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Production of Oxygen-Centered Radicals by Neutrophils and Macrophages as Studied by Electron Spin Resonance (ESR)

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Neutrophils and macrophages undergo a respiratory burst and an increase in the activity of the hexose monophosphate pathway in response to particulate or soluble agents. The increase in oxygen consumption was found to be associated with the production of oxygen-centered radicals. The ESR technique of spin trapping showed that besides a superoxide spin adduct, a hydroxyl spin adduct is also produced. ESR is considered to be the least ambiguous technique for the detection of free radicals. The spin-trapping agents used for oxygen-centered radical detection are usually nitrones. The most commonly used nitrone is 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), which reacts with $O_2^{\cdot-}$ to form 5,5-dimethyl-2-hydroperoxypyrroline-*N*-oxide (DMPO-OOH) and with $OH^{\cdot}$ to form 5,5-dimethyl-2-hydroxypyrroline-*N*-oxide (DMPO-OH). Although spin-adduct formation is considered to be the most direct technique for the detection of free radicals, some disadvantages are encountered.

There has been considerable interest in the isolation of the $O_2^{\cdot-}$ generating activity from phagocytic cells. The enzyme can be extracted with deoxycholate and gel filtration indicates that it is a high molecular weight complex. Maximum activity was between pH 7.0 and pH 7.5. The K_m value was 15.8 μM for NADPH and 434 μm for NADH, indicating that NADPH is the preferred substrate.

Introduction

Neutrophils undergo a respiratory burst in response to particulate or soluble agents, which results in the production of oxygen-centered radicals. The respiratory burst is not blocked by cyanide (*1*) and is associated with increased activity of the hexose monophosphate pathway (*2*). Although Iyer et al. (*3*) had shown that dioxygen was reduced to H_2O_2 during the respiratory burst of phagocytosing leukocytes, the linkage between the increase in oxygen consumption and production of oxygen-centered radicals was provided by the seminal discovery of Babior et al. (*4*) that neutrophils formed superoxide ($O_2^{\cdot-}$) when stimulated with latex particles. This was one of the major discoveries which followed the finding by McCord and Fridovich (*5*) that living cells contain superoxide dismutase and therefore must produce $O_2^{\cdot-}$.

The hexose monophosphate pathway in activated neutrophils produces reducing equivalents, in the form of NADPH, for the formation of $O_2^{\cdot-}$ from dioxygen

$$2O_2 + \text{NADPH} + H^+ \rightarrow 2O_2^{\cdot-} + \text{NADP}^+ \quad (1)$$

and for the detoxication of H_2O_2 that leaks into the cytoplasm of the cells, which is carried out preponderantly by the glutathione peroxidase–glutathione reductase system (*6*):

$$H_2O_2 + 2\,\text{GSH} \xrightarrow[\text{peroxidase}]{\text{GSH}} 2H_2O + \text{GSSG} \quad (2a)$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow[\text{reductase}]{\text{GSSG}} 2\,\text{GSH} + \text{NADP}^+ \quad (2b)$$

Dismutation of $O_2^{\cdot-}$ accounts for H_2O_2 production during the respiratory burst of neutrophils (*7*),

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

The conjugate acid of $O_2^{\cdot-}$, the hydroperoxyl radical ($HO_2^{\cdot}$), is responsible for the spontaneous dismutation of $O_2^{\cdot-}$, via the reactions (*8*):

$$HO_2^{\cdot} + HO_2^{\cdot} \rightarrow O_2 + H_2O_2$$
$$k_4 = 8.6 \times 10^5\ M^{-1}sec^{-1} \quad (4)$$

$$HO_2^{\cdot} + O_2^{\cdot-} \xrightarrow{H^+} O_2 + H_2O_2$$
$$k_5 = 1.0 \times 10^8\ M^{-1}sec^{-1} \quad (5)$$

The rate of dismutation of the total radical concentration ($[HO_2^{\cdot}] + [O_2^{\cdot-}]$) at a given pH is given by

$$k_6 = (k_4 + k_5X)/(1 + X)^2 \quad (6)$$

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where $X = K_{HO_2^{\cdot}}/[H^+]$. The p$K_a$ of $HO_2^{\cdot}$ is 4.69, i.e., $K_{HO_2^{\cdot}} = 2.04 \times 10^{-5}$ (*8*). Thus k_6 is approximately 1.9 $\times 10^5 M^{-1}sec^{-1}$ at pH 7.4. Neutrophils liberate $O_2^{\cdot-}$ extracellularly and into the phagosomes formed by interiorization of the plasma membrane. Intravacuolar fall of pH in the phagosomes (*9,10*) favors the formation of $HO_2^{\cdot}$, which is a more reactive species than $O_2^{\cdot-}$ (*11*), and the spontaneous dismutation of $O_2^{\cdot-}$. At pH 5.0, which is typical of phagosomal contents (*9*), k_6 is approximately 2.2 $\times 10^7 M^{-1}sec^{-1}$. Fall in pH is associated with phagolysosomal fusion. There is a transient rise in pH of the phagosome to pH 7.8 soon after phagocytosis (*12*). Initially, therefore, $HO_2^{\cdot}$ formation and spontaneous decay of $O_2^{\cdot-}$ in the phagosome are delayed.

The Superoxide-Generating System

An enzymatic basis for the oxidation of pyridine nucleotides in activated neutrophils was indicated by Patriarca et al. (*13*). The $O_2^{\cdot-}$-generating enzyme resides in the plasma membrane (*14*). Cohen et al. (*15*) found the enzyme in phagocytic vacuole walls. The enzyme activity is not expressed in membrane fractions from unstimulated neutrophils from normal subjects and from stimulated neutrophils from patients with chronic granulomatous disease (CGD) (*16*).

There has been considerable interest in the isolation of the $O_2^{\cdot-}$-generating activity from various phagocytic cells (*17–25*). Very little progress has been achieved until recently because of the use of procedures giving an extremely unstable enzyme. A procedure for the extraction and isolation of a highly active and stable enzyme from stimulated guinea pig neutrophils was reported by Bellavite et al. (*24*). The enzyme was extracted with deoxycholate and gel filtration indicated that it is a high molecule weight complex. Maximum activity was between pH 7.0 and pH 7.5. The K_m value was 15.8 μM for NADPH and 434 μM for NADH, indicating that NADPH is the preferred substrate.

A number of alternative enzyme activities have also been proposed as being responsible for the $O_2^{\cdot-}$-generating activity. These have included myeloperoxidase (MPO), D-amino acid oxidase, NADH oxidase, and cytochrome b. MPO is able to catalyse the oxidation of reduced pyridine nucleotides at low pH and in the presence of Mn^{2+} (*26*). However, a normal respiratory burst and NADPH oxidase activity are seen in neutrophils from MPO-deficient patients (*27,28*). A similar argument rules out D-amino acid oxidase. This enzyme activity is normally present in neutrophils from CGD patients which have no $O_2^{\cdot-}$-generating activity (*29*). The production of $O_2^{\cdot-}$ and H_2O_2 by an NADH oxidase in guinea pig neutrophils has been described by Badwey and Karnovsky (*30*). However, a rather modest decrease in the activity of the enzyme in neutrophils from CGD patients was observed in contrast to the total absence of NADPH oxidase activity.

The arguments presented so far clearly indicate that NADPH oxidase is the enzyme responsible for the production of $O_2^{\cdot-}$ by stimulated neutrophils. There is also considerable interest in the structural components, cofactors and mechanism of action of the enzyme. The enzyme has been characterized as a flavor protein (*19,23–25*). However, this has recently been disputed because various extensively purified preparations of the enzyme have been found to contain varying ratios of flavin to enzyme activity and recent preparations have been found to contain little flavin (*31*). A b-type cytochrome associated with the phagocytic vacuole wall has been implicated in the NADPH oxidase activity (*32*). A proposal that the cytochrome b may be the oxidase itself (*33*) is poorly supported because the cytochrome appears to be absent only in some CGD patients (*34,35*). It can be noted that cytochrome b autoxidation has been ruled out as a source of $O_2^{\cdot-}$ generation in mitochondria (*36*). The possibility that a b-type cytochrome forms part of the NADPH oxidase complex is, however, not excluded since the cytochrome copurifies with the enzyme activity and a constant ratio is maintained throughout the purification (*25*). Other components which have been claimed to be associated with the enzyme activity are calmodulin (*37,38*) and quinones (*39*). However, the exact involvement of all these components in the oxidase activity remains to be elucidated.

Macrophages behave metabolically in much the same way as neutrophils in response to phagocytic and a variety of surface stimuli, and the mechanism of $O_2^{\cdot-}$ production by these phagocytic cells is basically the same as in neutrophils (*40,41*).

Spin Trapping of the Oxygen-Centered Radicals

Reduction of ferricytochrome c, controlled by inhibition of the reaction by superoxide dismutase, as originally employed by Babior et al. (*4*) is commonly used to assay the production of $O_2^{\cdot-}$ by phagocytic cells. The reaction has the advantage that it can be followed continuously by optical spectroscopy (*42*). It should be borne in mind that regurgitation of MPO by phagocytosing neutrophils (*43*) may result in the reoxidation of ferricytochrome c, in the presence of H_2O_2 (*44*). Hydroxyl radicals ($OH^{\cdot}$), formed as a consequence of $O_2^{\cdot-}$ production, may also reduce ferricytochrome c by a mechanism whereby a radical formed after hydrogen is abstracted from the outer surface of the protein reduces the protein by electron tunnelling (*45*). Inhibition by superoxide dismutase alone would not distinguish this component of ferricytochrome c reduction.

Electron spin resonance (ESR) is considered to be the least ambiguous technique for the detection of free radicals. However, even when concentrations of $O_2^{\cdot-}$ exceed those normally required for detection (10^{-8} M) no ESR spectrum is observed in aqueous solution under physiological conditions. This is because of the very short relaxation time of the $O_2^{\cdot-}$ radical. However, the technique of spin trapping allows the formation of stable free radical products thereby permitting their detections. The main disadvantages of using the technique

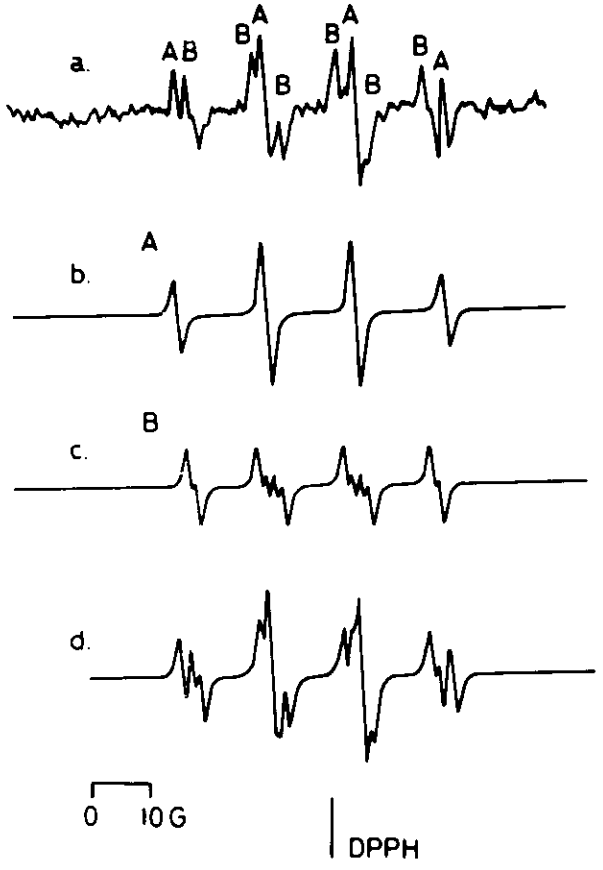

FIGURE 1. Bacille Calmette Guerin-elicited macrophages (4×10^6/mL) preincubated with 10 mg/mL PMA for 5 min at 37° C in the presence of 1 mM diethylenetriaminepentaacetic acid and 100 mM DMPO in Hanks' balanced salts solution, pH 7.4 produced two spin adducts in the spectrum which is an average of four scans from $t=5$ min to $t=9$ min. The adducts are labeled A and B. Computer-simulated spectra of components A and B (*b,c*) can be combined in propertions of 46%A and 54%B to give a composite spectrum (*d*) which is in good agreement with the experimental spectrum in (*a*). The ESR spectral parameters for the two adducts were found to be in agreement with those reported for DMPO-OH [component A in (*b*)] and for DMPO-OOH [component B, (*c*)] in Table 1. Reproduced with permission from Hume et al. (*71*).

for $O_2^{\dot{-}}$ detection are (i) most $O_2^{\dot{-}}$ spin adducts are relatively unstable and decay rapidly to nonradical species, and (ii) the rate constants for the spin trapping of $O_2^{\dot{-}}$ are usually low. A high concentration (60–200 mM) of spin trap is used to allow effective competition with spontaneous dismutation.

The spin-trapping agents used for $O_2^{\dot{-}}$ detection are usually nitrones and details of the technique have been reported (*46*). The most commonly used nitrone is 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) which reacts with $O_2^{\dot{-}}$ to form 5,5-dimethyl-2-hydroperoxypyrroline-*N*-oxide (DMPO-OOH) and with $OH^{\dot{}}$ to form 5,5-demethyl-2-hydroxypyrolline-*N*-oxide (DMPO-OH). A typical spectrum is given in Figure 1. The g factors, hyperfine components, and splitting constants for the spin adducts of DMPO and other nitrones are given in Table 1.

Green et al. (*47*) were the first to use the spin trap DMPO to detect $O_2^{\dot{-}}$ production by stimulated neutrophils. This work also represents the first use of spin trapping with intact cells. The ESR spectrum of neutrophils stimulated with phorbol myristate acetate (PMA) was consistent with that of a mixture of the products of the reaction of DMPO with $OH^{\dot{}}$ and $O_2^{\dot{-}}$. In neutrophils stimulated with IgG-coated latex particles, the ESR spectrum was that expected from the spin adduct DMPO-OH. In this system a contribution of the spin adduct DMPO-OOH was seen in the presence of azide. No spin adduct formation was observed in the presence of superoxide dismutase. However, the spectrum of the DMPO-OH was still observed, with reduction in intensity, in the presence of catalase.

Green et al. (*47*) and Rosen and Klebanoff (*48*) were able to detect only the DMPO-OH spin adduct with neutrophils stimulated with opsonized zymosan. The signal could be virtually abolished by superoxide dismutase, modestly decreased by catalase, and appreciably decreased by mannitol. Neutrophils from a patient with CGD did not show the DMPO-OH signal, while neutrophils from a patient with hereditary MPO deficiency showed an enhanced signal with 25% increase in intensity. The signal was reduced to the intensity level seen with normal neutrophils by addition of purified MPO to the incubation mixture (*48*). It was strongly suppressed by superoxide dismutase or mannitol but not by catalase.

It has been suggested that the work of Green et al. (*47*) and Rosen and Klebanoff (*48*) represents initial trapping of $O_2^{\dot{-}}$ (and $HO_2^{\dot{}}$) by DMPO to form DMPO-OOH and subsequent breakdown of DMPO-OOH to DMPO-OH, with only a small amount of DMPO-OH coming from the trapping of $OH^{\dot{}}$ itself (*49*). We can write:

$$\text{DMPO} + O_2^{\dot{-}} \xrightarrow{H_2O} \text{DMPO-OOH} + OH^- \qquad k_7 = 10\ M^{-1}sec^{-1} \tag{7}$$

$$\text{DMPO} + HO_2^{\dot{}} \rightarrow \text{DMPO-OOH} \qquad k_8 = 6.6 \times 10^3\ M^{-1}sec^{-1} \tag{8}$$

$$\text{DMPO} + OH^{\dot{}} \rightarrow \text{DMPO-OH} \qquad k_9 = 2.1 \times 10^9\ M^{-1}sec^{-1} \tag{9}$$

It may be noted that DMPO can best compete with spontaneous dismutation of $O_2^{\dot{-}}$ at high pH because of low pH the advantage of the higher rate constant of reaction (8) relative to reaction (7) is cancelled by the increased rate of dismutation of $O_2^{\dot{-}}$ (*49*). The rate constants shown for reactions (7) to (9) are those found by Finkelstein et al. (*50*), k_9 being the rate constant observed with $OH^{\dot{}}$ generation by a Fenton system. The spin adduct of DMPO-OOH is more stable at acid than alkaline pH. Half-life values of 80 sec at pH 6 and approximately 35 sec at pH 8 (*51*) have been reported for the adduct. Finkelstein et al. (*52*) observed that DMPO-OOH can decompose to yield DMPO-OH. In this work

Table 1. Hyperfine splitting constants of spin adducts.

Spin adducts	Hyperfine splitting constants (aqueous solutions)			
	g	a_N, G	a_H^β, G	a_H^γ, G
Superoxide spin adducts				
5-Dimethyl-2-hydroperoxypyrrolidino-*N*-oxide (DMPO-OOH)	2.0061	14.3	11.7	1.25
2-Hydroperoxy-3,5,5-trimethylpyrrolidino-*N*-oxide (TMPO-OOH)	2.0060	15.6	—	—
t-Butyl-α-hydroperoxybenzyl nitroxide (PBN-OOH)	2.0057	14.3	2.25	—
Hydroxyl spin adducts				
5,5-Dimethyl-2-hydroxypyrrolidino-*N*-oxide (DMPO-OH)	2.0050	14.9	14.9	—
2-Hydroxy-3,5,5-trimethylpyrrolidino-*N*-oxide (TMPO-OH)	—	15.7	—	—
t-Butyl-α-hydroxybenzyl nitroxide (PBN-OH)	2.0061	15.5	2.75	—

SOD depressed both DMPO-OOH and DMPO-OH spin adduct signals while catalase did not totally abolish the signal. This argues in favor of decomposition of DMPO-OOH to DMPO-OH; however, no such effect was observed with spin trapping of $O_2^{\dot{-}}$ from isolated guinea pig neutrophil NADPH oxidase (*38*) and macrophages stimulated with PMA (*71*).

Hydroxyl radical formation by phagocytosing neutrophils and macrophages is presumed to proceed by the Fenton reaction,

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^- \quad (10)$$

and redox cycling of the metal by $O_2^{\dot{-}}$,

$$Fe^{3+} + O_2^{\dot{-}} \rightarrow Fe^{2+} + O_2 \quad (11)$$

in phagocytosing neutrophils. The sum of reactions (10) and (11) is

$$O_2^{\dot{-}} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^- \quad (12)$$

Reaction (12) was called the Haber-Weiss reaction by Beauchamp and Fridovich (*53*), since it occurs in the reaction scheme of Haber and Weiss (*54*) to explain the catalytic decompostion of H_2O_2 by iron salts. This name is now sanctioned by usage. There are many difficulties concerning this reaction, not the least being the manner in which it occurs. The reaction is thermodynamically feasible (*55*) but kinetically hindered having a negligible rate constant (*56*). Metal catalysis of the reaction has been demonstrated with Fe-EDTA (*57,58*), the estimated rate constant of the catalyzed reaction being approximately $1.0 \times 10^3\ M^{-1}sec^{-1}$ (*57*). Of biological interest is the occurrence of the reaction with iron chelates of ADP (*59*) or ATP (*60*) and picolinic acid (*61*), with nonprotein-bound iron of body fluids (*62,63*), and with the iron associated with transferrin (*57,64,65*), lactoferrin (*66–68*), and ferritin (*69*).

The occurrence of the Haber-Weiss reaction in the presence of lactoferrin (Fig. 2) has a direct bearing in the production of $OH^{\cdot}$ radicals by phagocytosing neutrophils since these cells release lactoferrin into the phagolysosomes and extracellularly during degranulation (*70*). Ambruso and Johnston (*66*) showed that iron-saturated lactoferrin enhanced the production of $OH^{\cdot}$ radicals by human neutrophils stimulated with opsonized zymosan or PMA and by a particulate fraction prepared from the neutrophil homogenates. Production of $O_2^{\dot{-}}$ by purified NADPH oxidase obtained from guinea pig neutrophils (*24*), stimulated with PMA, has been demonstrated by spin trapping of the radical with DMPO. The signal observed was mainly that of DMPO-OOH (*38*). In the presence of iron-saturated transferrin (*64*), the intensity of the signal was reduced by about 40% indicating that the transferrin iron (Tr-Fe^{3+} was competing with DMPO for free $O_2^{\dot{-}}$ radicals,

$$Tr—Fe^{3+} + O_2^{\dot{-}} \rightarrow Tr—Fe^{2+} + O_2 \quad (13)$$

production of $OH^{\cdot}$ radicals resulted when H_2O_2 was added to the system,

$$Tr—Fe^{2+} + H_2O_2 \rightarrow Tr—Fe^{3+} + OH^{\cdot} + OH^- \quad (14)$$

The signal of the DMPO-OH spin adduct was resolved in the ESR spectrum by computer stimulation which gave $g = 2.0050$, $a_N = a_H = 14.9G$ (Fig. 3).

The Haber-Weiss reaction [reaction (12)], however catalyzed, should be inhibited by either superoxide dismutase or catalase. A large inhibition by catalase (~50%) of the intensity of the DMPO-OH spin adduct signal by human neutrophils stimulated with opsonized zymosan (*48*) and by Bacille Calmette-Guerin (BCG)-elicited mouse peritoneal macrophages activated with PMA (*71*). The intensity of the DMPO-OH spin adduct signal can be suppressed by about 70% by the $OH^{\cdot}$ radical scavengers mannitol (*48*), dimethyl sulfoxide (*71*) and the Fe^{3+} scavenger desferrioxamine (*71*) in stimulated neutrophils and macrophages. It is not clear why catalase does not totally abolish $OH^{\cdot}$ radical production. Catalase is however known to be ineffective as a scav-

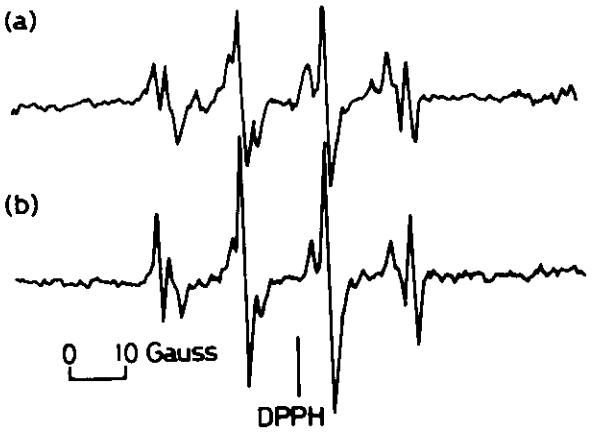

FIGURE 2. ESR spectra of DMPO-OH and DMPO-OOH generated from the xanthine-xanthine oxidase reaction. Reaction mixtures were (*a*) control: 40 mM xanthine, 0.090 μM/mL xanthine oxidase, 1mM diethylenetriaminepentaacetic acid and 100 mM DMPO in phosphate buffer, pH 7.8; (*b*) with lactoferrin: same as (*a*) but with 8.05μM lactoferrin. Reproduced with permission from Bannister et al. (*67*).

enger at low concentrations of H_2O_2. The use of $OH^{\cdot}$ radical scavengers still leaves 30% of the DMPO-OH spin adduct signal unaccounted for. Evidence for production of carbon-centered radicals in activated macrophages has been obtained by means of the lipophilic spin trap 5-octadecyl-5,3,3-trimethyl-1-pyrroline-*N*-oxide (*71*). However no clear relationship has been established between these radicals and the unaccounted DMPO-OH spin adduct signal.

An alternative mechanism to the iron-catalyzed Haber-Weiss reaction has been proposed to explain $OH^{\cdot}$ radical production by phagocytosing neutrophils. These cells release MPO from the azurophil granules during degranulation (*43*). This results in catalysis of the reaction:

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^- \qquad (15)$$

HOCl can react with $O_2^{\cdot -}$, possibly producing $OH^{\cdot}$ (*72*).

$$HOCl + O_2^{\cdot -} \rightarrow OH^{\cdot} + O_2 + Cl^- \qquad (16)$$

The rate constant of reaction (16), evaluated according to Long and Bielski (*72*), is $4.2 \times 10^6 M^{-1} sec^{-1}$ at pH 7.4, representing extracellular pH, and $7.5 \times 10^6 M^{-1} sec^{-1}$ at pH 5.0, representing phagolysosomal pH. Hill and Okolow-Zubkowska (*73*) pointed out that the sum of reactions (15) and (16) is reaction (12), the Haber-Weiss reaction. The evidence for this route to the $OH^{\cdot}$ radical is that azide, which inhibits MPO, caused the appearance of the signal attributable to the DMPO-OOH spin adduct in the ESR spectrum of phagocytosing neutrophils (*47*).

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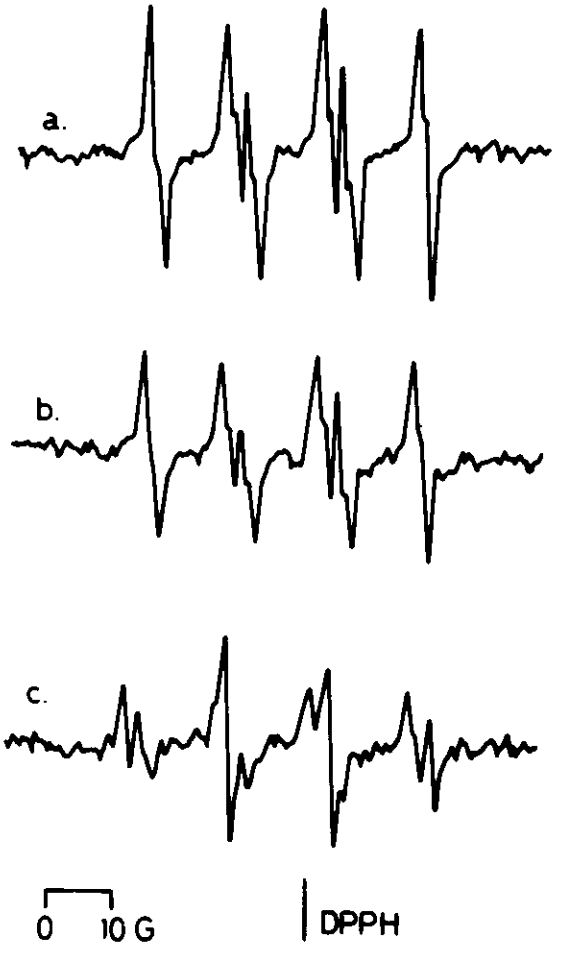

FIGURE 3. Spin trapping by 100 mM DMPO of (*a*) DMPO-OOH formed by NADPH oxidase; (*b*) DMPO-OOH formed by NADPH oxidase in the presence of transferrin; (*c*) DMPO-OOH formed by NADPH oxidase in the presence of transferrin and H_2O_2. Reproduced with permission from Bannister et al. (*64*).

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