ON THE DEVELOPMENT OF A WELL-DEFINED SOURCE OF SUPEROXIDE ION FOR STUDIES WITH BIOLOGICAL SYSTEMS*,**

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1. Introduction

Superoxide ion has recently been shown to be formed in several biochemical reactions involving molecular oxygen [1–8], and it has been proposed that this free radical is a potent cytogenic agent [9]. However, the latter point remains a matter os some debate, and while its chemistry has not been completely explored, most previous work suggests that superoxide ion is itself a fairly innocuous species [7,10–12]. In order to investigate the biochemical reactivity of O_2^- it is necessary to have reliable, simple, and well-understood methods for its synthesis and introduction to biological systems. The ideal source of O_2^- would: (1) be able to provide a pulse of a fairly high concentration ($\geq$1 mM) of O_2^-; (2) have no associated reactive substances; (3) allow spectrophotometric observation of the O_2^-; and (4) have no requirements for expensive, specialized equipment. Superoxide ion has been generated in situ by enzymatic [13], photochemical [1], and chemical [14] techniques but these are not well defined and they may involve reactive intermediates which would interfere in the processes under investigation. In the presence of suitable radical scavenging substances, pulse radiolytic techniques can provide a well defined solution of O_2^- but specialized equipment is required [15]. We report here in preliminary form, some of our results on the preparation and properties of O_2^- in some water-miscible organic solvents [16].

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2. Methods and results

2.1. *Stoichiometric studies*

Using the electrolytic procedure described by Maricle and Hodgson [17] and 0.1 M tetrabutylammonium bromide (TBA) as a carrier electrolyte we have prepared O_2^- in dry solutions of *N,N*-dimethylformamide (DMF, Fisher, Spectranalyzed), dimethylsulfoxide (DMSO, Mallinckrodt, AR), and acetonitrile (Matheson, Coleman and Bell, Spectroquality). The low temperature EPR spectrum of O_2^- in these solvents is shown in fig. 1. It is immediately apparent that the EPR spectrum of O_2^- in DMF is quite different from that of O_2^- in DMSO and CH_3CN; the latter spectra are very similar to those observed with frozen aqueous solutions [18]. The concentration of O_2^- in these solutions was estimated by double integration of the first derivative EPR spectrum and comparison to the spectrum from a 1 mM Cu^{2+}–EDTA solution; concentrations of $>$ 5 mM O_2^- were easily obtained.

When O_2^- is mixed with water a rapid and spontaneous dismutation proceeds according to $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, and George [19] has amply demonstrated that this is the dominant reaction of O_2^- in water. To test whether our solutions of O_2^- would accurately follow this stoichiometry, known amounts of O_2^- were infused into an aqueous solution using a syringe pump and the amount of oxygen formed was determined from the standardized response of an oxygen electrode. Immediately after infusion was stopped and the electrode stabilized, a small amount of catalase was added to convert H_2O_2 to O_2 which was also measured by the electrode response. The results are given in table 1. They show clearly that DMF solutions behave abnormally while DMSO and CH_3CN give the expected yields of O_2 and H_2O_2.

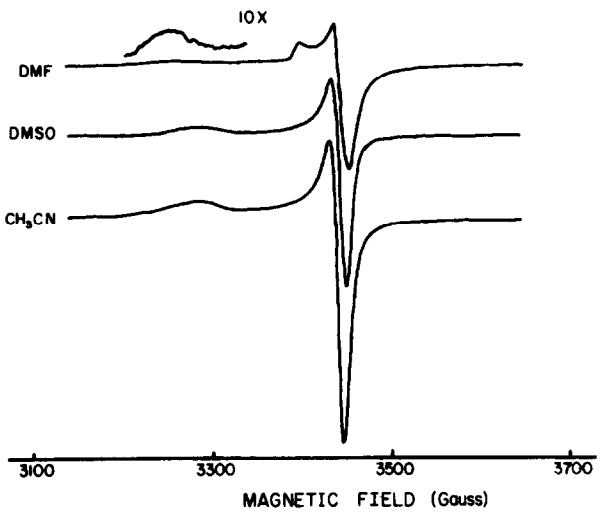

Fig. 1. Low temperature EPR spectrum of superoxide in various organic solvents. Spectra were recorded near −130°C with 100 kHz field modulation of 10 gauss and an approximate microwave frequency of 9.2 GHz. Solutions of O_2^- were produced at room temperature using a Hg pool as the cathode. A small d.c. voltage was applied to give a constant current of 1 mA. The time of electrolysis was generally less than one-half hour.

We have also recorded 60 MHz NMR spectra before and during electrolysis, and after spontaneous decay of the solutions (see below). It was found that the NMR spectra of DMSO and CH_3CN were unaltered, while the NMR spectrum of the DMF solution showed several new and intense resonances arising from substances formed during electrolysis.

2.2. *Spectral properties of O_2^- in CH_3CN*

Commercially available CH_3CN has a high optical transmission up to approximately 200 nm, and should thus serve as an appropriate solvent in which to observe the 250 nm absorbance band of O_2^- [15]. All attempts to do so, however, failed until it was brought to our attention by Prof. A.K. Covington that commercially available TBA was contaminated with iodide which absorbs ultraviolet radiation very strongly. We therefore synthesized TBA (20) and recrystallized this to a high degree of purity from ethyl acetate. Solutions of TBA (0.1 M) had high transmittance in the ultraviolet range and O_2^- in this solvent was observed by its 250 nm absorption band (fig. 2). The absorbance could be completely removed by the addition of a small amount of dilute HCl to the cuvette. The absorbance index of O_2^- at 250 nm was found to be $2580 \pm 300\ M^{-1} \cdot cm^{-1}$.

One of the major difficulties in handling the O_2^- solutions is their extreme sensitivity to protons which effect a spontaneous dismutation apparent as a slow decay. Without excessive precautions, however, such as the flaming of all glassware, these solutions were stable for periods of 2–5 hr, depending upon atmospheric humidity. This allowed ample time for mixing with an aqueous solution as in a stopped-flow experiment, for example, or for slow infusion into a rapidly agitated solution. Indeed, preliminary results of stopped-flow spectrophotometric studies show that the A_{250} disappears by a second-order process and this decay rate is enhanced by added superoxide dismutase.

Table 1
Oxygen release upon introduction of superoxide ion into aqueous solution.*

Solvent	μMoles O_2^- added**	μMoles O_2 from dismutation		μMoles O_2 from action of catalase	
		Observed***	Theory	Observed***	Theory
DMSO	0.45	0.23	0.23	0.10	0.12
CH_3CN	0.32	0.15	0.16	0.07	0.08
DMF	2.2	0.42	1.1	0†	0.55

* Superoxide solutions were deoxygenated before adding to 0.1 mM pH 7 phosphate buffer. Final organic solvent concentration < 10%.
** Determined by integration of low temperature EPR spectra.
*** The overall error in these representative data is approximately 20%.
† Same result was obtained in several independent experiments.

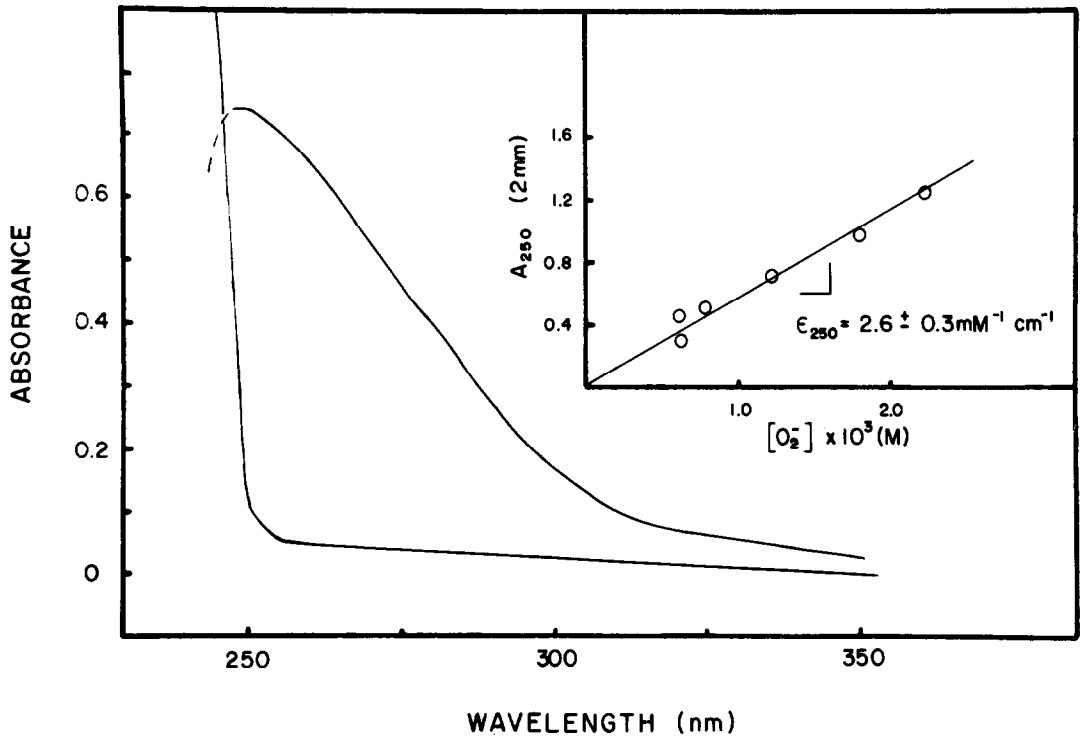

Fig. 2. Absorption spectrum of superoxide in CH_3CN. The lower tracing demonstrates the high transmittance of a 0.1 M solution of synthesized TBA (see text) and the upper curve is the spectrum of O_2^- in 0.1 M TBA *versus* 0.1 M TBA. *Inset*: Determination of the absorbance index of O_2^- in 0.1 M TBA/CH_3CN solution.

Several attempts to effect hydroxylation of an aromatic substance by introducing O_2^- into pure aqueous solutions of e.g., *p*-hydroxybenzoic acid in the presence and absence of H_2O_2 were unsuccessful. Apparently small amounts of redox active salts mediate hydroxylation (cf. Strickland and Massey [21]).

3. Discussion

The above results, which will be described in greater detail elsewhere [22], suggest that DMSO and acetonitrile solutions may serve as well behaved sources of superoxide ion for biochemical studies. In contrast, electrolyzed O_2/DMF solution probably contain only small amounts of O_2^- (as indicated in table 1) and substantial quantities of other unknown substances, at least one of which is a free radical (fig. 1). In the present experiments DMF solutions were electrolyzed for only a short period of time and were colorless in appearance. Longer times of electrolysis yielded intensely green solutions which were surely at an increased state of degradation. The use of such solutions as a source of O_2^- in an enzyme study may not lead to easily interpretable results [23, 24].

The absorption maximum and absorbance index of 250 nm and 2580 ± 300 $M^{-1} \cdot cm^{-1}$, respectively, for O_2^- are similar to values reported by other workers [15], showing that CH_3CN/0.1 M TBA is a good solvent for this free radical, and our preliminary work indicates that this will be a useful system for studying the mechanisms of the superoxide dismutase reactions by stopped-flow spectrophotometry as well as a means of exploring the chemical reactivity of this species.

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