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THE FORMATION OF SUPEROXIDE RADICAL ANIONS BY A REACTION BETWEEN O_2, OH^- AND DIMETHYL SULFOXIDE.

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SUMMARY : Addition of OH^- to air saturated dimethyl sulfoxide leads to the formation of the superoxide radical anion, as shown directly by electron paramagnetic resonance and ultra violet spectroscopy, and indirectly by superoxide dismutase inhibitable cytochrome c reduction. Superoxide production is related inversely to the water concentration of the dimethyl sulfoxide and solutions obtained are stable for up to three days. Reaction mechanisms are suggested and results are discussed in the light of the many uses of dimethyl sulfoxide as a solvent in both chemistry and biology.

INTRODUCTION : Interest in the role of reactive oxygen species in the initiation and progression of various types of tissue injury has increased rapidly in recent years (1,2). For example, superoxide anion radicals have been indicated in the inflammatory process (3), in paraquat intoxication (4), in haemolysis of erythrocytes (5), and in bacterial killing by polymorphonuclear leukocytes (6).

$O_2^{\cdot -}$ has been found to be generated in both chemical and biological systems, such as, pulse radiolysis (7), xanthine-xanthine oxidase (8), photochemically (9) by auto-oxidation mechanisms (10), or electrochemically (11).

We now report the production of $O_2^{\cdot -}$ by a reaction between OH^-, O_2 and dimethyl sulfoxide in a system which provides stable, reproducible concentrations of this radical.

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ABBREVIATIONS : DMSO, dimethyl sulfoxide ; $O_2^{\cdot -}$, superoxide anion radical ; SOD, superoxide dismutase ; K^+, $O_2^{\cdot -}$, potassium superoxide.

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The ability of this widely used solvent (12) to undergo radical reactions suggests that caution should be exercised when it is used in either chemical or biological experimentation.

MATERIALS AND METHODS

Chemicals : Cytochrome c (Boehringer), potassium superoxide (Fluka, Switzerland) dimethyl sulfoxide (Sigma). DMSO was purified by distillation over sodium sulphate at 48°C and 3mm Hg. Absorbed water was removed using a 4Å molecular sieve (Touzart and Malignon, Paris), dimethyl formamide (Prolabo, France) acetonitrile (Merck). Copper zinc superoxide dismutase was prepared according to the method of McCord and Fridovich (8).

Measurement of oxygen consumption : Studies of oxygen consumption were performed using a cylindrical glass chamber (vol. 1.4 ml ; Temp. = 20°C) equipped with a magnetic stirrer and Clarke electrode. The oxygen tension was recorded on a Gilson oxygraph. Air saturated DMSO was considered to contain 0.2 μ moles/ml of O_2 at 20°C.

Identification of $O_2^{\cdot -}$: a) Electron paramagnetic resonance spectra were recorded using a Bruker B-ER 420 apparatus. b) U.V. spectra were recorded on an Acta CIII recording spectrophotometer (Beckman). Reference cuvette held 3ml of DMSO containing 0.55M H_2O. Sample cuvette contained 0.55M H_2O and either 5mM NaOH or a small amount of K^+, $O_2^{\cdot -}$ dissolved in 3ml of DMSO. c) SOD inhibitable reduction of cytochrome c is a probe for the presence of $O_2^{\cdot -}$ (8). This system was used to detect $O_2^{\cdot -}$ by addition of volumes of DMSO containing 5mM NaOH and 0.55M H_2O to 1ml of 0.05M phosphate buffer pH 8.0 containing 2 x 10^{-5}M cytochrome c in the presence and absence of 1μg/ml SOD. Absorption was measured at 550 nm. As controls either pure DMSO was added to buffer containing cytochrome c, or alkaline DMSO was added to buffer alone, left for five minutes to allow for radical dismutation, and then cytochrome c added. In neither of the controls was cytochrome c reduction observed.

RESULTS : Addition of OH^- to DMSO provoked a large consumption of oxygen (Figure 1). This consumption appears to be specific for DMSO as there is no oxygen uptake on addition of OH^- to other aprotic solvents such as acetonitrile or dimethyl formamide. The rate of O_2 consumption was highly dependent on the H_2O concentration in the DMSO, oxygen uptake being inversly related to the H_2O content (Figure 2). The H_2O concentration could not be reduced below 0.25M due to the insolubility of NaOH in pure DMSO.

Reduction of O_2 normally occurs by a univalent pathway, the first step leading to the production of $O_2^{\cdot -}$. The fact that this radical is reasonably stable in DMSO (13) suggested that the oxygen consumption observed could have been caused by the one electron reduction of O_2 giving $O_2^{\cdot -}$. Figures 3 and 4 varify this hypothesis. The U.V. spectrum generated after addition of NaOH to DMSO is similar to that seen after dissolution of K^+, $O_2^{\cdot -}$ (Figure 3), both having an extinction maximum at 256nm. Also

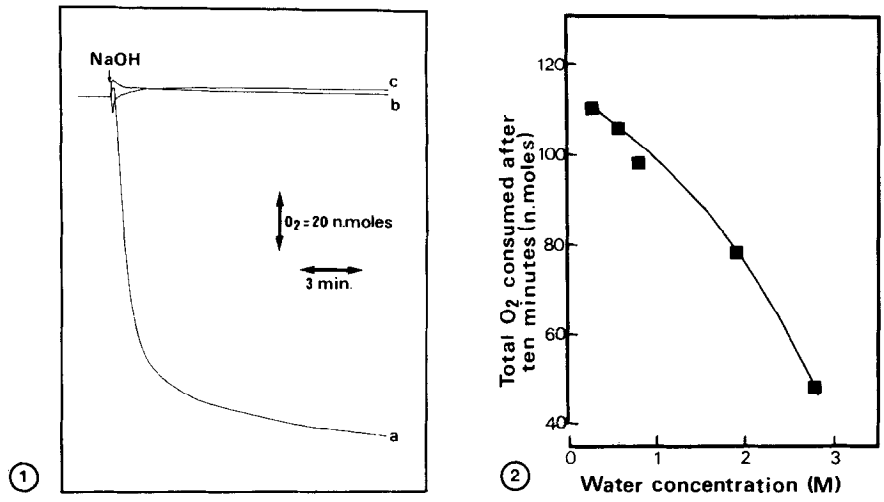


Fig. 1 Oxygen consumption associated with the addition of NaOH to DMSO (a), dimethyl formamide (b), and acetonitrile (c). The final NaOH concentration was 5mM and the H_2O concentration 0.55M.

Fig. 2 Effect of H_2O concentration on the oxygen consumption associated with the addition of NaOH (5mM) to DMSO. For H_2O concentrations above 0.55M (10 µl/ml DMSO), appropriate volumes of H_2O were initially equilibrated with the DMSO in the chamber of the oxygen electrode. Reactions were started by the addition of 10 µl/ml of 0.5M NaOH. For H_2O concentrations below 0.55M appropriate volumes of higher concentrations of NaOH were used.

the EPR spectrum obtained from alkaline DMSO at low temperature is that expected from $O_2^{\cdot -}$ (Figure 4). (14).

To investigate the kinetics of formation and stability of the generated $O_2^{\cdot -}$ SOD inhibitable reduction of cytochrome c was used as a

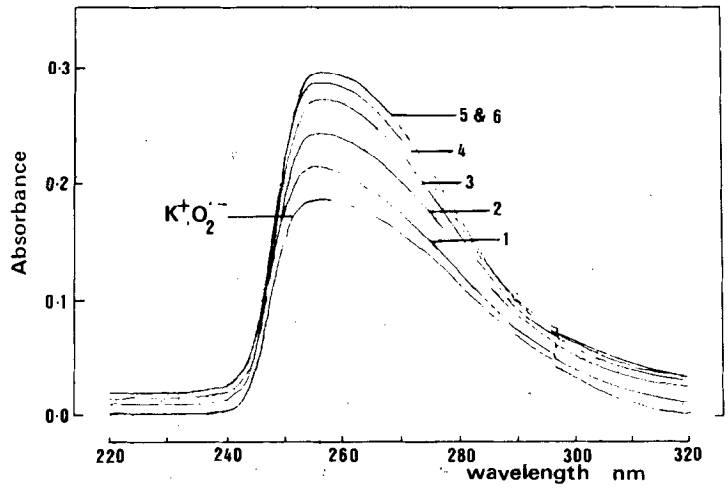


Fig. 3 Spectrum of $O_2^{\cdot -}$ in DMSO as generated by the addition of NaOH and K^+, $O_2^{\cdot -}$ to DMSO. Numbers represent times (seconds) at the peak of each curve, after addition of the NaOH. 1 = 30 ; 2 = 85 ; 3 = 185 ; 4 = 301 ; 5 = 600 ; 6 = 900.

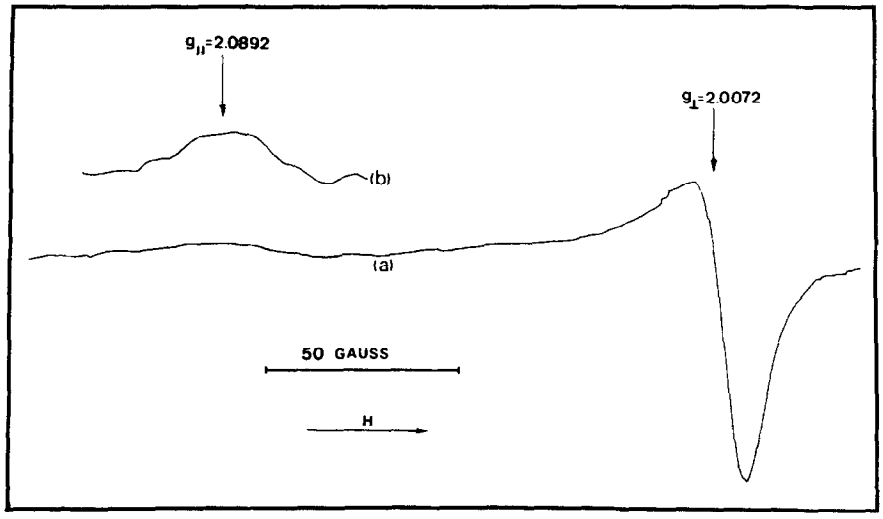


Fig. 4 EPR. spectra of $O_2^{\cdot -}$ generated on the addition of 10 µl of 0.5M NaOH/ml of DMSO. Spectra were performed at 130.K with a microwave frequency of 9.199 GHz, 1.6 Gauss modulation and 2.5mW microwave power. (a) gain = 5×10^4 ; (b) gain = 2.5×10^5.

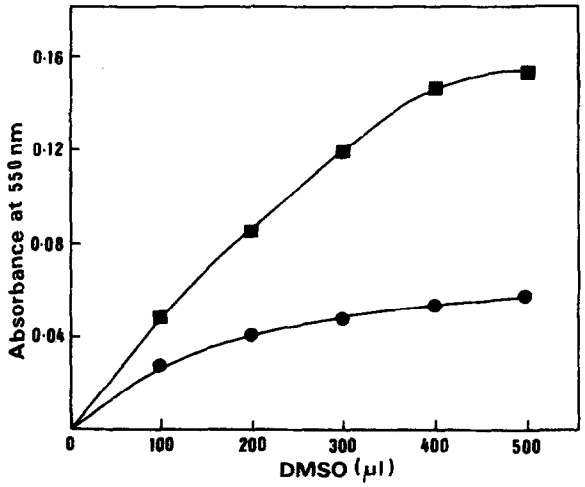


Fig. 5 SOD inhibitable reduction of cytochrome c by $O_2^{\cdot -}$ generated by OH^- in DMSO. $O_2^{\cdot -}$ was prepared by addition of 10 µl of 0.5M NaOH/ml of DMSO. These solutions were stored in tightly stoppered bottles for thirty minutes before use (to prevent H_2O absorbtion). Various volumes were added to buffer containing cytochrome c, (see methods). ●——● + SOD ; ■——■ − SOD.

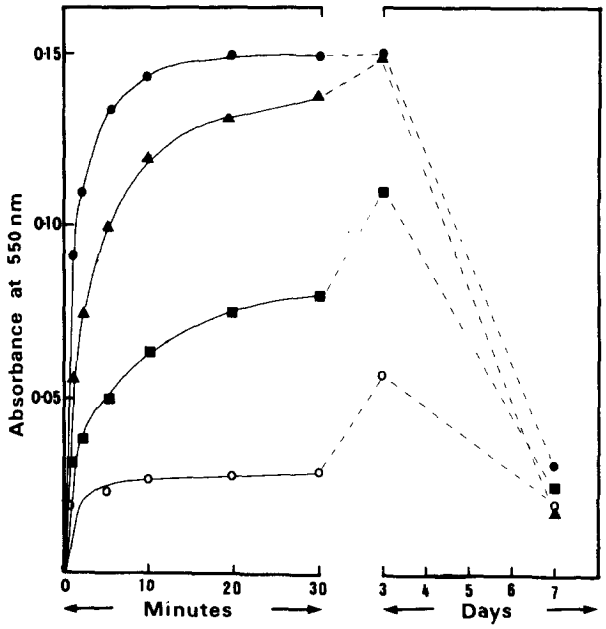
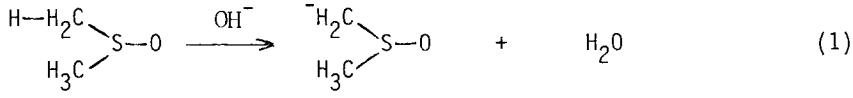


Fig. 6 The effect of H_2O concentration on the kinetics of formation and stabilty of $O_2^{\cdot -}$ formed in DMSO. NaOH was added to DMSO to give 5mM. 0.5 ml samples were removed as shown and added to buffer containing cytochrome c. Final H_2O concentrations were : ●——● 0.55M ; ▲——▲ 1.1M ; ■——■ 2.2M ; ○——○ 4.4M.

probe (8). Fig. 5 indicates that the reduction of cytochrome c is related to the volume of alkaline DMSO added and that the presence of 1µg/ml SOD inhibits the reduction by up to 60%. Fig. 6 shows that the $O_2^{\cdot -}$ produced is stable for up to 3 days and that the concentration is, like O_2 consumption, related inversly to the H_2O concentration in the DMSO.

DISCUSSION : Our results present positive evidence that the reaction between OH^-, O_2 and DMSO involves the transfer of one electron to molecular oxygen with the formation of $O_2^{\cdot -}$. Such a reaction could occur following proton extraction from the activated methyl group of DMSO this leading to the formation of the methyl sulphinyl carbanion (15) :



The formation of such a nucleophilic moiety could lead to the loss of an electron to O_2 and the formation of $O_2^{\cdot -}$.

Alternately, OH^- itself may be able to donate an electron to O_2 :

$$OH^- + O_2 \rightleftharpoons O_2^{\cdot -} + OH^{\cdot} \qquad (2)$$

In DMSO, $OH^{\cdot}$ is removed by the following sequence of reactions (16)

$$OH^{\cdot} + (CH_3)_2SO \longrightarrow CH_3SOOH + CH_3^{\cdot} \quad (3)$$

$$CH_3^{\cdot} + O_2 \longrightarrow CH_3O_2^{\cdot} \quad (4)$$

$$CH_3O_2^{\cdot} + CH_3O_2^{\cdot} \longrightarrow CH_3OOCH_3 + O_2 \quad (5)$$

The net reaction being :

$$2OH^- + 3O_2 + 2(CH_3)_2SO \longrightarrow 2CH_3SOOH + CH_3OOCH_3 + 2O_2^{\cdot -} \quad (6).$$

It has been proposed (17) that reaction (2) occurs in basic aqueous mediums when catalysed by the enzyme SOD. In our system such catalysis may not be necessary due to the increased reactivity of OH^- when present on DMSO (18). This, like other aprotic polar solvents, has the ability to solvate cations and leave anions unsolvated and highly reactive in solution, the acidity function of OH^- increasing by 10^9 when compared in H_2O and in DMSO (19). The dependence of the rate of formation of $O_2^{\cdot -}$ on the H_2O concentration of DMSO can therefore possibly be related to the increased solvation of OH^- as the H_2O concentration increases, leading to a decreased reactivity of this anion.

The lack of $O_2^{\cdot -}$ production in acetonitrile or dimethyl formamide may be explained either by the fact that these solvents are unable to scavenge $OH^{\cdot}$ or because they cannot undergo a reaction similar to that of reaction (1).

The stability of the $O_2^{\cdot -}$ produced in DMSO is high and this can be explained by the inability of $O_2^{\cdot -}$ to react with itself, dismutation only occuring in the presence of the protonated form HO_2 (20). At high pH and under aprotic conditions, concentrations of this species will be negligible.

Whatever the mechanism of $O_2^{\cdot -}$ production, the ability of DMSO to participate in radical forming reactions suggests that care should be taken when this solvent is used in both chemistry and biology. For instance, alkaline DMSO has been used for many years as a medium for chemical reactions, as the rate constants for these are greatly increased when compared to the same reactions performed in protic solutions (12). These increased rates have always been attributed to the increased reactivity of OH^- in DMSO (12), however our results suggest that radical catalysis may also play a role. Also in biology DMSO is used extensively for the dissolution of hydrophobic drugs to be used for both in vivo and in vitro investigations (15). This solvent can penetrate biological membranes (21)

and under such conditions may undergo similar radical producing reactions leading to lipid peroxidation and other radical induced membrane damage (22).

Taking heed of such events, the availability of a stable, easily produced, reproducible solution of $O_2^{\cdot -}$ may find many uses in both biology and chemistry.

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