 0.2 vol. of a 0.001 % solution of dithizone in CCl_4 (ref. 20). The positive test was completely eliminated, however, in the presence of 0.1 mM EDTA. Following these results, 2 mM EDTA was added to all subsequent enzymatic reactions to ensure that the non-enzymatic oxidation of sulfite both in the absence and presence of TTC was negligible.

The generation of free radicals of TTC by the effect of light[21] or chemical reduction by sulfite as the cause of TTC-induced sulfite oxidation was also shown to be unlikely. Thus the use of previously irradiated (sunlight) TTC solutions or carrying out the incubations in a bath, with water blackened with India ink, did not noticeably affect the results. None of the reactions described were accompanied by an apparent red colour, *e.g.* formazan, even under a gas phase of N_2 and at a high sulfite concentration (0.4 M). The cause, therefore, of TTC-induced sulfite oxidation is not clear.

As would be expected, however, the TTC-induced sulfite oxidation showed a sensitivity to various chain-breakers such as alcohols[12–14], aromatic amines[22–24], NADH (ref. 25), NADPH and microsomes[16].

Initiation of sulfite oxidation by microsomal NADPH oxidase system

In accordance with the observation of FRIDOVICH AND HANDLER[16], with rabbit-liver microsomes, the rat-liver microsomal NADPH oxidase system was also found to be capable of initiating sulfite oxidation (Fig. 2). The rate of O_2 uptake was initially higher, however, it decreased earlier with increasing concentrations of microsomes. It may be seen that added NADPH (0.3 μmole) was totally oxidized within 20 min or so in the presence of 4 mg of microsomes (×----×), when the sulfite oxidation also

stopped. Thus the various patterns observed may be interpreted as the net result of the rate of NADPH oxidation by microsomes and the chain-breaking action of microsomes themselves (*cf.* Fig. 4a).

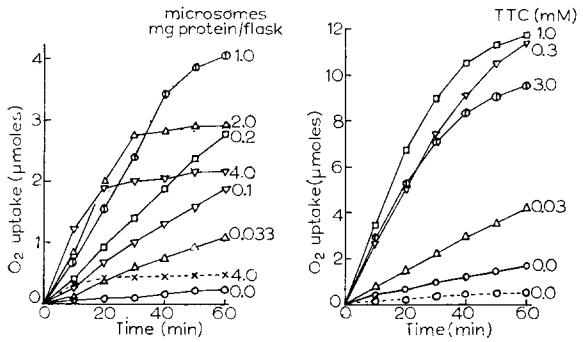

Fig. 2 (left). Initiation of sulfite oxidation by rat-liver microsomal NADPH oxidase system. Reaction mixture consisted of basic solution, 2 mM EDTA, 0.1 mM NADPH and microsomes as indicated. × denotes values in the absence of sulfite. Reactions were started by tipping in NADPH from side arms.

Fig. 3 (right). Stimulation by TTC of sulfite oxidation by rat-liver microsomal NADPH oxidase system. Reaction mixtures consisted of basic solution, 2 mM EDTA, 0.1 mM NADPH, 0.1 mg of microsomes and TCC as indicated. Dashed line, in the absence of microsomes. Reactions were started as in Fig. 2.

Stimulation by TTC of sulfite oxidation by microsomal NADPH oxidase system

Fig. 3 shows the effects of varying concentrations of TTC on sulfite oxidation by a microsomal NADPH oxidase system. It is evident that increasing addition of TTC enhanced the rate of O_2 uptake. This indicates that transfer of one electron from the oxidase system to molecular O_2 is facilitated in the presence of TTC. Thus, it is strongly suggested that TTC is involved in the reaction in a free-radical form. It is also observed that at high concentrations of TTC the rate of O_2 uptake decreased somewhat. Possibly, TTC itself is a weak chain-breaker. This assumption may also explain the slight inhibition of autoxidation of sulfite observed in the presence of 3 mM TTC.

Patterns of TTC-stimulation as influenced by varying the amount of either microsomes or NADPH, other components being kept constant

Fig. 4a shows the results obtained when the amount of microsomes was varied. The rate of O_2 uptake at the beginning was higher but slowed down rapidly with increasing amounts of microsomes. The leveling off of O_2 uptake with time may be due to the exhaustion of NADPH owing to its oxidation to $NADP^+$. This view is supported by the data given in Fig. 4b. Whereas the rate of NADPH oxidation, and that of sulfite oxidation accordingly, is expected to increase in proportion to the amount of microsomes, the fact that the initial rate of O_2 uptake did not increase proportionally to the amount of microsomes may suggest that chain-propagation was inhibited either by chain-breakers or by shortage of O_2, provided that enough NADPH was present. Since the formazan formed was slight, the relatively lower rate observed with a large amount of microsomes may be ascribed to the chain-breaking property of

microsomes themselves rather than to a deficiency of O_2. It may be added here that the presence of 40 mM sulfite and 2 mM EDTA did not affect the TTC-stimulated aerobic oxidation of NADPH by rat-liver microsomes, at least over a period of about 1 min when measured spectrophotometrically. After 1 min formation of formazan began to interfere with the absorbance at 340 mμ. This may be due to the rapid sulfite oxidation, which consumed the dissolved O_2 so quickly that the solution became anaerobic by that time.

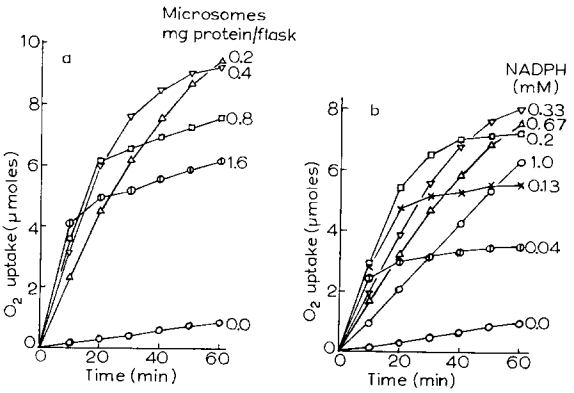

Fig. 4. Patterns of TTC-stimulated sulfite oxidation as influenced by varying the concentrations of microsomes (a) or NADPH (b). Reaction mixture consisted of basic solution, 2 mM EDTA, 3 mM TTC, 0.33 mM NADPH and rat-liver microsomes as indicated in (a); and basic solution, 2 mM EDTA, 1 mM TTC, 0.36 mg rat-liver microsomes and NADPH as indicated in (b). Reactions were started as in Fig. 2 in both cases.

Fig. 4b shows the results obtained when the amount of NADPH was varied. When the concentration of NADPH was high enough to remain as a source of hydrogen, the rate of O_2 uptake was lower but it did not decline so quickly. As the concentration of NADPH was decreased the initial rate of O_2 uptake became higher but slowed down more rapidly. These observations are in accord with those in Figs. 2 and 4a and indicate that NADPH is also a potent chain-breaker.

Specificity of NADPH

Just as TTC stimulation of aerobic oxidation of reduced nicotinamide adenine nucleotides by microsomes was specific for NADPH (ref. 1), so also the present TTC–sulfite system was ineffective when NADPH was replaced by NADH.

Inhibition by various substances of sulfite oxidation catalyzed by NADPH–microsomes–TTC system

Under the conditions comparable with Fig. 4b with 0.33 mM NADPH, *p*-aminophenol strongly inhibited sulfite oxidation, the concentration of 50 % inhibition being 0.08 mM. *p*-Phenylenediamine was inhibitory to the same extent. NADH (ref. 25) was also inhibitory as was NADPH, but the oxidized forms were not. Alcohols such as mannitol, at 20 mM, inhibited about 15 %. When the phosphate buffer of the reaction mixture was replaced by Tris–HCl buffer[24], the rate of sulfite oxidation was decreased by about 20 %. As was expected, the inhibition by 0.5 mM *p*-chloromercuribenzoate, an inhibitor of electron transfer, was nearly complete (93 %).

It is of particular interest that increasing addition of EDTA increasingly inhibited the rate of O_2 uptake in this enzymatic system (Fig. 5) as in the non-enzymatic one (Fig. 1b). Acetate (20 mM) had no effect. However, EDTA even at 20 mM did not affect the rate of NADPH (0.1 mM) oxidation by rat-liver microsomes (1.1 mg protein per ml) *plus* TTC (0.5 mM) as measured spectrophotometrically by the decrease in absorbance at 340 mμ; nor did EDTA (2 mM) interact with TTC (0.2 mM) as determined spectrophotometrically between 260 and 360 mμ. It was of interest,

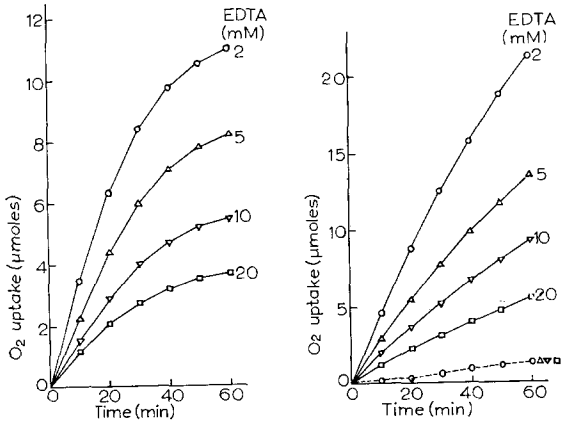

Fig. 5 (left). Inhibitory effect of EDTA on sulfite oxidation induced by NADPH–microsomes–TTC system. The reaction mixture consisted of basic solution, 0.2 mM NADPH, 3 mM TTC, 0.15 mg rat-liver microsomes and EDTA as indicated. Reactions were started as in Fig. 2.

Fig. 6 (right). Inhibitory effect of EDTA on sulfite oxidation initiated by hypoxanthine–xanthine oxidase system. Reaction mixture consisted of basic solution, 1.7 mM hypoxanthine, 0.1 mg milk xanthine oxidase and EDTA as indicated. Dashed line indicates values obtained in the absence of sulfite. Reactions were started by tipping in xanthine oxidase.

therefore, to test whether EDTA can inhibit sulfite oxidation initiated by the milk xanthine oxidase system, a system used by FRIDOVICH AND HANDLER[16] for testing the ability of a substance to act as a chain-breaker. As shown in Fig. 6 EDTA inhibited this system increasingly with increasing concentrations, but was without effect on the rate of O_2 uptake in the absence of sulfite. Preincubation of xanthine oxidase in the presence of 1 mM EDTA at 30° for 45 min (ref. 26) or the presence of 10 mM EDTA in the reaction mixture[27] has been shown to have no influence on its activity when measured with hypoxanthine or xanthine as substrate and O_2 as electron acceptor. All of these data lends support to the view that EDTA may act as a chain-breaker.

Although all the reported experiments were carried out with rat-liver microsomes, essentially the same observations were also made with microsomes from Ehrlich ascites tumor cells except that the enzymatic activities of the latter were several times less on a protein basis than the comparable activities of rat-liver microsomes.

DISCUSSION

The present study clearly showed that, although the microsomal NADPH oxidase system itself can initiate sulfite oxidation, addition of TTC markedly enhanced

this process. This fact strongly suggests that TTC participated in the reaction in the form of a reactive free-radical intermediate (TTC·). NADH could not replace NADPH. This shows that the electron-transfer path of this sulfite system is the same as that of the previously reported NADPH–microsomes–TTC system[1]. Therefore, generation and participation of TTC· in the latter system is most likely. The mechanism may be interpreted as follows: TTC will accept one electron to give TTC· presumably from X_1 (ref. 28), a component of the microsomal NADPH oxidase system. TTC· will then reduce molecular O_2 yielding superoxide anion, O_2^-, and be itself oxidized back to TTC. In the presence of sulfite, O_2^- will initiate sulfite oxidation[16]. In the absence of sulfite, O_2^- will take up a proton from the medium to form the perhydroxyl radical, HO_2, which will be rapidly converted to H_2O_2, thus resulting in stimulation of NADPH oxidation with concomitant O_2 uptake. Conversion of HO_2 to H_2O_2 may take place either by further univalent reduction by TTC· to peroxide anion, HO_2^-, followed by simultaneous uptake of a proton or by dismutation of 2 molecules of HO_2 to H_2O_2 and O_2. Thus, it is presumed that TTC plays its role as a catalyst by shuttling between TTC· and TTC. In the absence of O_2, TTC· will, of course, be reduced further to formazan.

In recent studies[5,9], generation of TTC free radicals in a NADPH–microsomes–TTC system was confirmed by electron spin resonance spectroscopy in collaboration with Dr. MASAMOTO IWAIZUMI of our university. Also H_2O_2 formation was confirmed.

The unexpected observation that TTC brought about autoxidation of sulfite non-enzymatically in the presence of concentrations of EDTA high enough to chelate metal impurities present in the reaction mixture was very troubling. As described earlier the reason is not yet clear.

On the other hand, this non-enzymatic reaction was sensitive to various chain-breakers, and was also sensitive to EDTA when in excess of the amount necessary to chelate metal impurities. Thus, EDTA might act as a chain-breaker. The investigation revealed that EDTA inhibited sulfite oxidations initiated not only by the NADPH–microsomes–TTC system but also by the milk xanthine oxidase system. However, EDTA had no effect on the aerobic oxidation of substrates, NADPH and hypoxanthine, by the respective oxidase systems in the absence of sulfite. These facts suggest that EDTA does not inhibit chain-initiation but inhibits chain-propagation. The latter view is in accord with that of LIPMANN and co-workers[30] that the protective effect of EDTA against uncoupling by thyroxine might be explained in terms of a free-radical mechanism. However, compared with other chain-breakers such as *p*-aminophenol, EDTA is not a potent breaker. It has neither primary nor secondary amino groups nor an alcohol group.

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