We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter

Essence of Reducing Equivalent Transfer Powering Neutrophil Oxidative Microbicidal Action and Chemiluminescence

Robert C. Allen

Abstract

Neutrophil leukocytes provide first-line phagocytic defense against infection. Phagocyte locomotion to the site of infection, identification, and phagocytosis of the infecting microbe results in metabolically driven O_2-dependent combustive microbicidal action. NADPH oxidase activity controls this respiratory burst metabolism. Its flavoenzyme character allows semiquinone-mediated crossover from two reducing equivalents (2RE) to 1RE transfer, as is necessary for univalent reduction of O_2 to the acid hydroperoxyl radical (HO_2) and its conjugate base, superoxide anion (O_2^-). RE transfer dynamics is considered from the perspectives of quantum and particle physics, as well as frontier orbital interactions. Direct disproportionation of HO_2-O_2^- yields electronically excited singlet molecular oxygen ($^1O_2{}^*$) and hydrogen peroxide (H_2O_2). Myeloperoxidase catalyzes H_2O_2-dependent 2RE oxidation of chloride (Cl^-) to hypochlorite (OCl^-). Direct nonenzymatic reaction of OCl^- with an additional H_2O_2 yields Cl^-, H_2O, and $^1O_2{}^*$. Thus, for two 2RE metabolized through NADPH oxidase, a total of three $^1O_2{}^*$ are possible. H_2O_2, OCl^-, and $^1O_2{}^*$ generated are all singlet multiplicity reactants and can participate in spin-allowed combustive oxygenations yielding light emission, that is, luminescence or chemiluminescence. The sensitivity of luminescence for measuring neutrophil redox activities is increased several orders of magnitude by introducing chemiluminigenic probes. Probes can be selected to differentiate oxidase from haloperoxidase activities.

Keywords: neutrophil, respiratory burst, reducing equivalent, combustion, frontier orbital, spin quantum number, NADPH oxidase, myeloperoxidase, Wigner spin conservation, Hund's maximum multiplicity rule, boson, fermion

1. Introduction

There is a complicated hypothesis, which usually entails an element of mystery and several unnecessary assumptions. This is opposed by a more simple explanation, which contains no unnecessary assumptions. The complicated one is always the popular one at first, but the simpler one, as a rule, eventually is found to be correct. This process

> *frequently requires 10–20 years. The reason for this long time lag was explained by Max Planck. He remarked that scientists never change their minds, but eventually they die.*

> John H. Northrop, 1961 [1].

Appreciating why combustion is not spontaneous, how electrons are transferred biologically, and the unusual nature of oxygen reactivity were difficult for me as a student. So, in addition to biochemical studies, my mentor Richard Steele suggested I study the writings of Herzberg and others. Although challenging, such exposure shook open the door to other perspectives. Fundamental quantum and particle physics considerations were entertained with regard to oxygen and biologic electron transfer. My epiphany was in recognizing that the polymorphonuclear neutrophil, a leukocyte familiar to me from clinical laboratory experience, might realize the electronegative potential of oxygen for combustive microbicidal action by changing the spin multiplicity of oxygen. The following, taken from a symposium abstract presented in 1972, succinctly describes that position [2]. "Recently, a chemiluminescence (CL) has been observed when human polymorphonuclear leukocytes (PMN) phagocytize bacteria or particulate matter. The CL response correlates well with the stimulation of the hexose monophosphate shunt, which results in the generation of NADPH. The PMN possesses both CN^--insensitive NADH and NADPH oxidases. Flavoproteins oxidases of this type are capable of univalent reduction of O_2. The reduced oxygen ($\cdot O_2^-$, $\cdot O_2H$) may then disproportionate yielding HOOH and singlet molecular oxygen $^1O_2$. The PMN also possesses a CN^--sensitive peroxidase, myeloperoxidase, which has microbicidal activity in the presence of HOOH and halide. In this reaction, the HOOH is reduced to OH^- with the oxidation of the halide to a reactive halogonium species. In cases where the halogonium formed is Cl^+ or Br^+, there is potential for further reaction with HOOH resulting in the generation of a haloperoxy anion. This unstable species can disintegrate to yield the original halide and $^1O_2$. $^1O_2$ has been demonstrated to be a potent microbicidal agent. Therefore, the biochemical generation of $^1O_2$ by the PMN might be closely associated with microbicidal activity. The CL response may be the result of the relaxation of excited carbonyl groups generated via $^1O_2$-mediated oxidations."

Neutrophil leukocytes and monocytes play an essential role in innate phagocytic defense against infection. Immune surveillance mechanisms detect the presence of potentially pathologic microbes and generate the chemical signals that mobilize circulating neutrophils and prime the expression of receptors necessary for neutrophil navigation and phagocytosis. Contact of a primed neutrophil with activated endothelium is followed by neutrophil diapedesis into the tissue interstitial space, and locomotion to the site of infection guided by concentration gradients of complement anaphylatoxin, microbial products, cytokines, and lipid factors. Once an immunologically primed neutrophil contacts an opsonin-labeled pathogen, phagocytosis occurs. Phagocytosis is associated with a constellation of metabolic changes classically referred to as the "respiratory burst" [3]. This presentation focuses on the neutrophil redox mechanisms necessary for microbicidal action, especially the roles of NADPH oxidase and myeloperoxidase (MPO) in lethal microbicidal oxygenations. The Merriam-Webster dictionary defines combustion as a chemical reaction that occurs when oxygen combines with other substances to produce heat and usually light. By changing the spin multiplicity of oxygen from triplet to doublet, and then to singlet, neutrophils remove the spin barrier to direct oxygenation, enabling direct oxygen combustive microbicidal action with associated light emission, that is, chemiluminescence or luminescence [4].

2. Respiratory burst

The neutrophil "respiratory burst" describes the large increases in glucose consumption via the hexose monophosphate shunt (aka, pentose pathway) [5, 6], and in nonmitochondrial molecular oxygen (O_2) consumption [7] associated with phagocytosis, and required for microbicidal action. Appreciating the underlying necessity for such metabolic changes provides perception into oxygen chemistry and biochemistry, radical reactivity and combustion in general. The character of electron transfer mediated by the dehydrogenases of the hexose monophosphate (HMP) shunt is common to cytoplasmic redox reactions. Such oxidation-reduction transfers typically involve movement of two reducing equivalents (2RE), that is, 2 electrons (e^-) and 2 protons (H^+), from an organic substrate catalyzed by a dehydrogenase. In turn, the dehydrogenase mobilizes the 2RE by transfer to nicotinamide adenine dinucleotide (phosphate) NAD(P)$^+$ generating its reduced form NAD(P)H. The cofactors NADPH and NADH serve as the cytoplasmic redox carriers for 2RE transfers between dehydrogenases and oxidases, and are common to various pathways of cytoplasmic metabolism. Consumption of 2RE carried by NADPH returns it to NADP$^+$. Availability of NADP$^+$ is rate limiting for HMP shunt dehydrogenase activity. Dehydrogenation is a type of oxidation that does not require or directly involve O_2. Glucose-6-phosphate (G-6-P) dehydrogenase, the initiator enzyme of the HMP shunt removes a total of 2RE and transfers the 2RE to NADP$^+$ producing NADPH. The point for emphasis is that 2RE are transferred, not one. Such 2RE transfer, sometimes referred to as hydride ion (H^-) transfer, is the rule for cytoplasmic redox reactions [8].

Respiratory burst metabolism results from the activation of NADPH oxidase. Like many oxidases, NADPH oxidase is a flavoenzyme. Flavoenzymes are mechanistically unique in that 2RE reduction, by cofactors such as NAD(P)H, is followed by a series of 1RE oxidations. In its 1RE form, the riboflavin prosthetic group of flavin adenine dinucleotide (FAD) is in the semiquinone state [9, 10]. This semiquinone capability, usually in combination with a cytochrome component, allows the oxidase to transition from 2RE transfer to 1RE transfers. As such, flavoenzymes are the junction enzymes where 2RE transfer proceeds as 1RE cytochrome transfers, for example, the mitochondrial electron transport system or the microsomal cytochrome-P450 mixed-function oxidase system [10, 11]. Flavoprotein oxidases are also capable of catalyzing the 1RE reduction of O_2 [12, 13]. As such, phagocytosis-associated activation of NADPH oxidase opens the possibility for univalent, that is, 1RE, reduction of O_2.

The molecular oxygen we breathe has unique physical-chemical characteristics. In its ground, that is, lowest energy state, oxygen is a diradical, paramagnetic molecule with triplet spin multiplicity [$^3O_2$; the preceding superscripted (^{3}) indicates multiplicity]. These spin characteristics guarantee a tendency for $^3O_2$ to participate in 1RE reduction yielding the doublet multiplicity hydroperoxyl radical ($^2HO_2$) and its conjugate base, the superoxide anion radical ($^2O_2^-$) [2, 4, 14, 15]. Such reduction does not produce radical character; it decreases such character.

2.1 Bosonic character of coupled fermionic electron transfer

Movement of 2RE is the transfer of an electron couple, that is, an orbital pair of electrons. Such 2RE transfers are the rule in cytoplasmic redox reactions. Considered from the perspective of particle physics, movement of a single electron (1RE) is quite different from paired electron (2RE) movement. Transfer of 1RE is a fermionic transfer. An electron is a fermion, and fermions have wave functions that are antisymmetric to exchange of particles; that is, $\Psi(a, b) = -\Psi(b, a)$. Fermions anti-commute; that is, $a \times b \neq b \times a$. Rotating a fermion through 360°,

Ψ — 360° → $-\Psi$, changes the phase, but does not return the fermion to its original state. An additional 360° rotation, $-\Psi$ —360° → Ψ, is required to return the anti-symmetric particle to its original state [16]. Fermions obey Fermi-Dirac statistics.

A fermionic electron is defined by its five quantum numbers: n, l, m_l, s, and m_s [17]. The spin number, s, describes the intrinsic angular momentum of the electron independent of orbital motion, and has a value of ½$\hbar$ (abbreviated to ½). This quality has no analogy in classical physics. The total spin angular momentum, S, of an atom or molecule is expressed by the equation $S = \sqrt{[s(s+1)]}\hbar$. s gives rise to the quantum number m_s, and only two values are allowed. When m_s = ½, the e^- is described as spin up (↑); when m_s = −½, the e^- is described as spin down (↓). The Pauli exclusion principle states that the total wave function for a system must be antisymmetric to the exchange of any pair of electrons. Differently stated, no two electrons of a given atom or molecule can have identical quantum numbers, and for two electrons to occupy an orbital, each electron must have opposite spins, that is, one orbital e^- must have an m_s = ½ (↑), the other orbital e^- must have an m_s = −½ (↓). Consequently, the total spin quantum number, S, for a filled orbital electron-couple is ½ + −½ = 0 (↑↓).

Bosons obey Bose-Einstein statistics, and have wave functions that are symmetric to exchange of a pair of particles; that is, $\Psi(a, b) = \Psi(b, a)$. They obey ordinary commutation, that is, $a \times b = b \times a$. Rotating a boson through 360°, Ψ — 360° → Ψ, returns it to its original state. Bosons, for example, photons are symmetric particles with integral spin. Likewise, a spin-balanced composite of fermionic particles, for example, an alpha particle with an S of 0, is bosonic. With regard to biochemical redox reactions, the coupling of antisymmetric fermions, for example, the coupled electrons of an orbital pair, result in a S = 0 state with bosonic symmetry. The transfer of 2RE describes the movement of a coupled electron pair with an S = 0 and is in essence a bosonic transfer.

2.2 Bosonic versus fermionic frontier orbital interactions

Chemistry is about the frontier orbital interactions of atoms and molecules [18]. The focus of frontier orbital theory is on the initial orbital conditions of the reactants and on reactive transition to product(s) with emphasis on the highest occupied atomic or molecular orbital (HO(A)MO) and the lowest unoccupied atomic or molecular (LU(A)MO) orbital. The frontier orbital of a radical reactant is neither empty nor completely filled, and as such, is described as a singly occupied atomic or molecular orbital (SOAO or SOMO). Atomic and molecular orbitals, including frontier orbitals, can have bosonic or fermionic character [19, 20]. A HO(A)MO has an S = 0. Such an atom or molecule has singlet spin multiplicity with nonradical, diamagnetic character. A radical SO(A)MO has an S = ½ or −½, and has doublet spin multiplicity with radical, paramagnetic character.

The bosonic character of the HOMO of a nonradical reactant differs fundamentally from the fermionic character of the SOMO of a radical reactant. The fermionic nature of a SOMA limits overlap possibilities with bosonic HOMO. If such reaction occurs, the fermionic character must be preserved in the product. The electronegative Fenton radical (^{2}OH) can extract 1RE from the HOMO of a singlet multiplicity nonradical substrate (^{1}substrate) yielding singlet multiplicity ^{1}H$_2$O, but in the process the HOMO of the substrate is converted to a SOMO, that is, the substrate becomes a doublet multiplicity radical (^{2}substrate). The symmetry of the reactants is preserved in the products. If a fermionic (doublet)-bosonic (singlet) reaction occurs, symmetry will be retained in the bosonic (singlet)-fermionic (doublet) products. Consistent with the Wigner-Witmer rules described in **Table 1**, spin symmetry is conserved [19–22].

The fermionic character of two radical reactants is eliminated in reactive bonding yielding a bosonic product. As described in **Table 1**, fermionic radical-radical, SOMO-SOMO reaction yields bosonic nonradical product. Simply stated, radicals

tend to react with radicals, and such doublet-doublet annihilations yield nonradical, that is, bosonic, product. Such reaction is responsible for terminating radical chain propagation reactions.

Molecular oxygen in its ground state has unique triplet spin multiplicity [23]. Its two degenerate, that is, equal energy, frontier orbitals are each populated by a single electron. These two SOMO electrons obey Hund's maximum multiplicity rule, that is, the electron in each degenerate SOMO will have the same spin [24]. As illustrated in **Figure 1**, the S value for molecular oxygen is ½ + ½ or −½ + −½, and thus, the multiplicity is triplet, that is, 2|1 or −1| + 1 = 3. This bi-radical, bi-fermionic character is responsible for the paramagnetic character of $^3O_2$. The high electronegativity of $^3O_2$ predicts potential for highly exergonic reactions with nonradical, singlet multiplicity organic molecules, but thermodynamic potential does not guarantee reactivity, and combustion is not spontaneous. Taking a different perspective, it is

Reactants	Products
Singlet + Singlet bosonic + bosonic	Singlet bosonic
Singlet + Doublet bosonic + fermionic	Doublet fermionic
Singlet + Triplet bosonic + bi-fermionic	Triplet bi-fermionic
Doublet + Doublet fermionic + fermionic	Singlet bosonic
Doublet + Triplet fermionic + bi-fermionic	Doublet fermionic
Triplet + Triplet bi-fermionic + bi-fermionic	Singlet bosonic

Spin multiplicity states with regard to the bosonic-fermionic character of reactants and products.

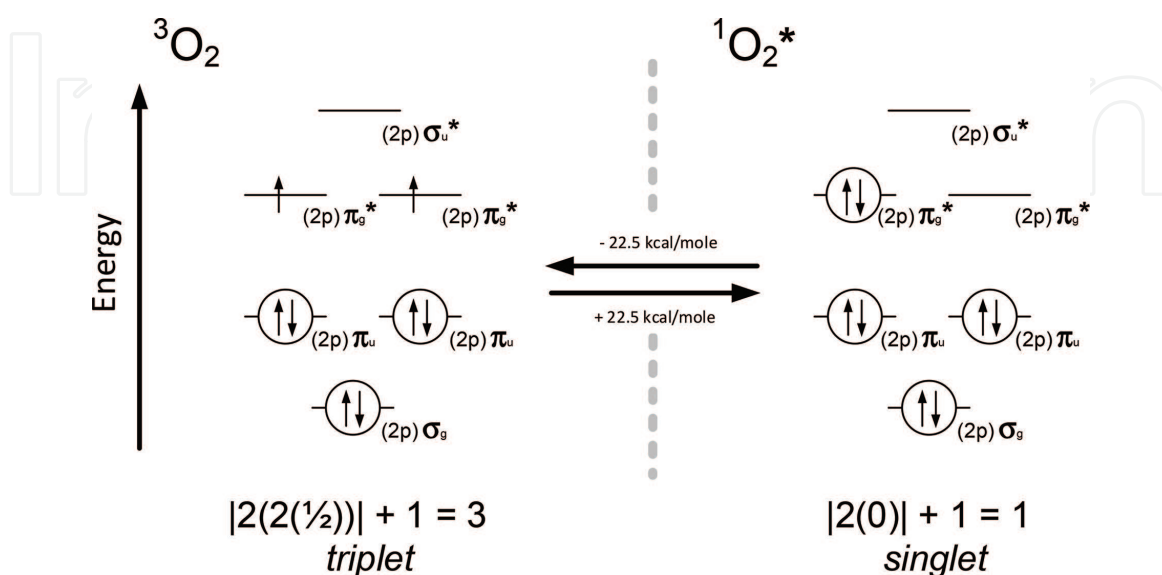**Table 1.**
Spin conservation rules.

Figure 1.
Triplet and electronically excited singlet molecular oxygen with emphasis on the π* (pi antibonding) frontier orbitals. The two π* are degenerate (same energy level). Hund's maximum multiplicity rule predicts lowest energy is achieved when each SOMO electron has the same spin, that is, the triplet state ($^3O_2$). The electronic energy of $^1O_2^*$ is 22.5 kcal/mol (94.2 kJ/mol) above $^3O_2$.

the bi-fermionic, bi-radical nature of $^3O_2$ that restricts its reactive potential. As per **Table 1**, the reaction of $^3O_2$ with a bosonic ^{1}substrate molecules is spin symmetry restricted, and could only result in the improbable generation of a bi-fermionic, triplet multiplicity product(s). However, the reaction of bi-fermionic $^3O_2$ with a fermionic (doublet multiplicity) radical can proceed via SOMO-SOMO overlap. As per **Table 1**, such a doublet-triplet reaction will generate a fermionic (doublet multiplicity) radical product. Thus, $^3O_2$ can participate in and be a necessary reactant in radical propagation reactions.

3. NADPH oxidase

NADPH oxidase controls "respiratory burst" metabolism, microbicidal action, and chemiluminescence [15, 25]. The oxidase (Nox2) is a complex flavoenzyme, and a member of the Nox family of enzymes involved in various biochemical activities [26–29]. More specifically, NADPH oxidase is a flavocytochrome enzyme composed of a large membrane-bound glycoprotein (gp91^{phox}) subunit associated with a smaller protein (p22^{phox}). The C-terminal portion of gp91^{phox} subunit contains the NADPH and flavin adenine dinucleotide (FAD) binding sites and an N-terminal portion that binds two heme groups. The activation of the oxidase is complex and involves other components. Association with the p67^{phox} component is essential for full activity. The present treatment will focus on the central role of the semiquinone state of the riboflavin component of FAD and heme involvement in splitting the 2RE from ^{1}NADPH and facilitating 1RE reduction of $^3O_2$.

As illustrated in **Figure 2**, the product of 1RE reduction of $^3O_2$ is the acid hydroperoxyl radical ($^2HO_2$) with an acid dissociation constant pK_a of 4.8 [30]. For comparison, the pK_a of $^1H_2O_2$ is 11.7. As the pH of the phagolysosomal space approaches the pK_a, the ratio of $^2HO_2$ to its conjugate base, the superoxide anion ($^2O_2^-$) approaches unity, and acid disproportionation, that is, reaction of $^2HO_2$ with $^2O_2^-$, approaches maximum reaction rate. At unity, anionic repulsion is no longer a problem. The rate constant for the reaction is 4.5×10^5 M^{-1} s^{-1} at pH 7.0 and reaches a maximum of 8.5×10^7 M^{-1} s^{-1} at pH 4.8 [30, 31]. From the frontier orbital perspective, this is a SOMO-SOMO reaction that yields the nonradical (singlet multiplicity) products $^1H_2O_2$ and $^1O_2^*$. As per **Table 1**, doublet-doublet annihilation yields single products [15, 32]. The reaction is sufficiently exergonic to yield $^1O_2^*$ with an energy of 22.5 kcal/mol (94.1 kJ/mol) above ground state $^3O_2$.
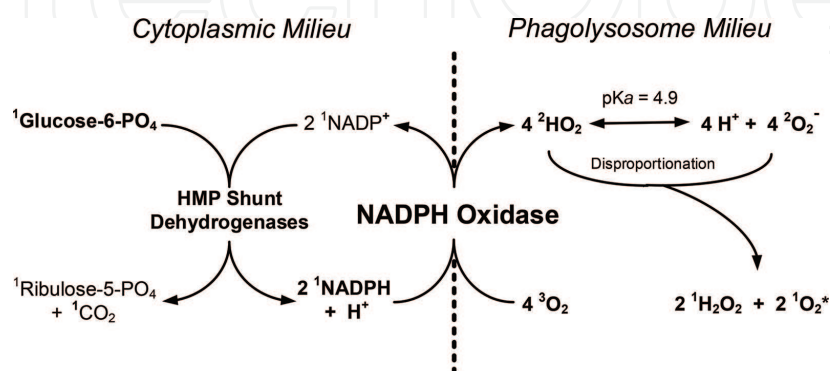

Figure 2.
Schema illustrating the central role of membrane-associated NADPH oxidase in respiratory burst metabolism. In the activated state, the Michaelis constant (K_M) of the oxidase for NADPH is decreased. NADP$^+$ availability controls the activities of glucose-6-PO$_4$ dehydrogenase and 6-phosphogluconate dehydrogenase of the HMP shunt. Each pass of the cycle generates two NADPH, that is, two 2RE. In the schema, the spin multiplicities of each molecule are indicated by the superscripted number preceding the molecular description, that is, ^{1}, ^{2}, and ^{3} for singlet, doublet, and triplet multiplicity, respectively.

In **Figure 2**, note that all reactions in the cytoplasmic milieu are singlet multiplicity nonradical reactions and that radical production is confined to the phagolysosomal milieu. The 2RE nature of cytoplasmic redox transfer provides a bosonic barrier to reaction with bi-fermionic $^3O_2$. Transfer of an orbital electron couple is nonradical, bosonic, and singlet multiplicity. In an atmosphere that is 20.9% $^3O_2$, the presence of a doublet multiplicity molecule is an opportunity for SOMO-SOMO overlap. The 2RE transfer from the HOMO of a reductant to the LUMO of an oxidant maintains the bosonic $S = 0$ condition.

The $S = 0$ condition is described by Dirac's statement that "If a state has zero total angular momentum, the dynamical system is equally likely to have any orientation, and hence spherical symmetry occurs" [33]. In addition to providing protection from the reactive consequences of fermionic 1RE transfer in an atmosphere high in $^3O_2$, 2RE transfer of a bosonic orbital electron couple may have additional advantage. Heisenberg's uncertainty principle states that the uncertainty of momentum (Δp) multiplied by the uncertainty of position (Δx) is always equal to or greater than $\frac{1}{2}\hbar$, that is, $\Delta p \Delta x \geq \frac{1}{2}\hbar$ [17]. With regard to 2RE transfer, the bosonic orbital electron couple has $S = 0$. Consequently, the positional uncertainty of the electron-couple must be proportionally large. The $S = 0$ nature of HOMO-LUMO redox transfer involving a 2RE orbital electron-couple opens the possibility that such transfer is facilitated by quantum tunneling. The nature of such transfer would be analogous to the emission of a bosonic $S = 0$ alpha particle from an atomic nucleus in alpha radiation decay [19, 20].

4. Myeloperoxidase

Myeloperoxidase (MPO) is a unique green cationic homo-dimeric glycosylated heme-a protein that is highly expressed in neutrophil leukocytes, making up about 5% of its dry mass [34, 35]. It is also synthesized to a lesser degree in monocytes and serves as a cellular marker for both neutrophils and monocytes. MPO synthesis occurs only during the promyelocyte phase of neutrophil development [36]. During the promyelocyte phase, MPO and other cationic lysosomal proteins are synthesized and stored in the azurophilic (aka primary) granules. Each mitotic division during the following myelocyte phase of development dilutes the azurophilic granule content per neutrophil by a half. Under normal conditions of hematopoietic production, these myelocytic phase mitoses are the rule, but under condition of neutrophil inflammatory consumption or G-CSF-stimulated marrow production, the promyelocyte pool is expanded, and there are fewer mitoses in the myelocyte phase of development. Neutrophils released into the circulation following a few days of myelopoietic stimulation show the effect of decreased myelocyte mitoses. These neutrophils are significantly increased in size due to greater azurophilic granule retention, and the MPO activity per neutrophil is severalfold higher than normal [37].

4.1 Electrochemistry of halide oxidation-reduction

MPO, like eosinophil peroxidase, lactoperoxidase and thyroperoxidase, is a haloperoxidase (XPO). However, MPO is unique in its ability to catalyze the pH-dependent oxidation of chloride [38–40]. Based on the Allen scale, fluorine (F) is the most electronegative element with a value of 4.19, followed by oxygen with a value of 3.61, then chlorine with a value of 2.87, bromine with a value of 2.69, and iodine with a value of 2.36 [41].

With regard to chloride oxidation, the Nernstian electrochemical possibilities and limitations are as follows [11, 42].

$$E = E_0 - (RT/nF) \ln [\text{reduced}]/[\text{oxidized}] \tag{1}$$

where E is observed potential (in volts), E_0 is the standard potential (in volts), R is the gas constant, T is the absolute temperature, F is a faraday (23 kcal/absolute volt equivalent), and n is the number of electrons/gram equivalent transferred.

Also, appreciate that hydrogen ion concentration, $[H^+]$, has an effect on redox chemistry.

$$E = (RT/F) \ln [H^+]/[P_{H2}]^{1/2} \tag{2}$$

P_{H2} is the partial pressure of H_2 gas

$$E = (2.3RT/F) \log [H^+] = 0.06 \log [H^+] = -0.06 \text{ pH} \tag{3}$$

For the reaction, $A_{red} + B_{ox} \leftrightarrow B_{red} + D_{ox}$, the half reaction equations become:

$$E = E_0{}^A - (RT/nF) \ln [A_{red}]/[A_{ox}] \tag{4}$$

$$E = E_0{}^B - (RT/nF) \ln [B_{red}]/[B_{ox}] \tag{5}$$

$$E_0{}^B - E_0{}^A = (RT/nF) \left[\ln [B_{red}]/[B_{ox}] - \ln [A_{red}]/[A_{ox}] \right] \tag{6}$$

$$\Delta E_0 = (RT/nF) \left[\ln [A_{ox}][B_{red}]/\ln [A_{red}][B_{ox}] \right] \tag{7}$$

$$\Delta E_0 = (RT/nF) \ln K_{eq} \tag{8}$$

K_{eq} is the equilibrium constant. The change in potential (ΔE) can be expressed in terms of Gibbs free energy (ΔG).

$$\Delta G^0 = -RT \ln K_{eq} \tag{9}$$

$$\Delta G^0 = -nF \Delta E_0 \tag{10}$$

The schema of **Figure 3** depicts the MPO-catalyzed H_2O_2 oxidation of Cl^- to HOCl. Chloride serves as the reductant and undergoes a 2RE oxidization yielding a chloronium intermediate (Cl^+) that reacts with ^1H_2O to generate hypochlorous acid with a pKa of 7.5.

$$^1Cl^- \rightarrow {}^1Cl^+ + 2RE \tag{11}$$

$$^1Cl^+ + {}^1H_2O \rightarrow {}^1HOCl + H^+ \tag{12}$$
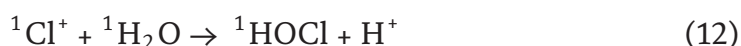
Note that $^1H_2O_2$ is the oxidant for the MPO-catalyzed reaction undergoing 2RE reduction yielding two waters. One ^1H_2O is consumed in the reaction described by Eq. (12).

$$^1H_2O_2 + 2RE \rightarrow 2^1H_2O \tag{13}$$

The reactants and products of this MPO-catalyzed redox reaction are exclusively singlet multiplicity, that is, nonradical [2, 15].

As depicted in **Figure 4**, increasing acidity, that is, lowering pH, increases the ΔE (i.e., $E_{H_2O_2} - E_{X^-}$) and the Gibbs free energy for all halides. The exergonicity of MPO-catalyzed 2RE dehydrogenation of Cl^- increases with increasing acidity. The required potentials for the various halides are consistent with their electronegativities. Dehydrogenation of Cl^- is more difficult than Br^-, but dehydrogenation of I^- is relatively easy. Whereas MPO is capable of dehydrogenating Cl^-, Br^-, and I^-, eosinophil peroxidase (EPO), lactoperoxidase, and thyroperoxidase are only capable of dehydrogenating Br^- and I^-.

The plots of **Figure 4** illustrate that increasing acidity increases the exergonicity of MPO-catalyzed $^1H_2O_2$-dependent oxidation of halides. This is especially import for MPO-catalyzed oxidation of chloride. Conversely, increasing alkalinity increases the exergonicity of the nonenzymatic $^1OCl^-$-$^1H_2O_2$ reaction yielding $^1O_2^*$ as depicted in **Figure 5**. The combined $^1H_2O_2$-driven haloperoxidase plus $^1H_2O_2$-driven $^1OCl^-$ generation of $^1O_2^*$ can be considered as a net disproportionation reaction, as depicted in **Figure 6**. $^1H_2O_2$ is the reactant common to both MPO-catalyzed reaction of **Figure 4** and the chemical reaction of **Figure 5**. The Gibbs free energies shown in
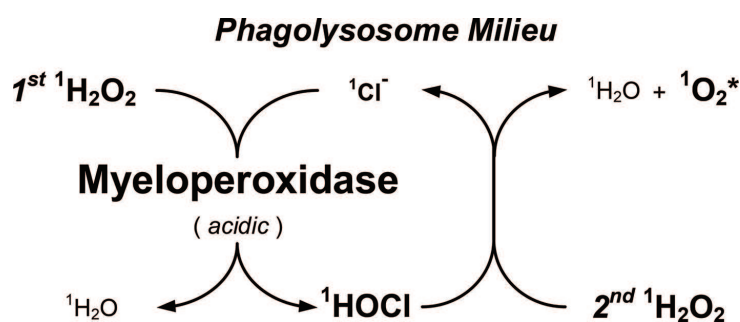
Figure 3.
Schema depicting myeloperoxidase-catalyzed H_2O_2-dependent oxidation of chloride to hypochlorite, and its reaction with a second H_2O_2 to generate $^1O_2^*$. The spin multiplicity of each molecule is indicated by the superscripted number preceding the molecular description.
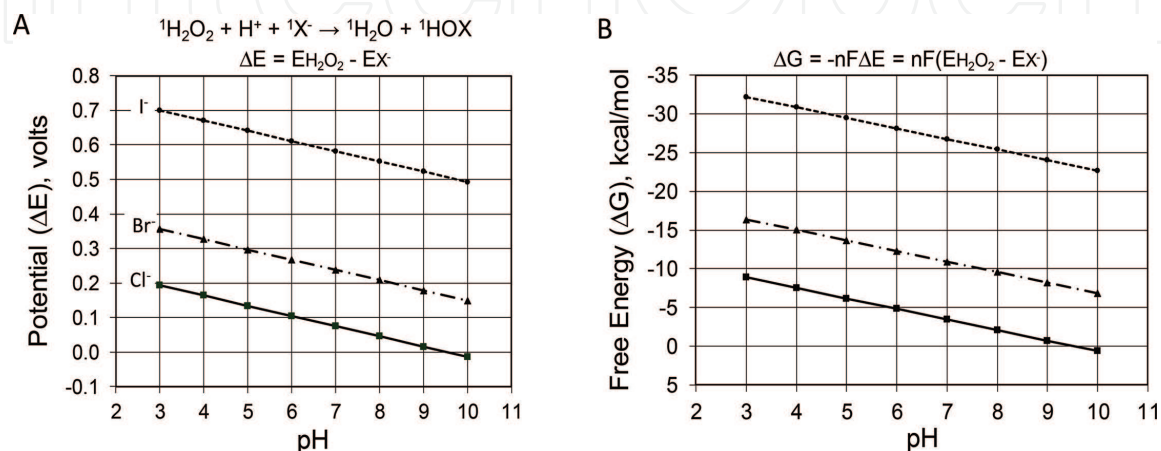
Figure 4.
*Graph **A** plots changes in potential (ΔE) and graph **B** plots change in Gibbs free energy against pH for various halides. From bottom to top, the plotted lines represent chloride (lowest), bromide (middle) and iodide (highest).*
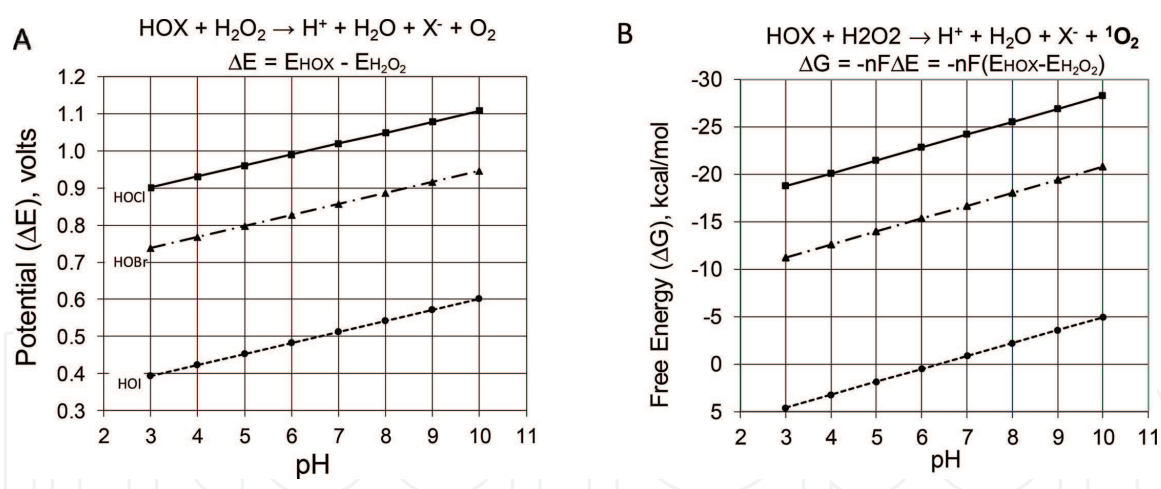
Figure 5.
*Graph **A** plots changes in potential (ΔE) and graph **B** plots change in Gibbs free energy with respect to pH for various halides for the reaction of $^1H_2O_2$ with $^1OCl^-$. In graph **B** the Gibbs free energies are adjusted for the 22.5 kcal mol^{-1} retained as the electronic energy of $^1O_2{}^*$, that is, the difference separating $^3O_2$ from $^1O_3{}^*$.*
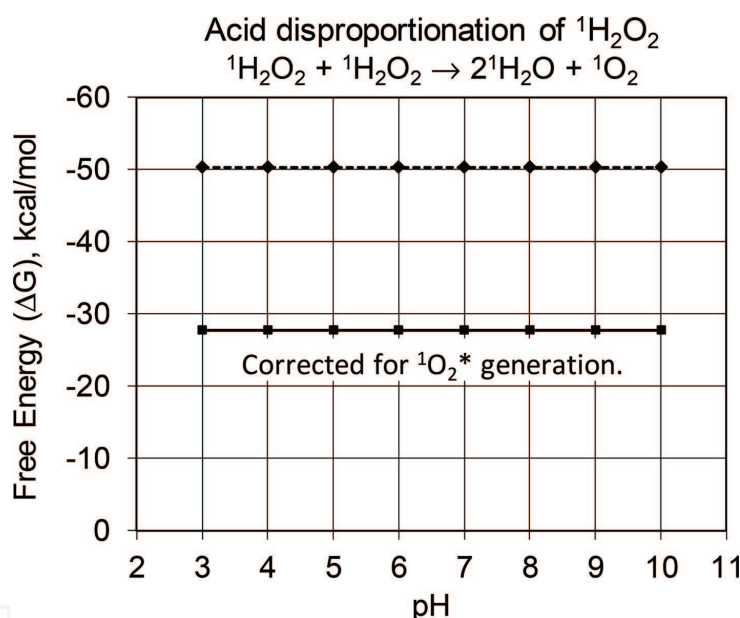

Figure 6.
*Plot of free energy against pH for the net $^1H_2O_2$ disproportionation reaction as described in **Figure 2**. The free energy results are expressed with (ΔG = −27.8 kcal mol^{-1}) and without (ΔG = −50.3 kcal mol^{-1}) adjustment for the energy electronically conserved in oxygen excitation (ΔG = −22.5 kcal mol^{-1}).*

Figure 6 have been adjusted to reflect the energy conserved in electronically excited $^1O_2{}^*$. The overall net free energy is independent of the halide employed and independent of pH.

Since the reactants involved are all singlet multiplicity, the products of reaction, that is, ^1H_2O, $^1Cl^-$, and $^1O_2{}^*$, are all singlet multiplicity. This provides a spin symmetry explanation as to why pouring bleach ($^1OCl^-$) into $^1H_2O_2$ causes rapid reactive release of $^1O_2{}^*$ gas and a red chemiluminescence [23]. Caution, rapid release of gas is potentially explosive. When the concentration of $^1O_2{}^*$ is sufficiently high, $^1O_2{}^*$-$^1O_2{}^*$ collision with simultaneous relaxation yields red chemiluminescence. The relaxation of one $^1O_2{}^*$ emits a 1270 nm photon; simultaneous relaxation of two $^1O_2{}^*$ emits a 635 nm photon. As such, this red emission is second order with respect to $^1O_2{}^*$, that is, $dh\nu_{635nm}/dt = k[^1O_2{}^*]^2$, and is relatively short-lived.

The double dehydrogenation of [1]glucose-6-PO_4 produces [1]ribulose-5-PO_4 plus [1]CO_2 plus two 2RE, that is, two bosonic electron couples carried as 2NADPH. As illustrated in **Figure 2**, NADPH oxidase reduces four [3]O_2 in four one 1RE reduction steps, ultimately yielding two [1]O_2* and two [1]H_2O_2. As illustrated in **Figure 3**, MPO uses one [1]H_2O_2 for oxidation of Cl^- to OCL^-, and this OCL^- reacts with the other [1]H_2O_2 to generate an additional [1]O_2*. Thus, two NADPH have the potential to drive the generation of three [1]O_2*. Steinbeck et al. have reported experiments using glass beads coated with 9,10-diphenylanthracene, a [1]O_2*-specific trap, for measurements of neutrophil [1]O_2* production [43]. Neutrophils were allowed to phagocytose the beads for an hour. The endoperoxide trapped indicated that at least 11.3 ± 4.9 nmol [1]O_2*/1.25×10^6 neutrophils were produced. When the neutrophils were chemically activated with phorbol-12-myristate-13-acetate (PMA), at least 14.1 ± 4.1 nmol [1]O_2*/1.25×10^6 neutrophils were produced. Based on their trapping results, [1]O_2* production accounted for at least $19 \pm 5\%$ of the total oxygen consumed. Although the quantities of [1]O_2* measured using this difficult trapping approach are lower than expected; this study provides direct empirical evidence of significant neutrophil [1]O_2* production.

Quantifying cellular production of [1]O_2* by measuring the 1270 nm near-infrared photon emitted on [1]O_2* relaxation to [3]O_2 is also problematic. Although highly specific for [1]O_2*, this infrared proton emission approach is highly insensitive in biological system measurements. The fact that a 1270 nm photon is measured is proof that [1]O_2* did not participate in chemical reaction. Considering the variety of reactive substrates available in biological milieux, electrophilic reaction is favored over relaxation.

4.2 Myeloperoxidase-binding specificity focuses combustive activity

[1]O_2* is a potent electrophilic reactant with a high probability for participation in spin-allowed reaction with electron-dense biological substrates. The lifetime of metastable electronically excited [1]O_2* restricts its reactive possibilities [44]. In biological milieux, [1]O_2* has a reactive lifetime of about 4–6 microseconds [45, 46]. This lifetime restricts reactivity to within a radius of about 0.2–0.3 µm (microns) from its point of generation. In the case of MPO generation of [1]O_2*, these temporal and spatial restrictions can be advantageous.

MPO selectively binds to all gram-negative bacteria and most gram-positive bacteria tested, but MPO binding is weak for gram-positive lactic acid bacteria (LAB) [44, 47]. LAB are common members of the normal flora of the mouth, vagina, and colon, and include streptococci, lactobacilli, and bifidobacteria. These LAB cannot synthesize cytochromes and produce lactic acid as a metabolic end product. They are typically microaerophilic, and often produce [1]H_2O_2 as a metabolic product. The green hemolysis associated with colonies of viridans streptococci on blood agar plates results from the production of [1]H_2O_2 by the streptococci. When a pathogen, such as *Staphylococcus aureus* or *Escherichia coli*, is contacted with a nonpathogen LAB, such as *Streptococcus viridans*, the pathogen overwhelmingly inhibits the LAB, but when a small quantity of MPO is added to a mixture, the pathogen is inhibited allowing LAB dominance. This phenomenon repeats even when erythrocytes are added to the mix at a ratio of 10 erythrocytes per bacteria. MPO selectively binds to the *S. aureus* and *E. coli* with essentially no binding to [1]H_2O_2-producing *Strep. viridans*. Thus, LAB-produced [1]H_2O_2 drives MPO microbicidal action that is restricted to the surface of the MPO-bound pathogen. MPO combustive microbicidal action is focused on the pathogen with minimum damage to the [1]H_2O_2-producing LAB, and without hemolytic damage to the added erythrocytes, that is, no bystander injury.

Specificity of MPO binding results in specificity of microbicidal action. Binding specificity allows synergistic MPO-LAB interaction and suppression of pathogens. It also suggests a role for MPO in the selection and maintenance of LAB in the normal flora [48]. Healthy human adults release about a hundred billion MPO-rich neutrophils into the circulating blood each day. The circulating lifetime of the neutrophil is reportedly less than a day. The neutrophils then leave the blood and enter a tissue and body cavity phase lasting a few days [36]. Migration of MPO-rich neutrophils into the mouth and vagina is well-known [49, 50]. When quantified, the neutrophil count of the mouth is proportional to the blood neutrophil count. These spaces typically provide an acidic milieu. Neutrophil disintegration with MPO release may provide LAB with a selective advantage in such body spaces.

5. Microbicidal combustion and chemiluminescence

Reactions of $^1O_2^*$ with singlet multiplicity substrates (^{1}Sub) are spin-allowed and highly exergonic. The exergonicities of most biochemical reactions are sufficient for rotational and vibrational excitation, but not electronic excitation. Dioxygenation reactions are sufficiently exergonic for electronic excitation. Oxygenations producing singlet multiplicity endoperoxide and dioxetane intermediates are excellent candidates for luminescence [51]. The disintegrations of such intermediates generate **nπ*** electronically excited products, that is, an electron from the nonbonding (**n**) orbital of oxygen populates the pi antibonding (**π***) orbital of the carbonyl. Singlet multiplicity **nπ*** excited molecules have short lifetimes. Electronic transition from the **π*** of the carbonyl to the **n** of oxygen with photon emission is spin-allowed.

In addition to direct reaction of $^1O_2^*$ with ^{1}Sub, other singlet multiplicity reactants such as $^1OCl^-$ can react with ^{1}Sub to yield chloramine products (^{1}Sub-Cl) or dehydrogenated products (^{1}Sub$_{-2RE}$). Such products can in turn react with $^1H_2O_2$ yielding endoperoxide or dioxetane intermediates with subsequent disintegration to **nπ***-excited carbonyl products relaxing by photon emission [52, 53]. The fundamental principle is that all reactants and products are singlet multiplicity nonradicals.

Dioxygenations yielding intermediate endoperoxide and dioxetanes disintegrate yielding an **nπ*** electronically excited carbonyl. **Figure** 7 illustrates the energy and orbital differences that characterize the carbonyl states. Physical generation of a **nπ*** electronically excited carbonyl occurs when a fluorescent compound in its ground state absorbs a photon of appropriate energy. Because the ground state of the carbonyl is singlet, an electronically excited singlet multiplicity carbonyl undergoes rapid spin-allowed relaxation to ground state with a lifetime of less than 10^{-8} second [51]. Fluorescence describes photon-generated excitation followed by photon emission. Chemiluminescence or luminescence describes chemically generated electronic excitation followed by photon emission.

The metabolic changes of the respiratory burst describe the movement of RE required to change the spin multiplicity of $^3O_2$ from triplet to doublet ($^2HO_2$), and ultimately to singlet, that is, $^1H_2O_2$ and $^1O_2^*$. MPO catalyzes the 2RE oxidation of $^1Cl^-$ to ^1HOCl followed by chemical reaction with a second $^1H_2O_2$ to generate $^1O_2^*$. Changing the bi-fermionic $^3O_2$ to bosonic $^1O_2^*$ eliminates the spin barrier to direct dioxygenation of bosonic singlet multiplicity biological molecules. If intermediate endoperoxides and dioxetanes are generated, their disintegration yields electronically excited **nπ*** carbonyl functions that relax by photon emission. By changing the spin multiplicity of oxygen, neutrophil leukocytes realize its electronegative potential for combustive microbicidal action. Such combustion generates electronically excited products emitting light in the visible range of the spectrum.
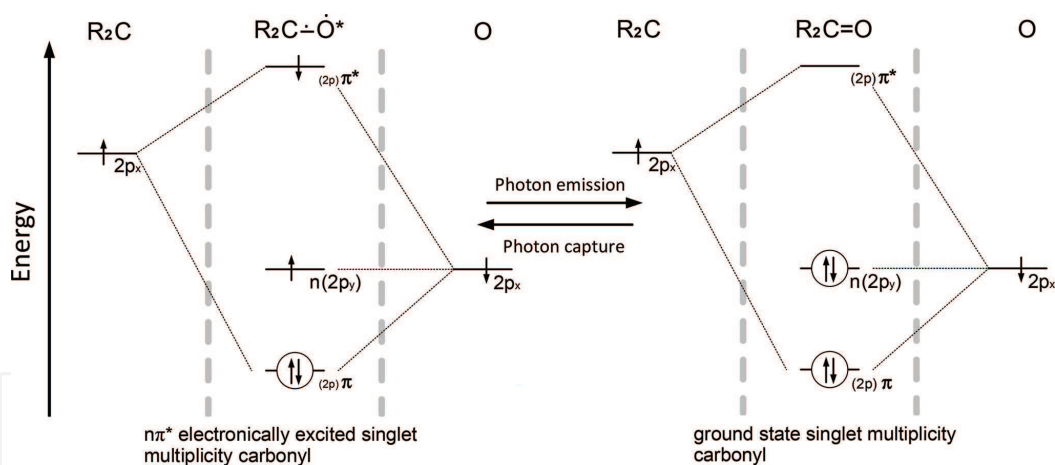
Figure 7.
*Orbital diagram plot depicting the **nπ*** electronically excited singlet state and the singlet ground state of a carbonyl. The gray dashed brackets indicate the carbonyl with the participating carbon and oxygen atoms shown on to the left and right, respectively. In the carbonyl diagram on the left, the **nπ*** notation indicates that an electron of the nonbonding (**n**) orbital of the carbonyl oxygen atom has been excited to the pi antibonding (**π***) orbital of the carbonyl. Although excited, the electrons remained paired and the excited state is singlet multiplicity. Electron relaxation from **π***-to-**n** yields photon emission.*

6. Chemiluminigenic probes

The native chemiluminescence of neutrophils is proportional to respiratory burst activity [4, 54]. Since the luminescence resulting from microbicidal combustion is proportional to dioxygenations, especially those yielding endoperoxide and dioxetane intermediates, it follows that native neutrophil luminescence is influenced by the molecular composition of the microbe combusted. Native luminescence from phagocytosing neutrophils can be detected using less than a million neutrophils. For perspective, a milliliter of normal human blood contains about 4 million neutrophils. The native luminescence product of neutrophil combustive action is of low intensity. However, electronic excitation and the resultant luminescence is unambiguous evidence of neutrophil combustive dioxygenation action. Native luminescence has been usefully applied to measurement of neutrophil metabolic defects, e.g., chronic granulomatous disease [54, 55], and neutrophil responsiveness to humoral immune factors, such as complement and immuno-globulins [56].

Inclusion of high quantum yield chemiluminigenic substrates as probes (CLP) of neutrophil dioxygenation activities greatly increases the sensitivity and, to some degree, the specificity for detecting such activities [52, 57, 58]. With regard to increasing sensitivity, a CLP must be susceptible to neutrophil dioxygenation activities. This is achieved when endoperoxide or dioxetane intermediate are produced. The breakdown of such intermediates yields electronically excited **nπ*** carbonyl functions that relax by light emission. Use of a CLP typically increases the sensitivity for detecting dioxygenation activity by several orders of magnitude. Selecting a CLP with reactive specificity also provides information with regard to the nature of neutrophil activity measured.

6.1 Probing reductive oxygenation activity with lucigenin

Phagocytic or chemical activation of neutrophil respiratory burst metabolism can be tested using the dye nitro-blue tetrazolium (NBT) [59]. The NBT reaction measures neutrophil reduction activity, not neutrophil oxidation activity. A positive NBT result requires neutrophil respiratory burst activity resulting in reduction of

the tetrazolium ring of the dye to a dark blue water-insoluble formazan precipitate. NBT is a large complex nitrogen heterocyclic compound with abundant resonance and electron delocalization possibilities. That NBT reduction might be linked to neutrophil univalent reduction of molecular oxygen was considered, and we observed that adding a small grain of potassium superoxide (KO_2) to a solution of NBT resulted in immediate reduction of the dye to a dark blue formazan precipitate [15]. Normal neutrophils reduce NBT upon activation of NADPH oxidase. The neutrophils of chronic granulomatous disease patients have defective NADPH oxidase, and as such, are incapable of NBT reduction [60].

Lucigenin (aka, bis-*N*-methylacridinium nitrate, or dimethyl biacridinium nitrate ($^1DBA^{+2}$)) is a heterocyclic organic compound known to generate chemiluminescence as a product of base-catalyzed peroxidation [61]. If sufficiently alkaline, singlet multiplicity ^{1}lucigenin reacts with the conjugate base of peroxide ($^1HO_2^-$) producing a dioxetane (^{1}lucigenin-dioxetane) intermediate that disintegrates to a **nπ***-excited carbonyl function that relaxes to ground state by **π***-to-**n** transition with photon emission. The pK_a of $^1H_2O_2$ is 11.7. As previously considered, $^1H_2O_2$ is the sum product of two RE reductions of $^3O_2$. Consequently, lucigenin chemiluminescence is the product of reductive dioxygenation. Both lucigenin and peroxide are singlet multiplicity reactants. Spin restriction is not a problem. Alkalinity favors the formation of $^1HO_2^-$ and dioxygenation yielding a dioxetane.

Lucigenin is a heterocyclic compound with resonance and electron delocalization possibilities, and can undergo one RE reduction yielding a doublet multiplicity product (^{2}lucigenin$_{+RE}^+$). Such reduction may involve $^2O_2^-$ or some other 1RE reductant. The product radical, ^{2}lucigenin$_{+RE}^+$, can now react with $^2O_2^-$ by SOMO-SOMO overlap, that is, a doublet-doublet annihilation, producing a singlet multiplicity product, the ^{1}lucigenin-dioxetane intermediate. As depicted in **Figure 8**, the disintegration of this unstable dioxetane yields chemiluminescence [52, 58, 62, 63].

Reduction of lucigenin by 2RE, that is, by a bosonic orbital electron couple, maintains singlet multiplicity. Such a reduced ^{1}lucigenin$_{+2RE}$ can react with $^1O_2^*$, but not $^3O_2$, to produce chemiluminescence [64]. As shown in **Figure 8**, the state of lucigenin reduction determines the deoxygenating agent required. All reactions shown satisfy the spin conservation rules.

The radical product of 1RE reduction of lucigenin, ^{2}lucigenin$_{+RE}^+$, can react with the radical product of NADPH oxidase, $^2O_2^-$, resulting in intermediate dioxetane formation with breakdown to a **nπ*** electronically excited carbonyl with relaxation by light emission, and as such, lucigenin can be applied as a chemiluminigenic probe for measurement of NADPH oxidase activity [52, 58, 63]. MPO haloperoxidase activity does not yield lucigenin-luminescence.

Chicken blood phagocytes, that is, heterophil leukocytes, have oxidase activity, but are deficient in haloperoxidase. Chemical or phagocytic stimulation of these heterophil leukocytes results in lucigenin-dependent luminescence responses comparable to those observed from human neutrophils under similar test conditions and using similar stimuli [58, 65]. However, the luminol-dependent luminescence responses of MPO-deficient chicken heterophils are a hundredfold lower than those observed from MPO-rich human neutrophils. In addition, azide (N_3^-), a known inhibitor of MPO, inhibits the luminol-dependent luminescence responses of MPO-rich human neutrophil. Azide shows no inhibitory action against the luminol or the lucigenin luminescence responses of MPO-deficient chicken heterophils [66]. These chicken heterophil results plus the previously described macrophage results [57] experimentally support the position that luminol provides a very sensitive measure of MPO activity. However, the weaker luminol-luminescence measured is evidence for haloperoxidase-independent oxidase activity.
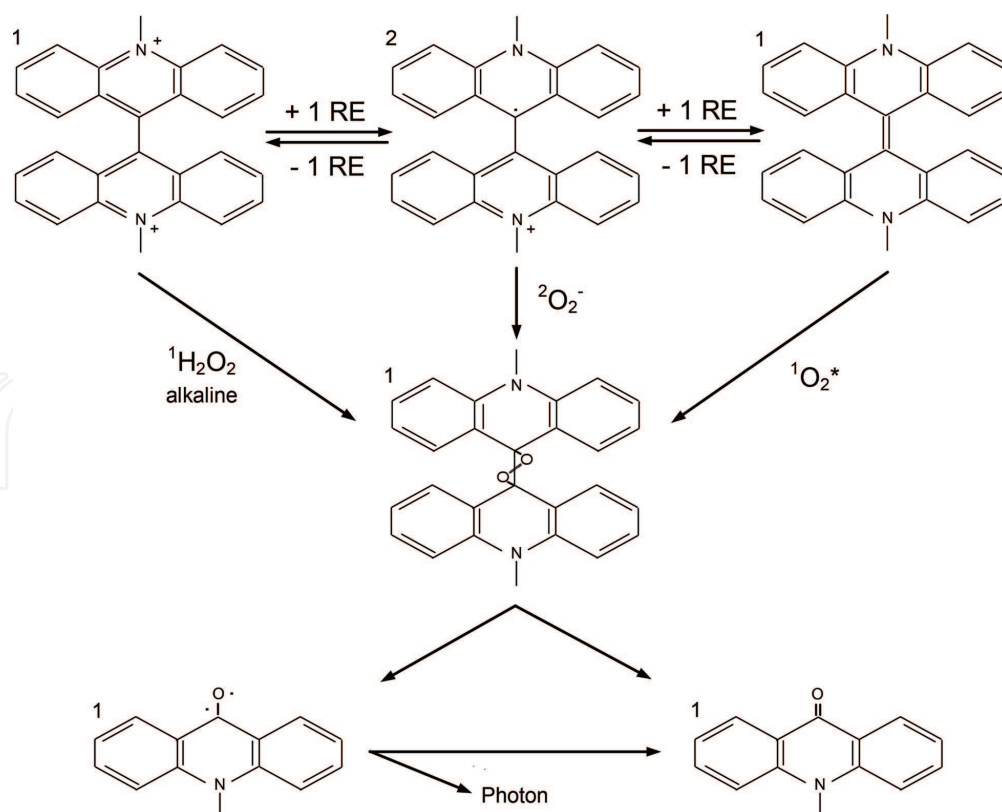
Figure 8.
Oxygenating reactions yielding lucigenin chemiluminescence. Spin multiplicity is shown by the superscript value that precedes the reactant, and 1RE indicates one reducing equivalent.

6.2 Probing oxygenation activities with cyclic hydrazides

Luminol chemiluminescence is a well-established phenomenon, but the mechanisms responsible for luminol-luminescence are diverse [67]. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is a nonradical, cyclic hydrazide [68]. Luminol dioxygenation is thought to involve an intermediate endoperoxide with disintegration yielding the **nπ*** electronically excited aminophthalate that relaxes by photon emission. Albrecht first described the blood-catalyzed luminol-luminescence [69]. Like lucigenin, alkalinity and $^1H_2O_2$ are required, but luminol-luminescence has an additional requirement for a catalyst, for example, blood or peroxidase. To appreciate how these CLS differ, compare, and contrast the net reactions responsible for luminol-luminescence and lucigenin-luminescence. Luminol-luminescence is a dioxygenation:

$$^1\text{luminol} + {}^3O_2 \text{—X} \rightarrow {}^1\text{aminophthalate} + {}^1N_2 + \text{Photon} \qquad (14)$$

The reaction of ^{1}luminol with $^3O_2$ (Eq. (14)) is not spin allowed, but reaction with $^1O_2{}^*$ (Eq. (15)) is spin allowed producing **nπ*** electronically excited ^{1}aminophthalate* plus $^1N_2$, and ultimately, ground state ^{1}aminophthalate plus a photon.

$$^1\text{luminol} + {}^1O_2{}^* \rightarrow {}^1\text{aminophthalate} + {}^1N_2 + \text{Photon} \qquad (15)$$

Lucigenin-luminescence is a reductive dioxygenation.

$$^1\text{lucigenin} + {}^1H_2O_2 \rightarrow 2\,{}^1\text{N–methylacrodone} + \text{Photon} \qquad (16)$$
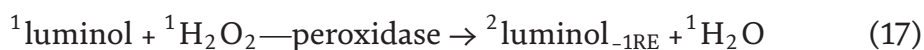
As per Eq. (16), lucigenin-luminescence requires the spin-allowed reactive addition of molecular oxygen plus 2RE, that is, $^1H_2O_2$. The product of this reductive

dioxygenation is a dioxetane intermediate that breaks down to one ground state ^{1}N-methylacridone and one **nπ***electronically excited ^{1}N-methylacridone*. Relaxation of the ^{1}N-methylacridone* yields a photon.

Luminol dioxygenation is not reductive. The net dioxygenation incorporates molecular oxygen to produce an endoperoxide intermediate with the breakdown release of ^{1}N$_2$ and formation of a **nπ***electronically excited aminophthalate. As indicated by Eq. (14), ^{1}luminol does not react with ground state oxygen. Spin conservation and frontier orbital overlap problems restrict such direct reaction. As illustrated in **Figure 1**, the frontier orbitals of ^{3}O$_2$ are its two degenerates **π*** SOMOs. Hund's maximum multiplicity rule is satisfied when the electrons of each SOMO have the same spin. Each of the two **π*** orbitals of ^{3}O$_2$ have fermionic character that restricts overlap with the bosonic frontier orbitals of luminol. By contrast, the frontier **π*** orbitals of ^{1}O$_2$* are bosonic and include one LUMO **π*** orbital and one HOMO **π*** orbital. Overlap of the LUMO of ^{1}O$_2$* with the HOMO of ^{1}luminol satisfies the symmetry requirements for reaction.

There are three mechanistic possibilities for ^{1}luminol reactions yielding luminescence. The fermionic (doublet multiplicity/radical) pathway requires two steps as illustrated by Eqs. (17) and (18).

$$^1\text{luminol} + {}^1\text{H}_2\text{O}_2\text{---peroxidase} \rightarrow {}^2\text{luminol}_{-1\text{RE}} + {}^1\text{H}_2\text{O} \qquad (17)$$

The radical ^{2}luminol$_{-1RE}$ can participate in SOMO-SOMO reaction with superoxide (^{2}O$_2^-$) yielding singlet multiplicity electronically excited aminophthalate (^{1}aminophthalate*) that relaxes with photon emission.

$$^2\text{luminol}_{-1\text{RE}} + {}^2\text{O}_2^- \rightarrow {}^1\text{aminophthalate} + {}^1\text{N}_2 + \text{Photon} \qquad (18)$$

The bosonic (singlet multiplicity/nonradical) pathway can occur by a single reaction as illustrated by Eq. (19),

$$^1\text{luminol} + {}^1\text{O}_2^* \rightarrow {}^1\text{aminophthalate}^- + {}^1\text{N}_2 + \text{Photon} \qquad (19)$$

The bosonic (singlet multiplicity/nonradical) pathway can also occur by a two-step reaction as illustrated by Eqs. (20) and (21).

$$^1\text{luminol} + {}^1\text{OCl}^- \rightarrow {}^1\text{luminol}_{-2\text{RE}} + {}^1\text{Cl}^- \qquad (20)$$

$$^1\text{luminol}_{-2\text{RE}} + {}^1\text{H}_2\text{O}_2 \rightarrow {}^1\text{aminophthalate} + {}^1\text{N}_2 + \text{Photon} \qquad (21)$$

Although luminol is versatile with regard to reactive mechanism, dioxygenation is ultimately required for chemiluminescence. In an alkaline milieu, classical peroxidase or hemoglobin can catalyze ^{1}H$_2$O$_2$-dependent luminol-luminescence. The peroxidase-catalyzed mechanism of luminol-luminescence described by Dure and Cormier illustrates the kinetics of the fermionic pathway [70]. For such reaction, a classical peroxidase is first oxidized by ^{1}H$_2$O$_2$, that is, 2RE are transferred to ^{1}H$_2$O$_2$ producing two ^{1}H$_2$O as described in Eq. (22).

$$\text{peroxidase} + {}^1\text{H}_2\text{O}_2 \rightarrow \text{Cpx } 1_{-2\text{RE}} + 2\,{}^1\text{H}_2\text{O} \qquad (22)$$
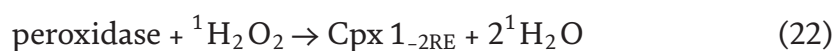
This 2RE oxidized peroxidase, referred to as complex 1 (Cpx 1), can now readily oxidize ^{1}luminol by removing 1RE producing ^{2}luminol$_{-1RE}$, as per Eq. (23).

$$^1\text{luminol} + \text{Cpx 1}_{-2RE} \rightarrow \text{Cpx 2}_{-RE} + {}^2\text{luminol}_{-1RE} \qquad (23)$$
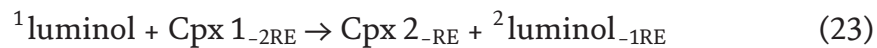
The reaction of complex 2 (Cpx 2_{-RE}) with another ^{1}luminol is slow and rate limiting with regard to luminescence, but this reaction is necessary for regeneration of the starting peroxidase, as per Eq. (24).

$$^1\text{luminol} + \text{Cpx 2}_{-RE} \rightarrow \text{peroxidase} + {}^2\text{luminol}_{-1RE} \qquad (24)$$

Disproportionation of the two radical ^{2}luminol$_{-1RE}$ can proceed as a spin allowed SOMO-SOMO reaction, that is, a doublet-doublet annihilation, yielding the nonradical ^{1}luminol (starting reactant) and nonradical 2RE-oxidized luminol (^{1}luminol$_{-2RE}$).

$$2\,^2\text{luminol}_{-1RE} \rightarrow {}^1\text{luminol} + {}^1\text{luminol}_{-2RE} \qquad (25)$$
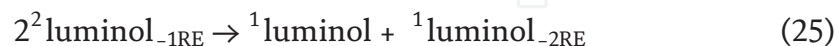
As per Eq. (21), the spin-allowed reaction of ^{1}luminol$_{-2RE}$ with ^{1}H$_2$O$_2$ yields electronically excited aminophthalate (^{1}aminophthalate*) that relaxes by photon emission.

Metalloenzymes and cytochromes are suited to 1RE transfers and under proper reaction conditions can catalyze the 1RE oxidation of a ^{1}substrate producing ^{2}substrate$_{-1RE}$. The ^{1}H$_2$O$_2$-dependent oxidation of peroxidase to Cpx 1_{-2RE} allows it to catalyze the initial fermionic 1RE oxidation of luminol in an alkaline milieu. Hemoglobin has peroxidase activity under alkaline conditions, thus explaining the sensitivity of luminol-luminescence for detecting the presence of blood erythrocytes by alkaline peroxide methods. Luminol-luminescence by the classical plant peroxidase-catalyzed reactions of Eqs. (22)–(25) is sensitive to pH, decreasing with increasing acidity. Acidification of the reaction milieu to a pH of about 5 ± 1 effectively eliminates classical peroxidase-catalyzed luminol luminescence. This is quantitatively demonstrated in the Michaelis-Menten enzyme kinetic analyses of luminol-luminescence for myeloperoxidase and horse radish peroxidase presented in **Table 2** [71].

Alkaline pH favors the fermionic luminol-luminescence reactions catalyzed by plant peroxidase, hemoglobin, and heavy metals. The pKa of ^{1}H$_2$O$_2$ is 11.75. The ferricyanide-catalyzed luminol luminescence reaction is most efficient in the pH range from 10.4 to 10.8 [72]. In **Table 2**, note that no significant luminescence is observed from HRP-catalyzed luminol reaction at pH 4.9. The maximum luminescence velocity (V_{max}) values are low and standard errors (SE) are high. However, a relatively weak but significant luminescence is observed at pH 7.0, that is, Michaelis-Menten analysis of the HRP luminescence shows a low V_{max}, but an acceptable SE.

Of special note, Michaelis-Menten kinetic analysis indicates that the HRP-catalyzed luminol-luminescence velocity is first order with respect to H$_2$O$_2$ concentration, but second order with respect to luminol concentration, that is, the luminescence velocity is directly proportional to the square of the luminol concentration. These results are consistent with those reported by Dure and Cormier [70], and with the fermionic radical reactive pathway described in Eqs. (22)–(25) and Eq. (21).

Although luminol solubility becomes a problem at low pH, acidity favors the bosonic haloperoxidase luminol-luminescence catalyzed by MPO. Note that bosonic, haloperoxidase-catalyzed luminol luminescence is first order with respect to luminol, chloride, or bromide, but second order with respect to H$_2$O$_2$, that is, luminescence activity is proportional to the square of the H$_2$O$_2$ concentration.

The MPO-catalyzed luminol-luminescence kinetic finding is the opposite of those observed for HRP-catalyzed luminol-luminescence, and are consistent with the bosonic reactive pathway for luminol-luminescence via ^{1}O$_2$* reaction described

	Substrate [S], variable (conc. range)	pH	Substrates, constant				Michaelis-Menten kinetics		
			H_2O_2, mM	Cl^-, mEq/L	Br^-, mEq/L	Luminol, µM	M-M equation	Km ± SE	Vmax ± SE
Haloperoxidase: Myeloperoxidase	H_2O_2 (0.01–1.4 mM)	5.0	variable	90	0	77	$v = Vmax[S]^2/(Km + [S])^2$	2.82 ± 0.05	3900 ± 1
	H_2O_2 (0.01–1.4 mM)	5.0	variable	0	4.5	77	$v = Vmax[S]^2/(Km + [S])^2$	0.58 ± 0.03	2932 ± 2
	Cl^- (0.2–7.7 mEq/L)	5.0	2.27	variable	0	45	$v = Vmax[S]/Km + [S]$	7.60 ± 2.60	1105 ± 253
	Br^- (14–882 µEq/L)	5.0	2.27	0	variable	45	$v = Vmax[S]/Km + [S]$	0.68 ± 0.05	2280 ± 96
	Luminol (0.0018–15 µM)	4.9	2.27	90	0	variable	$v = Vmax[S]/Km + [S]$	8.80 ± 0.77	1490 ± 70
	Luminol (0.0018–0.47 µM)	7.0	2.27	90	0	variable	$v = Vmax[S]/Km + [S]$	0.10 ± 0.02	3252 ± 219
Classical Peroxidase: Horse Radish Peroxidase	H_2O_2 (0.01–1.4 mM)	5.0	variable	90	0	77	$v = Vmax[S]/Km + [S]$	31.02 ± 0.05	280 ± 55
	H_2O_2 (0.01–1.4 mM)	5.0	variable	0	4.5	77	$v = Vmax[S]/Km + [S]$	17.49 ± 3.84	156 ± 34
	Cl^- (0.2–900 mEq/L)	5.0	2.27	variable	0	45	$v = Vmax[S]/Km + [S]$	0.0 ± 0.7	0 ± 0
	Br^- (14–882 µEq/L	5.0	2.27	0	variable	45	$v = Vmax[S]/Km + [S]$	6.23 ± 2.68	3 ± 0
	Luminol (0.0147–30 µM)	4.9	2.27	0	0	variable	$v = Vmax[S]^2/(Km + [S])^2$	0.0 ± 0.0	0 ± 0
	Luminol (0.0018–7.5 µM)	7.0	2.27	0	0	variable	$v = Vmax[S]^2/(Km + [S])^2$	0.90 ± 0.17	189 ± 2

Reaction milieu was 50 mM acetate buffer (pH 5.0, 4.9) or phosphate buffer (pH 7.0) in a 0.3 mL volume. The indicated conc. of Cl^- or Br^- was added in a 0.3 mL volume.

The enzymes, 78 pmol MPO and 10 pmol HRP as indicated, were added in a 0.1 mL volume. The final concentration was 78 nM for MPO and 10 nM for HRP.

The luminescence reaction was initiated by injecting the indicated concentration of H_2O_2 in a 0.3 mL volume. The final volume was 1.0 mL.
Chemiluminescence velocity (v) and Vmax are expressed as peak kilocounts of relative light units (RLU × 10–3) per sec measured during the initial 20 sec post H_2O_2 injection.

Table 2.
Michaelis-Menten enzyme kinetic analyses of classical peroxidase (horse radish peroxidase) and haloperoxidase (myeloperoxidase) activities with regard to H_2O_2, halide (Cl^- or Br^-), luminol, and pH.

in Eq. (19) or the sequential bosonic pathway described in Eqs. (20), (21). By either pathway, and consistent with the second order findings, two $^1H_2O_2$ are required for luminol dioxygenation.

Under alkaline conditions, luminol-luminescence provides high sensitivity for detection of classical peroxidase catalysts or $^1H_2O_2$, but relatively low specificity. Under acid conditions, the luminol-luminescence provides a method for specific quantification of haloperoxidase-dependent dioxygenation activity. In **Table 2**, note that Cl⁻ or Br⁻ is required for MPO-catalyzed luminol-luminescence, that the requirement is first order with respect to halide, and that the Michaelis constant (K_M) for the more electronegative Cl⁻ is expectedly greater that for Br⁻. Haloperoxidase activity is exclusively bosonic. Reactants are all singlet multiplicity, involving HOMO-LUMO frontier orbital interaction.

Luminol was the first, and remains the most common, chemiluminigenic probe used for measurement of phagocyte oxygenation activities. Its original application was an attempt to amplify the relatively weak native luminescence signal from stimulated macrophages. Comparing the luminol-luminescence responses of neutrophils with those of macrophages illustrates that the MPO-rich neutrophils responses are several magnitudes greater than the luminol-luminescence responses from MPO-deficient macrophage [57].

Comparing MPO-rich human neutrophils with the MPO-deficient heterophile leukocytes of chickens further illustrates how chemiluminigenic probing can be used as a sensitive method for quantifying and differentiating the oxygenating activities of phagocytes [58, 65]. The luminol-dependent activities of MPO-positive human neutrophil leukocytes are a hundredfold higher than those of MPO-negative chicken heterophil leukocytes. Despite the diminution in luminol-luminescence, dioxygenation activity is still quantifiable from MPO-negative phagocytes. Such activity is not inhibited by the MPO inhibitor azide (N₃⁻) [66]. In the absence of haloperoxidase, luminol-luminescence most probably reflects the type of fermionic oxidase-dependent reactions described in reactions Eqs. (17)–(18).

7. Circulating neutrophils reflect the state of inflammation

Under normal conditions, large numbers of neutrophils are produced by the hematopoietic marrow and released into the circulating blood each day, highlighting the importance of neutrophils for innate host protection against infection. To accomplish its microbicidal role, neutrophils undergo specific degranulation and mobilization of appropriate membrane receptors in response to a constellation of microbial peptides, complement activation products, cytokines, interleukins, and lipid activators. Such activities prepare neutrophils for phagocytosis, but do not directly trigger respiratory burst activity [73]. Priming actuates neutrophil locomotion and increases neutrophil recognition of and phagocytic response to opsonin-labeled microbes [56, 74, 75].

Activation of systemic inflammation in response to infection directly affects circulating blood neutrophils. The chemical signals of inflammation alter the state of neutrophil alert. As such, the state of neutrophil priming reflects the state of host immune activation [76]. Selective *in vitro* measurement of unprimed and maximally-primed circulating blood neutrophil activities by sensitive chemiluminigenic probing allows rapid multi-metric analysis using less than a half drop of anticoagulated whole blood. Analysis of such blood neutrophil luminescence metrics using classification statistical approaches, especially discriminant function analysis, allows assessment of the *in vivo* state of immune activation. The state of neutrophil priming gauges the state of host systemic inflammation [77, 78].

Acknowledgements

Conflict of interest

I am the inventor of pending and issued patents related to diagnostic applications of chemiluminescence for quantifying neutrophil function and for gauging systemic immune activation, and patents related to therapeutic applications of haloperoxidases.

Author details

Robert C. Allen
Department of Pathology, Creighton University School of Medicine,
Omaha, NE, USA

*Address all correspondence to: robertallen@creighton.edu

IntechOpen

References

[1] Northrop JH. Biochemists, biologists, and William of Occam. Annual Review of Biochemistry. 1961;**30**(1):10

[2] Allen RC, Stjernholm RL, Benerito RR, Steele RH. Functionality of electronic excitation states in human microbicidal activity. In: Chemiluminescence and Bioluminescence. Athens, GA. New York: Plenum Press; 1973. p. 498

[3] Sbarra AJ, Karnovsky ML. The biochemical basis of phagocytosis. I. Metabolic changes during the ingesting of particles by polymorphonuclear leukocytes. Journal of Biological Chemistry. 1959;**234**(4):1355-1362

[4] Allen RC, Stjernholm RL, Steele RH. Evidence for the generation of an electronic excitation state (s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochemical and Biophysical Research Communications. 1972;**47**(4):679-684

[5] Bazin S, Delanney A, Avice C. Le glycogène intraleucocytaire et ses variations au cours de la phagocytose. Annales de l'Institut Pasteur. 1953;**85**:774-783

[6] Stahelin H, Suter E, Karnovsky ML. Studies on the interaction between phagocytes and tubercle bacilli. I. Observations on the metabolism of Guinea pig leucocytes and the influence of phagocytosis. Journal of Experimental Medicine. 1956;**104**(1):121-136

[7] Baldridge CW, Gerard RW. The extra respiration of phagocytosis. American Journal of Physiology. 1932;**103**(1):235-236

[8] Suelter CH, Metzler DE. The oxidation of a reduced pyridine nucleotide analog by flavins. Biochimica et Biophysica Acta. 1960;**44**:23-33

[9] Michaelis L, Schwarzenbach G. The intermediate forms of oxidation-reduction of the flavins. Journal of Biological Chemistry. 1938;**123**:527-542

[10] Beinert H. Spectral characteristics of flavins at the semiquinoid oxidation level 1. Journal of the American Chemical Society. 1956;**78**(20):5323-5328

[11] Mahler HR, Cordes EH. Biological Chemistry. 2nd ed. New York: Harper and Row; 1971

[12] Massey V, Strickland S, Mayhew SG, Howell LG, Engel PC, Matthews RG, et al. The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochemical Biophysical Research Communications. 1969;**36**(6):891-897

[13] Knowles P, Gibson J, Pick F, Bray R. Electron-spin-resonance evidence for enzymic reduction of oxygen to a free radical, the superoxide ion. Biochemical Journal. 1969;**111**(1):53-58

[14] Allen RC. Oxygen-dependent microbe killing by phagocyte leukocytes: Spin conservation and reaction rate. Studies in Organic Chemistry. 1987;**33**:425-434

[15] Allen RC, Yevich SJ, Orth RW, Steele RH. The superoxide anion and singlet molecular oxygen: Their role in the microbicidal activity of the polymorphonuclear leukocyte. Biochemical and Biophysical Research Communications. 1974;**60**(3):909-917

[16] Sudbery A. Quantum Mechanics and the Particles of Nature. Cambridge: Cambridge University Press; 1986

[17] Matthews P. Quantum Chemistry of Atoms and Molecules. Cambridge: Cambridge University Press; 1986

[18] Fukui K, Yonezawa T, Shingu H. A molecular orbital theory of reactivity in aromatic hydrocarbons. Journal of Chemical Physics. 1952;**20**(4):722

[19] Allen RC. Neutrophil leukocyte: Combustive microbicidal action and chemiluminescence. Journal of Immunology Research. 2015;**15**:794072. DOI: 10.1155/2015/794072

[20] Allen RC. Molecular oxygen (O2): Reactivity and luminescence. In: Bioluminescence and Chemiluminescence: Progress & Current Applications. Cambridge UK: World Scientific; 2002. pp. 223-232

[21] Wigner E, Witmer EE. Ober die struktur der zweiatomigen molekelspektren nach der quantenmechanik. Zeitschrift für Physik. 1928;**51**:859-886

[22] Herzberg G. Molecular Spectra and Molecular Structure. Spectra of Diatomic Molecules. New York: Van Nostrand Reinhold; 1950

[23] Kasha M, Khan A. The physics, chemistry, and biology of singlet molecular oxygen. Annals of the New York Academy of Sciences. 1970;**171**:1-33

[24] Katriel J, Pauncz R. Theoretical interpretation of Hund's rule. Advances in Quantum Chemistry. 1977;**10**:143-185

[25] Allen RC. Reduced, radical, and excited state oxygen in leukocyte microbicidal activity. Frontiers of Biology. 1979;**48**:197-233

[26] Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Archives of Biochemistry and Biophysics. 2002;**397**(2):342-344

[27] Brandes RP, Weissmann N, Schroder K. Nox family NADPH oxidases: Molecular mechanisms of activation. Free Radical Biology and Medicine. 2014;**76**:208-226

[28] Jones RD, Hancock JT, Morice AH. NADPH oxidase: A universal oxygen sensor? Free Radical Biology and Medicine. 2000;**29**(5):416-424

[29] Skonieczna M, Hejmo T, Poterala-Hejmo A, Cieslar-Pobuda A, Buldak RJ. NADPH oxidases. Insights into selected functions and mechanisms of action in cancer and stem cells. Oxidative Medicine and Cellular Longevity. 2017;**2017**. Article ID 9420539

[30] Bielski BHJ, Allen AO. Mechanism of the disproportionation of superoxide radicals. The Journal of Physical Chemistry. 1977;**81**(11):1048-1050

[31] Behar D, Czapski G, Rabani J, Dorfman LM, Schwarz HA. Acid dissociation constant and decay kinetics of the perhydroxyl radical. The Journal of Physical Chemistry. 1970;**74**(17):3209-3213

[32] Khan AU. Singlet molecular oxygen from superoxide anion and sensitized fluorescence of organic molecules. Science. 1970;**168**(3930):476-477

[33] Dirac PAM. The Principles of Quantum Mechanics. 4th ed. Oxford: Oxford University Press; 1958

[34] Schultz J, Kaminker K. Myeloperoxidase of the leukocyte of normal human blood. I. Content and location. Archives of Biochemistry and Biophysics. 1962;**96**(3):465-467

[35] Agner K. Crystalline myeloperoxidase. Acta Chemica Scandinavica. 1958;**12**(1):89-94

[36] Bainton DF. Developmental biology of neutrophils and eosinophils. In: Gallin JI, Snyderman R, editors. Inflammation Basic Principles and Clinical Correlates. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 1999. pp. 13-34

[37] Allen RC, Stevens PR, Price TH, Chatta GS, Dale DC. In vivo effects of recombinant human granulocyte colony-stimulating factor on neutrophil oxidative functions in normal human volunteers. Journal of Infectious Diseases. 1997;**175**(5):1184-1192

[38] Allen RC. Halide dependence of the myeloperoxidase-mediated antimicrobial system of the polymorphonuclear leukocyte in the phenomenon of electronic excitation. Biochemical and Biophysical Research Communications. 1975;**63**(3):675-683

[39] Allen RC. The role of pH in the chemiluminescent response of the myeloperoxidase-halide-HOOH antimicrobial system. Biochemical and Biophysical Research Communications. 1975;**63**(3):684-691

[40] Klebanoff SJ. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. Journal of Bacteriology. 1968;**95**(6):2131-2138

[41] Allen LC. Electronegativity is the average one-electron energy of the valence-shell electrons in ground-state free atoms. Journal of the American Chemical Society. 1989;**111**(25):9003-9014

[42] Pourbaix M. Atlas of Electrochemical Equilibria in Aqueous Solutions. Houston TX: National Association of Corrosion Engineers; 1974

[43] Steinbeck MJ, Khan AU, Karnovsky MJ. Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap. Journal of Biological Chemistry. 1992;**267**(19):13425-13433

[44] Allen RC, Stephens JT Jr. Myeloperoxidase selectively binds and selectively kills microbes. Infection and Immunity. 2011;**79**(1):474-485

[45] Skovsen E, Snyder JW, Lambert JD, Ogilby PR. Lifetime and diffusion of singlet oxygen in a cell. Journal of Physical Chemistry B. 2005;**109**(18):8570-8573

[46] Redmond RW, Kochevar IE. Spatially resolved cellular responses to singlet oxygen. Photochemistry and Photobiology. 2006;**82**(5):1178-1186

[47] Allen RC, Stephens JT Jr. Reduced-oxidized difference spectral analysis and chemiluminescence-based Scatchard analysis demonstrate selective binding of myeloperoxidase to microbes. Luminescence. 2011;**26**(3):208-213

[48] Allen RC, Stephens JT Jr. Role of lactic acid bacteria-myeloperoxidase synergy in establishing and maintaining the normal flora in man. Food and Nutrition Sciences. 2013;**4**:67-72

[49] Wright DG, Meierovics AI, Foxley JM. Assessing the delivery of neutrophils to tissues in neutropenia. Blood. 1986;**67**(4):1023-1030

[50] Cauci S, Guaschino S, De Aloysio D, Driussi S, De Santo D, Penacchioni P, et al. Interrelationships of interleukin-8 with interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women. Molecular Human Reproduction. 2003;**9**(1):53-58

[51] Turro NJ, Ramamurthy V, Scaiano JC. Principles of Molecular Photochemistry. Sausalito: University Science Books; 2009

[52] Allen RC. Biochemiexcitation: Chemiluminescence and the study of biological oxygenation. In: Adam W, Cilento G, editors. Chemical and Biological Generation of Excited States. New York: Academic Press; 1982. pp. 310-344

[53] Kearns DR, Khan AU. Sensitized photooxygenation reactions and the role

of singlet oxygen. Photochemistry and Photobiology. 1969;**10**(3):193-210

[54] Allen RC, Stjernholm RL, Reed MA, Harper TB, Gupta S, Steele RH, et al. Correlation of metabolic and chemiluminescent responses of granulocytes from three female siblings with chronic granulomatous disease. Journal of Infectious Diseases. 1977;**136**(4):510-518

[55] Allen RC, Mills EL, McNitt TR, Quie PG. Role of myeloperoxidase and bacterial metabolism in chemiluminescence of granulocytes from patients with chronic granulomatous disease. Journal of Infectious Diseases. 1981;**144**(4):344-348

[56] Allen RC. Evaluation of serum opsonic capacity by quantitating the initial chemiluminescent response from phagocytizing polymorphonuclear leukocytes. Infection and Immunity. 1977;**15**(3):828-833

[57] Allen RC, Loose LD. Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochemical and Biophysical Research Communications. 1976;**69**(1):245-252

[58] Allen RC. Phagocytic leukocyte oxygenation activities and chemiluminescence: A kinetic approach to analysis. Methods in Enzymology. 1986;**133**:449-493

[59] Park BH, Fikrig SM, Smithwick EM. Infection and Bitroblue-tetrazolium reduction by neutrophils: A diagnostic aid. The Lancet. 1968;**292**(7567):532-534

[60] Ochs HD, Igo RP. The NBT slide test: A simple screening method for detecting chronic granulomatous disease and female carriers. The Journal of Pediatrics. 1973;**83**(1):77-82

[61] Gleu K, Petsch W. Die Chemiluminescenz der

dimethyl-diacridyliumsalze. Angewandte Chemie. 1935;**48**(3):57-59

[62] Totter JR. The quantum yield of the chemiluminescence of dimethylbiacridinium nitrate and the mechanism of its enzymatically induced chemiluminescence. Photochemistry and Photobiology. 1964;**3**:231-241

[63] Allen RC. Lucigenin chemiluminescence: A new approach to the study of polymorphonuclear leukocyte redox activity. In: Bioluminescence and Chemiluminescence Basic Chemistry and Analytical Applications. New York: Academic Press; 1981. pp. 63-73

[64] McCapra F, Hann RA. The chemiluminescent reaction of singlet oxygen with 10, 10′-dimethyl-9, 9′-biacridylidene. Journal of the Chemical Society D: Chemical Communications. 1969;**9**:442-443

[65] Merrill GA, Bretthauer R, Wright-Hicks J, Allen RC. Oxygenation activities of chicken polymorphonuclear leukocytes investigated by selective chemiluminigenic probes. Laboratory Animal Science. 1996;**46**(5):530-538

[66] Merrill GA, Bretthauer R, Wright-Hicks J, Allen RC. Effects of inhibitors on chicken polymorphonuclear leukocyte oxygenation activity measured by use of selective chemiluminigenic substrates. Comparative Medicine. 2001;**51**(1):16-21

[67] Roswell DF, White EH. The chemiluminescence of luminol and related hydrazides. Methods in Enzymology. 1978;**57**:409-423

[68] Lind J, Merenyi G, Eriksen TE. Chemiluminescence mechanism of cyclic hydrazides such as luminol in aqueous solutions. Journal of the American Chemical Society. 1983;**105**(26):7655-7661

[69] Albrecht HO. Über die chemiluminescenz des aminophthalsäurehydrazids. Zeitschrift für Physikalische Chemie. 1928;**136U**:321-330

[70] Dure LS, Cormier MJ. Studies on the bioluminescence of Balanoglossus biminiensis extracts. Journal of Biological Chemistry. 1964;**239**:2351-2359

[71] Allen RC. Haloperoxidase Acid Optimum Chemiluminescence Assay System. USPO. Patent US005556758A1996. pp. 1-46

[72] Seitz RW. Chemiluminescence detection of enzymically generated peroxide. Methods in Enzymology. 1978;**57**:445-462

[73] Allen RC, Stevens DL. The circulating phagocyte reflects the in vivo state of immune defense. Current Opinion in Infectious Diseases. 1992;**5**(3):389-398

[74] Madonna GS, Allen RC. Shigella sonnei phase I and phase II: Susceptibility to direct serum lysis and opsonic requirements necessary for stimulation of leukocyte redox metabolism and killing. Infection and Immunity. 1981;**32**(1):153-159

[75] Allen RC, Lieberman MM. Kinetic analysis of microbe opsonification based on stimulated polymorphonuclear leukocyte oxygenation activity. Infection and Immunity. 1984;**45**(2):475-482

[76] Stevens DL, Bryant AE, Huffman J, Thompson K, Allen RC. Analysis of circulating phagocyte activity measured by whole blood luminescence: Correlations with clinical status. Journal of Infectious Diseases. 1994;**170**(6):1463-1472

[77] Taylor F, Haddad P, Kinasewitz G, Chang A, Peer G, Allen RC.
Luminescence studies of the phagocyte response to endotoxin infusion into normal human subjects: Multiple discriminant analysis of luminescence response and correlation with phagocyte morphologic changes and release of elastase. Journal of Endotoxin Research. 2000;**6**(1):3-15

[78] Allen RC, Dale DC, Taylor FB. Blood phagocyte luminescence: Gauging systemic immune activation. Methods in Enzymology. 2000;**305**:591-629