BBA 45740

FREE-RADICAL MECHANISM BY WHICH TRIPHENYLTETRAZOLIUM CHLORIDE STIMULATES AEROBIC OXIDATION OF NADPH BY MICROSOMES

SHOJIRO SATO* AND MASAMOTO IWAIZUMI**

*The Research Institute for Tuberculosis, Leprosy and Cancer**, *and The Chemical Research Institute of Non-Aqueous Solutions***, *Tohoku University, Sendai (Japan)*

(Received May 31st, 1968)
(Revised manuscript received September 3rd, 1968)

SUMMARY

1. Free-radical intermediate of 2,3,5-triphenyltetrazolium chloride (TTC) (TTC•) in the NADPH–microsomes–TTC system has been detected by means of ESR spectroscopy.

2. In contrast to reduced TTC (triphenylformazan), TTC• is easily autoxidizable. The product of reduced O_2 is H_2O_2.

3. The mechanism by which TTC stimulates the aerobic oxidation of NADPH by microsomes has been interpreted based on the above findings.

4. Autoxidizability of TTC• may also shed some light on the controversies about the mechanisms of enzymatic reduction of TTC.

INTRODUCTION

Previous studies showed that 2,3,5-triphenyltetrazolium chloride (TTC) stimulates the microsomal NADPH-oxidase system[1] and that the NADPH–microsomes–TTC system can initiate sulfite oxidation[2]. Since the latter fact indicates the involvement of TTC in the reaction in a reactive free-radical intermediate, generation and participation of TTC• in the former reaction are strongly suggested. The present paper shows that the above suggestion proved to be the case by means of ESR spectroscopy. TTC• was found to be reactive with molecular O_2. This property of TTC• may explain the mechanism by which TTC stimulates the microsomal NADPH oxidation and the well known difference in formazan formation by enzymatic preparations between under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Rat-liver microsomes and supernatant fractions in 1.15% KCl were prepared by differential centrifugation by the method of MITOMA *et al.*[3]. Nitrogen was determined by micro-Kjeldahl method.

Abbreviations: TTC, 2,3,5-triphenyltetrazolium chloride; TTC•, one-electron-reduced TTC (free radical); reduced TTC, two-electron-reduced TTC (triphenylformazan); ESR, electron spin resonance.

ESR spectrum was measured with a Hitachi MPU-3B type X-band spectrometer with 100 kcycles magnetic field modulation, at modulation amplitude of about 5 gauss. Nitrogen gas passed through a heat-exchanger in liquid N_2 kept the resonant cavity temperature at about —150°, at which temperature all samples were assayed in glass tubes (3 mm internal diameter). Each spectrum was calibrated with an auxiliary proton resonance probe as the spectrum was being recorded. The spectra shown in the figures are first derivative curves of the actual absorption. Magnetic field increases from left to right. Conditions for reactions prior to placing samples into the cavity will be given in the legends to the figures.

O_2 uptake was measured by a conventional Warburg apparatus. Catalase activity was determined by the method of CHANCE[4]. H_2O_2 formed in the reaction mixture was detected indirectly using a system similar to that of KEILIN AND HARTREE[5] as applied by GILETTE, BRODIE AND LA DU[6]. Formaldehyde was measured by the chromotropic acid method of MAC FADYEN[7].

TTC was obtained from the Sigma Chemical Co. (St. Louis), nicotinamide nucleotide coenzymes, milk xanthine oxidase (EC 1.2.3.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and beef-liver catalase (EC 1.11.1.6) from Boehringer and Soehne (Germany).

RESULTS

Detection and identification of ESR signal in NADPH–microsomes–TTC system

Microsomes themselves only showed a singlet signal at $g = 2.005$ under the present experimental conditions (Fig. 1a). The signal was assumed to be due to flavin[8]. When mixtures of NADPH, TTC and microsomes were shaken aerobically, transferred to tubes, and ESR spectroscopy was carried out, the signal ($g = 2.0047$) appeared abruptly and at the highest intensity when the reaction mixture had become pale pink in color (Figs. 1b, c and d). This was true when shaking time was 4 min instead of 15 sec (not shown). The signal, once appeared under the conditions, seems relatively stable and tended to decay slowly with time (Figs. 1e and f). These results show that ample O_2 inhibits the appearance of the signal. That the mixture system consumes O_2 actively has been shown previously[1] (*cf.* Table I).

Mixtures which lacked either NADPH or TTC or microsomes failed to give the signal. With heated microsomes (70° for 10 min), no signal was observed. Further, the signal appearance was not affected by 3.3 mM EDTA, but was completely abolished by 0.5 mM *p*-chloromercuribenzoate.

Reduction of TTC with hydrosulfite (Fig. 1T) or with NADH *plus* xanthine oxidase (Fig. 1X) gave spectra ($g = 2.004$ and 2.005, respectively), whose hyperfine structures were, however, unsatisfactorily resolved. (In the latter case, the spectrum was reversed in phase, but this is unimportant.) Replacing NADH with either hypoxanthine or xanthine did not improve the resolution.

Recently, DEGUCHI AND TAKAGI[9] reduced TTC chemically in an organic solvent and assayed it at room temperature. A nine-line hyperfine structure, indicative of even distribution of the unpaired electron on the four nitrogen atoms of the ring, with a splitting constant of 6.4 gauss was observed at $g = 2.0037$. Our seven-line spectra obtained in the microsomal system are similar to this in *g*-value and in the number of lines (two outermost lines may be too low to be recorded but see Fig. 1d

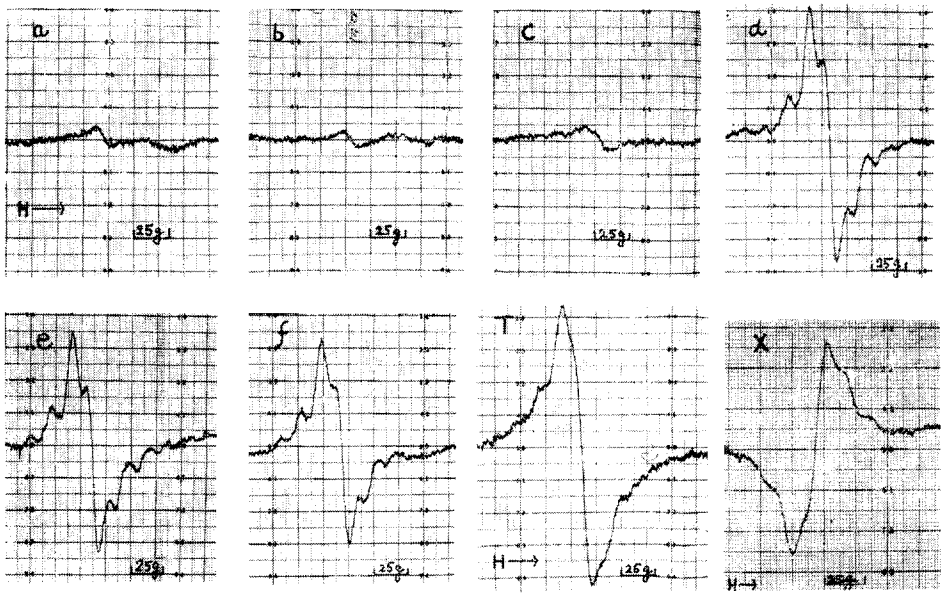

Fig. 1. ESR spectra of TTC-free radicals. a–f. Produced in the microsomal system. Solution A: A microsomal suspension (12.6 mg protein per ml) in 40 mM potassium phosphate (pH 7.4) *plus* 0.12 M KCl. Kept in ice-water. Solution B: A mixture consisting of 10.8 mM NADPH and 30 mM TTC in 40 mM potassium phosphate (pH 7.4). Kept in the dark. a. Solution A alone. b. 0.5 ml of Solution A was added to 0.1 ml of Solution B contained in a test tube (14 mm × 110 mm) (final concns.: NADPH, 1.8 mM; TTC, 5 mM) and after vigorous shaking for 15 sec by hand, transferred to a tube and immediately placed in the cavity. No red color had developed. The total time that elapsed from mixing to placing was about 40 sec. c–f. The same procedure was followed up to the step of transferring the mixture into a tube and allowing it to stand at room temperature (23°) for 20, 30, 60 and 120 sec for c, d, e and f, respectively, and then placing it in the cavity. The colors of the mixture were: questionable, pale pink, pink and bright red for c, d, e and f, respectively. Instrument settings were the same for a–f. T. Hydrosulfite-reduced. 1 mg each of $Na_2S_2O_4$ and TTC were successively dissolved in 0.5 ml of Solution A which lacked microsomes and immediately measured. The solution was red. X. Produced in a xanthine oxidase system consisting of 6 mM NADPH, 3 mM TTC, 80 mM KCl, 40 mM phosphate (pH 7.5) and 0.2 mg of xanthine oxidase in total volume of 0.6 ml. Instrument settings for T and X were similar to those for a–f.

and Fig. 2f), but are different from it in the splitting constant, which was about 14.5 gauss in average (lines were not strict equally spaced). Reason for this discrepancy is not clear. However, the consideration that our spectra were taken at −150° and that the varieties of method of reduction and of reducing agents gave more or less similar spectra (g-value, whole line-width) may permit the signals observed to be ascribed to TTC-free radical.

Reactivity with O_2 and fate in the absence of O_2 of TTC•

In this series of experiments (Fig. 2), in contrast to those in Fig. 1, about twice as much NADPH as TTC was employed. When the frozen reaction mixture, which was pale pink in color and high in signal (Fig. 2b), was further warmed for 6 min by rubbing in hand, it became bright red and considerably low in signal (Fig. 2c). In contrast, when the mixture was continuously shaken for 6 min (almost colorless) and assayed after 1 min standing at room temperature (pink), a signal of about the same intensity as that of Fig. 2b was obtained (Fig. 2d). This indicates that shaking

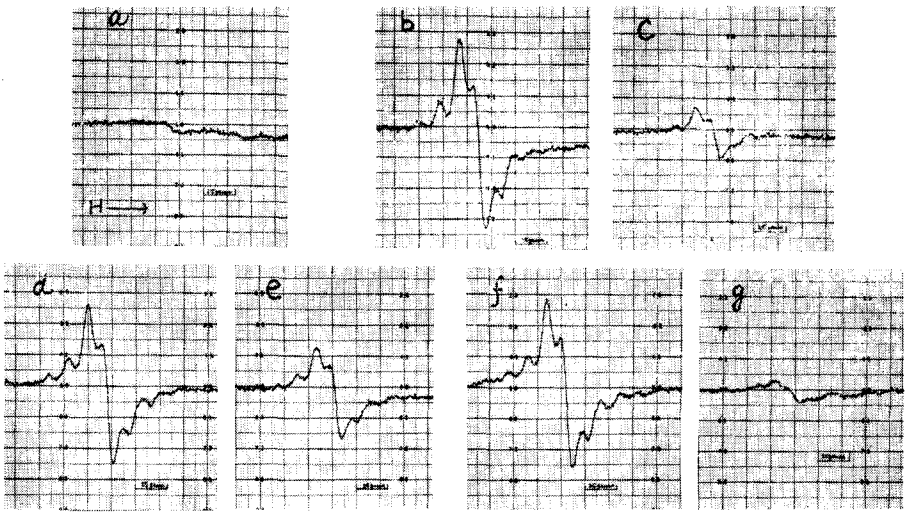

Fig. 2. Change in intensity of spectra as the reaction proceeded aerobically or anaerobically. Solutions A and B: The same as in Fig. 1 except that microsomes was 9.0 mg protein per ml in Solution A and NADPH and TTC were 35 and 18 mM, respectively, in Solution B. a. Solution A alone. b. 0.5 ml of Solution A was added to 0.1 ml of Solution B (final concns.: NADPH, 5.8 mM; TTC, 3 mM), vigorously shaken for 15 sec, transferred into a tube and placed in the cavity after 45 sec standing at room temperature (22°) (pink). c. The tube of b was taken out from the cavity, warmed for 6 min by rubbing in hand and placed again in the cavity. The color had changed to bright red. d. Solution A and Solution B were mixed as in b and continuously shaken for 6 min, transferred to a tube and placed in the cavity after 1 min standing (pink). e. The same as d, but standing period was 6 min (bright red). f and g. 1.0 ml of Solution A was added to 0.1 ml of Solution B. After shaking for 15 sec, the mixture was divided into two tubes. One was measured after 1 min standing (pale pink, f), and the other after 15 min standing (bright red, g). Instrument settings were the same for all spectra.

TABLE I

DETECTION OF H_2O_2 IN NADPH–MICROSOMES–TTC SYSTEM

Complete system consisted of 40 mM potassium phosphate (pH 7.4), 80 mM KCl, 0.2 mM $NADP^+$, 6.7 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (1.4 units), 50 mM methanol, catalase (15000 units), 10 mM semicarbazide, 15 mM nicotinamide, 2 mM EDTA and microsomes (6 mg protein). When indicated, 3 mM TTC was present. Total volume, 3 ml. Incubated for 30 min at 30° under air. The detection of H_2O_2 depends on the following reactions:

$$NADPH + H^+ + O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + H_2O_2$$

$$CH_3OH + H_2O_2 \xrightarrow[\text{(large amount)}]{\text{Catalase}} \text{formaldehyde} + 2\,H_2O$$

Reaction mixture	*O_2 uptake* (μmoles)*		*Formaldehyde formed** (μmoles)*	
	TTC (−)	*TTC (+)*	*TTC (−)*	*TTC (+)*
Complete system	0.73	0.2	0.49	5.1
Complete system — catalase	0.59	4.0	0.31	0.78
Complete system — methanol	0.49	3.9	0.39	0.40
Complete system — $NADP^+$	0.10	0.0	0.07	0.06
Complete system — glucose-6-phosphate dehydrogenase	0.21	0.29	0.22	0.22
Complete system — catalase — methanol	0.40	3.3	0.0	0.0

* O_2 uptakes were linear with time in all runs.
** Complete system which lacked both catalase and methanol were taken as blanks.

under air prevented the formazan formation but did not bring about a corresponding accumulation of the radical. All these data strongly suggest the easy reactivity of TTC$^{\bullet}$ with molecular O_2. It is clear that when standing time was prolonged, the signals (Figs. 2d and f) gradually decayed (Fig. 2e) to disappear almost completely (Fig. 2g), suggesting the completion of further reduction of TTC$^{\bullet}$ to reduced TTC, which is diamagnetic. However, in further experiments similar to those of Fig. 2b and c, but using 2 mM NADPH and 6 mM TTC, the high signal observed at the beginning was also almost lost after 1 h standing at room temperature in the dark (not shown). Since the amount of NADPH was one-third that of TTC in this case, the dismutation of 2 molecules of TTC$^{\bullet}$ to TTC and formazan is strongly indicated.

Free-radical concentrations corresponding to the higher signals given in Figs. 1 and 2, as measured by comparing with those given by known concentrations of diphenylpicryl hydrazyl in benzene, were roughly of the order of 2 to 3 μM. However, the steady-state concentration must be much lower than these values, since it failed to detect signals when assayed immediately after shaking.

Detection of hydrogen peroxide in NADPH–microsomes–TTC system

A typical experiment of several ones is shown in Table I. Only complete system with TTC brought about a distinct increment of O_2 uptake and formaldehyde formation. The ratio of formaldehyde formed to O_2 uptake was 0.82 (5.1/6.2), which should theoretically be one. In contrast to the results reported by GILETTE, BRODIE AND LA DU[6], the reaction mixtures which lacked either catalase or methanol also showed residual, but definite, amounts of formaldehyde formation. When the higher value (0.78 μmole) is also included in the blank, the above ratio becomes 0.7. On the other hand, the increment of O_2 uptake, 2.9 μmoles (6.2—3.3), was 88 % of the theoretical one, 3.3 μmoles. It may be added that catalase activity of microsomes used was found to be roughly 13 units per mg protein, which was high enough to decompose generating H_2O_2 catalatically. In the absence of TTC, the values were too small to be discussed in consideration of the relatively large values observed with incomplete reaction mixtures. In a similar experiment in which 9500 $\times$ g rat-liver supernatant was used instead of microsomes and glucose-6-phosphate dehydrogenase O_2 uptake was exactly doubled, whereas formaldehyde detection was 75 % of O_2 consumed. Thus, the (approximate) doubling of O_2 uptake was accompanied by formaldehyde detection of about 70 to 80 % of the expected. The missing portion of formaldehyde may be due, at least partly, to the fact that formed formaldehyde can undergo further 'coupled oxidation' (ref. 10). In conclusion, almost all of the O_2 taken up may be accounted for as H_2O_2.

Mechanism by which TTC stimulates aerobic oxidation of NADPH by microsomes

Microsomal NADPH oxidase system will reduce TTC by one-electron to give TTC$^{\bullet}$ (Reaction a), which will then reduce molecular O_2 to yield superoxide anion, $O_2^{\bullet-}$, and thereby be itself oxidized back to TTC (Reaction b). $O_2^{\bullet-}$ will take up a proton instantaneously from the medium to form perhydroxyl radical, $HO_2^{\bullet}$ (Reaction c), which will be rapidly converted to H_2O_2 either by dismutation reaction to form $\frac{1}{2}$ ($H_2O_2 + O_2$) (Reaction d) or by further univalent reduction by TTC$^{\bullet}$ to peroxide anion, HO_2^- (Reaction e), followed by simultaneous uptake of a proton to form H_2O_2 (Reaction f), or by both. Thus, TTC plays its role as a catalyst by shuttling

between TTC$^{\bullet}$ and TTC. In the absence of O_2, TTC$^{\bullet}$ will further be reduced to formazan (Reaction g). TTC$^{\bullet}$ may also disappear from the system by dismutation (Reaction h). Reaction a is assumed to be rapid compared with NADPH oxidase activity and so are Reactions b–f compared with Reactions a, g, and h, so the over-all reaction, $NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O_2$, is stimulated by TTC and formazan formation will be inhibited strongly under aerobic conditions.

DISCUSSION

The present experiment leaves little doubt that the free radical detected by ESR spectroscopy in the NADPH–microsomes–TTC system is derived from TTC. The hyperfine structure observed may suggest that, in the main, TTC$^{\bullet}$ is not bound to microsomes but exists freely in solution (*cf.* ref. 11) and its concentration is not high. TTC$^{\bullet}$ is easily reactive with molecular O_2, like the free radical of dihydroxyfumarate generated during peroxidatic reaction[11]. ASHLEY *et al.*[12] observed that when oxidation of triphenylformazan to TTC was effected with lead tetraacetate in a closed system there was a marked O_2 uptake by the reacting formazan. Thus, it may be said that autoxidizability is an inherent property of TTC$^{\bullet}$. Further, it was observed that TTC$^{\bullet}$ can disappear under anaerobic conditions by dismutation reaction. We often noticed that the pale color of the sample had deepened more or less distinctly by the end of the assay, especially when it took much time. Since the enzymatic reaction will not proceed at temperatures as low as −150°, the phenomenon was strange to us, but may now be explained in terms of dismutation.

Based on these findings, the mechanism of TTC-stimulation of microsomal NADPH oxidase system has been interpreted. This will, in turn, explain the previously reported methylene-blue-like action of TTC in stimulating the pentose phosphate pathway in ascites tumor cells[13].

It is well known[14–19] that enzymatic preparations which actively reduce TTC to formazan under anaerobic conditions produce less formazan under the aerobic and aerobic formazan formation is strongly inhibited by respiratory inhibitors such as KCN. In an effort to explain these puzzling problems various views have been postulated[16,18,19]. However, none of them seems to be satisfactory. It is felt that the concept of autoxidizability of TTC$^{\bullet}$ may answer these problems reasonably.

ACKNOWLEDGEMENTS

We should like to express our thanks to Professor TARO ISOBE of the Chemical Research Institute of Non-Aqueous Solutions for many helpful suggestions.

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