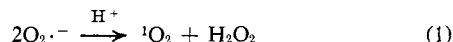


Singlet Molecular Oxygen and Superoxide Dismutase<sup>1</sup>

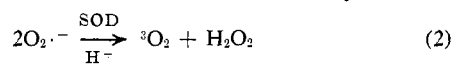
Sir:

The role of singlet molecular oxygen (<sup>1</sup>O<sub>2</sub>) and of the superoxide anion radical (O<sub>2</sub><sup>·-</sup>) in enzyme-mediated peroxidation reactions *in vivo* has been an area of considerable interest in recent years. We recently reported that <sup>1</sup>O<sub>2</sub> is produced by the adrenodoxin reductase system.<sup>2</sup> Singlet oxygen is formed by the dismutation reaction of superoxide anion radicals (eq 1). Stauff<sup>3</sup>



and Arneson<sup>4</sup> have described the formation of <sup>1</sup>O<sub>2</sub> by the xanthine oxidase-xanthine system. Howes and Steele<sup>5</sup> have reported the singlet oxygen-mediated chemiluminescence obtained from rat liver microsomes, NADPH, and O<sub>2</sub>.

Superoxide dismutase (SOD), a metalloprotein containing 2 g-atoms of Cu and Zn each, catalyzes the dismutation reaction of O<sub>2</sub><sup>·-</sup> to give *ground state* molecular oxygen (eq 2).<sup>6</sup> SOD has been found in many aerobic



organisms including bovine and human erythrocytes, yeast, chicken liver, *Streptococcus* mutants, and *Escherichia coli*. It is thought that SOD may serve to protect these systems from the deleterious effects of the highly reactive O<sub>2</sub><sup>·-</sup>.

Several investigators have suggested that superoxide dismutase is also an efficient singlet oxygen quencher. Paschen and Weser<sup>7</sup> have observed that SOD inhibits the chemiluminescence obtained from a mixture of luminol and K<sub>3</sub>CrO<sub>8</sub>. They interpret these results in terms of SOD quenching of the <sup>1</sup>O<sub>2</sub> produced from K<sub>3</sub>CrO<sub>8</sub>. These workers find that this quenching is observable at SOD concentrations as low as 10<sup>-10</sup> M. They conclude that the main physiological function of SOD is to scavenge singlet oxygen rather than catalyze the dismutation reaction of O<sub>2</sub><sup>·-</sup>. Agro<sup>8</sup> and Arneson<sup>4</sup> have also suggested that SOD is a singlet oxygen quencher on the basis of SOD inhibition of the xanthine oxidase-xanthine chemiluminescence.

We now describe the results of experiments which indicate that SOD does *not* significantly quench singlet oxygen at concentrations of 1 mg/ml (10<sup>-5</sup> M). We have studied the effect of SOD on the reaction of <sup>1</sup>O<sub>2</sub> with α-lipoic acid (1)<sup>9</sup> and 9,10-diphenylanthracene-2,3-dicarboxylic acid (2)<sup>10</sup> in aqueous Tris buffer (pH 8.8).

(1) A. P. Schaap, presented in part at the International Conference on the Excited States of Biological Molecules, Lisbon, April 1974.

(2) K. Goda, J. Chu, T. Kimura, and A. P. Schaap, *Biochem. Biophys. Res. Commun.*, **52**, 1300 (1973).

(3) J. Stauff, *Photochem. Photobiol.*, **4**, 1199 (1965).

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

(5) R. M. Howes and R. H. Steele, *Res. Commun. Chem. Pathol. Pharmacol.*, **3**, 349 (1972).

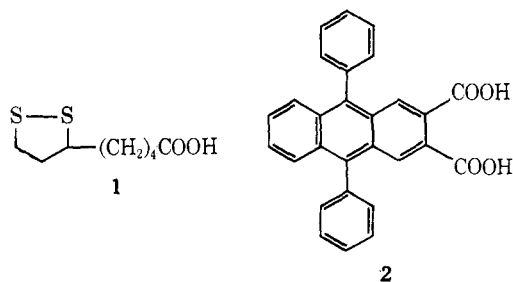
(6) R. Zimmermann, L. Flohe, U. Weser, and H. Hartmann, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **29**, 117 (1973) and references contained therein.

(7) W. Paschen and U. Weser, *Biochim. Biophys. Acta*, **327**, 217 (1973).

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

(9) Singlet oxygen reacts with α-lipoic acid to yield thiosulfonates and thiosulfonates: R. W. Murray, F. E. Story, and S. L. Jundal, Abstracts, 1st National Meeting of the American Society for Photobiology, Sarasota, Fla., June 1973, MAM-B9.

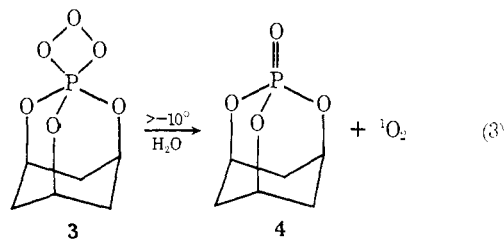
(10) Derivatives of 9,10-diphenylanthracene undergo photooxidation to yield endoperoxides: K. Gollnick and G. O. Schenck in "1,4-Cycloaddition Reactions," J. Hammer, Ed., Academic Press, New York, N. Y., 1967, p 255.



Singlet oxygen was generated photochemically with the heterogeneous sensitizer, ⊖-Rose Bengal,<sup>11</sup> and with the water-soluble 1-phospha-2,8,9-trioxadamantane ozonide (3).<sup>12</sup> The use of water as the solvent for these studies was important as SOD is probably denatured in organic solvents.

Photooxidation of α-lipoic acid (1) in water (Tris buffer, pH 8.8) with the insoluble, polymer-bound Rose Bengal was monitored by the disappearance of the absorption at 333 nm. A 5-ml solution of 1 (3.43 × 10<sup>-3</sup> M) in the buffer with 50 mg of ⊖-Rose Bengal under oxygen and mechanically stirred was irradiated at 560 nm using a Bausch and Lomb grating monochromator and SP-200 mercury light source. After 188 min, 50% of 1 had been consumed. Absorption spectra before and after photolysis indicated that no Rose Bengal was leached into the reaction solution. Photolysis of 1 in the absence of ⊖-Rose Bengal gave no change in the concentration of 1. Superoxide dismutase<sup>13</sup> was added to the reaction solution and the photooxidation of 1 carried out as above. With 1 mg/ml (10<sup>-5</sup> M) of SOD, photooxidation of 1 was unaffected within experimental error (51.8% reaction after 188 min). It was further established that SOD is not denatured under the reaction conditions by photolysis with 560 nm radiation in the presence of ⊖-Rose Bengal and oxygen.<sup>14</sup> The addition of 1,4-diazabicyclo[2.2.2]octane, a singlet oxygen quencher<sup>15</sup> (3.7 × 10<sup>-2</sup> M), inhibited the photooxidation of 1 with 26% reaction after 188 min.

The reaction of 1 with <sup>1</sup>O<sub>2</sub> in the presence of SOD was also studied utilizing the thermal decomposition of the ozonide 3 as the source of singlet oxygen. A solu-



tion of 3 in CH<sub>2</sub>Cl<sub>2</sub> was prepared by the addition of

(11) E. Blossey, D. C. Neckers, A. L. Thayer, and A. P. Schaap, *J. Amer. Chem. Soc.*, **95**, 5820 (1973).

(12) 1-Phospha-2,8,9-trioxadamantane ozonide (3), formed at -78° by the addition of ozone to the phosphite, spontaneously decomposes (*k* = 1.06 × 10<sup>-3</sup> sec<sup>-1</sup> at 17° in CH<sub>2</sub>Cl<sub>2</sub>) to phosphate and singlet oxygen. Trapping experiments in CH<sub>2</sub>Cl<sub>2</sub> with <sup>1</sup>O<sub>2</sub> acceptors have shown that <sup>1</sup>O<sub>2</sub> is formed in >95% yield. Decomposition studies at a series of temperatures give an activation energy of 19 kcal/mol: A. P. Schaap, K. Kees, and A. L. Thayer, Abstracts, 6th Central Regional Meeting of the American Chemical Society, Detroit, Mich., April 1974.

(13) Bovine erythrocyte superoxide dismutase was purchased from Miles Laboratories.

(14) The activity of SOD was checked by the procedure of Fridovich: J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).

(15) C. Ovannes and T. Wilson, *J. Amer. Chem. Soc.*, **90**, 6527 (1968).

ozone to 1-phospha-2,8,9-trioxaadamantane. Removal of the solvent from a portion of this solution under vacuum at  $-40^\circ$  gave the solid ozonide **3** (0.06 mmol). A 5-ml solution of **1** ( $2.8 \times 10^{-3} M$ , 0.014 mmol) in the Tris buffer was added to the solid ozonide at  $-10^\circ$ . The resulting solution was allowed to warm to ambient temperature over a period of 15 min. The uv spectrum of the solution indicated 63.1% reaction of **1**. A series of four experiments gave an average consumption of **1** of  $63.7 \pm 3\%$ . The reaction in the presence of 5 mg of SOD gave 63.3% conversion.

We have used the water-soluble 2,3-dicarboxylic acid derivative of 9,10-diphenylanthracene to further test our conclusion that SOD does not quench  $^1O_2$ . A solution (10 ml) of **2** ( $8.1 \times 10^{-5} M$ ) in aqueous Tris buffer (pH 8.8) with 50 mg of  $\oplus$ -Rose Bengal was irradiated with stirring under  $O_2$  at 560 nm. The consumption of **2** was monitored by the decrease in the absorption at 380 nm. After 825 min, 50% of **2** had been consumed. The addition of SOD (1 mg/ml,  $10^{-5} M$ ) had no effect on the rate of disappearance of **2** with 49% reaction after 825 min.

The decomposition of ozonide **3** is attended with chemiluminescence. We attribute this chemiluminescence to the singlet oxygen "dimol" emission.<sup>16</sup> We have observed that SOD does not quench the luminescence produced by the decomposition of **3** in  $H_2O$ . The details of these experiments will be reported shortly.

We conclude on the basis of the results of the above experiments that superoxide dismutase does *not* quench singlet oxygen. SOD probably protects biological systems from oxidative damage by  $^1O_2$  by removing  $O_2^{\cdot-}$  via the SOD catalyzed dismutation reaction (eq 2) and preventing the formation of singlet oxygen.

**Acknowledgment.** Financial support from the U. S. Army Research Office-Durham, Eli Lilly and Company, and the National Institutes of Health, (AM-12713, CA-15874) is gratefully acknowledged.

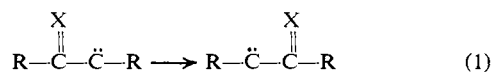
(16) A. U. Khan and M. Kasha, *J. Amer. Chem. Soc.*, **88**, 1574 (1966).  
 (17) Alfred P. Sloan Research Fellow, 1974-1976.

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### Evidence for the Discrete Existence of a Bicyclo[4.1.0]heptatriene

Sir:

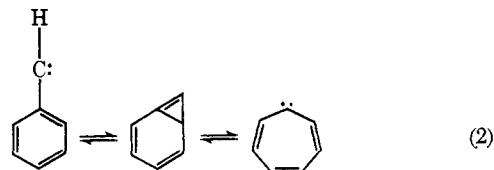
Carbene-carbene rearrangements in which a group migrates to the reaction site with generation of a new carbene (eq 1) are well documented in the now familiar



interconversion of phenylcarbene and its derivatives with cycloheptatrienylenes.<sup>1,2</sup> Although a number

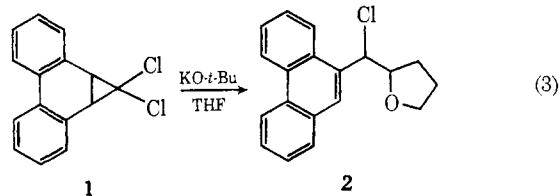
(1) (a) R. C. Joines, A. B. Turner, and W. M. Jones, *J. Amer. Chem. Soc.*, **91**, 7754 (1969); (b) J. A. Myers, R. C. Joines, and W. M. Jones, *ibid.*, **92**, 4740 (1970); (c) W. J. Baron, M. Jones, Jr., and P. P. Gaspar, *ibid.*, **92**, 4739 (1970); (d) P. O. Schissel, M. E. Kent, M. J. McAdoo, and E. Hedaya, *ibid.*, **92**, 2147 (1970); (e) E. Hedaya and M. E. Kent, *ibid.*, **93**, 3285 (1971); (f) T. Mitsuhashi and W. M. Jones, *ibid.*, **94**, 677 (1972); (g) G. G. Vander Stouw, A. R. Kraska, and H. Schechter, *ibid.*,

of attractive mechanisms have been considered,<sup>3</sup> one which involves a bicyclo[4.1.0]heptatriene (eq 2) as an



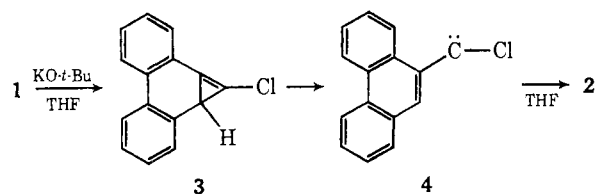
intermediate (or transition state) seems to have gained the most support. In this communication we report evidence for the discrete existence of a bicyclo[4.1.0]heptatriene derivative.

Treatment of 7,7-dichlorodibenzo[*a;c*]bicyclo[4.1.0]heptane (**1**)<sup>4</sup> with a suspension of KO-*t*-Bu (2 equiv) in THF at  $0^\circ$  gives a yellow viscous oil identified as **2**<sup>5,6</sup> (mixture of diastereomers) in 72% yield (eq 3).



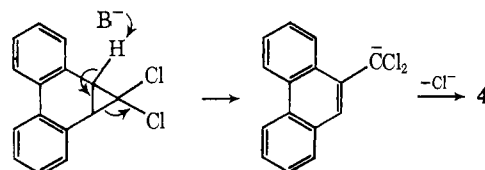
This observation is readily understood in terms of the dibenzobicycloheptatriene (**3**) shown in Scheme I,

Scheme I



although a mechanism which by-passes **3** is shown in Scheme II. Formation of the solvent insertion product

Scheme II



can, of course, be taken as evidence for formation of the carbene **4**.

In order to distinguish between these two mechanisms, an attempt was made to trap the cyclopropene

**94**, 1655 (1972); (h) K. E. Krajca, T. Mitsuhashi, and W. M. Jones, *ibid.*, **94**, 3661 (1972); (i) W. M. Jones, R. C. Joines, J. A. Myers, T. Mitsuhashi, K. E. Krajca, E. E. Waali, T. L. Davis, and A. B. Turner, *ibid.*, **95**, 826 (1973), and references cited therein.

(2) For similar rearrangements involving nitrenes see: C. Wentrup and K. Wilczek, *Helv. Chim. Acta*, **53**, 1459 (1970); W. D. Crow and C. Wentrup, *Chem. Commun.*, 1387 (1969); R. J. Sundberg and S. R. Suter, *J. Org. Chem.*, **35**, 827 (1970), and references cited therein.

(3) For several views see ref 1a-g.

(4) Prepared as described by Joshi; see G. C. Joshi, N. Singh, and L. M. Pande, *Synthesis*, 317 (1972).

(5) Spectral data are: nmr ( $CCl_4$ )  $\delta$  1.70-2.20 (m, 4 H), 3.65-4.10 (m, 2 H) 4.40-4.80 (m, 1 H), 5.50-5.71 (one diastereomer exhibits a doublet at 5.53, 1 H,  $J = 5$  Hz); the remaining diastereomer has the doublet at 5.63 (1 H,  $J = 5$  Hz) and 7.30-8.80 (m, 9 H); ir (neat) 745 (s), 1060 (s), 1450 (s), 1498 (m), 1690 (s)  $cm^{-1}$ ; elemental composition was provided by high resolution mass spectroscopy, 296.0984 ( $m^+$ ), calcd 296.0967.

(6) Using nmr as a limit of detection insertion into the  $\beta$  position in THF was not observed.