of both a one-electron oxidation and a one-electron reduction for the former molecule and four one-electron reduction steps for the latter molecule. Attempts to prepare, isolate, and characterize these species are presently in progress.

Acknowledgments. This research was supported by the Petroleum Research Fund, administered by the American Chemical Society, and by the National Science Foundation (No. GP-19175X). The use of the UNIVAC 1108 computer at the Academic Computing Center, University of Wisconsin (Madison), was made available through partial support of the National Science Foundation and the Wisconsin Alumni Research Foundation administered through the University Research Committee.

Supplementary Material Available. A listing of atomic coordinates will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W. Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JACS-74-4019.

Robert S. Gall, Cynthia Ting-Wah Chu, Lawrence F. Dahl*
Department of Chemistry, University of Wisconsin
Madison, Wisconsin 53706

Received February 14, 1974

Singlet Oxygen. The Suppression of Its Production in Dismutation of Superoxide Ion by Superoxide Dismutase

Sir:

We have recently shown that the homogeneous electron transfer between superoxide ion, $O_2\cdot^-$, and a radical cation, $R\cdot^+$ (*e.g.*, ferricenium cation), produces singlet oxygen.[1] The occurrence of both superoxide ion and singlet oxygen in biological systems has been a topic of recent interest. Fridovich[2] discovered the enzyme superoxide dismutase (SOD, also called erythrocuprein), which catalyzes the dismutation reaction (1)

$$2O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2 \tag{1}$$

and apparently serves in the defense mechanism of organisms against the potential toxicity of superoxide ion or radicals generated from its decomposition. Stauff and coworkers[3] observed a very weak emission at 630 nm, characteristic of the dimeric $^1\Delta_g$ state of O_2, from the dismutation of superoxide ion in the presence of proton donors. They proposed the following reaction scheme to account for the results.

$$O_2\cdot^- + H^+ \longrightarrow HO_2\cdot \tag{2}$$

$$HO_2\cdot + O_2\cdot^- \longrightarrow HO_2^- + {}^1O_2 \tag{3}$$

$$HO_2\cdot + HO_2\cdot \longrightarrow H_2O_2 + {}^1O_2 \tag{4}$$

$$^1O_2 + {}^1O_2 \longrightarrow ({}^1O_2)_2 \quad \text{(gas phase)} \tag{5}$$

$$({}^1O_2)_2 \longrightarrow 2O_2 + h\nu \quad \text{(gas phase)} \tag{6}$$

In the most recent work of Stauff and coworkers,[4] emission during dismutation of superoxide ion was absent, and they suggest the previously observed emission in the blue-green region was attributable to contamination by atmospheric CO_2 and the intermediate involvement of carbonate or bicarbonate radicals.

We report here, by the use of 1,3-diphenylisobenzofuran (DPBF) as a trapping agent, that singlet oxygen is produced by the dismutation of superoxide ion and that this production is greatly decreased in the presence of SOD. The results further show that SOD does not quench singlet oxygen directly but apparently interferes with its production in the dismutation step itself.

Solutions of superoxide ion were produced by reducing oxygen electrolytically with a platinum gauze electrode in a 0.2 *M* solution of tetra-*n*-butylammonium perchlorate (TBAP) in dry acetonitrile (35 ml). The reduction was carried out at a potential of −1.6 V *vs.* saturated calomel electrode and the electrolysis of 100 min duration was discontinued after 18 mF were passed. To this solution of superoxide ion, 150 mg (0.55 mmol) of DPBF was added and then the cell was covered with a black cloth to prevent possible photochemical reactions. Ten milliliters of water were added slowly with a syringe. The product, *o*-dibenzoylbenzene (DBB), was isolated by column and thin-layer chromatography and identified by comparison of ir spectra and R_F values with an authentic sample.

The experiments were repeated utilizing identical conditions as before except that the water (10 ml) contained 0.5 mg of SOD (from bovine red blood cells). Finally, since DPBF can react slowly with ground state oxygen, control experiments were performed to determine the contribution from this pathway. Oxygen was bubbled through a solution of 0.2 *M* TBAP in acetonitrile for 20 min. Then 150 mg of DPBF was added and the flask was covered with a black cloth. Water (10 ml) was added slowly and the yield of DBB was determined. The results are summarized in Table I. Control experiments described in our paper[1] showed that a reaction between $O_2\cdot^-$ and DPBF to form appreciable amounts of DBB does not occur.

Table I. Yield of *o*-Dibenzoylbenzene (DBB) for Superoxide Dismutation in Absence and Presence of SOD

Conditions	Mean yield DBB (mg)	Standard deviation (mg)	% DPBF conversion	No. of trials
$O_2\cdot^- + DPBF + H_2O$	5.1	1.9	3.1	3
$O_2\cdot^- + DPBF + H_2O + SOD$	0.5	<0.1	0.3	2
Control experiment $O_2 + H_2O + DPBF$	1.5	1.0	0.9	3

The data in Table I indicate that singlet oxygen is produced from the dismutation of superoxide ion and that SOD has a definite inhibitory effect upon its formation. Emission studies at 630 (the $^1\Delta_g{}^1\Delta_g$ state) and at 478 nm (the $^1\Sigma_g{}^+{}^1\Delta_g$ state), upon water addition to the $O_2\cdot^-$ solution contained in a cell in an Aminco-Bowman spectrophotofluorometer equipped with a Hamamatsu R456 photomultiplier tube, were unsuccessful.

---

[1] E. A. Mayeda and A. J. Bard, *J. Amer. Chem. Soc.*, **95**, 6223 (1973).

[2] I. Fridovich, *Accounts Chem. Res.*, **5**, 321 (1972).

[3] J. Stauff, H. Schmidkeenz, and G. Hartmann, *Nature* (*London*), **198**, 281 (1963).

[4] J. Stauff, U. Sander, and W. Jaeschke in "Chemiluminescence and Bioluminescence," M. J. Cormier, D. M. Hercules, and J. Lee, Ed., Plenum Press, New York, N. Y., 1973, p 131.

Table II. Calculation of the Energy of Dismutation Reactions of Superoxide Ion

Half-reactions	Thermodynamic data	Ref	$\Delta G°$, kcal
	(A) $O_2\cdot^-$–HO_2 Reaction		
$O_2 + H_2O + 2e \rightleftarrows HO_2^- + OH^-$	$E° = -0.076$ V	*a*	+1.7
$-2(O_2 + e \rightleftarrows O_2\cdot^-)$	$E° = -0.563$ V	*a*	−25.8
$HO_2\cdot + OH^- \rightleftarrows O_2\cdot^- + H_2O$	$K = 10^{9.2}$	2	−12.7
$HO_2\cdot + O_2\cdot^- \rightleftarrows HO_2\cdot^- + O_2$		$\Delta G° = -36.8$ $\Delta H° = \sim -35$ kcal[b]	
	(B) $HO_2\cdot$–HO_2 Reaction		
$HO_2\cdot + H^+ + e \rightleftarrows H_2O_2$	$E° = +1.495$ V	*a*	−29.6
$-(O_2 + e \rightleftarrows O_2\cdot^-)$	$E° = -0.563$ V	*a*	−12.9
$HO_2\cdot \rightleftarrows O_2\cdot^- + H^+$	$K = 10^{4.8}$	2	+6.5
$2HO_2\cdot \rightleftarrows H_2O_2 + O_2$		$\Delta G° = -36.0$ $\Delta H° = \sim -34$ kcal[b]	

[a] A T. DeBethune and W. A. S. Loud, "Standard Aqueous Electrode Potentials and Temperature Coefficients," Hampel, Skokie, Ill., 1964.
[b] Assuming $T\Delta S° \approx 2$ kcal.

The formation of oxygen in an electronically excited state follows the theory of electron transfer chemiluminescence,[5] if the reaction involves fast electron transfer between $O_2\cdot^-$ and $HO_2\cdot$ or between two $HO_2\cdot$ molecules. Calculation of the free energies of these reactions is given in Table II. The excited singlet states of oxygen are the $^1\Delta_g$ and $^1\Sigma_g{}^+$ states, which lie 22 and 38 kcal, respectively, above the ground state.[6] Hence, the $^1\Delta_g$ is energetically accessible by either reaction 3 or 4, but the $^1\Sigma_g{}^+$ state appears not to be. The direct uncatalyzed dismutation reaction thus is envisioned as a rapid protonation followed by the electron transfer reaction 3 or 4. A rapid electron transfer often favors the production of electronically excited states rather than the thermal dissipation of the reaction energy.[7] For the case of the SOD-catalyzed dismutation, however, the overall reaction probably involves intermediate elementary steps of lower energy involving the enzyme; in this case the thermal dissipation of the reaction enthalpy is much more favorable and singlet oxygen is not produced.

Another possibility, however, is that SOD simply behaves as a direct quencher of singlet oxygen. Indeed, several previous reports have demonstrated quenching of chemiluminescence by SOD, for example, in the xanthine–xanthine oxidase system, and have postulated such a direct quenching reaction.[8–10] To test this hypothesis, we examined the trapping by DPBF of singlet oxygen produced by the well-established method used in photosensitized oxygenations.[11] Methylene Blue was irradiated in an ethanolic solution saturated with oxygen in the presence of DPBF and the decrease of the visible absorption peak of DPBF at 410 nm was monitored. The rate of decrease of this peak was identical for solutions containing no SOD or 0.1 or 1.0 mg/ml of SOD. The extinction coefficient of SOD at 635 nm is very small,[2] so that SOD itself is probably unaffected by irradiation. The hydrogen peroxide–sodium hypochlorite system[12] was also employed. The amount of DPBF trapped upon the mixing of these reactants in the absence or presence of SOD was the same. Solutions containing both DPBF and hydrogen peroxide in ethanol show essentially no reaction in 10–15 min. This experiment is somewhat more ambiguous than the photosensitization one, however, because the stability of SOD in this reaction mixture under these conditions is unknown. The results of both experiments, however, strongly suggest that SOD does not quench singlet oxygen.

Finally, the very low concentrations of SOD (as small as 10^{-8} *M*) said to be effective in quenching obviously low ($<10^{-4}$ *M*) concentrations of singlet oxygen would require quenching rate constants that were larger than those for diffusion controlled reactions in the media. Indeed, the recent results of Teng and Smith[13] cast some doubt on the previously reported intermediacy of singlet oxygen in several enzyme oxidations.

In summary the results suggest that the dismutation of superoxide ion produces singlet oxygen, while the SOD-catalyzed dismutation does not.[14,14a] In biological systems, then, SOD not only aids in the protection of the organism from superoxide ion but may also prevent formation of singlet oxygen during its removal as well.[16]

---

(5) See, *e.g.*, A. J. Bard, C. P. Keszthelyi, H. Tachikawa, and N. E. Tokel in ref 4 p 193, and references contained therein.

(6) A. U. Khan and M. Kasha, *J. Amer. Chem. Soc.*, **92**, 3293 (1970).

(7) R. A. Marcus, *J. Chem. Phys.*, **43**, 2654 (1965).

(8) A. F. Agro', C. Giovagnoli, P. DeSote, L. Calabrese, G. Rotilio, and B. Mondovi, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **21**, 183 (1972).

(9) R. M. Arneson, *Arch. Biochem. Biophys.*, **136**, 352 (1970).

(10) U. Weser and W. Paschen, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **27**, 248 (1972).

(11) C. S. Foote and S. Wexler, *J. Amer. Chem. Soc.*, **86**, 3879 (1964).

(12) C. S. Foote, S. Wexler, W. Avdo, and R. Higgins, *J. Amer. Chem. Soc.*, **90**, 975 (1968).

(13) J. I. Teng and L. L. Smith, *J. Amer. Chem. Soc.*, **95**, 4060 (1973).

(14) A reviewer has suggested that hydroxyl radicals formed by reaction of $O_2\cdot^-$ with any hydrogen peroxide produced in the original dismutation step[15] might also react with DPBF to produce DBB.

(14a) NOTE ADDED IN PROOF. The possibility of hydroxyl radical reacting with DPBF to form DBB was investigated by forming $HO\cdot$ using either Fenton's reagent ($FeSO_4 + H_2O_2$) or by thermolysis of H_2O_2. To stirred solutions of DPBF (0.555 mmol) and $FeSO_4$ (1.1 or 10 mmol in 5 or 10 ml of water) in acetonitrile (35 ml), H_2O_2 (5.3 or 10.6 mmol) was slowly added dropwise. Comparison of the melting point and ir spectra for the isolated product indicated that it was not DBB. A solution of DPBF (0.555 mmol) and H_2O_2 (5.3 mmol) was refluxed for 30 min and again the isolated product was not DBB. We therefore conclude that $HO\cdot$ is not the reactive intermediate leading to the formation of DBB.

(15) S. A. Goscin and I. Fridovich, *Arch. Biochem. Biophys.*, **153**, 778 (1972).

(16) We thank Professor Irwin Fridovich for a sample of SOD and helpful comments. Research was supported by the Robert A. Welch Foundation and the National Science Foundation (GP-31414X).

Edward A. Mayeda, Allen J. Bard*
Department of Chemistry, The University of Texas
Austin, Texas 78712

Received December 31, 1973