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Direct Generation of Superoxide Anions by Flash Photolysis of Human Oxyhemoglobin*

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The results presented in this report suggest that human oxyhemoglobin can directly form methemoglobin and superoxide anion when flashed with low intensity (38 joules) white light. The effect only occurred in quartz but not glass (cut off λ ≅ 300 nm) cuvettes.

The formation of O_2^- was established by observing the reduction of oxidized cytochrome *c* concomitant with MetHb formation at pH 9, and by showing that superoxide dismutase and catalase inhibit cytochrome *c* reduction at that pH. The inhibition of cytochrome *c* reduction by catalase led us to explore the possibility that H_2O_2 might reduce oxidized cytochrome *c* at pH 9. We show that H_2O_2 does reduce oxidized cytochrome *c* at that pH but not at pH 7. Furthermore, catalase, but not superoxide dismutase, almost completely inhibited this reduction process. These experiments serve to confirm our interpretation of the effect of catalase on the reduction of oxidized cytochrome *c* in the photolytic experiments, thus establishing that H_2O_2 was also formed. In addition, we were able to identify the production of O_2^- and H_2O_2 due to the photolysis of water in agreement with the results of McCord and Fridovich ((1973) *Photochem. Photobiol. 17,* 115-121). Production of O_2^- from this source was considerably less than that observed when HbO_2 was present. Addition of MetHb to aerated solutions of oxidized cytochrome *c* did not cause additional reduction, unlike addition of HbO_2.

The production of MetHb was found to have at least two components. One component was the primary photolytic process, and the second was a strongly pH-dependent reattack of HbO_2 by O_2^-. Addition of superoxide dismutase inhibited this second component, but did not significantly effect the primary photolytic process.

Superoxide anion can be generated from several sources. A detailed understanding of the mechanisms by which it is generated, as well as its subsequent reactions are currently of considerable chemical and biochemical interest (1-3). One process which has been shown to generate O_2^- is the autooxidation of HbO_2 (4-7). Discussions of the mechanism involved in this process have depended to varying degrees on a knowledge of the electronic structure of the oxygenated heme. An often cited proposal suggests that the oxygenated heme has some portion of an iron *d* electron transferred to the unoccupied π_z* orbital of the bound oxygen molecule (8-10). Through spin-spin coupling of the iron and oxygen unpaired electrons, HbO_2 would remain diamagnetic and would be "best described" as O_2^- bound to low spin ferric heme (10, 11).

We report experiments which may have a bearing on this proposal. We have found that flashing solutions of HbO_2 with low intensity white light causes the immediate formation of MetHb. This observation is analogous to the finding of Possani *et al.* (12) where white light was shown to markedly effect the net amount of oxygen "utilized" by a solution of HbO_2. They suggested that the bound oxygen molecule in HbO_2 was activated by the light absorbed by the heme forming singlet oxygen ($^1\Delta g$) and that singlet oxygen was the active oxidizing agent responsible for the extra consumption of oxygen in the oxidation of HbO_2 under illumination. An alternative possibility would be that the light caused direct lysis of HbO_2, yielding MetHb and O_2^- as photoproducts. Experiments were designed to test this possibility and the results are presented in this report.

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MATERIALS AND METHODS

Human HbO_2 was isolated as described elsewhere (13, 14). Superoxide dismutase, catalase, and oxidized cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, Mo.). Reagents used were of analytical grade. Flash illumination of the various samples was performed using a constant output (38 joules) camera strobe (Ultrablitz, model Metor SPGH, West Germany, equipped with a xenon lamp) placed about 2 inches from the cuvettes which were mounted in an aluminum foil-lined box with one open face. The constancy of the intensity of the flash source over the number of flashes used in most of this work was checked in a way which employed the effect we report below (*i.e.* MetHb formation by flashing HbO_2). Three quartz cuvettes were filled with exactly the same HbO_2 solution at pH 9. The optical spectrum of one cuvette was initially measured and it was then subjected to 30 flashes. The spectrum was measured again and the amount of MetHb formed was calculated. We then flashed the strobe ninety times away from any of the cuvettes. The next 30 flashes were directed at the second cuvette containing 100% HbO_2, after initially measuring the spectrum. This process was repeated on the third sample of 100% HbO_2, after the 200th flash of the strobe. These experiments showed that exactly the same amount of MetHb was formed at each of these widely separated points over 230 flashes and indicated that the light output characteristics of this strobe do not change significantly with use.

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When MetHb formation and cytochrome *c* reduction were to be followed, we employed a tandem flash approach. Two matched quartz cuvettes were used with the sample cell containing HbO_2 and cytochrome *c* and the reference cell containing exactly the same concentration of HbO_2. When enzymes were added, the same enzyme concentration was also present in both cuvettes. Spectra were recorded in a Beckman Acta CV spectrophotometer from 630 nm to 500 nm on the sample cell and the reference cell. A difference spectrum was also recorded (sample minus reference). Several checks on the validity of this approach were made during each experiment. First, MetHb formation was monitored at several isosbestic points for oxidized and reduced cytochrome *c* in the sample cell and compared with the amount of MetHb formed in the reference cell. There were no significant differences at pH 9. Second, the difference spectrum between the sample and reference cell was carefully checked to make sure that all of the oxidized *versus* reduced isosbestics for cytochrome *c* were present. Once again, there were no significant deviations and the proper isosbestics were obtained at pH 9. We also monitored autooxidation of the same hemoglobin samples which were being flashed. This was always considerably slower than the amount of MetHb produced by flashing (<5% of that formed by flashing). In addition, we measured the temperature in the open box containing the cuvettes during each short flash interval of these low intensity flashes. We observed no measurable difference from the ambient temperature (25 ± 2°).

In order to see if MetHb formation from low intensity photolysis of HbO_2 required the presence of oxygen, we prepared a quartz cuvette containing carbon monoxide-bound hemoglobin at pH 9 in 0.01 M phosphate buffer. The cell was sealed and flushed with pure CO (Matheson, Inc., East Rutherford, N. J.) to remove any free oxygen. We then flashed the cell the same number of times that we flashed HbO_2 and recorded the spectrum at appropriate intervals. As an additional control, we prepared aerated solutions of MetHb according to the method of Gibson *et al.* (15) and flashed it in the presence of oxidized cytochrome *c*. This latter experiment was performed in the event that the light could cause some group on the protein to dissociate an electron and reduce O_2 to O_2^-.

Stopped flow kinetic experiments were performed using a Durrum-Gibson stopped flow apparatus, interfaced to an On-Line-Instruments-Systems (OLIS, Athens, Ga.) data acquisition system. The system is equipped with a Data General Nova 2/10 computer with 24 K of core memory. Typical experiments involved sampling 900 data points/reaction and accumulating and averaging at least three reactions/experimental condition. The data were analyzed, plotted, and read out, all under computer control. Reactions were studied using a 2-cm path length cuvette at 25°.

RESULTS AND DISCUSSION

MetHb Formation and Cytochrome c Reduction with Flash Photolysis – When HbO_2 was flashed at pH 9 in 0.01 M phosphate buffer, MetHb progressively formed (Fig. 1*B*). Control experiments showed no MetHb formation from the autooxidation process at this pH and temperature over the period of time necessary to execute these experiments. Also, flashing these solutions in glass cuvettes (cut off $\lambda \cong 300$ nm) showed no significant spectral changes over the same range. This result would indicate that the ultraviolet component of the white light ($\lambda \leq 300$ nm) is required. The isosbestics observed for this process (590 and 525 nm) are those for HbO_2 *versus* MetHb at pH 9. We observed no measurable denaturation of hemoglobin with these low intensity flashes. Flashing a cuvette containing the same concentration of HbO_2 as in Fig. 1*B* (27 μM) and also containing 12 μM of oxidized cytochrome *c* in tandem with the HbO_2 solution of Fig. 1*B*, caused the formation of MetHb and the concomitant reduction of oxidized cytochrome *c* (Fig. 1, *A* and *C*). Fig. 1*C* shows the reduction of cytochrome *c* observed by taking a difference spectrum between cuvettes A and B. The isosbestics are those of oxidized *versus* reduced cytochrome *c*. When we attempted to perform the same tandem flash experiments at pH values below 9, we were unable to observe the proper isosbestics in the difference spectrum. The pseudo-isosbestics observed were shifted in a way which indicated that at lower pH values, less MetHb was being formed in the cuvette containing the oxidized cytochrome *c*. As we will see shortly, the reattack of superoxide anion on HbO_2 to form MetHb is minimal at pH 9, whereas it does become significant below that pH (16–18). When reattack is occurring, having oxidized cytochrome *c* present would effectively compete with HbO_2 for O_2^-. Thus, at the lower pH, the cuvette containing oxidized cytochrome *c* could form less MetHb than the one without.

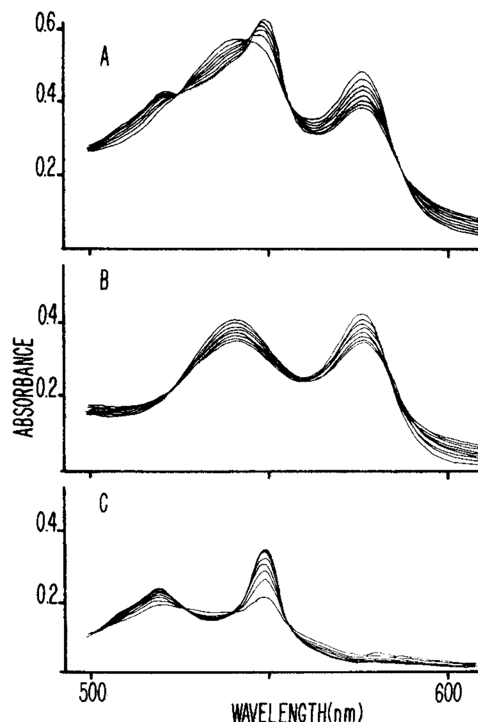

FIG. 1. Effect of low intensity (38 joules/flash) photolysis using white light in quartz cuvettes on solutions of (*A*) oxidized cytochrome *c* plus HbO_2 and (*B*) HbO_2 alone. Spectrum *C* is the difference spectrum recorded between *A* and *B*. Reaction mixtures contained 27 μM (heme) of HbO_2 and 12 μM of oxidized cytochrome *c* initially. The reactions were in 0.01 M phosphate buffer, pH 9; T 25°. The path length of the cuvettes was 1 cm.

Cytochrome c Reduction in Absence and Presence of HbO_2 and Effect of Superoxide Dismutase and Catalase – Fig. 2*A* shows control experiments where aerated solutions of oxidized cytochrome *c* were flashed. Flashing such solutions alone at pH 9 did lead to some reduction. This reduction process appears to be nearly equally due to the action of O_2^- and H_2O_2, since both superoxide dismutase and catalase caused inhibition. Addition of both enzymes produced still further inhibition.

McCord and Fridovich (19) have previously demonstrated that ultraviolet light can lyse water to form H· and OH·. The former radical attacks oxygen in solution to form perhydroxyl radical which dissociates to proton and O_2^- (pK 4.8) (20). They also followed their reactions by monitoring the reduction of oxidized cytochrome *c*. However, no effect of catalase was observed at the pH employed (7.4). In contrast to the generally accepted view that H_2O_2 does not reduce oxidized cytochrome *c* (21), we reproducibily observed an effect of catalase in our experiments at pH 9. It seemed possible that the reduction of oxidized cytochrome *c* by H_2O_2 could occur at pH 9 due to the lower oxidiation-reduction potential of cytochrome *c* (22). In order to test this, we investigated the reaction of oxidized

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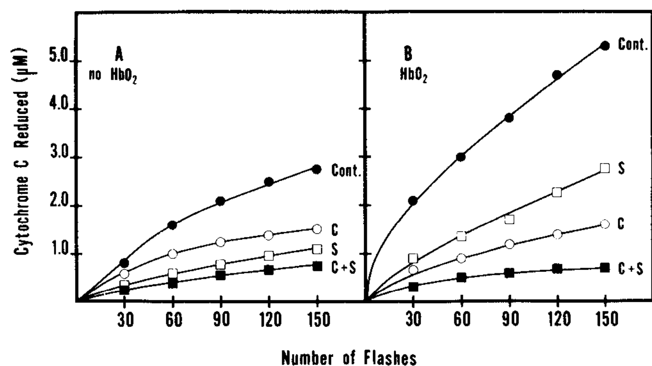

FIG. 2. Reduction of oxidized cytochrome *c* in the absence and presence of HbO_2, superoxide dismutase, catalase, and superoxide dismutase plus catalase. See text for the manner by which this data was obtained. *A*, no HbO_2: ●, (*Cont.*) control; ○, *C* plus 1200 units/ml of catalase; □, *S* plus 20 μg/ml of superoxide dismutase; ■, *C* + *S* plus 1200 units/ml of catalase and 20 μg/ml of superoxide dismutase. *B*, with 27 μM HbO_2 present: ●, (*Cont.*) control; ○, *C* plus 1200 units/ml of catalase; □, *S* plus 10 μg/ml of superoxide dismutase; ■, *C* + *S* plus 1200 units/ml of catalase and 20 μg/ml of superoxide dismutase. The initial concentration of oxidized cytochrome *c* in all cases was 12 μM. All of these reactions showed good difference spectra like those in Fig. 1*C*, and the results were calculated using the absorbance changes at 550 nm and a value of $\Delta\epsilon_{550}$ for oxidized *versus* reduced cytochrome *c* of 21.1 mM^{-1} cm^{-1}. The solution conditions in all cases were 0.01 M phosphate buffer, pH 9, 25°. The reproducibility of these measurements was ±5% of the mean values for each point.

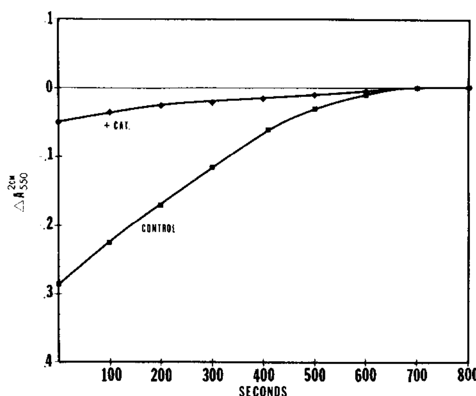

FIG. 3. Stopped flow kinetic measurements of the reaction of oxidized cytochrome *c* with H_2O_2 at pH 9. The concentrations of oxidized cytochrome *c* and H_2O_2 before the mix were 50 μM and 14 μM, respectively. The reactions were studied at 25° in 0.05 M phosphate buffer, pH 9, at 550 nm with a 2-cm path length. *CONTROL* = 50 μM oxidized cytochrome *c* alone; + *CAT.* = 500 units/ml of catalase added to 50 μM of oxidized cytochrome *c*.

cytochrome *c* with H_2O_2 at pH 9 *versus* pH 7 in the stopped flow apparatus. When 14 μM H_2O_2 was mixed with 50 μM oxidized cytochrome *c* in the stopped flow at pH 9, we observed a substantial increase in the absorbance at 550 nm (Fig. 3). Investigation of this reaction as a function of wavelength showed a kinetic difference spectrum which clearly indicates that we are observing the reduction of oxidized cytochrome *c* (Fig. 4). When the same reaction was studied at pH 7, we observed no reduction of oxidized cytochrome *c*. Furthermore, addition of 500 units/ml of catalase to 50 μM of cytochrome *c* caused a substantial diminution in the amount of cytochrome *c* reduced (Fig. 3). Addition of 20 μg/ml of superoxide dismutase did not cause an inhibition of this reduction reaction. These results offer direct evidence that H_2O_2 does reduce oxidized cytochrome *c* at pH 9, thus confirming the interpretation of the results shown in Fig. 2.

When HbO_2 was added to solutions containing oxidized cytochrome *c*, the amount of cytochrome *c* reduced at pH 9, increased by about a factor of 2 (Fig. 2*B*). Once again, catalase and superoxide dismutase caused substantial inhibition of cytochrome *c* reduction. When the same concentration of MetHb was added to aerated solutions of cytochrome *c* (12 μM), no additional reduction was observed at pH 9. Finally, flashing oxygen-free solutions of carbon monoxide-bound hemoglobin at pH 9 showed no significant MetHb formation (<1% after 200 flashes), in marked contrast to HbO_2 under otherwise similar conditions.

Evidence Suggesting Presence of at Least Two Components in MetHb Formation Process with Flash Photolysis —The results presented above showed that adding HbO_2 to oxidized cytochrome *c* solutions and flashing produced more reduced cytochrome *c* at pH 9 than was generated without HbO_2. The fact that superoxide dismutase and catalase both caused inhibition (Fig. 2*B*) would suggest that flashing a solution of HbO_2 not only causes MetHb to form (Fig. 1*B*), but also directly generates additional O_2^-, with H_2O_2 being largely produced

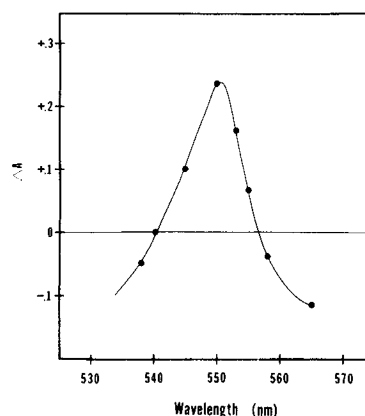

FIG. 4. Kinetic difference spectrum for the reaction of oxidized cytochrome *c* with H_2O_2 at pH 9 in the absence of catalase. The conditions used here are the same as those given in the legend to Fig. 3.

through the spontaneous dismutation process. The recent reports by Lynch *et al.* (16), Winterbourn *et al.* (17), and Sutton *et al.* (18) showed that when O_2^- was generated from a second source in a solution, it, or H_2O_2, could attack HbO_2 and form MetHb, with this attack being pH-dependent. Based on these results, we might expect MetHb formation in the photolysis experiments to contain at least two components. One component would be MetHb formation and O_2^- generation directly caused by photolysis, with the second component resulting from a pH-dependent reattack of O_2^- or H_2O_2 on HbO_2. This pH-dependent reattack process should be more prominent early in the photolytic conversion where more HbO_2 is present. The results of Fig. 5 show the yield of MetHb/flash *versus* the number of flashes as a function of pH. At lower pH, the initially high yield of MetHb decreased as the number of flashes increased. At pH 9 the yield was nearly constant throughout the photolytic conversion process. These results would be consistent with the proposal that the photolytic production of MetHb consists of at least two components.

It is generally accepted (1) that those processes which generate O_2^- should not be affected by superoxide dismutase, whereas those processes which involve this radical as a reactant would be expected to be inhibited by the enzyme, provided the kinetics of the enzyme catalyzed and competing reactions

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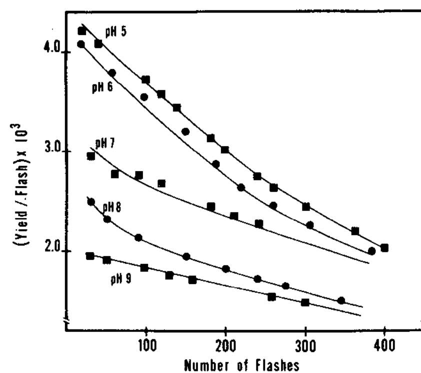

FIG. 5. Yield of MetHb formed/flash *versus* the number of flashes at various pH values. The appearance of MetHb was calculated by measuring the absorbance changes at 577 nm after each sequence of flashes from spectra like those shown in Fig. 1*B*. Since the optical spectra of both MetHb and, to a lesser extent, HbO_2 are pH-dependent, it was necessary to measure the spectra of 100% HbO_2 and 100% MetHb at each pH studied, in order to obtain proper extinction coefficients for each pH. The yield of MetHb/flash is defined as MetHb formed divided by the total heme concentration, with that ratio being divided by the total number of flashes. The buffer used was 0.01 M phosphate and the temperature was 25°. The initial concentration of HbO_2 in these reactions was 60 μM (heme).

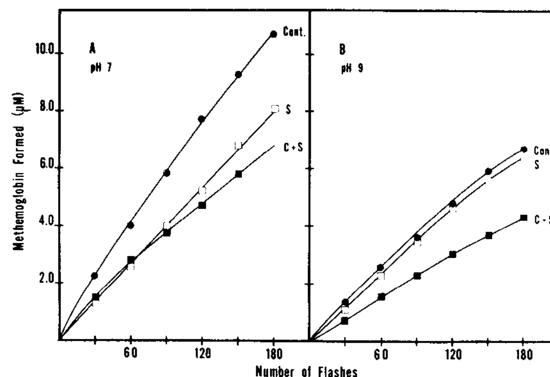

FIG. 6. Effect of superoxide dismutase (*S*) and catalase plus superoxide dismutase (*C* + *S*) on MetHb formation at pH 7 and 9. *A*, pH 7: ●, (*Cont.*) control; □, *S* plus 20 μg/ml of superoxide dismutase; ■, *C* + *S* plus 1200 units/ml of catalase plus 20 μg/ml of superoxide dismutase. *B*, pH 9: ●, (*Cont.*) control; □, *S* plus 20 μg/ml of superoxide dismutase; ■, *C* + *S* plus 1200 units/ml of catalase and 20 μg/ml of superoxide dismutase. All reactions initially contained 27 μM (heme) HbO_2 and were in 0.01 M phosphate; 25°. MetHb formation was measured as described in the legend to Fig. 5.

favor inhibition. Thus, if the reattack process is more prominent at lower pH than at pH 9, we would expect the effect of superoxide dismutase to be greater at lower pH. The results shown in Fig. 6 seem to support this expectation. There was a significantly larger effect of superoxide dismutase on MetHb formation at pH 7 than at pH 9, where the enzyme was almost without effect. When catalase was added along with superoxide dismutase, we observed almost no additional inhibition at pH 7, but significantly more at pH 9. These results would suggest that the reattack of O_2^- is more pronounced at lower pH and almost absent at pH 9. At pH 9, the O_2^- formed largely undergoes spontaneous dismutation to produce hydrogen peroxide when HbO_2 is present alone. The additional inhibition by catalase at pH 9 (Fig. 6*B*) would come from an inhibition of this H_2O_2 from attacking HbO_2. The diminished effect of adding catalase at pH 7 (Fig. 6*A*), may be a result of the inability of catalase to effectively catalyze the relatively large amounts of H_2O_2 generated and/or the inability of catalase to compete kinetically with HbO_2 for H_2O_2.

Some of the MetHb formed by flashing HbO_2 may be due to the direct attack of OH ·, a strong oxidant. We flashed HbO_2 in the absence and presence of 17 mM mannitol at pH 9 to test this. Mannitol will effectively scavenge OH · (19). We observed no significant effect of mannitol on MetHb formation.

Concluding Remarks — The MetHb formed in the presence of catalase and superoxide dismutase at pH 9 arises directly from photolysis of HbO_2. The effect only occurred in quartz cuvettes suggesting that the effective wavelength is less than the cut off wavelength for glass cuvettes (<300 nm), but greater than the cut off wavelength for quartz (>180 nm). There is some recent infrared spectroscopic evidence which appears to suggest that the heme-bound oxygen of HbO_2 exists as O_2^-. Caughey *et al.* (23) have shown that the infrared stretching frequency for oxygen bound to hemoglobin is almost identical to that of free O_2^- and is quite different from unbound oxygen or peroxide. If the ground state exists as an essentially formed ferric · superoxide complex, it is possible that ultraviolet light may cause photooxidation by eliminating a barrier which normally prevents dissociation of O_2^-. For example, Folin *et al.* (24) have observed that irradiation of oxymyoglobin with visible light can lead to specific photooxidation of both the heme-linked and the distal histidines. These histidines may well be important in the reversible oxygenation process (11, 25) and their photooxidation could explain our observation that flashing HbO_2 with ultraviolet light caused the direct formation of MetHb and O_2^-.

The demonstration that ultraviolet light can photooxidize human HbO_2 yielding O_2^- as a primary photoproduct, may be directly relevant to studies dealing with the photohemolysis of human erythrocytes by ultraviolet light (26). It has been shown that ultraviolet light can cause cells to lyse, with the action spectrum showing a peak at about 280 nm. Although erythrocyte membrane proteins probably absorb in this region, the results presented in this report would strongly suggest that if hemoglobin absorbs a substantial portion of the incident ultraviolet light, MetHb and O_2^- will also form.

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