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The Tetrazolium Dyes MTS and XTT Provide New Quantitative Assays for Superoxide and Superoxide Dismutase

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The tetrazolium dyes MTS and XTT were reduced to their soluble formazans by superoxide radical anions (O_2^-) produced by the oxidation of xanthine by xanthine oxidase under standard conditions. These reactions were compared to the well-known reductions of NBT and cytochrome c by the xanthine/xanthine oxidase system. Reduction of the dyes was completely inhibited by superoxide dismutase (SOD). Rate constants for the reaction of MTS and XTT with O_2^- were estimated at $1.3 \pm .1 \times 10^5$ $M^{-1}s^{-1}$ and $8.6 \pm .8 \times 10^4$ $M^{-1}s^{-1}$ respectively. The stable MTS and XTT formazans have high extinction coefficients in the visible range which enable sensitive detection and quantification of superoxide radicals, avoiding some of the problems inherent in assays based on production of the insoluble NBT formazan. MTS and XTT have considerable potential both for the quantitative assay of radical production in living tissues and for the assay of superoxide dismutase activity in tissue extracts. Implications for the interpretation of cell culture growth assays which employ these dyes are discussed.

Keywords: MTS, NBT, XTT, cytochrome c, superoxide assay, tetrazolium dyes

INTRODUCTION

The reduction of the dye nitroblue tetrazolium (NBT) by the superoxide radical (O_2^-) has been widely used to detect generation of the radical in biological systems and in indirect assays of the enzyme superoxide dismutase (SOD).[1] The product of this reduction is an insoluble blue diformazan. To indicate that the reduction observed is due to a reaction with O_2^-, and not due to other cellular processes, inhibition of colour formation by the presence of active SOD is generally demonstrated. While the insolubility of the blue formazan is useful for the localisation of radical generation in tissues, it renders quantitative measurement and kinetic studies very difficult. For these investigations, assays relying on soluble products, such as the production of reduced cytochrome c[2], are favoured.

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A wide array of tetrazolium dyes are known and their chemistry has been recently reviewed.[3] Histologists have recognised for some time that many of these dyes are subject to reduction by superoxide and that tissue SOD levels can be a source of interference in some tetrazolium-based enzyme assays.[3] In contrast, free radical researchers have not systematically investigated other tetrazolium dyes apart from NBT for their potential as radical indicators.

We are currently engaged in research into O_2^- production during the pathogenic challenge of higher plant cells. Many of the earliest events in pathogen recognition, including active oxygen generation, appear to take place at the outer surface of the plasma membrane, releasing radicals into the periplasmic space between the membrane and the cell wall.[4,5] To examine these events, we have been searching for a O_2^- assay based on a diffusable low molecular weight reagent that produces a stable, soluble product which can be measured spectrophotometrically in cell culture supernatants. Both the reagent and its reduction product must be able to diffuse through the cell wall unhindered.

Two candidates for such a role have been synthesised in the last decade. The monotetrazolium dye XTT(2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide, sodium salt),[6] was developed for rapid screening of *in vitro* growth of animal cell lines cultured in microtitre plates.[7] Since the synthesis of XTT, other novel tetrazolium derivatives with similar characteristics have been investigated[8], leading to the emergence of an alternative cell viability assay based on MTS(5-(3-carboxymethoxyphenyl)-2-(4,5,-dimethylthiazolyl)-3-(4-sulphophenyl)tetrazolium, inner salt)[9], a derivative of MTT (3- (4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium). MTS generates a charged, water-soluble formazan when reduced,[8] and MTS is more stable than XTT in the presence of phenazine methosulphate (PMS), a redox intermediate required in both assays.[10] MTS has recently been used to assay NAD(P)H dependant dehydrogenases.[11]

Both reagents appear to satisfy the criteria for quantitative O_2^- assay. Their monoformazan reduction products are water soluble and these products have strong absorbance peaks in the visible range. Furthermore, these dyes require the presence of a redox intermediate, such as PMS, in order to be rapidly reduced by the action of reductases and dehydrogenases in growing animal cells. In the absence of a redox intermediate, reduction rates of the dyes by cellular enzymes are low.[10]

Here we report initial investigations into the reaction of these dyes with O_2^-, generated by the oxidation of xanthine by xanthine oxidase. Rate constants have been determined and the reactions of MTS and XTT with O_2^- have been compared to that of NBT and cytochrome c in the same *in vitro* system.

MATERIALS AND METHODS

MTS was obtained from Promega, Madison, WI, USA. A pure sample of MTS formazan was provided by Prof T.C. Owen of the University of South Florida. XTT was obtained from Diagnostic Chemicals Ltd, Charlottetown, PEI, Canada. Unless otherwise indicated all reagents and enzymes were obtained from Sigma Chemical Co., St. Louis, MO. Solutions were made up in high purity water drawn from a Millipore MilliQ system.

A flux of O_2^- was generated during the oxidation of xanthine to urate by xanthine oxidase,[2] with standard conditions set such that a solution of 10μM cytochrome c was reduced at a rate of 0.025 ± 0.001 absorbance units/min at 550nm. Briefly, the reaction was carried out in 3ml of 0.05M potassium phosphate buffer at pH 7.8 containing 0.1mM DTPA, 0.1mM xanthine and sufficient xanthine oxidase (approx. 0.01 units as per supplier) to produce a linear rate of urate formation equivalent to 0.017 absorbance units per minute at 295nm. Reactions were commenced by the addition of xanthine oxidase to a mix of all other components and then monitored for 4 min-

utes in a Shimadzu Model UV 2100 recording spectrophotometer. During this time the observed rate of urate production was linear.

Reduction of the tetrazolium dyes MTS, NBT (nitroblue tetrazolium, i.e. 3,3' (3,3'-dimethoxy-4,4'-biphenylylene)-bis-(2-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride)), and XTT in the presence of xanthine/xanthine oxidase was monitored at 490nm, 560nm and 470nm respectively, the observed absorbance maxima of their formazan products at pH 7.8. NBT (0.0012M) and XTT (0.012M) stock solutions were maintained at approximately 45°C prior to addition to avoid trace precipitate formation. The effects of catalase (150 units/ml) and of a range of hydrogen peroxide (H_2O_2) concentrations (2.5μM – 1 mM) on rates of dye reduction in the presence and absence of xanthine/xanthine oxidase were also observed.

Quantities of dye added were varied to determine levels required for maximum rates of reduction. Inhibition of dye reduction by superoxide dismutase (SOD) was estimated following addition of varying enzyme concentrations to the reaction mixture approximately 1 minute after xanthine addition. Control experiments using SOD which had been inactivated at 100°C for ten minutes, were also carried out. These results were used to establish an apparent rate constant for the reaction of MTS and XTT with O_2^-.[12,13] Assays to monitor SOD activity were carried out using the reduction of cytochrome c.[2]

Estimation of the extinction coefficient for MTS formazan was conducted following rigorous drying under vacuum and assumed a formula weight of 569g, which includes two waters of hydration (*Prof. T. C. Owen, personal communication*).

RESULTS

The reduction of XTT and MTS by the xanthine oxidase reaction was compared with the well-known reduction of NBT *(Fig. 1)*. While similar concentrations of MTS and NBT were required to reach maximum rates of reduction, higher concentrations of XTT were required to obtain the same effect. At these concentrations the rates of reduction were linear over the time course of the experiment and the soluble monoformazan reduction products were stable in solution. In contrast, NBT formazan, although produced linearly, subsequently precipitated from solution onto the sides of the cuvette. Neither MTS, NBT or XTT had any effect on the rate of urate production by the enzyme.

The addition of increasing concentrations of SOD progressively decreased the reduction of MTS and XTT by xanthine oxidase activity until dye reduction was completely inhibited. In contrast, the enzyme was unable to completely inhibit NBT reduction *(Fig. 2)*. Inactivated SOD had no effect on reduction of the dyes and active SOD had no effect on the rate of xanthine oxidation to urate.

Catalase did not affect the reduction of MTS and XTT by the xanthine oxidase system. Addition of a range of H_2O_2 concentrations (2.5μM – 1 mM) to MTS (100mM) and XTT (400mM) at pH 7.8 did not result in formazan formation (results not shown), except in the case of XTT exposed to 1mM H_2O_2, the highest concentration tested, where a low rate of 0.0003 absorbance units/min was observed. Addition of H_2O_2 to the xanthine/xanthine oxidase reaction in the presence of the dyes significantly inhibited reduction of MTS and XTT in a concentration-dependent manner. However this inhibition could be entirely accounted for by the direct inhibitory effects of H_2O_2 on O_2^- generation by the enzyme, indicated by a fall in urate production (observed at 295nm—results not shown).

From a series of replicated SOD inhibition curves similar to those in *Figure 2*, the apparent rate constants (k_{dye}) for the reaction of MTS and XTT with O_2^- were estimated (*Table I*), assuming a rate constant (k_{SOD}) for the SOD reaction of $2 \times 10^9 M^{-1} s^{-1}$[14], according to the equation

$$V_o / V_s = 1 + \frac{k_{SOD} \cdot [SOD]}{k_{dye} \cdot [dye]}$$

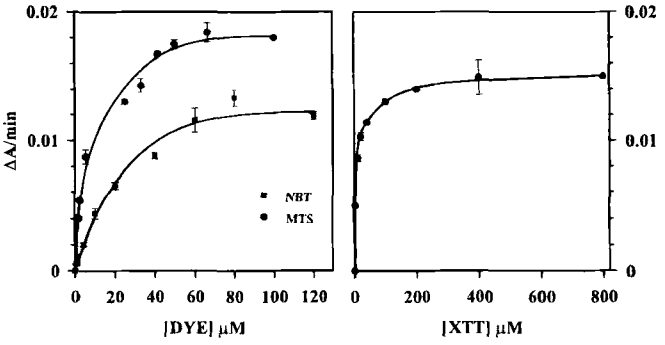

FIGURE 1 Reduction of MTS, NBT and XTT by xanthine oxidase. ΔA/min measured at 490nm, 560nm and 470nm respectively, in the presence of O_2^- generated by oxidation of xanthine by xanthine oxidase under standard conditions (see Methods). Individual points are x ± s.e. of 2–6 replicates.

where V_o is the rate without SOD and V_s is the rate plus SOD. For comparative purposes we also calculated the apparent rate constant for the reaction of cytochrome c with O_2^- under these conditions (*Table I*). The concentration dependence of cytochrome c reduction under these standard conditions and the scavenging of all O_2^- ions at 10μM cytochrome c was confirmed (results not shown). Our estimate of $4.82 \pm .73 \times 10^5 M^{-1}s^{-1}$ is consistent with published values.[14]

In order to determine absolute rates of dye reduction and therefore an estimate of the rate of O_2^- generation in this system, the extinction coefficient of MTS formazan was estimated as 26,900 ± 200 $M^{-1}cm^{-1}$ (n = 3 independent experiments) in 50mM potassium phosphate buffer at pH 7.8, containing 0.1 mM DTPA, using a range of formazan concentrations. A linear relationship between absorbance and concentration was maintained to an absorbance value of 1.0.

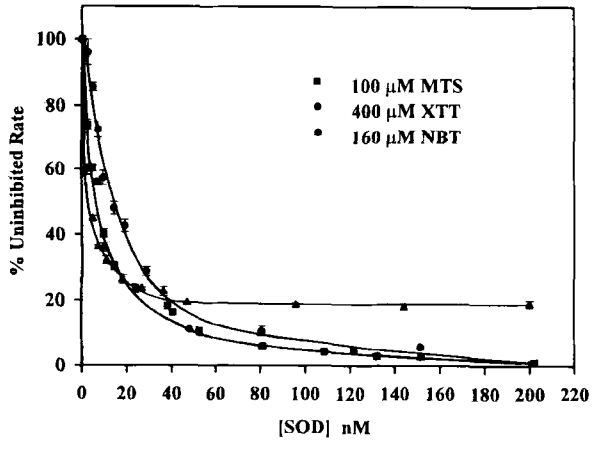

FIGURE 2 Inhibition by SOD of the reduction of MTS, NBT, and XTT by xanthine oxidase. Individual points are x ± s.e of 2–5 replicates.

DISCUSSION

MTS and XTT are reduced by xanthine oxidase during the oxidation of xanthine. These reductions are completely inhibited by active SOD, indicating that the agent responsible for reduction of the dyes is O_2^- (*Fig. 3*).

The potential advantages of a stable, low molecular weight water-soluble formazan either for the assay of O_2^- production by chemical or cellular processes or for the assay of SOD are clear. Unlike the ditetrazolium and monotetrazolium dyes originally developed for histological use (e.g. INT, NBT and MTT),[3] the formazan reduction products of MTS and XTT are freely soluble, allowing accurate spectrophotometric analysis of their production in culture supernatants or tissue perfusates without the necessity of solvent extractions.

We have calculated an extinction coefficient of 26,900 $M^{-1}cm^{-1}$ for the MTS formazan at its

TABLE I Rate Constants for Reaction of O_2^- with MTS, XTT, and Cytochrome c

REACTANT	$^1K_{dye}$ ($M^{-1}s^{-1}$)
MTS	$1.31 \pm .10 \times 10^5$ (n = 3)
XTT	$8.59 \pm .81 \times 10^4$ (n = 4)
Cytochrome c	$4.82 \pm .73 \times 10^5$ (n = 2)

[1] Value represents mean ± std error from *n* independent experiments. For regression lines, r^2 values were > 0.89 for every replicate.

absorbance peak of 490nm in 50mM phosphate buffer at pH 7.8. Given that the mean rate of MTS reduction under the conditions of the standard cytochrome c assay was 0.0174 abs units/min (from *Fig. 1*), then the rate of production of the formazan was 1.57×10^{-6}M.min^{-1}. Under these standard conditions, given that the extinction coefficient of cytochrome c is 21,000 $M^{-1}cm^{-1}$ at 550nm[15] and the mean measured rate of cytochrome c reduction in matched experiments was 0.0244 abs units/min, then the observed ratio of the MTS reduction rate to the cytochrome c reduction rate is 0.558. This value is consistent

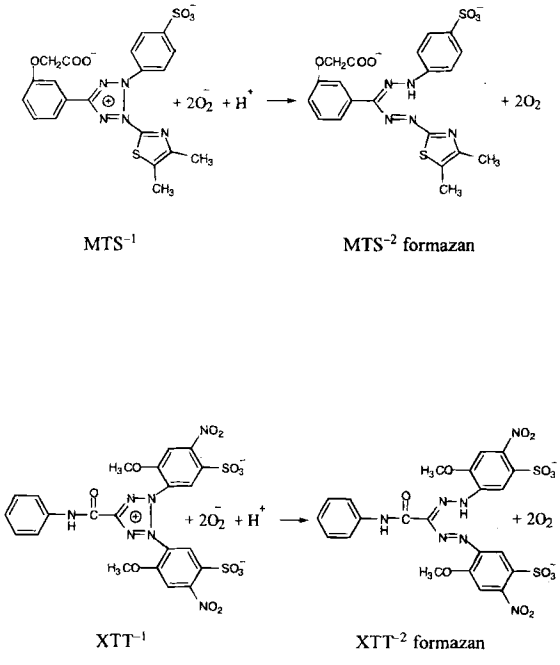

FIGURE 3 Dye Structures.

with the fact that MTS (and XTT) reduction requires two electrons[6,8] while cytochrome c reduction requires only one. Hence the yield of O_2^- scavenged by MTS is similar to, if not higher than, that scavenged by cytochrome c under saturating conditions. A pure sample of XTT formazan was not available to us for this study. However if the maximum rate of XTT reduction observed in *Figure 1* is compared to that for MTS, then in 50mM phosphate buffer at pH 7.8, XTT formazan has an extinction coefficient of approximately 21,600 $M^{-1}cm^{-1}$ at its absorbance peak of 470nm, assuming the kinetics of the reduction of the two dyes are similar. This estimate is not dissimilar to the value of 23,800 $M^{-1}cm^{-1}$ determined in pure water at 475nm[6].

We observed, as have others,[16] that saturating concentrations of SOD did not completely inhibit the reduction of NBT by the xanthine oxidase system. As a result, the calculation of a rate constant for the reaction of NBT with O_2^- is not possible by the indirect method employed in this study.[12] Other studies have established a value of 5.9×10^4 $M^{-1}s^{-1}$ for this reaction.[14] In contrast SOD was able to completely inhibit the reduction of MTS, XTT and cytochrome c, a result consistent with O_2^- being the sole reducing species. Neither MTS or XTT was reduced by H_2O_2 at peroxide concentrations likely to be found in tissues. The apparent rate constants of $1.3 \pm .1 \times 10^5$ $M^{-1}s^{-1}$ and $8.6 \pm .8 \times 10^4$ $M^{-1}s^{-1}$ for the reaction of O_2^- with MTS and XTT respectively, indicate that both dyes react somewhat faster with superoxide than does NBT and are thus more efficient scavengers of the radical. These measurements depend on the commercial SOD preparation used in this study being at full activity. The fact that our estimation of the rate constant for the reaction of cytochrome c with O_2^- ($4.8 \pm .7 \times 10^5$ $M^{-1}s^{-1}$) falls within the range of published values,[14] confirms that this was the case. This indirect method of studying tetrazolium reduction by O_2^- is unable to shed light on details of the reaction mechanisms involved and possibly underestimates the rate constants due to side

reactions.[12] To address this issue, detailed direct kinetic studies have commenced.

In the absence of PMS, reduction of either MTS or XTT by cultured animal cells is slow.[10] Thus pathological processes or stress responses which generate O_2^- in cells should accelerate the rate of XTT or MTS reduction over these background levels and permit quantitative assay of the radical. Our finding that O_2^- readily reacts with these two dyes suggests that investigators using MTS or XTT as a monitor of cell metabolism in the presence of drugs or other cell treatments, should note the possibility that interactions between cells and treatments which lead to superoxide production have the potential to give false positive results in viability or cell growth assays. A recent report[17] indicating that up to 30% of direct intracellular MTT reduction (i.e. in the absence of PMS) by human tumor cells may be due to reaction with O_2^-, supports the case for further investigation of this possibility.

With regard to the reduction of XTT by non-radical processes in plant cells in the absence of PMS, we have observed similarly low rates to those in animal cells. We are currently completing a study of XTT reduction by tobacco suspension cells challenged by zoospores of an incompatible race of the fungus *Phytophthora parasitica* var. *nicotianae*. When disease resistant genotypes of host cells are challenged in the presence of XTT, rapid SOD-inhibitable reduction of the dye is observed, consistent with the oxidative burst which occurs in resistant host plants undergoing a hypersensitive response.[5,18] Cells unchallenged by zoospores, or susceptible cells infected with zoo-spores, show only low levels of dye reduction which are not inhibited by SOD. These results are being submitted for publication elsewhere.

This study has revealed potential new tools for quantitative kinetic assay of O_2^- production. Furthermore these tetrazolium salts appear suitable for use in standard indirect assays of SOD activity. They have particular potential for use as replacements for cytochrome c in the standard xanthine/xanthine oxidase-based SOD assay, where the tissue to be assayed contains components which interfere with the reduction of cytochrome c by superoxide.[19] XTT may be particularly useful for replacing cytochrome c in high sensitivity SOD assays, where its slower rate of reaction with O_2^- should allow a significant drop in the lower limit of enzyme detection.

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References

[1] Beauchamp, C. and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, **44**, 276–287.
[2] McCord, J. M. and Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244**, 6049–6055.
[3] Seidler, E. (1991). The tetrazolium-formazan system: design and histochemistry. *Progress in Histochemistry and Cytochemistry*, **24**, 1–86.
[4] Apostol, I., Heinstein, P. F. and Low, P. S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiology*, **90**, 109–116.
[5] Sutherland, M. W. (1991). The generation of oxygen radicals during host plant responses to infection. *Physiological and Molecular Plant Pathology*, **39**, 79–93.
[6] Paull, K. D., Shoemaker, R. H., Boyd, M. R., Parsons, J. L., Risbood, P. A., Barbera, W. A., Sharma, M. N., Baker, D. C., Hand, E., Scudiero, D. A., Monks, A., Alley, M. C. and Grote, M. (1988). The synthesis of XTT: A new tetrazolium reagent that is bioreducible to a water-soluble formazan. *Journal of Heterocyclic Chemistry*, **25**, 911–914.
[7] Scudiero, J. D., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D. and Boyd, M. R. (1988). Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensi-

tivity in culture using human and other tumor cell lines. *Cancer Research*, **48**, 4827–4833

[8] Barltrop, J. A., Owen, T. C., Cory, A. H. and Cory, J. G. (1991). 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide(MTT) reducing to purple water-soluble formazans as cell-viability indicators. *Bioorganic & Medicinal Chemistry Letters*, **1**, 611–614.

[9] Cory, A. H., Owen, T. C., Barltrop, J. A. and Cory, J. G. (1991). Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Communications*, **3**, 207–212.

[10] Goodwin, C. J., Holt, S. J., Downes, S. and Marshall, N. J. (1995). Microculture tetrazolium assays: A comparison between two new tetrazolium salts, XTT and MTS. *Journal of Immunological Methods*, **179**, 95–103.

[11] Dunigan, D. D., Waters, S. B. and Owen, T. C. (1995). Aqueous soluble tetrazolium/formazan MTS as an indicator of NADH- and NADPH-dependent dehydrogenase activity. *BioTechniques*, **19**, 640–649.

[12] Goldstein, S., Michel, C., Bors, W., Saran, M. and Czapski, G. (1988). A critical reevaluation of some assay methods for superoxide dismutase activity. *Free Radical Biology & Medicine*, **4**, 295–303.

[13] Goldstein, S. and Czapski, G. (1991). Comparison between different assays for superoxide dismutase-like activity. *Free Radical Research Communications*, **12–13**, 5–10.

[14] Bielski, B. H. J, Cabelli, D. E., Arudi, R. L. and Ross, A. B. (1985). Reactivity of HO_2/O_2^- radicals in aqueous solution. *Journal of Physical and Chemical Reference Data*, **14**, 1041–1100.

[15] Massey, V. (1959). The microestimation of succinate and the extinction coefficient of cytochrome c. *Biophysica Biochimica Acta*, **34**, 255–256.

[16] Spitz, D. R. and Oberley, L. W. (1989). An assay for superoxide dismutase activity in mammalian tissue homogenates. *Analytical Biochemistry*, **179**, 8–18.

[17] Burdon, R. H., Gill, V. and Rice-Evans, C. (1993). Reduction of a tetrazolium salt and superoxide generation in human tumor cells (HeLa). *Free Radical Research Communications*, **18**, 369–380.

[18] Hammond-Kosack, K. E. and Jones, J. D. G. (1996). Resistance gene-dependent plant defense responses. *The Plant Cell*, **8**, 1773–1791.

[19] Azzi, A., Montecucco, C. and Richter, C. (1975). The use of acetylated ferricytochrome c for the detection of superoxide radicals produced in biological membranes. *Biochemical and Biophysical Research Communications*, **65**, 597–603.