The Production of Superoxide Radical during the Decomposition of Potassium Peroxochromate(V)†

E. K. Hodgson and I. Fridovich*

ABSTRACT: The decomposition of potassium tetraperoxochromate(V) in aqueous solutions generates a reductant of ferricytochrome *c* as well as an oxidant of ferrocytochrome *c*. The reductant was intercepted by superoxide dismutase and is presumed to be O_2^-. The oxidant was scavenged by azide or histidine, which are known to react rapidly with singlet oxygen, and is therefore presumed to be the singlet oxygen which has already been demonstrated to be a product of this decomposition. Decomposition of the perchromate ion was accompanied by a luminescence in the visible region which was dependent upon the presence of carbonate and was inhibited by superoxide dismutase. Carbonate also augmented the rate of oxidation of ferrocytochrome *c*. These observations indicate that the decomposition of perchromate ion in aqueous solutions liberates both O_2^- and singlet oxygen and that the luminescence observed was due to an excited species generated from carbonate. Superoxide dismutase is shown to be unable to intercept photochemically generated singlet oxygen.

Superoxide dismutases have been shown to catalyze the reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, at a rate ($k_2 = 2 \times 10^9$ M^{-1} sec^{-1}) which approaches the theoretical diffusion limit (Klug *et al.*, 1972; Rotilio *et al.*, 1972). Since several enzymatic (McCord and Fridovich, 1968, 1969; Fridovich, 1970; Hirata and Hayaishi, 1971; Massey *et al.*, 1969) and nonenzymatic (Ballou *et al.*, 1969; McCord and Fridovich, 1970; Misra and Fridovich, 1972a,b,c, 1971; Nishikimi *et al.*, 1972; Orme-Johnson and Beinert, 1969; Nilsson *et al.*, 1969; Wever *et al.*, 1973; Heikkila and Cohen, 1973) reactions, of relevance to biological systems, generate O_2^-, it was reasonable to propose (McCord *et al.*, 1971) that O_2^- is one of the causes of oxygen toxicity and that superoxide dismutase is the defense which minimizes the danger from this reactive radical. This theory has been supported by studies of its distribution (McCord *et al.*, 1971), by studies of mutants with a temperature-sensitive defect in this enzyme (McCord *et al.*, 1973) and by the observations that its induction in bacteria (Gregory and Fridovich, 1973), yeast (Gregory *et al.*, 1974), and rat lung (Crapo and Tierney, 1973) was correlated with enhanced resistance toward hyperbaric oxygen.

A counter proposal has been advanced which suggests that the true biological function of superoxide dismutase is that of catalytically scavenging singlet oxygen (Finazzi-Agro *et al.*, 1972; Weser and Paschen, 1972; Joester *et al.*, 1972; Zimmerman *et al.*, 1973). There is no sound experimental support for this proposal. Nevertheless a recent study of the decomposition of potassium tetraperoxochromate(V) in aqueous solutions has purported to have demonstrated unequivocally that superoxide dismutase does specifically "decontaminate" singlet oxygen (Paschen and Weser, 1973). This was done by observing that superoxide dismutase inhibits the chemiluminescence of solutions of luminol plus the perchromate. It has, in fact, been shown that the chemiluminescence of luminol in buffered aqueous media, induced by any of a wide range of oxidants, always involves superoxide radical and is thus always strongly inhibited by superoxide dismutase (Hodgson and Fridovich, 1973). It thus seemed likely that the conclusions of Paschen and Weser (1973) were not supported by their observations and that superoxide dismutase may have inhibited luminescence, in their reaction mixtures, by virtue of its ability to scavenge superoxide radicals, rather than by any action on singlet oxygen. It nevertheless seemed worthwhile to investigate more carefully the decomposition of the peroxochromate ion, since this reaction has been shown to produce singlet oxygen and is thus intrinsically interesting, and because it is important to determine the extent to which superoxide dismutase can catalyze the quenching of singlet oxygen, if at all.

Materials and Methods

Methylene Blue, horse heart cytochrome *c* type III, bovine pancreatic trypsin, type III, and beef liver catalase were obtained from Sigma Chemical Co. Ferrocytochrome *c* was prepared by reduction with sodium dithionite followed by anaerobic dialysis. Crystalline potassium tetraperoxochromate(V) was prepared by treating CrO_3 with 30% hydrogen peroxide, in the presence of concentrated KOH, according to the method of Riesenfeld (Riesenfeld *et al.*, 1905). Pyrophosphate buffers were prepared by adding concentrated sulfuric acid to a sodium pyrophosphate solution until the desired pH was obtained. Superoxide dismutase was prepared from bovine erythrocytes and was assayed in terms of its ability to inhibit the superoxide-dependent reduction of cytochrome *c* by xanthine oxidase (McCord and Fridovich, 1969). Trypsin was assayed by measuring the rate of hydrolysis of *p*-tolyl-L-arginine methyl ester (Hummel, 1959), and catalase was assayed by following the disappearance of hydrogen peroxide spectrophotometrically (Beers and Sizer, 1952).

Spectrophotometric assays were performed with a Gilford Model 2000 recording spectrophotometer equipped with a thermostated cell compartment. Absorption spectra were obtained with an Aminco DW-2 spectrophotometer. Photosensitized oxidations were performed by exposing reaction mixtures to a 200-W incandescent lamp at a distance of 25 cm. These photooxidations were performed in 25-ml erlenmeyer flasks, which were agitated in a Dubnoff incubator at the specified temperature. Chemiluminescence was monitored using a Nuclear-Chicago Mark I liquid scintillation counter with the coincidence circuit turned off. Polyethylene counting vials were

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received April 11, 1974.* This work was supported in full by Research Grant GM 10287 from the National Institutes of Health.

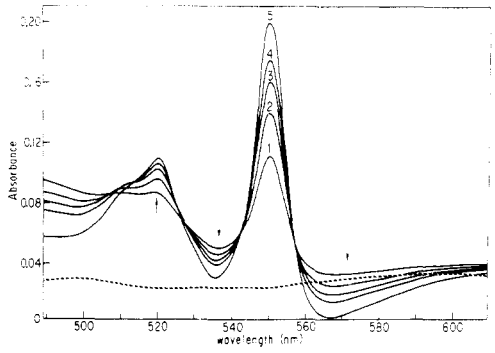

FIGURE 1: Reduction of ferricytochrome *c* during the decomposition of K_3CrO_8. Approximately 0.5 mg (7×10^{-4} M) of solid K_3CrO_8 was added to a cuvet containing 3 ml of 2.3×10^{-5} M ferricytochrome *c* in 0.05 M pyrophosphate buffer at pH 10.2 and 18°. Repetitive scans (23 sec/scan) were recorded at (1) 34, (2) 68, (3) 102, (4) 136, and (5) 234 sec after addition of the perchromate. The dashed line is the base line recorded before addition of K_3CrO_8.

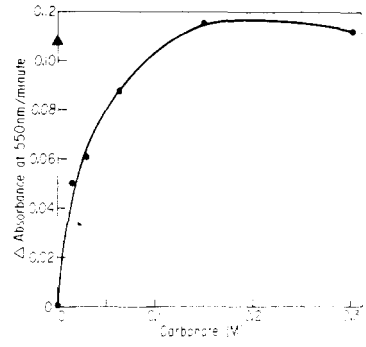

FIGURE 3: Effect of carbonate on the oxidation of ferrocytochrome *c*. The oxidation of 1.5×10^{-5} M ferrocytochrome *c* by 7×10^{-4} M K_3CrO_8 in 0.05 M pyrophosphate buffer at pH 10.2 and 25° was monitored by following the decrease in absorbance at 550 nm. Carbonate (pH 10.2) at the indicated concentrations was also present and the total reaction volume was 3 ml. Plotted on the ordinate is the initial rate of oxidation, *i.e.*, the initial change in absorbance per minute. The single point (▲) on the ordinate was obtained in the presence of 15 μg/ml of superoxide dismutase.

used and the reaction was started by adding a weighed amount of solid K_3CrO_8 to the assay mixture, which was being stirred magnetically. Counting was started 20 sec after addition of the K_3CrO_8. Each vial was preincubated in the counting chamber, which was maintained at approximately 8°, before the perchromate was added, and the background counting rate was subtracted from the signal given by the luminescent reaction.

Results

The Reduction of Cytochrome c by Perchromate. If the decomposition of perchromate produces O_2^-, then perchromate should cause the reduction of ferricytochrome *c* and superoxide dismutase should inhibit the reduction. In contrast, singlet oxygen, being an electronically excited, rather than a reduced form of oxygen, might cause the oxidation of ferrocytochrome *c* but could not cause the reduction of ferricytochrome *c*. As shown in Figure 1 decomposition of the tetraperoxochromate ion did cause a time dependent reduction of ferricytochrome *c*. Superoxide dismutase at 5.0 μg/ml caused 100% inhibition of this reduction. It is thus established that perchromate does, in decomposing in aqueous solutions, generate O_2^-.

Effect of Carbonate on the Reduction of Cytochrome c. In the course of these measurements it was observed that carbonate depressed the rate of reduction of cytochrome *c* by perchromate. Since carbonate does not significantly effect the reduction of cytochrome by O_2^- generated by the xanthine oxidase reaction, this effect of carbonate promised to unmask the presence of additional reactive species produced by the decomposition of perchromate. Figure 2 illustrates the inhibition, by carbonate, of cytochrome *c* reduction in the perchromate reaction mixtures. Curve 7 in Figure 2 also demonstrates the inhibition by superoxide dismutase. In these experiments, cytochrome *c* was at first reduced in these reaction mixtures and was then more slowly reoxidized. Since these reaction mixtures initially contained 2.5×10^{-5} M ferricytochrome *c*, complete reduction should have given an increased absorbancy at 550 nm of 0.5, yet the maximum seen was 0.3 (curve 1) and this was decreased by carbonate. The net *decrease* in A_{550} nm, seen in the presence of superoxide dismutase (line 7), should also be noted. These results suggested that cytochrome *c* was being both reduced and oxidized in these reaction mixtures. The reduction was due to O_2^- and when superoxide dismutase eliminated O_2^- then the oxidation was fully unmasked. The ferricytochrome *c* used in these studies did actually contain 6% of ferrocytochrome *c* whose oxidation accounts for the net decrease in A_{550} nm seen in the presence of 5 μg/ml of superoxide dismutase. In this light we could suppose that carbonate appeared to inhibit the reduction of ferricytochrome *c* only because it somehow augmented the opposing oxidation of ferrocytochrome *c*. This hypothesis was directly tested by preparing reduced cytochrome *c* and by investigating the effect of carbonate on its rate of oxidation during the decomposition of perchromate.

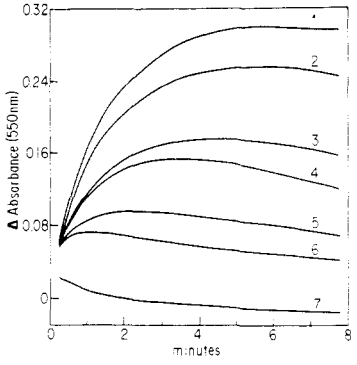

FIGURE 2: Effect of carbonate on the reduction of ferricytochrome *c*. The reduction of 2.5×10^{-5} M ferricytochrome *c* by 7×10^{-4} M K_3CrO_8 in buffers at pH 10.2 and 18° was monitored by following the change in absorbance at 550 nm. In each case the total concentration of buffer (pyrophosphate and carbonate) was 0.05 M. The carbonate concentration was (1) zero, (2) 8.3×10^{-4} M, (3) 5×10^{-3} M, (4) 8.3×10^{-3} M, (5) 1.8×10^{-2} M, and (6) 4.8×10^{-2} M. Curve 7 was obtained when the reaction was carried out in 0.05 M pyrophosphate in the presence of 5 μg/ml of superoxide dismutase. Reactions were initiated by addition of solid K_3CrO_8 to the cuvets containing 3 ml of the reaction mixture.

Effect of Carbonate on the Oxidation of Cytochrome c. Figure 3 demonstrates that carbonate did expose the ability of perchromate to oxidize ferrocytochrome *c* and that the rate of this oxidation did increase with the concentration of carbonate. Was an oxidant generated even in the complete absence of carbonate? This question was answered in the affirmative by the observation that superoxide dismutase unmasked a rapid oxidation of cytochrome *c*, in the absence of carbonate. This is shown by the single point on the ordinate of Figure 3. It is thus clear that the decomposition of peroxochromate liberates both a reductant, which is O_2^-, and an oxidant whose action on ferrocytochrome *c* was somehow augmented by carbonate. Since Peters *et al.* (1972) have shown that perchromate decomposition does produce singlet ($^1\Delta g$) oxygen it seemed likely that

TABLE I: Effects of Azide, Histidine, and Superoxide Dismutase on the Oxidation and Reduction of Cytochrome *c* by K_3CrO_8.[a]

Buffer	Ferricytochrome *c* (M)	Ferrocytochrome *c* (M)	Additions	$v \times 10^6$
Pyrophosphate	3.6×10^{-5}			+14.7
Pyrophosphate	3.6×10^{-5}		0.02 M azide	+18.8
Carbonate	3.6×10^{-5}			0
Carbonate	3.6×10^{-5}		0.5 M histidine	+11.3
Carbonate	3.6×10^{-5}		0.02 M azide	+16.5
Carbonate		3.2×10^{-5}		−8.7
Carbonate		3.2×10^{-5}	0.5 M histidine	−5.4
Carbonate		3.2×10^{-5}	0.02 M azide	0
Carbonate		3.2×10^{-5}	15 μg/ml of SOD	−18.3

[a] Reaction mixtures contained 7×10^{-4} M K_3CrO_8 in 0.05 M buffer at pH 10.2 and 25°. Total volume was 3 ml and additional components are listed in the table. The reactions were monitored by following the change in absorbance at 550 nm, and *v* is the initial molar change in cytochrome *c* concentration per minute. A plus sign indicates a net reduction of cytochrome *c* whereas a minus sign is used to indicate a net oxidation.

this was the oxidant. Since singlet oxygen is known to react rapidly with the imidazole ring of histidine (Wasserman, 1970; Tomita *et al.*, 1969; Wasserman *et al.*, 1968) and with azide (Hasty *et al.*, 1972; Nilsson and Kearns, 1973) the effects of these substances on the reduction and on the oxidation of cytochrome *c*, by perchromate, were tested. If singlet oxygen and O_2^- were both produced in this system then scavengers of singlet oxygen, such as histidine and azide, should augment the net rate of reduction of ferricytochrome *c* and should inhibit the net rate of oxidation of ferrocytochrome *c*. Superoxide dismutase by eliminating the reducing action of O_2^- should have the opposite effects, *i.e.*, it should inhibit the reduction while accelerating the oxidation of cytochrome *c*. Table I demonstrates that this is indeed what happened in regard to both the oxidation and the reduction of cytochrome *c*. We conclude then that the decomposition of perchromate generates both O_2^- and singlet oxygen, and that the oxidation of ferrocytochrome *c* is in some way greatly increased by the presence of carbonate.

Chemiluminescence during the Decomposition of Peroxochromate. Since carbonate enhanced the oxidation of ferrocytochrome *c* during the decomposition of peroxochromate, it appeared possible that some kind of oxidizing radical, derived from carbonate, might be formed during the decomposition. In the light of the experiments of Stauff (1973), in which he obtained a blue-green luminescence from reactions of perhydroxyl radicals in the presence of carbonate, which he proposed resulted from the formation of carbonate radicals ($CO_3\cdot^-$), we attempted to determine if the decomposition of peroxochromate in the presence of carbonate would result in luminescence in the visible region. Figure 4 demonstrates that there is indeed light produced by this system. Increasing the level of carbonate increased the luminescence, and in the absence of carbonate no significant luminescence was observed. Also, curve 7 in Figure 4 shows that the luminescence seen in the presence of carbonate was completely inhibited by superoxide dismutase. Therefore the observed luminescence resulting from the decomposition of peroxochromate was dependent upon both carbonate and O_2^-.

Does Superoxide Dismutase Scavenge Singlet O_2? Aerobic oxidations photosensitized by Methylene Blue have been shown to be largely mediated by singlet oxygen. If superoxide dismutase could scavenge singlet oxygen it should inhibit such photosensitized oxidations. It has already been reported that the holosuperoxide dismutase of bovine erythrocytes is not susceptible to such photosensitized oxidation but that the corresponding apoenzyme was susceptible. It was also shown that holoenzyme, present in the same solution, did not protect the apoenzyme (Forman *et al.*, 1973). It follows that O_2^- was not involved in this photosensitized oxidation and that singlet oxygen, which is the important agent of these oxidations (Foote, 1968), is not scavenged by holosuperoxide dismutase. It seemed necessary to emphasize this conclusion by examining the effects of superoxide dismutase on other photosensitized oxidations. Figure 5 demonstrates that, whereas azide did protect trypsin against photosensitized oxidation, superoxide dismutase was entirely without effect. Similar results were obtained when the photosensitized oxidation of catalase was examined. Oxygen was required for these oxidations and superoxide dismutase, at 27 μg/ml, did not protect. Furthermore, control experiments showed that the superoxide dismutase was itself unaffected by exposure to light plus the photosensitized dye, under the conditions of these experiments.

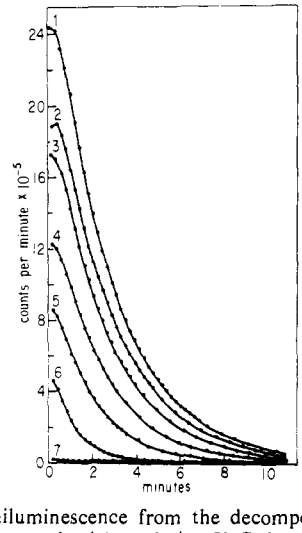

FIGURE 4: Chemiluminescence from the decomposition of K_3CrO_8. Reaction mixtures contained 1×10^{-4} M K_3CrO_8, 1×10^{-4} M EDTA, and the following concentration of carbonate: (1) 0.5 M, (2) 0.45 M, (3) 0.32 M, (4) 0.23 M, (5) 0.14 M, (6) 0.05 M, and (7) zero (contained 0.05 M pyrophosphate buffer). Curve 7 was also obtained when the reaction was carried out in 0.5 M carbonate containing 7 μg/ml of superoxide dismutase. The total reaction volume was 10 ml and each reaction mixture was buffered at pH 10.2 and 8°.

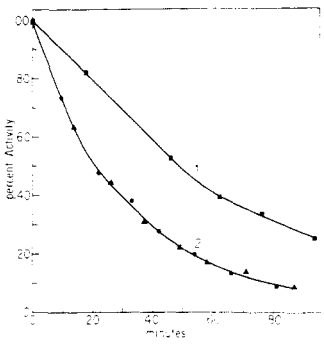

FIGURE 5: Photooxidation of trypsin. Reaction mixtures containing 1 × 10^{-5} M Methylene Blue, 0.1 M potassium phosphate, and 1 mg/ml of trypsin in a total volume of 3 ml at pH 7.5 were exposed, at a distance of 25 cm, to the light of a 200-W incandescent lamp. Additional components present were: (●) none; (▲) 25 μg/ml of superoxide dismutase; and (■) 0.01 M sodium azide. These mixtures were agitated in a water bath at 30° throughout the period of illumination and aliquots were withdrawn at intervals for assay of the residual trypsin activity, and also for superoxide dismutase activity when it was present.

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received April 11, 1974.* This work was supported in full by Research Grant GM 10287 from the National Institutes of Health.

TABLE II: Effect of Superoxide Dismutase on the Reduction and Oxidation of Cytochrome *c*.[a]

Buffer	Superoxide Dismutase	v (M min^{-1})
Pyrophosphate		-0.3×10^{-4}
Pyrophosphate	15 μg/ml	-1.3×10^{-4}
Carbonate		-1.0×10^{-4}
Carbonate	15 μg/ml	-2.0×10^{-4}

[a] The decomposition of 7 × 10^{-4} M K_3CrO_8 in buffered solutions containing 5 × 10^{-5} M ferricytochrome *c* and 5 × 10^{-5} M ferrocytochrome *c* was followed spectrophotometrically at 550 nm, and the initial rate of cytochrome *c* oxidation (minus sign) or reduction (plus sign) was determined. In each case buffer was present at a concentration of 0.05 M and pH 10.2 and 25°, and the total reaction volume was 3 ml. In a corresponding experiment, the rate of decomposition of this level of K_3CrO_8 (7 × 10^{-4} M) was determined using the reported (Brown *et al.*, 1968) $\Delta\epsilon_M$ at 372 nm of 3200 for the difference in the molar absorptivities of K_3CrO_8 and the product CrO_4^{2-}. The conditions were identical with those used to obtain the data in this table except for the absence of cytochrome *c*. Under these conditions the initial rate of K_3CrO_8 decomposition, which was found to be the same in carbonate and pyrophosphate buffers and to be unaffected in either buffer by the presence of superoxide dismutase, was found to be approximately 0.8 × 10^{-4} M min^{-1}.

Discussion

The decomposition of peroxochromate generates a reductant which can reduce ferricytochrome *c* and which can be intercepted by superoxide dismutase. We conclude that this reductant is O_2^-. Indeed Peters *et al.* (1972), who demonstrated the production of singlet oxygen by perchromate, suggested that O_2^- is probably also generated during the perchromate decomposition. The decomposition of this ion in basic solution has been shown to occur according to the equation (Riesenfeld *et al.*, 1905)

$$4CrO_8^{3-} + 2H_2O \longrightarrow 4CrO_4^{2-} + 7O_2 + 4OH^- \quad (1)$$

Also the kinetics of this reaction have been investigated (Brown *et al.*, 1969) and the rate of decomposition was found to be first order with respect to both peroxochromate ion and to hydrogen ion. From the experiment summarized in Table II, in which the decomposition of K_3CrO_8 was carried out in a solution containing 5 × 10^{-5} M ferricytochrome *c* and 5 × 10^{-5} M ferrocytochrome *c* in pyrophosphate buffer at pH 10.2, we can conclude that approximately one O_2^- was formed per K_3CrO_8 decomposed. Thus, in the absence of any other additions, the rate of oxidation and the rate of reduction were almost in balance. When superoxide dismutase was added the net oxidation rate increased by 1 × 10^{-4} M min^{-1}, which is approximately equal to the rate of decomposition of K_3CrO_8, which was 0.8 × 10^{-4} M min^{-1}. Since the effect of superoxide dismutase would be to unmask one oxidation event for each O_2^- scavenged, we can conclude that the rate of O_2^- production was approximately equal to the rate of K_3CrO_8 decomposition. In Table II it can be seen that the rate of oxidation of ferrocytochrome *c* in the presence of carbonate plus superoxide dismutase corresponds to two oxidations per K_3CrO_8 decomposed and that the effect of superoxide dismutase in the presence of carbonate was the same as in its absence. It thus appears that one O_2^- and one singlet oxygen are released during the course of the decomposition of CrO_8^{3-} to CrO_4^{3-}: the O_2^- being the reductant of ferricytochrome *c* and the singlet oxygen being the oxidant of ferrocytochrome *c*. The stoichiometry of eq 1, however, indicates 7/4 O_2 released for each CrO_8^{3-} decomposed. This stoichiometry could be achieved by a reaction of some of the O_2^- with intermediates of this decomposition.

Carbonate ion augmented the oxidation of ferrocytochrome *c* and caused a luminescence in the visible which was inhibitable by superoxide dismutase. We propose that carbonate is oxidized by perchromate or by one of its decomposition intermediates to the carbonate radical which is then reduced by O_2^- to yield electronically excited CO_2 which, in its return to the ground state, emits visible light. Stauff (1973), who also observed a carbonate-dependent luminescence in the visible region, has proposed that excited CO_2 was the emitter. The carbonate radical should be a powerful oxidant which could oxidize ferrocytochrome *c*. In balance we would thus generate two cytochrome *c* oxidizing equivalents per CrO_8^{3-} in the presence of carbonate but only one in its absence.

It is obvious that the decomposition of K_3CrO_8 can be very complex, especially in the presence of extraneous reactants such as ferri- and ferrocytochrome *c* and carbonate. It is, however, unequivocally demonstrated that O_2^- is a product of the decomposition of CrO_8^{3-} and that superoxide dismutase does not serve to scavenge singlet oxygen.

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