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USE OF DESFERRIOXAMINE AS A 'PROBE' FOR IRON-DEPENDENT FORMATION OF HYDROXYL RADICALS

EVIDENCE FOR A DIRECT REACTION BETWEEN DESFERAL AND THE SUPEROXIDE RADICAL

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Abstract—The iron chelator desferal is a powerful inhibitor of lipid peroxidation and of hydroxyl radical formation dependent on the presence of iron salts. Desferal also reacts with superoxide radical with a second-order rate constant approximately equal to $3 \times 10^2\,M^{-1}\,s^{-1}$ at pH 10.2 and approximately $9 \times 10^2\,M^{-1}\,s^{-1}$ at physiological pH. It is concluded that this slow reaction of desferal with O_2^- is unlikely to influence the interpretation of experiments in which the chelator is used. The ability of desferal to react with hydroxyl radical (k_2 approximately $10^{10}\,M^{-1}\,s^{-1}$) is a far more likely source of error in the interpretation of results using this chelating agent.

Oxygen supplied at concentrations greater than those in normal air has long been known to have toxic effects upon aerobic organisms [1]. Biochemical mechanisms responsible for oxygen toxicity include increased rates of lipid peroxidation and increased formation of the superoxide radical, O_2^-, *in vivo* [2, 3]. Superoxide radical is not highly reactive in aqueous solution and many aspects of its toxicity seem to be due to O_2^--dependent formation of more reactive species, of which the principal candidate is hydroxyl radical, OH· [2, 4]. Both lipid peroxidation and O_2^--dependent formation of hydroxyl radicals are dependent on the presence of transition metal ions, of which iron is likely to be the most important *in vivo* [4]. Iron complexes capable of catalysing such radical reactions are known to be present *in vivo* [4–8].

The iron chelator desferrioxamine, produced by *Streptomyces pilosus*, is a powerful inhibitor of both lipid peroxidation [9, 10] and hydroxyl radical formation [10] dependent on the presence of iron salts. Since large doses of this chelator, available commercially as Desferal® (desferrioxamine B methanesulphonate), can be injected into animals and humans, it has been proposed for use as a probe to study the *in vivo* importance of iron-dependent oxygen radical reactions. Since its introduction to the oxygen radical field [10], desferal has been used in whole animal systems to study the role of oxygen radical reactions in inflammatory joint disease [11], ethanol metabolism [12], complement-mediated lung injury [13], alloxan toxicity [14] and eradication of malarial parasites [15]. It has also been used in several *in vitro* systems (e.g. refs. 16, 17). In using desferal as a probe for iron-dependent oxygen radical reactions, it must be borne in mind that both desferal [18, 19] and its complex with iron (III) [18] are powerful scavengers of the hydroxyl radical. Careful control of concentrations [18] and appropriate control experiments [18, 13] are necessary to allow for this effect.

An additional complication has been raised by Sinaceur *et al.* [12, 20], who have claimed that desferal reacts directly with O_2^- radical. No such reaction was observed in previous pulse radiolysis studies with desferal [21], but pulse radiolysis is not capable of measuring low reaction rates. In view of the widespread use of desferal *in vivo* and *in vitro* it was thought important to investigate in detail this proposed reaction of desferal with O_2^- and to determine the extent to which it might influence the interpretation of experiments with this chelating agent.

MATERIALS AND METHODS

Reagents. Xanthine oxidase was from Boehringer, cytochrome *c* (horse-heart type VI), nitro-blue tetrazolium, hypoxanthine and superoxide dismutase (bovine) were from Sigma, desferal® (desferrioxamine B methanesulphonate) was from CIBA-Geigy. All other reagents were of the highest quality available from BDH Chemicals Ltd. (Poole, U.K.).

Solutions of desferal were made up in distilled water and adjusted to the pH required immediately before use; concentrations were calculated assuming a M_r of 657. Cytochrome *c* was dissolved in 1 mM KH_2PO_4–KOH buffer pH 7.4 and its concentration in solution calculated by measuring A_{550} before and after addition of excess sodium dithionite, using a molar (reduced minus oxidised) extinction coefficient of 1.85×10^4 at 550 nm [22]. Hypoxanthine was dissolved to a final concentration of 30 mM in 50 mM KOH immediately before use; the amount of KOH added to the reaction mixtures together with hypoxanthine produced no significant change in pH in any of these experiments. The iron(III) desferal complex was made by mixing a solution of desferal of molarity x with an equal volume of a fresh solution of $Fe(NO_3)_3$ with molarity 0.9 x. This ensured that the uncomplexed desferal was always in slight molar excess over the iron since uncomplexed iron or iron-EDTA interfere with assays of cytochrome *c* or nitro-blue tetrazolium reduction [23, 24].

Assays. Experiments were carried out at 25 ± 1°. For studies at pH 10.2 a final concentration of 50 mM Na_2CO_3 buffer adjusted to pH 10.2 with HCl was used; for studies at pH 7 to 7.8 50 mM KH_2PO_4 buffer adjusted to the required pH with KOH was used. Reaction mixtures contained, in a final volume of 3 ml, buffer, 10 μM hypoxanthine (final concentration) 10 μM EDTA and either oxidised cytochrome *c* or nitro-blue tetrazolium at the concentrations reported in the text. For studies of xanthine oxidase activity the rise in A_{290} (due to urate production) was measured in the absence of cytochrome *c* or nitro-blue tetrazolium. For studies of cytochrome *c* reduction the rise in A_{550} was measured and for studies of nitro-blue tetrazolium reduction the rise in A_{560} was measured. Rate constants were calculated using simple competition kinetics [18].

RESULTS

Initial experiments using the technique of pulse radiolysis showed no obvious reaction between desferal or the Fe(III)-desferal complex and O_2^- at any pH value in the range 7–10.2. From the data it could be concluded that the second-order rate constant for the reaction of desferal or Fe(III) desferal with O_2^- is less than $2 \times 10^5 M^{-1} s^{-1}$.

To look for slower reaction rates 'indirect' assay systems were used. Superoxide generated by a mixture of hypoxanthine and xanthine oxidase is allowed to react with a 'detector' molecule. If the concentration of detector is insufficiently high to react with all the O_2^- formed, the remainder will undergo non-enzymic dismutation at a rate depending on the pH [25]. Preliminary experiments showed that neither desferal nor Fe(III) desferal at the concentrations used in these studies had any effect on xanthine oxidase activity.

Cytochrome *c* and nitro-blue tetrazolium were chosen as detector molecules since their rate constants for reaction with O_2^- are well established [22, 26]. Reaction mixtures also contained EDTA at a final concentration of 10 μM to ensure that any Cu^{2+} ions contaminating the reagents (especially Na_2CO_3 [23] or cytochrome *c* [22]) were bound in an inactive Cu-EDTA complex [23]. Reaction rate constants determined in the absence of EDTA were the same as in its presence (data not shown).

Studies at pH 10.2 using NBT and cytochrome c

pH 10.2 is frequently used for assays of superoxide dismutase activity [27] and the addition of chelating agents such as desferal to reaction mixtures has been recommended to prevent interference from contaminating metal ions in tissue extracts [4, 23, 24]. The ability of desferal to react with O_2^- at pH 10.2 was therefore tested.

At pH 0.2 the rate of non-enzymic dismutation of O_2^- is low [25] and so even small concentrations of detector molecule are sufficient to intercept essentially all the O_2^- generated. Table 1 shows the effect of 'detector' concentration on the rates of cytochrome *c* reduction (measured as absorbance rise at 550 nm) or NBT reduction (measured as absorbance rise at 560 nm) by a mixture of hypoxanthine and xanthine oxidase at pH 10.2. These rates were measured against control reaction mixtures containing 300 units (defined as in ref. 28) of bovine superoxide dismutase, to correct for any direct reductions of cytochrome *c* or NBT by xanthine oxidase. Preliminary experiments showed that these direct reduction rates were less than 10% of the overall rates measured. The rates of absorbance change

Table 1. Detection of superoxide radical at pH 10.2

Final concn. of detector in reaction mixture (μmol/l)	Detector: NBT Initial rate ($A_{560}\,sec^{-1}$)	Detector: Cytochrome *c* Initial rate ($A_{550}\,sec^{-1}$)
10	9.63×10^{-3}	8.6×10^{-3}
20	9.19×10^{-3}	1.05×10^{-2}
30	9.76×10^{-3}	1.13×10^{-2}
40	1.26×10^{-2}	1.07×10^{-2}
50	1.26×10^{-2}	1.05×10^{-2}
60	1.05×10^{-2}	—
70	1.11×10^{-2}	—
100	1.15×10^{-2}	1.19×10^{-2}
200	1.09×10^{-2}	1.07×10^{-2}

Experiments were carried out at pH 10.2 as described in the Materials and Methods section. The initial rates of absorbance change were recorded. Temperature was 25 ± 1°. NBT—nitro-blue tetrazolium.

The rates of absorbance change achieved at 'saturating' concentrations of NBT and cytochrome *c* are determined by the rate of O_2^- generation, which differs when different batches of xanthine oxidase are used. In several experiments using different batches of enzyme the rates of NBT and cytochrome *c* reduction varied, but the concentrations of these detectors required to achieve the maximum rates of reduction were identical with those in the typical experiment shown.

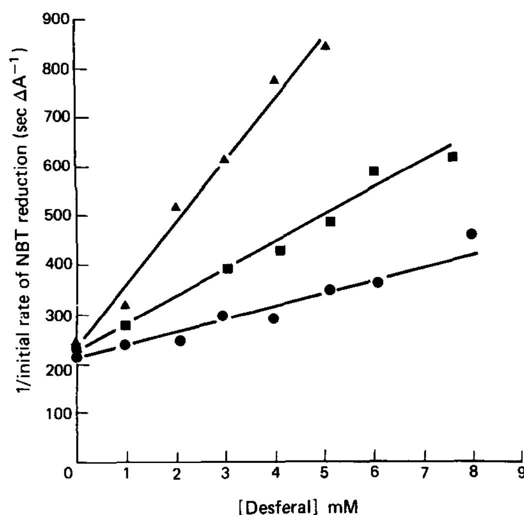

Fig. 1. Action of desferal on the reduction of NBT by superoxide radical at pH 10.2. Experiments were carried out as described in the Materials and Methods section using NBT at final concentrations of ▲ 10 μM, ■ 20 μM, ● 50 μM. Similar results were obtained using different batches of xanthine oxidase that produced different rates of NBT reduction.

achieved at 'saturating' concentrations of NBT or cytochrome *c* differ slightly depending on the activity of the batch of xanthine oxidase used in generating O_2^-, but in several experiments with different batches of enzyme the concentrations of NBT or cytochrome *c* needed to give maximum reduction rates (i.e. to intercept essentially all the O_2^- generated) were identical to those shown in Table 1.

If desferal reacts with O_2^-, addition of it to the reaction mixtures should intercept some of the O_2^- produced and decrease the rate of absorbance change of the detector molecule. Since under the reaction conditions used the initial rates of NBT or cytochrome *c* reduction were essentially independent of their concentration (Table 1), the rate of non-enzymic O_2^- dismutation must have been negligible and the effects of added desferal on the absorbance changes can be analysed in terms of a simple competition between desferal and detector for the O_2^- generated.

Figure 1 shows that such competition kinetics could indeed be demonstrated, using NBT as a detector molecule. Assuming a second-order rate constant of $6 \times 10^4 M^{-1} s^{-1}$ [26] for the reaction of NBT with O_2^- at pH 10.2, analysis of experiments of the type shown in Fig. 1, using different batches of xanthine oxidase, allowed calculation of the rate constant for reaction of desferal with O_2^-. The value was found to be $2.97\ (\pm 0.36) \times 10^2 M^{-1} s^{-1}$ (mean ± S.D. of 4 experiments).

To check the value obtained, experiments similar to those in Fig. 1 were performed using cytochrome *c* as detector molecule and again competition kinetics were observed (Fig. 2). The rate constant for reaction of desferal with O_2^- was found to be $2.67\ (\pm 0.40) \times 10^2 M^{-1} s^{-1}$ (mean ± S.D. of 3 determinations) assuming a second-order rate constant of $6 \times 10^4 M^{-1} s^{-1}$ for the reduction of cytochrome *c* by O_2^- at pH 10.2 [22].

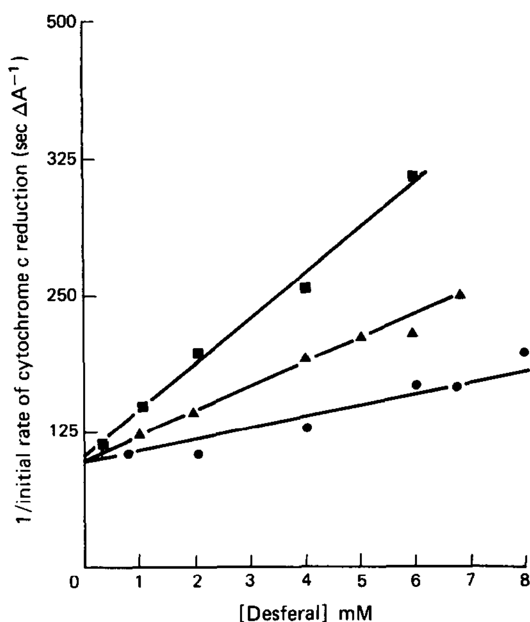

Fig. 2. Action of desferal on the reduction of cytochrome *c* by superoxide radical at pH 10.2. Experiments were carried out as described in the Materials and Methods section using horse-heart cytochrome *c* at final concentrations of ■ 10.05 μM, ▲ 20.1 μM, ● 30.15 μM. Similar results were obtained using different batches of xanthine oxidase that produced different rates of cytochrome *c* reduction.

Studies at pH 7.8 using NBT and cytochrome c

Since desferal is often used in experiments *in vivo* (see the Introduction Section), its reaction with O_2^- at pH values in the range 7.0–7.8 was investigated. Results were identical over this pH range.

At pH 7–7.8 the rate of non-enzymic dismutation of O_2^- is much faster than at pH 10.2 [25]. Table 2 shows that it was possible to find concentrations of cytochrome *c* that essentially intercepted all the O_2^- produced, but the rate of NBT reduction increased with NBT concentration over the whole concentration range tested. This may be because of the great complexity of the reaction between O_2^- and NBT at pH 7.0–7.8 [26].

Figure 3 shows that added desferal competed with cytochrome *c* for O_2^-. It was noticed that the absorbance at 550 nm of reaction mixtures containing high concentrations (>5 mM) of desferal together with cytochrome *c* increased gradually in the absence of xanthine oxidase, and the data in Fig. 3 are calculated using concentrations at which this background rate was insignificant. Assuming a second-order rate constant of $2.6 \times 10^5 M^{-1} s^{-1}$ for the reduction of cytochrome *c* by O_2^- at pH values of 7–7.8 [22], the rate constant for reaction of desferal with O_2^- was found to be $9.13\ (\pm 0.85) \times 10^2 M^{-1} s^{-1}$ (mean ± S.D. of 4 determinations). The inhibition of NBT reduction in the hypoxanthine-xanthine oxidase system by desferal also decreased as the concentration of NBT was raised. The apparent inability of NBT to intercept all the O_2^- generated (Table 2) makes calculation of

Table 2. Detection of superoxide radical at pH 7.8

Final concn. of detector in reaction mixture (μmol/l)	Detector	
	NBT Initial rate ($A_{560}\,sec^{-1}$)	Cytochrome *c* Initial rate ($A_{560}\,sec^{-1}$)
10	7.93×10^{-4}	2.25×10^{-3}
20	1.70×10^{-3}	2.79×10^{-3}
30	2.16×10^{-3}	2.61×10^{-3}
40	2.50×10^{-3}	2.69×10^{-3}
50	3.03×10^{-3}	2.71×10^{-3}
60	3.28×10^{-3}	—
70	3.31×10^{-3}	—
80	3.58×10^{-3}	—
90	3.85×10^{-3}	—
100	3.85×10^{-3}	—
150	4.92×10^{-3}	—
200	5.51×10^{-3}	—

Experiments were carried out at pH 7.8 as described in the Materials and Methods section. The initial rates of absorbance change were recorded. Temperature was 25 ± 1°. NBT—nitro-blue tetrazolium

The rate of absorbance change achieved at 'saturating' cytochrome *c* concentrations depends on the rate of O_2^- generation, which differs when different batches of xanthine-oxidase are used. The concentration of cytochrome *c* required to give maximum absorbance change was the same whatever batch of xanthine oxidase was used, however. The rate of O_2^- production by hypoxanthine-xanthine oxidase at pH 7.8 was smaller than that at pH 10.2, as based on the rates of cytochrome *c* reduction at saturating cytochrome *c* (Table 1).

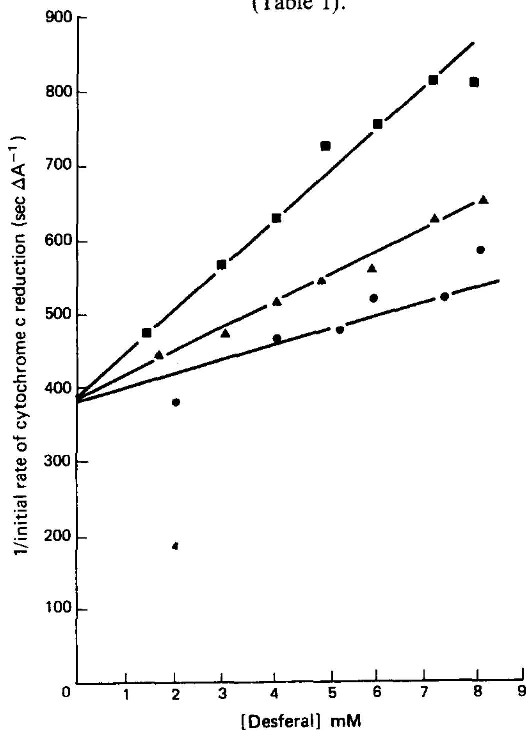

Fig. 3. Action of desferal on the reduction of cytochrome *c* by superoxide radical at physiological pH. The experiment was carried out using phosphate buffer pH 7.8 as described in the Materials and Methods section; identical results were obtained using buffers of pH 7.0 or 7.5. Reaction mixtures contained horse-heart cytochrome *c* at final concentrations of ■ 20.1 μM, ▲ 30.2 μM, ● 40.2 μM. Similar results were obtained using different batches of xanthine oxidase that produced different rates of cytochrome *c* reduction.

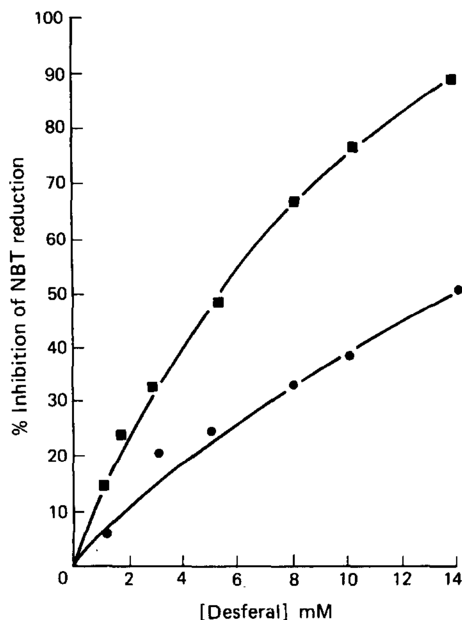

Fig. 4. Action of desferal on the reduction of NBT by superoxide radical at pH 7.8. The experiment was carried out as described in the Materials and Methods section; identical results were obtained using buffers of pH 7.0 or 7.5. NBT was present in the reaction mixtures at final concentrations of ■ 100 μM, ● 500 μM. The rates of absorbance change at 560 nm corresponding to 0% inhibition (no added desferal) were $2.70 \times 10^{-3}\,sec^{-1}$ at 100 μM NBT and $4.67 \times 10^{-3}\,sec^{-1}$ at 500 μM NBT in this experiment.

a rate constant from the data in Fig. 4 almost impossible, but the data are consistent with a reaction between desferal and O_2^-.

DISCUSSION

On the basis of its ability to competitively inhibit reduction of cytochrome *c* or NBT by O_2^- generated by a hypoxanthine-xanthine oxidase system, I conclude that desferal reacts with O_2^- at pH 10.2 with k_2 approximately equal to $3 \times 10^2 M^{-1}s^{-1}$. At pH values of 7–7.8, based on inhibition of cytochrome *c* reduction, k_2 was slightly greater at approx. $9 \times 10^2 M^{-1}s^{-1}$.

Desferal is added to reaction mixtures in order to probe for the role of iron salts in oxygen radical reactions such as lipid peroxidation and hydroxyl radical formation. The most likely artefact it can produce is a direct scavenging of hydroxyl radical with a second-order rate constant of about 10^{10} $M^{-1}s^{-1}$ [18, 19]. If concentrations of desferal that are too low to scavenge OH· are used, as recommended [18], it is obvious that the reaction of desferal with O_2^-, proceeding at a rate that is nearly eight orders of magnitude less and considerably smaller than the overall rate of non-enzymic dismutation of O_2^- at physiological pH [25] is scarcely likely to interfere either. I conclude that the reaction of desferal with O_2^-, although demonstrable in sensitive detector systems, can be safely ignored in normal uses of this chelating agent.

The use of inhibition of cytochrome *c* reduction as a means of testing the reactivity of molecules with O_2^- has been criticised [29], because any substance that reduces cytochrome *c* directly can produce an apparent inhibition. This artefact was looked for in the present work and not found to be significant (see text). The fact that similar competition kinetics and rate constants were obtained using NBT, a completely different detector molecule, argues against the inhibition being an artefact. Suppose however that the effect of desferal in these systems were some artefact; this would not detract from the conclusion that its rate of reaction with O_2^- is extremely small.

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REFERENCES

1. J. D. Balentine, *Pathology of Oxygen Toxicity*. Academic Press, New York (1982).
2. B. Halliwell, in *Age Pigments* (Ed. R. S. Sohal), pp. 1–62. Elsevier, Amsterdam (1981).
3. I. Fridovich, *Ann. Rev. Biochem.* **44**, 147 (1975).
4. B. Halliwell and J. M. C. Gutteridge, *Biochem. J.* **219**, 1 (1984).
5. B. Halliwell, *Biochem. J.* **205**, 461 (1982).
6. R. A. Floyd, *Biochem. biophys. Res. Commun.* **99**, 1209 (1981).
7. R. A. Floyd, *Arch. Biochem. Biophys.* **225**, 263 (1983).
8. J. M. C. Gutteridge, *Biochem. Int.* **8**, 89 (1984).
9. E. D. Wills, *Biochem. J.* **113**, 325 (1969).
10. J. M. C. Gutteridge, R. Richmond and B. Halliwell, *Biochem. J.* **184**, 469 (1979).
11. D. R. Blake, N. D. Hall, P. A. Bacon, P. A. Dieppe, B. Halliwell and J. M. C. Gutteridge, *Ann. rheum. Dis.* **42**, 89 (1983).
12. R. Sinaceur, C. Ribiere, C. Abu-Murad, J. Nordmann and R. Nordmann, *Biochem. Pharmacol.* **32**, 2371 (1983).
13. P. A. Ward, G. O. Till, R. Kunkel and C. Beauchamp, *J. clin. Invest.* **72**, 789 (1983).
14. K. Grankvist and S. L. Marklund, *Life Sci.* **33**, 2535 (1983).
15. I. A. Clark and N. H. Hunt, *Infect Immun.* **39**, 1 (1983).
16. A. I. Cederbaum and E. Dicker, *Biochem. J.* **210**, 107 (1983).
17. H. Nohl, W. Jordan and D. Hegner, *Hoppe-Seylers Z. Physiol. Chem.* **363**, 599 (1982).
18. S. Hoe, D. A. Rowley and B. Halliwell, *Chem. Biol. Interact.* **41**, 75 (1982).
19. R. L. Willson, in *Free Radicals, Lipid Peroxidation and Cancer* (Eds. D. C. H. McBrien and T. F. Slater), pp. 275–303. Academic Press, London (1982).
20. J. Sinaceur, C. Ribiere, J. Nordmann and R. Nordmann, *Biochem. Pharmac.* **33**, 1693 (1984).
21. J. Butler and B. Halliwell, *Arch. Biochem. Biophys.* **218**, 174 (1982).
22. J. Butler, W. H. Koppenol and E. Margoliash, *J. biol. Chem.* **257**, 10747 (1982).
23. B. Halliwell, *FEBS Lett.* **56**, 34 (1975).
24. G. R. Buettner, L. W. Oberley and S. W. H. C. Leuthauser, *Photochem. Photobiol.* **28**, 693 (1978).
25. B. H. Bielski, *Photochem. Photobiol.* **28**, 645 (1978).
26. B. H. J. Bielski, G. G. Shive and S. Bajuk, *J. phys. Chem.* **84**, 830 (1980).
27. T. W. Kirby and I. Fridovich, *Anal. Biochem.* **127**, 435 (1982).
28. J. M. McCord and I. Fridovich, *J. biol. Chem.* **224**, 6049 (1969).
29. J. Diguiseppi and I. Fridovich, *Arch. Biochem. Biophys.* **203**, 145 (1980).