Bull. Chem. Soc. Jpn., 58, 2051—2055 (1985)

Reduction Mechanism of 2,3,5-Triphenyltetrazolium Chloride and 1,3,5-Triphenylformazan

Kisaburo Umemoto
Department of Pure and Applied Sciences, College of General Education, The University of Tokyo, Komaba, Meguro-ku, Tokyo 153
(Received March 14, 1985)

The reduction mechanism of 2,3,5-triphenyltetrazolium chloride(TTC) and 1,3,5-triphenylformazan was studied by electrochemical, ESR, and spectrophotometric methods. In non-aqueous aprotic solvent, 2,3,5-triphrnyltetrazolium ion was reduced to formazan anion either by two-electron reaction or by disproportionation reaction of tetrazolinyl radical generated by one-electron reduction of TTC. The same formazan anion was generated by one-electron reduction of 1,3,5-triphenylformazan. In the presence of proton donor, the formazan anion was converted to formazan by protonation reaction and cyclic regeneration of formazan was observed in the reduction of formazan. These results indicate that the quantitative determination of reducing materials by using TTC may possibly lead to erroneous evaluation of reductants unless the strength of electron donating property of reductants is established.

Tetrazolium salts have been used for demonstrating dehydrogenase activities,[1–4] for detecting hyperoxide radical,[5–7] and for determination of inorganic reducing materials.[8] Upon reduction tetrazolium salts, generally colorless, are converted to highly colored formazans.

Although tetrazolium salts have merits as qualitative indicators, their use as quantitative reagents has been less successful.[4,5] In the estimation of microbial damage by using TTC, the coloration was dependent on the quality as well as the quantity of microflora.[4] In the examination of using TTC as a quantitative reagent for the determination of hyperoxide, poor reproducibility was revealed in the intensity of the coloration of the solution.[9]

In addition to the ambiguity involved in the quantitative use of tetrazolium salts, there appeared an uncertain relationship between the color of the solution and the reaction intermediate: There has been either or not a relationship between the color of the solution and the intensity of ESR signals.[10,11] Nevertheless, there have been a few works on the systematic studies of the reduction mechanism of tetrazolium salts.[5,11–14]

The purpose of the present work is to elucidate the problem involved in the quantitative use of TTC by clarifying the stepwise reduction pathways of TTC and its formazan.

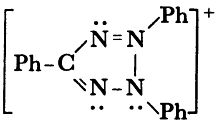
Experimental

TTC obtained from Tokyo Kasei was recrystallized from mixture of ethanol and diethyl ether and 1,3,5-triphenylformazan of the same source was recrystallized from aqueous ethanol solution under nitrogen. These materials were dried at 70 °C under vacuum. Dimethyl sulfoxide (DMSO) was treated with activated alumina and distilled twice under nitrogen at reduced pressure. Tetraethylammonium perchlorate(TEAP) was prepared according to the usual method.

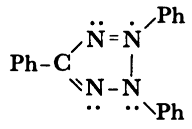
Absorption spectra were measured with a Hitachi 100-60 spectrophotometer. ESR spectra were measured by a Japan Electron Optics Lab. Co. JES-PE-3X spectrometer. Cyclic voltammograms were recorded with a laboratory made potentiostat.[15] The Pt electrode used for cyclic voltammetry was soaked in aqua regia for 30 s and heated in a blue flame.[15] Controlled potential electrolysis was carried out in an H-type cell equipped with a sintered glass separator. The cathode was a Pt net and the anode was a spiral Pt wire. Potentials were all referred to an SCE equipped with a salt bridge.[15] All experiments were carried out at room temperature (20 °C).

Results and Discussion

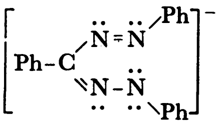
Molecular structures and abbreviations of molecular species appeared in this paper are summarized as follows:



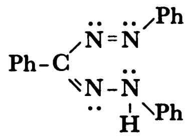
2,3,5-triphenyltetrazolium
(R^+)



2,3,5-triphenyltetrazolinyl
($R\cdot$)



1,3,5-triphenylformazan anion
(R^-)



1,3,5-triphenylformazan
(RH)

Reduction of TTC. A cyclic voltammogram of TTC is shown in Fig. 1. Two cathodic and two anodic waves were observed. By carrying out the potential reversal immediately after the first cathodic wave, the first redox couple was found to be A/A′.

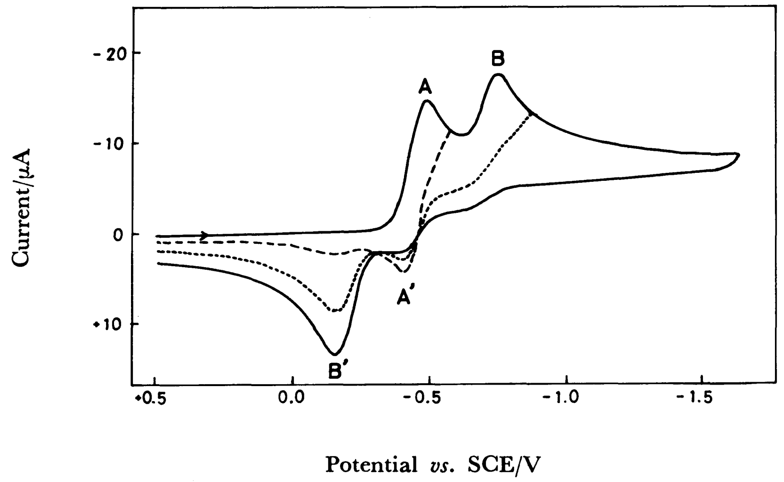


Fig. 1. Cyclic voltammogram of TTC: 1.0×10^{-3} mol dm^{-3} in DMSO containing 0.1 mol dm^{-3} TEAP. Scan rate: 0.05 Vs^{-1}.

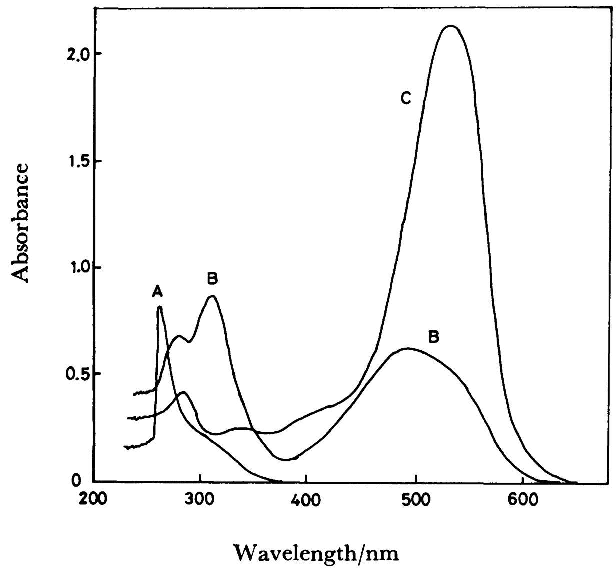


Fig. 2. Absorption spectra of (A): TTC (3.5×10^{-5} mol dm^{-3}), (B): 1,3,5-triphenylformazan (3.2×10^{-5} mol dm^{-3}), and (C): 1,3,5-triphenylformazan anion generated from (A) by controlled potential electrolysis at −1.09 V in DMSO.

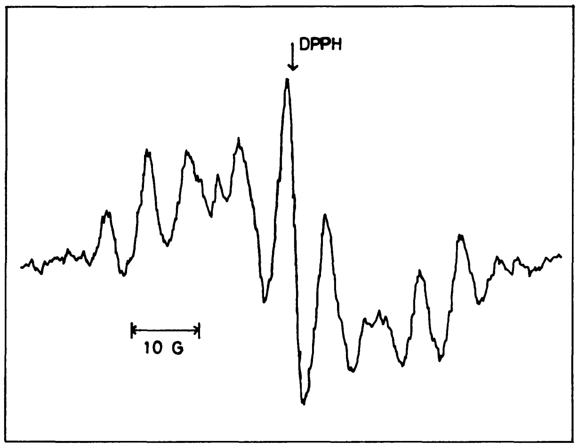


Fig. 3. ESR spectrum of tetrazolinyl radical generated by electrochemical reduction of TTC in DMSO.

The peak potential separation of the first redox couple was *ca.* 70 mV, being close to a reversible one-electron reaction. When constant potential electrolysis at −0.95 V corresponding to the second reduction step was performed to completeness, overall two electrons (1.98 electrons were found by experiment) were found to be required for each molecule of TTC and a highly red colored solution was obtained. The absorption spectrum of the solution is given in Fig. 2 along with absorption spectra of TTC and 1,3,5-triphenylformazan. The absorption band with its maximum at 538 nm was identified to that of formazan anion R^-, since an identical spectrum was obtained when R^- was liberated by adding tetraethylammonium hydroxide to the DMSO solution of 1,3,5-triphenylformazan. Taken together with these results the following stepwise electron transfer reactions are given:

$$R^+ + e \rightleftharpoons R\cdot \tag{1}$$

$$R\cdot + e \longrightarrow R^- \tag{2}$$

In order to obtain a solution of tetrazolinyl radical R·, constant potential electrolysis was carried out at −0.55 V corresponding to the first reduction wave. For the complete electrolysis, two electrons (experimentally 1.96 electrons were found), contrary to the probable one electron, were found to be required for each molecule of TTC. The resulted solution was highly red colored and gave no ESR signal, but gave exactly the same absorption spectrum as that of R^- shown in Fig. 2C. In contrast with the complete electrolysis, the partially electrolyzed solution gave an ESR spectrum as shown in Fig. 3 irrespective of the electrolysis potential being either −0.55 V or −0.95 V. The ESR spectrum was identified to that of 2,3,5-triphenyltetrazolinyl radical.[10,11,16] The same radical was also generated by adding TTC to the solution containing R^- alone. These results provide sufficient evidence for supporting the following equilibrium disproportionation reaction of $R\cdot$.

$$R\cdot + R\cdot \rightleftharpoons R^+ + R^- \qquad (3)$$

Judging from the result that the electron-transfer reaction to the radical (Eq. 2) is observable at the second cathodic wave on the cyclic voltammogram of TTC, the rate of disproportionation reaction is not very fast, since if this rate is too fast, only the first cathodic wave raised twice in height should be observed.

On the absorption spectrum of the solution containing tetrazolinyl radical, any band characteristically attributable to the radical was not observed. However, by subtracting the bands of TTC and formazan anion, a small absorption band was admitted in a range from 300 to 400 nm. Recently, by pulse radiolysis of 2-propanol solution of TTC, the absorption band of transiently lived radical was found to have its maximum at 365 nm.[14] The present observation that the absorption band of the radical was so weak indicates the equilibrium constant of disproportionation reaction (Eq. 3) being large in DMSO.

Cyclic voltammograms obtained in a series of reduction of TTC and subsequent re-oxidation of formazan anion are given in Fig. 4. The peak potentials of formazan anion (curve C) coincided with those of the second anodic wave and the two cathodic waves observed for TTC. In order to verify the probable regeneration of tetrazolium ion, constant potential electrolysis was carried out at +0.20 V. Upon completion of the electrolysis two electrons (experimentally 1.94 electrons were found) were found to be released for each formazan anion, and as shown in Fig. 4D, the original tetrazolium ion was recovered in high efficiency by the following oxidation reaction:

$$R^- - 2e \longrightarrow R^+ \qquad (4)$$

In this oxidation reaction, the stepwise two one-electron reactions were not observed as shown in Fig. 4C. The large potential separation of the second redox couple observed for TTC (B/B′ in Fig. 1) indicates a large activation energy being needed for the cyclization of the open chain structure of formazan anion, bringing the potential of electron release of R^- more positive than the oxidation potential of $R^{\cdot}$. Accordingly, the second electron release would follow immediately after the first electron release of R^- and leaving no place for the accumulation of $R\cdot$ as a reaction intermediate.

Additional support for the reversible interconversion of R^+ and R^- was obtained in the study of absorption spectra. The absorption band of R^+ was substituted by that of R^- on completion of reduction of R^+, and on re-oxidation the absorption band of R^+ was recovered on consumption of that of R^-. The cycle of the complete reduction and oxidation was successively repeated and the interconversion of R^+ and R^- was found to be reproducible in high efficiency.

Formazan anion was stable in unhydrous DMSO, but when proton donors such as water and acids were added into the solution, the anion turned to formazan by the following protonation reaction:

$$R^- + H^+ \longrightarrow RH \qquad (5)$$

The absorption spectrum obtained by addition of proton donors into the DMSO solution of R^- agreed

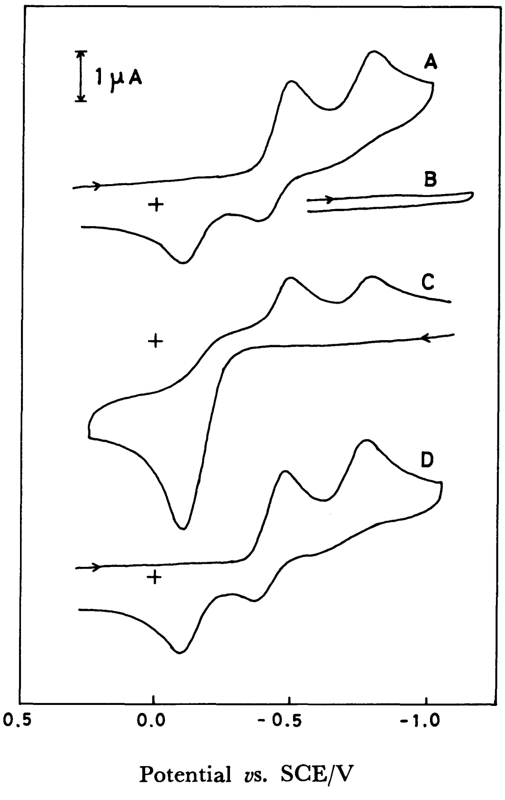


Fig. 4. Cyclic voltammogram of TTC taken (A): Before electrochemical reduction, (B) and (C): After complete electrochemical reduction, and (D): After electrochemical re-oxidation of formazan anion.

well with that of formazan shown in Fig. 2B. The rate of protonation reaction became faster with the stronger acidity of proton donors.

Reduction of 1,3,5-Triphenylformazan. The oxidation of formazan to tetrazolium salts is known to be the most characteristic property of formazan.[1,17–19] The electrochemical oxidation of formazans has been studied in acetonitrile and it was found that formazans are converted to tetrazolium salts with high efficiency.[17–19] However, the electrochemical study of reduction of formazans is quite few.[18] The reason seems to be due to the circumstances that the oxidation of formazans usually affords valuable pathways to prepare tetrazolium salts, while the reduction of formazans has not been praised for the preparative method of their decomposition products, such as hydrazine, amidrazone,amines, and aniline.

As formazan is indeed reducible, the reduction of formazan should be carefully studied, since formazan is the key substance in the analytical use of tetrazolium salt for determining reducing substances.

A cyclic voltammogram of 1,3,5-triphenylformazan is shown in Fig. 5. The first scan showed one cathodic and one anodic waves. Upon sweep reversal at *a* for the second scan additional two cathodic waves appeared at potentials where TTC gave its reduction waves. Constant potential electrolysis at −1.09 V revealed one electron (experimentally 1.06 electron was found) being required for complete electrolysis of each molecule of formazan. After the completion of the electrolysis, a highly red colored solution was obtained and the absorption spectrum as well as the cyclic voltammogram of the solution agreed well with those of formazan anion shown in Fig. 2C and in Figs. 4B and 4C, respectively. Subsequent constant potential electrolysis at +0.20 V revealed two electrons (experimentally 1.91 electrons were found) being released for the complete electrolysis of each formazan anion. After the electrolysis was completed, both the cyclic voltammogram and the absorption spectrum of the solution agreed well with those of TTC. The efficiency of conversion of 1,3,5-triphenylformazan to 2,3,5-triphenyltetrazolium ion was estimated to be over 95%. These results indicate that 1,3,5-triphenylformazan is reduced to formazan anion R^- by one-electron reaction accompanied by the elimination of hydrogen atom as shown below:

$$\mathrm{RH} + \mathrm{e} \longrightarrow \mathrm{R}^- + \mathrm{H} \qquad (6)$$

When formazan is reduced in the presence of proton donors, the regeneration of formazan is expected, since the protonation reaction shown by Eq. 5 would follow after the generation of R^-. In order to examine the probable cyclic regeneration of formazan, constant potential electrolysis at −1.09 V was carried out in an aqueous DMSO solution. Water was chosen as the proton donor, since acids were reduced at this potential, while water did not affect the electrolysis. The results are given in Fig. 6. In contrast with the asymptotic approach of the electrolysis current to the background level in unhydrous DMSO, the curent was observed to reach a stationary level in an aqueous DMSO solution, showing a stationary state being attained between the reduction and the regeneration of formazan in an aqueous DMSO solution. After 60 min of electrolysis, the peak heights of the absorption band and the reduction wave of formazan were almost the same with those recorded before the electrolysis was started, indicating that the concentration of formazan was

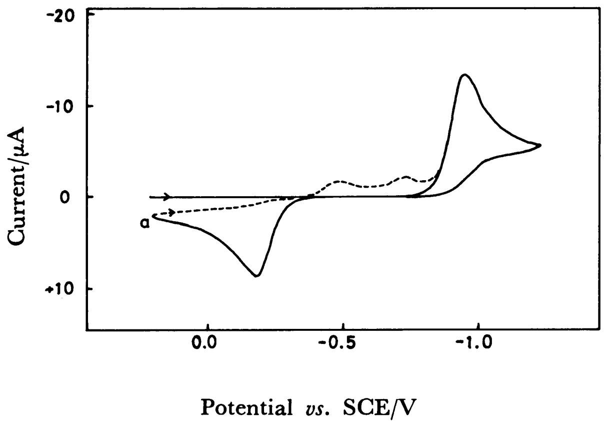


Fig. 5. Cyclic voltammogram of 1,3,5-triphenylformazan: 1.0×10^{-3} mol dm^{-3} in DMSO containing 0.1 mol dm^{-3} TEAP. Scan rate: 0.05 Vs^{-1}.

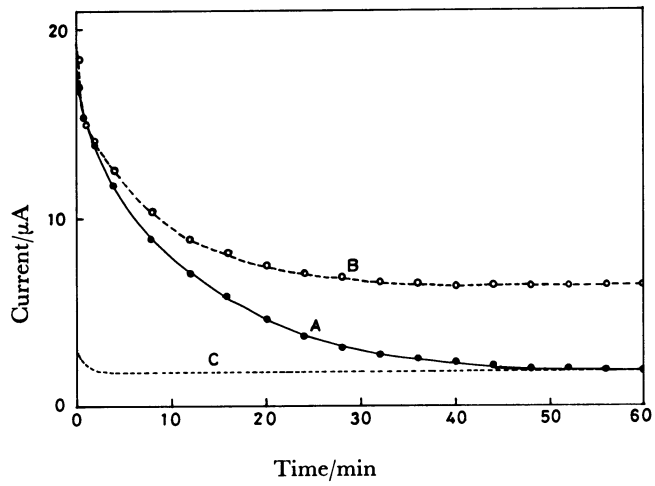


Fig. 6. Current-time curves taken in electrolysis of 1,3,5-triphenylformazan in (A): Unhydrous DMSO, (B): Aqueous DMSO (DMSO : water = 92 : 8 in volume), and (C): base current (extrapolated after 10 min.). 1×10^{-7} mol of formazan was taken in *ca.* 4 dm^{-6} of solution containing 0.1 mol dm^{-3} TEAP. Potential: −1.09 V *vs.* SCE.

little affected in spite of formazan being really accepting electrons under conditions examined.

The results presented in this work provide an explanation for the problem encountered in the use of TTC as an electron acceptor. As 1,3,5-triphenylformazan is reduced at a potential (−0.95 V) not far more negative than the second reduction potential of TTC (−0.75 V), a stronger electron donor would reduce not only TTC (according to Eq. 1—4) but also formazan (according to Eqs. 5 to 6). However, the amount of electron donor used for the reduction of formazan is uncertain, since formazan is regenerated by the protonation reaction. The effect of regeneration of formazan depends both on the electron donor and the used solvent system; the stronger the electron donor and the stronger the proton donating property of the solvent system, the more effective the contribution of reduction of formazan, leading to erroneous estimation of electron donors by using TTC. In this sence, the electron donating property of reducing agents should be well established prior to the application of TTC for the particular system.

The author thanks Drs. Ryo Hirasawa and Kazuo Sakamoto for their kind permission and outstanding assistance in using their ESR spectrometer.

References

1) A. W. Nineham, *Chem. Rev.*, **55**, 355 (1955).

2) F. P. Altman and G. R. Butcher, *Histochemie*, **37**, 333 (1973); R. G. Butcher and F. P. Altman, *ibid.*, **37**, 351 (1973); F. P. Altman, *ibid.*, **38**, 155 (1974).

3) P. S. N. Rao and U. B. S. Swami, *Curr. Sci.*, **48**, 818 (1979).

4) V. A. Kalaganov, V. Z. Zakirova, and Z. M. Kartavtseva, *Khim. Tekhnol. Topl. Masel*, **1983**, 40; *CA*, **98**, 146184h (1983).

5) B. H. J. Bielski, G. G. Shiue, and S. Bajuk, *J. Phys. Chem.*, **84**, 830 (1980).

6) I. Saito, T. Matsuura, and K. Inoue, *J. Am. Chem. Soc.*, **105**, 3200 (1983).

7) E. Lee-Ruff, *Chem. Soc. Rev.*, **6**, 195 (1977).

8) A. I. Lazarev, V. I. Lazarev, and I. P. Kharlamov, *Zab. Lab.*, **46**, 291 (1980); *CA*, **93**, 36356e (1980).

9) K. Umemoto and N. Okamura, unpublished data.

10) Y. Deguchi and H. Takagi, *Tetrahedron Lett.*, **33**, 3179 (1967).

11) O. W. Maender and G. C. Russell, *J. Org. Chem.*, **31**, 442 (1966); F. A. Neugebauer and C. A. Russell, *ibid.*, **33**, 2744 (1968).

12) F. A. Neugebauer, *Tetrahedron*, **26**, 4843 (1970).

13) K. M. Dyumaev, G. V. Formin, and Z. K. Kriminskaya, *Khim. Vys. Energ.*, **16**, 558 (1982); *CA*, **98**, 81387e (1983).

14) Z. K. Kriminskaya, K. M. Dyumaev, L. S. Vischipanova, Yu. V. Ivanov, and A. K. Pikaev, *Khim. Vys. Energ.*, **18**, 40 (1984); *CA*, **100**, 174049w (1984).

15) K. Umemoto, T. Saeki, and N. Matsuura, *Bull. Chem. Soc. Jpn.*, **55**, 746 (1982).

16) N. Azuma, K. Mukai, and K. Ishizu, *Bull. Chem. Soc. Jpn.*, **43**, 3960 (1970).

17) M. Lacan, T. Tabakovic, and Z. Cekovic, *Tetrahedron*, **30**, 2911 (1974).

18) I. Tabakovic, M. Trkovnik, and Z. Gruic, *J. Chem. Soc., Perkin Trans. 2*, **1979** 166.

19) V. F. Gryazev, E. S. Karavaeva, I. N. Borukhova, and N. P. Bednyagina, *Khim. Geterosikl. Soedin.*, **1982**, 1274; *CA*, **98**, 4517v (1983).