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The detection of superoxide anion from the reaction of oxyhemoglobin and phenylhydrazine using EPR spectroscopy[1]

B. Goldberg[2], A. Stern[3], J. Peisach and W.E. Blumberg

Department of Pharmacology, New York University School of Medicine, New York (N.Y. 10016, USA), Departments of Molecular Pharmacology and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx (N.Y. 10461, USA), and Bell Laboratories, Inc., Murray Hill (New Jersey 07974, USA), 3 July 1978

Summary. The low temperature EPR spectrum of a quickly reacted mixture of oxyhemoglobin and phenylhydrazine was studied. With the use of a computer, the spectral contribution of methemoglobin in the region of $g=2$ was subtracted. The remaining spectrum was that of an axial free radical ($g_{\perp}=2.00$, $g_{\parallel}=2.06$) having the magnetic parameters of superoxide anion. In the presence of superoxide dismutase, this axial radical was not seen, confirming that superoxide anion is indeed generated by the reaction.

It has been shown by chemical means[4] that the interaction of phenylhydrazine with HbO_2 leads to the generation of superoxide anion, O_2^-, a partially reduced form of molecular oxygen. Rather than being derived from the bound oxygen of hemoglobin, it was found that O_2^- arises from the reaction of molecular O_2 with phenyldiazine, a partially oxidized form of phenylhydrazine which is produced as the primary reaction product of HbO_2 and phenylhydrazine[5].

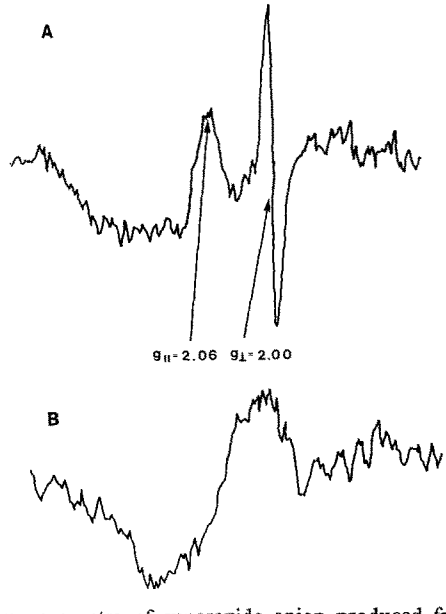
Although superoxide anion has a single unpaired spin, its reactivity in aqueous solution prevents an accumulation of this species in sufficiently reasonable concentration so that it can be detected by conventional EPR methods. It is possible though, to use stopped flow techniques in order to physically demonstrate the formation of this species.

In this present study, we demonstrate for the first time, using a combination of stopped flow and computer facilitated difference EPR techniques, the formation of superoxide anion in a reaction mixture containing HbO_2 and phenylhydrazine. We also show that in the presence of superoxide dismutase, O_2^- does not accumulate so that the EPR spectrum characteristic of this species is not seen.

Materials and methods. Oxyhemoglobin was prepared from a red cell lysate by a standard procedure. For the EPR study, 2 solutions were prepared. The first consisted of HbO_2 (0.8 mM) in 50 mM Na-PO_4 buffer, pH 7.0, containing EDTA (100 µM). The second, phenylhydrazine (3.2×10^{-3} M) in the same aerated buffer. The solutions were individually transferred to 5 ml hypodermic syringes which were components of a manually operated stopped flow apparatus. The syringe effluents were simultaneously combined, mixed in a Gibson-Durrum type mixing chamber and squirted into an EPR cavity[6] precooled in liquid N_2.

EPR spectra were taken at 1.4° on an X-band spectrometer described by Feher[7] operating near 9200 mc/sec and with a sweep rate of 1000 G per 2.5 min. Data obtained near $g=2$ in the first 1.6 min of the sweep were digitized as 1024 data points and were stored for computer processing. The spectrum in the region of $g=6$ was also recorded. The EPR spectrum of an oxyhemoglobin solution to which buffer was added was obtained in the same way. Similar experiments were performed using HbO_2 solutions as above, but to which 0.143 mg/ml of bovine superoxide dismutase (Truett Laboratories) was added.

Results and discussion. Due to the short lifetime of O_2^- in aqueous solution, EPR examination of this species is performed in the frozen state. Using a technique of rapid mixing of reagents followed by fast freezing, it has been possible to examine the EPR spectrum of this species[8]. The experiments described in this present work concerning O_2^-



EPR difference spectra of superoxide anion produced from the reaction of oxyhemoglobin and phenylhydrazine. Spectra were obtained by rapid mixing of reagents followed by quick freezing in an EPR cavity. In A, the spectrum was recorded after phenylhydrazine addition and from which the EPR spectral contribution of methemoglobin was subtracted. In B, the same procedure as in A was followed, but in the presence of superoxide dismutase. The broad, unresolved feature observed to lower field of $g=2$ arises from Cu(II) in the superoxide dismutase.

generation by the reaction of phenylhydrazine with HbO_2 are complicated by the formation of methemoglobin during the reaction. The EPR of this species extends from $g=6$ to $g=2$ and overlaps the region of EPR absorption ascribable to superoxide anion. One can overcome this problem, though, by resorting to a computerized difference spectroscopic technique where the spectral contribution from the methemoglobin is subtracted. One can ascertain the spectral contribution of the methemoglobin in the region of $g=2$ for the phenylhydrazine reacted protein from the amplitude of the $g=6$ resonance. The proportionality between the amplitude of the $g=6$ feature and the $g=2$ feature is obtained from the spectrum of the HbO_2 solution mixed only with buffer and which contains a small methemoglobin impurity. The spectrum of unreacted HbO_2 in the region near $g=2$ multiplied by an appropriate factor indicative of methemoglobin content is then subtracted from the spectrum of HbO_2 reacted with phenylhydrazine, making a correction for frequency differences, so that there is a correspondence of g in the data that are processed by computer.

As can be seen in the figure, A, an EPR signal having the features $g_\perp = 2.00$ and $g_\parallel = 2.06$ is obtained. The axial EPR spectrum is characteristic of superoxide anion[8-10]. Similar experiments carried out in the presence of superoxide dismutase do not lead to the generation of the EPR signal (figure, B). These results are thus confirmatory of the original assertion based solely on indirect chemical methods of detection that superoxide anion is generated from the reaction of oxyhemoglobin and phenylhydrazine.

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A new type of mutant of *Euglena* which produces permanently bleached progeny by darkness

Y. Ohki, A. Musashi and Y. Tsubo

Department of Physiological Chemistry, Kobe Women's College of Pharmacy, Higashinada, Kobe (Japan), and Department of Biology, College of General Education, Kobe University, Nada, Kobe (Japan), 28 July 1978

Summary. A new type of mutant of *Euglena gracilis* strain Z was isolated. In the light-grown culture, it contains 5–20% of bleached cells which have irreversibly lost the ability of chloroplast formation. When once grown in the dark, differing from the case with the wild type, it segregates only bleached cells, probably due to the inability of the replication of proplastids in darkness. Cell multiplication under the inhibition of chlorophyll synthesis or photosynthesis in the light also produces bleached cells.

Light-grown cells of *E. gracilis* strain Z have 8 or 10 chloroplasts which are said to degenerate into proplastids in the dark[1,2]. The dark grown cells look completely colourless, and reversible formation of plastids is induced by light. In the course of the investigation on the mutagenesis in *Euglena*, we obtained a new type of mutant, a so-called conditional one, named U.

Cells were grown in the modified Hutner's medium[3], containing acetate-Na, citrate-3Na and glutamate-Na as carbon and nitrogen sources. Continuous illumination was supplied for light culture by a fluorescent lamp of FL-20 PG, Matsushita Electric Ind. Co. (ca. 70 $\mu W\ cm^{-2}$ at the surface of the culture). Temperature of incubation was 23–25 °C. Colony counting of green and bleached cells was made on the plates medium solidified by 1% agar. Colonies were visible after 7 days incubation in the light.

The culture of U, when grown in the light, always contained a certain amount (5–20%) of permanently bleached cells. But in contrast to the light culture, the frequency of bleached cells in the dark grown culture increased with the culture age, and reached nearly 100% after 4.5 generations when the cell number reached plateau (figure 1). However, when cells of U were maintained in a 'resting' medium[4], where no cell multiplication was permitted, no bleached cells were produced even after a longer incubation in the dark. In the case of Z, the wild strain, bleached cells did not appear in any one of these experimental conditions.

The relation between light itensity supplied and the effi-

Table 1. Frequencies of bleached cells of U produced after 4 generations in various light sources

Light sources (wavelength, nm)	Bleached cells (%)
FL-20 PG (400–680)	4.8
FL-20 BF (420–500)	5.2
FL-20 GF (510–560)	99.3
FL-20 YF (560–640)	3.6
FL-20 RF (620–670)	4.3

All the lamps were the product of Matsushita Electric Ind. Co.

Table 2. Frequencies of bleached cells produced after 4 generations in the presence or absence of DCMU in the light

DCMU addition	Bleached cells (%)	
	Z	U
0	0.1	1.4
10^{-4} M	9.0	99.1