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Mineral-Induced Formation of Reactive Oxygen Species

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INTRODUCTION

The term reactive oxygen species, ROS, is defined by the US National Library of Medicine (NIH 2006) as:

> *"Molecules or ions formed by the incomplete one-electron reduction of oxygen. These reactive oxygen intermediates include singlet oxygen; superoxides; peroxides; hydroxyl radical; and hypochlorous acid. They contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and the oxidative damage to nucleic acids; proteins; and lipids."*

This chapter explores the role of minerals in the formation of reactive oxygen species. Five different mechanisms by which minerals may promote the formation and transformation of ROS species are explored (Fig. 1). These are:

1. *Mineral release of metal ions:* Metals that are released into body fluids via congruent or incongruent mineral dissolution can act as catalysts. In this mechanism minerals are a source of metals, but are not directly involved in any of the reactions.
2. *Surface-bound metal-promoted reactions:* Insoluble metal-containing minerals can catalyze formation of ROS from molecular oxygen. In this mechanism the conversion of molecular oxygen takes place on the mineral surface, with the mineral surface itself, or adsorbed species, acting as an electron donor.
3. *Intrinsic or mechanically-induced surface defects:* Defects on the mineral surface, either intrinsic to the mineral structure or generated by crushing, can react to form ROS. In this mechanism, highly reactive defects combine with water, molecular oxygen, or carbon dioxide to form ROS.
4. *Inflammatory cell/mineral interactions:* Insoluble particles that deposit in the airways and alveoli of the lung may activate airway epithelial cells or macrophages as a result of binding to the cell surface or engulfment mechanisms that bring the particles into the cell interior; these processes can lead to production of secondary cellular ROS, including hydroxyl radicals and other very reactive entities. Hence, minerals that do not promote ROS formation via the first three mechanisms *in vitro* may still show upregulation of ROS *in vivo*.

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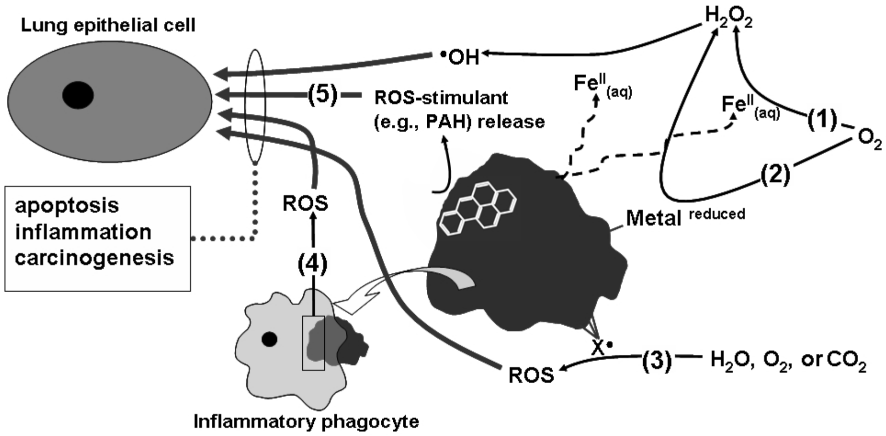


Figure 1. Mineral-induced ROS formation and transformations. Numbers refer to mechanisms listed in text. [Used by permission of Corey Cohn and Martin Schoonen, Stony Brook University, 2006]

Table 1. Abbreviations used in this chapter.

8-oxodG	8-oxodeoxyguanosine
Amplex UltraRed	10-acetyl-3,7-dihydroxyphenoxazine, hydrogen peroxide indicator
APF	3'-(*p*-aminophenyl) fluorescein, $C_{26}H_{17}NO_5$, fluorogenic spintrap
BMPO	5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline N-oxide, spin trap
DCFH	2',7'-dichlorofluorescin, reactive oxygen species indicator
DFO	Desferrioxamine, ligand
DMPO	5,5-dimethyl-1-pyrroline N-oxide, spintrap
EDTA	ethylenediaminetetraacetic acid, $C_{10}H_{16}N_2O_8$, ligand
EPR	Electron paramagnetic resonance spectroscopy
ESR	Electron spin resonance spectroscopy
HRP	Horseradish Peroxidase
MPO	myeloperoxidase
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NHE	Normal Hydrogen Electrode
PAH	Polycyclic aromatic hydrocarbon
PBN	N-*tert*-Butyl-α-phenylnitrone; $C_{11}H_{15}NO$, spintrap
PF	Proxyl fluorescamine; 5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one; $C_{26}H_{28}N_2O_5^-$
POBN	N-*tert*-Butyl-α-(4-pyridyl)nitrone N-oxide
RCS	Reactive Chlorine Species
Resorufin	$C_{12}H_6NNaO_3$
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	superoxide dismutase
Tempo-9-ac	(4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl); $C_{23}H_{26}N_3O_2$, fluorogenic spintrap

5. *Mineral surface-sorbed compounds:* Minerals may act as "carriers" of ROS-inducing compounds (e.g., PAH). Sorption onto or aggregation of non-mineral ROS-inducing compounds with minerals can transform inhaled minerals into carriers. Dissolution of the carrier species into the alveolar fluid may induce formation of ROS. Hence, in this mechanism the mineral acts as a substrate for a ROS stimulant (e.g., PAH). Mineral surface charge, surface functionalities, and hydrophobicity/hydrophilicity are important factors that dictate the interaction between a ROS stimulant and the mineral.

In reality a mineral may promote ROS formation or transformation via a combination of the mechanisms listed above. In this chapter, we use the term "mineral-induced" without implying a specific mechanism. In addition, the term "Earth material" will be used for all natural particles including minerals, glasses, and composite materials such as coal, soil and sediments.

The second part of the definition provided by the US National Library of Medicine provides a powerful motivation for the study of the role of minerals in ROS formation or transformations. While it is well documented that exposure to a range of particles of various composition can lead to ROS formation, the factors that contribute to mineral-induced ROS formation are far from completely understood. ROS and related oxidants are integral components of the normal function of the human body and important in the human immune system, signal transduction and gene expression. However, the concentrations of ROS are tightly regulated in the body by a balance between pro-oxidants and anti-oxidants (Fujimoto et al. 1996; Heiser and Elstner 1998; Babior 2000). ROS can cause cell death by disrupting cell membranes (necrosis) or initiating programmed cell death (apoptosis or "cellular suicide"). In sublethal concentrations, ROS can upregulate production of cytokines and other inflammatory mediators and can promote mutagenesis and carcinogenesis. It should be remembered that ROS generated by inhaled particulate materials can be active either extracellularly or intracellularly. Within the tightly regulated intracellular microenvironment even small and transient increases in ROS levels can markedly alter cell metabolism. Particle characteristics (e.g., metallic content, surface area, particle size and morphology) as well as cellular characteristics (e,g, embryologic origin [epithelial cells (lung-surface cells) vs. phagocytic mesenchymal cells (innate immune system cells)], state of differentiation, and pre-existing levels of activation) combine to produce a broad spectrum of responses.

Chronic inflammation associated with mineral-generated ROS production has been linked to a number of diseases including cardiovascular disease (Brook et al. 2003), lung diseases (i.e., silicosis, asbestosis) (Kamp et al. 1992), and lung cancer (Knaapen et al. 2004). Oxidative stress has been linked to memory loss and learning impairment (Fujimoto et al. 1996). Hence, it is important to understand which minerals can induce formation of primary ROS or transformations to secondary ROS. Furthermore, from a biological standpoint, it is important to study the cellular responses to mineral exposures, including the upregulation of ROS or impairment of antioxidant defense systems.

This chapter is divided into four parts. The first part provides background. It consists of a short description of ROS species, thermodynamic data related to ROS species, a summary of common analytical methods and assays for the determination of ROS in cell-free (acellular) systems and within cellular systems. Part II is a detailed discussion of the five mechanisms listed above. Part III discusses silicosis in detail to illustrate the complexities involved in unraveling the relation between a mineral exposure and a disease. Closing remarks are presented in Part IV.

PART I: BACKGROUND

The objective of this part of the chapter is to briefly provide background information that is of importance in understanding the mechanisms discussed in Part II of the chapter. This section is written with the geochemist in mind. Hence, we have made an attempt to place the

Table 2. List of selected terms used in this chapter.

Term	Explanation
apoptosis	Programmed cell death (PCD) of an unwanted cell in a multicellular organism. The apoptotic cell usually undergoes phagocytosis
chemokine	Chemokines are small, secreted protein signals. Chemokines are chemoattractants for leukocytes, recruiting monocytes and neutrophils from the blood to sites of infection or damage
coal worker's pneumoconiosis	CPW, Coal worker's pneumoconiosis, is a lung condition caused by the inhalation of dust, characterized by formation of fibrotic changes in lungs
cytokine	Cytokines are proteins produced by inflammatory and other cell types that are involved in immune responses
deoxyribonucleic acid	Deoxyribonucleic acid (DNA) is a nucleic acid—usually in the form of a double helix—that contains the genetic instructions specifying the biological development of all cellular forms of life
endocytosis	Internalization of particles or fluid by cells without forming a vesicle around the engulfed material
endogonic reaction	Energetically unfavorable chemical reactions
exogonic reaction	Energetically favorable chemical reactions
granulocyte	A leucocyte that has small granules in the cytoplasm
inflammation	First response of the immune system to infection or irritation
leukocytes	Leukocytes or white blood cells are a component of blood. They defend the body against infectious agents and foreign materials as part of the immune system
macrophage	A type of white blood cell that surrounds and kills microorganisms, removes dead cells, and stimulates the action of other immune system cells
monocyte	A large white blood cell that is formed in the bone marrow, enters the blood, and migrates into the connective tissue where it differentiates into a macrophage
necrosis	Unprogrammed death of cells and results in production of extracellular debris which can in turn be proinflammatory
neutrophil	A granulocyte leucocyte that is the chief phagocytic white blood cell.
phagocytes	An immune system cell that can surround and kill microorganisms and remove dead cells. Phagocytes include macrophages
phagocytosis	Phagocytosis is a form of endocytosis wherein large particles are enveloped by the cell membrane of a cell and internalized to form a phagosome. Phagocytosis is performed by macrophages and granulocytes
point mutation	type of genetic mutation that causes the replacement of a single base nucleotide with another nucleotide
Ribonucleic acid	Ribonucleic acid, (RNA), consists of nucleotide monomers.. It is transcribed from DNA by enzymes called RNA polymerases. RNA serves as the template for translation of genes into proteins, transferring amino acids to the ribosome to form proteins, and also translating the transcript into proteins
Silicosis	Lung disease caused by inhalation of crystalline silica dust, and is marked by inflammation and scarring

various forms of ROS in the context of concepts familiar to most geochemists. The role of ROS in human health is an active area of biomedical research with broad implications. It is far beyond the scope of this chapter to even attempt to summarize the biomedical literature on this topic. Instead the focus here is to provide the necessary background that will allow geochemists to appreciate how minerals may form, transform, or interact with ROS. Literature sources, such as Gilbert and Colton (1999), provide extensive background information on the role of ROS in human physiology and disease.

Formation, function and reactivity of ROS species and related oxidants

In this section, formation, function and chemical reactivity of ROS and related oxidants are briefly discussed. The emphasis in this chapter will be on mineral-induced reactions that lead to formation of primary ROS or to transformation to secondary ROS; however, it is useful to start with a short overview of the formation of ROS and related oxidants within the human body and their function to provide the necessary context. Besides molecular oxygen and its intermediate reduction products (superoxide, hydrogen peroxide, and hydroxyl radical), the oxidants nitric oxide, peroxynitrite and hypochlorite are also introduced. These latter species are often referred to as reactive nitrogen species (RNS) and reactive chlorine species (RCS). The RNS and RCS are included in this overview because there are several important reactions in which ROS are transformed into RNS or RCS or react with RNS or RCS to yield secondary species.

Enzymatic formation of oxidants

White blood cells or leukocytes are the first line of defense against invading pathogens. Specialized leukocytes participate in a robust, nonspecific, defense system known as the innate immune response that deploys enzymatically-generated oxidants to kill invading pathogens. Stimulated either by the presence of an invader or via cell signaling, four enzymes [NADPH oxidase, superoxide dimutase (SOD), nitric oxide synthase (NOS), and myeloperoxidase (MPO)] produce four primary oxidants: $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, HOCl (Babior 2000). These oxidants may be formed in response to internalization of pathogens by phagocytic cells (monocytes, macrophages, and neutrophils) or to binding of pathogens to the cell surface. The primary oxidants can either directly detoxify the pathogens or can react to form other oxidants and radicals that inactivate pathogens. Figure 2 shows some of the key transformation reactions involving the four primary oxidants. A deficiency in any of the four enzymes that produce the primary oxidants leads to weakening of the innate immune system.

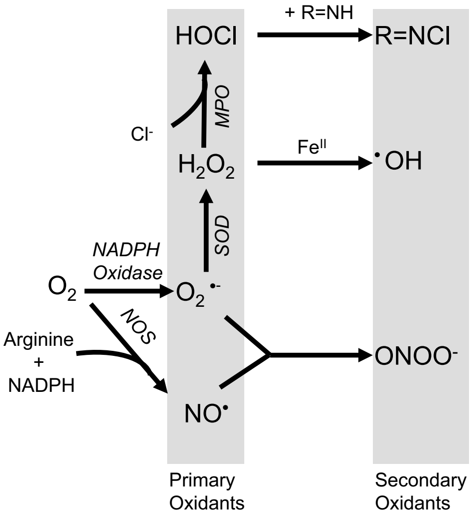


Figure 2. Formation pathways of ROS within human body. Primary oxidants are formed enzymaticaly, secondary oxidants are formed via subsequent reactions.

It is beyond the scope of this chapter to discuss the biochemistry involved in the regulation of the enzyme activity and the enzymatic mechanisms in any level of detail. The reviews by Babior (2000) and by Heiser and Elstner (1998) provide excellent introductions to this topic. In the remainder of this section the origin and function of each of the primary oxidants are discussed and the roles of molecular oxygen and the hydroxyl radical and peroxynitrite, two of the most reactive secondary oxidants are also considered.

It should be noted that the generation of primary oxidants leads to free radical chain reactions with organic molecules that will produce a myriad of radicals. For example, as illustrated in Equation (1) below, hydroxyl radical, $^{\bullet}OH$, may react via hydrogen abstraction and create a new radical carbon species.

$$^{\bullet}OH + R\text{-}C\text{-}H \rightarrow H_2O + R\text{-}C^{\bullet} \tag{1}$$

Secondary, carbon-based radicals may be longer lived and act as important chain propogators.

Molecular oxygen. Molecular oxygen, O_2, has two unpaired electrons in its $\pi*$ orbitals. Despite the fact that it is a bi-radical species, molecular oxygen is relatively stable. Molecular oxygen can accept four electrons and the overall reaction (Eqn. 2) leads to water.

$$O_2(aq) + 4H^+ + 4e^- \rightarrow 2H_2O \tag{2}$$

However, the reduction of oxygen, especially when catalyzed by metal-containing enzymes, proceeds via intermediate steps in which electrons are transferred singly in a stepwise fashion.

Provoked by the presence of an invader or stimulated by cellular signaling mediators, phagocytes will convert molecular oxygen into superoxide, see below. This is the first of a series of reactions outlined in Figure 2 that lead to the formation of the four primary oxidants. Hence, as the innate immune system is engaged, molecular oxygen is consumed.

Superoxide. The first step in the reduction of molecular oxygen leads to superoxide, $O_2^{\bullet-}$ (Eqn. 3), a radical.

$$O_2(aq) + e^- \rightarrow O_2^{\bullet-}(aq) \tag{3}$$

The superoxide anion has an unpaired electron and is the conjugate base of the weak acid HO_2 (Eqn. 4).

$$HO_2^{\bullet}(aq) = H^+ + O_2^{\bullet-}(aq) \qquad pK = -4.88 \tag{4}$$

Given the value of the acid dissociation constant for $HO_2^{\bullet}$, it is expected that at pH values in excess of 4.88 $O_2^{\bullet-}$ will be the dominant form. Hence in lung fluid where the pH is buffered at a value of 7.4, less than 1% of the total superoxide concentration will be present as $HO_2^{\bullet}$. Superoxide can be stabilized as a potassium salt KO_2, which is often used as a source of superoxide *in vitro*.

As pointed out by Sawyer and Valentine (1981), the name superoxide may lead one to believe that this is a very reactive species. In fact, superoxide is rather stable and does not possess the reactivity one may associate with a radical. Superoxide shows little reactivity toward organic matter; it is a single-electron reductant of iron (Eqn. 5).

$$Fe^{3+}(aq) + O_2^{\bullet-}(aq) \rightarrow Fe^{2+} + O_2(aq) \tag{5}$$

In the lungs, ferric iron may be complexed with organic ligands, which increases the rate of this rate and facilitate redox cycling of iron within cells and intracellular fluids (Rose and Waite 2005).

Superoxide does play an important role in the immune system. It is produced by the enzyme NADPH oxidase as the first step in the immune system's response against pathogens, Figure 2. The enzyme superoxide dismutase (SOD) facilitates a reaction between two superoxide molecules yielding hydrogen peroxide and molecular oxygen (Eqn. 6):

$$2\,O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \tag{6}$$

SOD is an extremely efficient biocatalyst, it has the highest turnover number of any known enzyme (Babior 2000). Hydrogen peroxide, one of the products of this reaction, is not a radical, but is nevertheless a crucial component of pathways of ROS generation as it can undergo subsequent reactions leading to several radicals (Fig. 2).

Hydrogen peroxide and peroxide anion. The acid dissociation equilibrium of hydrogen peroxide favors the protonated form under most environmental and physiological pH conditions (Eqn. 7).

$$H_2O_2 = HO_2^- + H^+ \qquad pK = 11.65 \tag{7}$$

H_2O_2 is not a radical like superoxide and it is also rather unreactive toward biomolecules. For example, RNA is stable in dilute hydrogen peroxide solutions (Cohn et al. 2004). Most free radicals of biological relevance react within a few molecular diameters from where they are formed; however, hydrogen peroxide can be expected to be transported over much longer distances before it reacts. For example, most natural waters have nanomolar concentrations of hydrogen peroxide (Cooper and Zika 1983); higher concentrations are found in waters exposed to sunlight (Cooper et al. 1988; Emmenegger 1998; Wilson et al. 2000b). Hydrogen peroxide is injected into groundwater and added to wastewater to promote the degradation of the organic pollutants by forming hydroxyl radicals via reaction with ferrous iron (EPA 1998; Watts et al. 1999a; Kwan and Voelker 2003; Ma et al. 2005; Watts and Teel 2005). The reactivity of hydroxyl radicals is discussed below.

The SOD-catalyzed conversion of superoxide to hydrogen peroxide and molecular oxygen is an especially prominent reaction in mononuclear phagocytes (monocytes and macrophages). Given that hydrogen peroxide is a key reactant that leads to very reactive oxidants, such as hydroxyl radicals and hypochlorous acid (Fig. 3), its intracellular concentration is tightly controlled. The enzyme catalase is used to protect cells from hydrogen peroxide-induced damage by facilitating the conversion of hydrogen peroxide to water and oxygen.

Hydroxyl radical. With a bond dissociation energy of 146 kJ/mole, the O-O bond in hydrogen peroxide is relatively weak compared to other covalent bonds (e.g., C-C 357 kJ/mole, S-S 231 kJ/mole). Homolytic cleavage of this bond leads to the formation of hydroxyl radical, $^{\bullet}OH$ (Eqn. 8).

$$H_2O_2 \rightarrow 2^{\bullet}OH \tag{8}$$

The hydroxyl radical is one of the most reactive species in nature. It reacts nonspecifically with most organic molecules. Reactions take place within nanoseconds after $^{\bullet}OH$ formation.

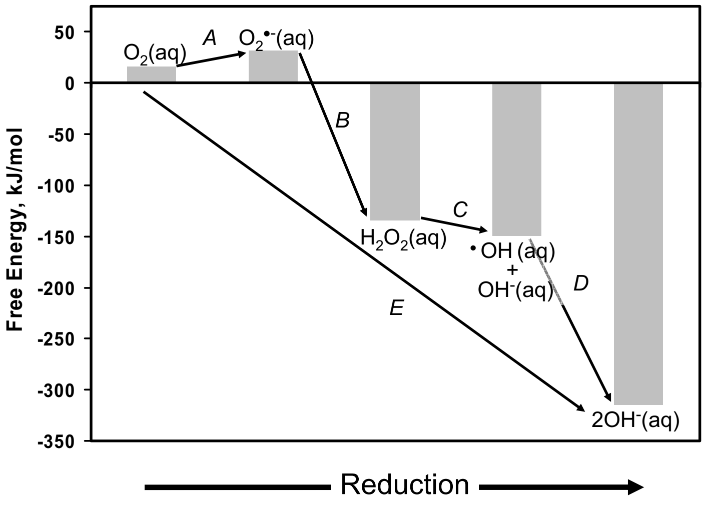


Figure 3. Free Energy diagram for (step-wise) O_2 reduction. See Table 3 for reactions and thermodynamic data.

Hence, reactions involving $^{\bullet}$OH take place within a few molecular diffusion distances from the sites of formation (Pryor 1986).

The homolytic cleavage of hydrogen peroxide requires either thermal activation, exposure to UV, or the presence of a catalyst. At a temperature of 160 °C, half of the hydrogen peroxide present will undergo homolysis in one hour. By contrast, capture of a UV photon leads to the slow dissociation of hydrogen peroxide at room temperature (this is the reason H_2O_2 solutions are typically stored in the dark). The addition of a reduced transition metal, such as ferrous iron and cupric copper leads to a heterolytic decomposition with OH^- and $^{\bullet}$OH as products. This is exploited in the so called Fenton reaction (Eqn. 9) in which hydrogen peroxide is mixed with a solution of a ferrous iron salt (Fenton 1894; Walling 1975).

$$H_2O_2(aq) + Fe^{2+} \rightarrow {}^{\bullet}OH(aq) + OH^-(aq) + Fe^{3+} \tag{9}$$

Given its reactivity, the formation of hydroxyl radical in biological systems is tightly controlled. This is accomplished in cellular systems by limiting the availability of labile ferrous iron and controlling the concentration of hydrogen peroxide, the two Fenton reactants (Valko et al. 2005).

Nitric oxide and peroxynitrite. Nitric oxide, $NO^{\bullet}$, is a radical species which can further react with superoxide to form peroxynitrite, $ONOO^-$ (Vásquez-Vivar et al. 1996; Burney et al. 1999). Nitric oxide is present in the environment as an air pollutant emitted by combustion engines (Berner and Berner 1996). Nitric oxide, although it is a free radical, is nevertheless relatively unreactive toward biomolecules and can, therefore, be transported through lipid membranes in and out of cells. Because of its relatively low chemical reactivity and its capacity to pass from cell to cell, nitric oxide is now recognized as an especially important signaling molecule (Burney et al. 1999). For example, nitric oxide levels in the inner lining of blood vessel signal the smooth muscle around the vessel to contract or dilate, thereby controlling blood flow. The formation of nitric oxide is synthesized by at least three different nitric oxide synthase (NOS) enzymes (Vásquez-Vivar et al. 1998) associated with endothelial cells (eNOS), neurons (nNOS), and inflammatory leukocytes (inducible or iNOS). Given its relatively low reactivity, it has been suggested that the conversion of nitric oxide to the much more highly reactive peroxynitrite anion is a critical step in the defense mechanism against invading bacteria (Pou et al. 1995; Vásquez-Vivar et al. 1996; Burney et al. 1999). The peroxynitrite species is a strong oxidant, capable of oxidizing nucleic acids and lipids among other biomolecules (Vásquez-Vivar et al. 1996).

Peroxynitrite is a weak acid ($pK_1 = 6.8$). Hence, at pH 7.4, the anionic form will be slightly more abundant than the acid form. Below pH 6.8, the acid form will dominate. The reaction between superoxide ions and nitric oxide that results in formation of peroxynitrite proceeds at a near diffusion-limited rate ($k = 6.7\ 10^9\ M^{-1}\ s^{-1}$) and may compete effectively with the SOD catalyzed dismutase of superoxide (Burney et al. 1999).

Hypochlorous acid. Hypochlorous acid, HOCl, and its conjugate base hypochlorite, OCl^-, is used as household bleach and is the most widely used mild disinfectant in water treatment (Stumm and Morgan 1995). At physiological pH (7.4), about 50% of the acid will be present as OCl^-. Hypochlorous acid reacts with a broad spectrum of biomolecules and causes cell death (hence its effectiveness as a disinfectant) (Whiteman et al. 2002). The broad-spectrum reactivity of hypochlorous acid is exploited in the immune system. Hypochlorous acid is synthesized within the neutrophil, a type of phagocytic leukocyte that is especially active in engulfing and killing bacteria. The enzyme myeloperoxidase (MPO) catalyzes the oxidation of halides by hydrogen peroxide (Whiteman et al. 2002; Fig. 2). Given that chloride is the most abundant halide in cell fluid, the predominant product is HOCl (Babior 2000):

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^- \tag{10}$$

Thermodynamics of ROS

The thermodynamics of the stepwise reduction of molecular oxygen have been studied extensively. The most important thermodynamic data are summarized in this section. This provides the necessary background for discussing the kinetics and catalysis of ROS formation and transformation reactions.

The relative thermodynamic stability of ROS acts as an important constraint on their reactivity. In Figure 3, the stepwise reduction of molecular oxygen is presented in a free energy diagram. In this diagram, all one-electron transfer reactions are written so that only electrons and protons are consumed in the reactions. Since, by convention, the free energy of formation for both the proton and electron equal zero, the free energy differences among the different ROS can be considered to account fully for the difference in free energy for the reactions in which these species are transformed. The complete reactions and their equilibrium constants are summarized in Table 3. There are several important observations to be made here.

The first step in the stepwise reduction of dissolved molecular oxygen is thermodynamically unfavorable (endergonic), with a positive ΔG_r^o of 15.52 kJ/mole (Table 3). In essence, adding the first electron to molecular oxygen is an activation step along the stepwise reduction path. This activation barrier explains in part the relative inertness of molecular oxygen. All other steps are thermodynamically favorable (exergonic); with the reduction of superoxide to hydrogen peroxide as well as the hydroxyl radical to hydroxide steps as the most energetically favorable reactions. Hence, these two steps provide a very strong oxidative driving force. In other words, superoxide and hydroxyl radical have the thermodynamic potential to oxidize a wide range of compounds (whether these species react rapidly depends on the reaction mechanism and cannot be predicted on the basis of the thermodynamics). The reduction of hydrogen peroxide to hydroxyl radical and hydroxide is exergonic but requires the dissociation of an O-O bond. This bond has a dissociation energy of 146 kJ/mole (Atkins and de Paula 2002), which represents a formidable activation barrier. It is this activation barrier that explains the relative stability of hydrogen peroxide.

The free energy diagram presented in Figure 3 also suggests that there are two steps within the cascade that could be catalyzed. Catalysis that allows two electrons to be added to molecular oxygen in one step would lead to hydrogen peroxide and avoids the endergonic first step in the cascade. For example, oxidation of pyrite, FeS_2, is known to produce hydrogen peroxide (Ahlberg and Broo 1997; Cohn et al. 2005c). As a semiconductor mineral, pyrite can transfer two electrons in concert and avoid the formation of a superoxide intermediate. The second reaction that benefits from catalysis is the dissociation of hydrogen peroxide. For example, the addition of ferrous or ferric iron is known to facilitate the decomposition of hydrogen peroxide [the Fenton reaction referred to above; Fenton (1894)]. The exact mechanism has been a matter

Table 3. Thermodynamic data for selected reactions involving ROS[a].

	Reaction	ΔG_r^o (kJ mol^{-1})	log K_{25}	E^o (pH = 7) (mV)
A	$O_2(aq) + e^- = O_2^{\bullet -}(aq)$	15.52	−2.7	−161
B	$O_2^{\bullet -}(aq) + e^- + 2H^+ = H_2O_2(aq)$	−165.9	29.1	892
C	$H_2O_2(aq) + e^- = OH^-(aq) + {}^\bullet OH(aq)$	−15.42	2.71	575
D	${}^\bullet OH(aq) + e^- = OH^-(aq)$	−165.0	28.91	2126
E	$O_2(aq) + 4\,e^- + 2H^+ = 2\,OH^-(aq)$	−330.9	57.97	3432

[a] Thermodynamic data taken from Stumm and Morgan (1995)

of debate for close to a century, but it is thought to involve an activated reaction complex that involves a ferrous iron cation and a hydrogen peroxide. The ferrous iron transfers an electron to hydrogen peroxide, forcing the dissociation of the O-O bond into a hydroxyl radical and a hydroxide ion. It has been suggested that as an intermediate a ferryl [Fe(IV)] species forms (Pierre and Fontecave 1999; Alegría et al. 2003). Ferrous iron acts as a catalyst in the Fenton reaction (Eqn. 9) if the ferric iron product is reduced. Some ferric iron is reduced by hydrogen peroxide back to ferrous iron (Eqn. 11) (Alegría et al. 2003).

$$Fe^{3+}(aq) + H_2O_2\,(aq) = Fe^{2+} + HO_2^{\bullet} + H^+ \tag{11}$$

This reaction is slow, but leads to regeneration of ferrous iron which can then further promote the Fenton reaction. For example, it has been shown that adding ferric iron to a hydrogen peroxide solution leads to an induction period in which no hydrogen peroxide is decomposed. After the induction period the reaction proceeds at the same rate as when the reaction is initiated with ferrous iron (Alegría et al. 2003). In many systems the reduction of ferric iron takes place with organic reductant. Other reducible transition state metals (e.g., Cu, Co, V, Cr) can also catalyze the Fenton reaction (Pierre and Fontecave 1999; Valko et al. 2005). The deleterious effects of ROS are predominantly related to reactions involving hydroxyl radicals, which are more reactive than superoxide. Hence, controlling the concentration of metals that can catalyze the Fenton reaction is crucial. Chelation with a ligand that prevents the formation of a ternary complex between hydrogen peroxide, metal ion, and the ligand is a key requirement (Graf and Goldsmith 1955). It has been shown that ternary ligand-metal-H_2O_2 complexes will actually accelerate the Fenton reaction (Graf and Goldsmith 1955; Alegría et al. 2003) whereas desferrioxamine (DFO) and phytate can complex iron and inhibit the formation of the ternary ligand-metal-H_2O_2 complex (Graf et al. 1984).

It is common to analyze the thermodynamics of redox reactions involving ROS on the basis of a comparison of standard potentials for the redox couples involved. While this approach is convenient, it can lead to some serious misconceptions, as pointed out by Pierre and Fontecave (1999). To illustrate the problems with this approach consider the reduction of hydrogen peroxide to hydroxyl radical (Eqn. 9), the molecular oxygen/superoxide couple (Eqn. 6), and several other relevant reactions. It is customary to construct a redox ladder, which is nothing more than the standard potentials for the reactions of interest at a common set of conditions, e.g., pH 7 (Stumm and Morgan 1995). The vertical scale of the ladder can be either in mV or Volts with respect to the NHE or p*e*. It is common to use the relative position of two half reactions on a redox ladder to evaluate in which direction an overall reaction between the two half cells will proceed spontaneously (the higher redox couple will accept electrons; whilst the lower couple will donate electrons). For example, the standard potential of the $O_2(aq)/O_2^{\bullet-}$ redox couple suggests that any redox couple involving ferric and ferrous iron with a standard potential in excess of -160 mV can lead to reduction of the ferric iron to ferrous iron (Fig. 4). Hence, one would predict that $Fe(H_2O)_6^{3+}$ as well as Fe(III)-EDTA will be reduced to ferrous iron. However, this only holds if the superoxide activity (or effective concentration) is equal to that of dissolved molecular oxygen. As pointed out by Pierre and Fontecave (1999), the concentration of superoxide in a normal cell is around 10^{-11} M and the concentration of molecular oxygen will be around 3.5×10^{-5} M. Applying the Nernst Equation, the actual $O_2(aq)/O_2^{\bullet-}$ ratio leads to a redox potential of +230 mV, an upward shift of 390 mV. As a result, a 50/50 Fe(II)-EDTA/Fe(III)-EDTA solution is no longer expected to be reduced by the $O_2(aq)/O_2^{\bullet-}$ couple. Conversely, upregulation of superoxide production at the expense of molecular oxygen will shift the actual potential provided by the $O_2(aq)/O_2^{\bullet-}$ activity ratio down. When interpreting which oxidation reactions the $H_2O_2/{}^{\bullet}OH+OH^-$ couple can drive, it should be kept in mind that the activity of ${}^{\bullet}OH$ will be many orders of magnitude smaller than that for H_2O_2, effectively raising the position of this couple upward. For example, if the activity of the radical is a factor of 10^{10} lower than that for hydrogen peroxide, the position of

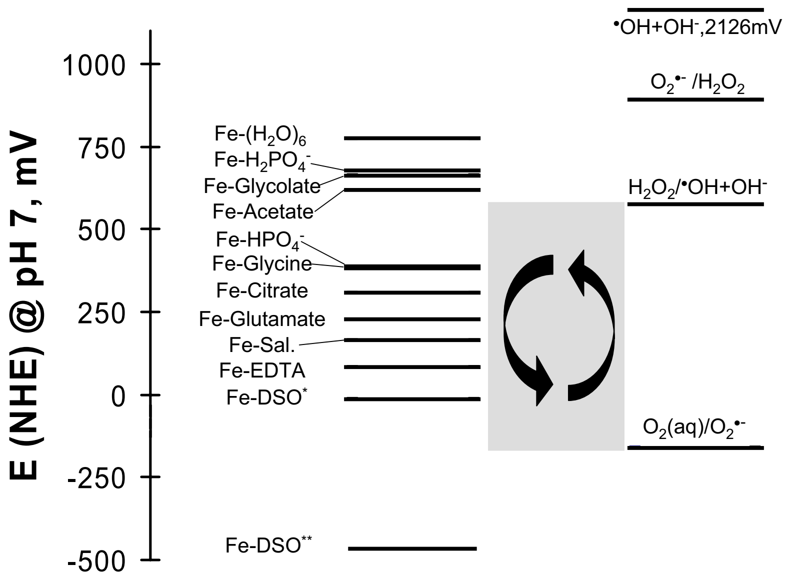


Figure 4. Standard redox potentials for couples involving dissolved iron species and ROS.

the effective position of the redox couple will be raised by 590 mV. As a result this couple can drive almost any oxidation reaction. It is also important to keep in mind that reactions that are feasible energetically may not proceed at significant rates due to kinetic inhibitions.

Analytical methods to determine ROS

The reactivity of ROS, particularly hydroxyl radical, presents a considerable analytical challenge. To meet this challenge several strategies have been developed for the analysis of ROS in acellular and cellular studies. For very reactive species (e.g., hydroxyl radical), there are two different approaches. One can add a scavenger to the system of interest and analyze the conversion of the scavenger. In this strategy the scavenger is added in sufficiently high concentration to effectively out compete all other radical-consuming reactions. An example of a common scavenger is benzoic acid, which is converted to salicylic acid as it reacts with hydroxyl radical (Winterbourn 1987). An alternative strategy is to use a radical probe. Probes, such as nitrobenzene and anisole, are in essence radical scavengers but they are deployed in low concentrations (typically less than 5 μM) and their reaction does not affect the steady state concentration of radicals. The radical concentration in the experimental system is determined on the basis of loss or conversion of probe molecules. This strategy is often deployed in photochemical studies in which the interaction of photons with the system of interest produces radicals and the objective is to determine the steady state radical concentration under illumination (Zepp et al. 1992; Kwan and Voelker 2002).

While scavenging techniques relying on the conversion of organic molecules is widely used it is also possible to use structural changes to biomolecules to demonstrate the presence of radicals. For example, Cohn and coworkers have developed a protocol that uses strand breakage in yeast RNA as an assay that is suitable for the study of mineral-induced hydroxyl radical formation in mineral slurries and coal slurries (Cohn et al. 2005b). In brief, this method makes use of a molecular probe specific to RNA (Ribogreen from Invitrogen). The probe will fluoresce when bound to RNA. However, if the yeast RNA strand is shortened due to interaction with hydroxyl radical, the molecular probe will no longer fluoresce. Thus, the loss of Ribogreen fluorescence serves as a measure of hydroxyl radical-induced strand breakage. In addition to the method developed by Cohn, there are a number of other standard assays that are all based on radical-induced strand breakage of nucleic acids. In most cases, such methods make use of DNA, rather than RNA. The strand breakage is often detected by studying the mobility of the nucleic acid in a gel electrophoresis experiment. The mobility of a nucleic acid in an agarose gel depends on its size and structure. A tightly coiled DNA can unwind as it reacts with a hydroxyl radical. This (partial) unwinding as a result of the interaction with a radical will result in increased hydrodynamic radius of the linear polymer and a consequent lowered mobility. Conversely, fragmentation of nucleic acids will lead to shorter pieces that will have a higher mobility. Several examples of the use of gel electrophoresis will be given later in the chapter. In addition to introducing nucleic acid strand breaks, the hydroxyl radical may also oxidize the bases. Guanine is particularly susceptible and its main oxidized product, 8-oxodeoxyguanosine (8-oxodG) is often used as a marker for hydroxyl radical-induced oxidation.

Radicals can also be detected by forming a stable adduct that can be detected. This strategy is often referred to as spin trapping. The concentration of the stable adduct is often determined using electron spin resonance spectroscopy (ESR) or in some cases by fluorescence measurements. For cellular systems it is also useful to analyze for biomarkers that are indicative of the upregulation of ROS. In this section, we briefly present some of the most commonly used strategies and techniques. In addition we discuss in more detail assays our group have developed or adapted for studies involving mineral suspension ROS detection. The first part of this section is devoted to ESR and spin trapping, followed by a section on the challenges related to ROS detection in cellular systems. Table 4 summarizes techniques that are used commonly for ROS detections.

Table 4. Common ROS detection methods[*].

Probe	Detects	Equipment	Sensitivity	Literature[#]
DMPO	$^{\bullet}OH$, $(O_2^{\bullet})^-$	ESR	nM range	1
DCFH	H_2O_2, $^{\bullet}OH$, $ONOO^-$, $NO^{\bullet}$	fluorometer	nM range	2
APF	$^{\bullet}OH$, ^-OCl	fluorometer	nM range	3
PF	$^{\bullet}OH$, $(O_2^{\bullet})^-$	fluorometer	nM range	4
Tempo-9-ac	$^{\bullet}OH$, $(O_2^{\bullet})^-$	fluorometer	nM range	5
Amplex Red	H_2O_2	fluorometer	10 nM	6
LCV	H_2O_2	UV-Vis	μM range	7
Cu-DMP	H_2O_2	fluorometer	1.0 μM	8
Scopoletin	H_2O_2	fluorometer	25 nM	9
Benzoic acid	$^{\bullet}OH$	GC	nM range	10
Luminol and Lucigenin chemiluminescence	H_2O_2, $^{\bullet}OH$, $(O_2^{\bullet})^-$	luminometer	nM range	11

[*] The ROS detection methods listed here have been used by our group; however, there are many other methods available. The literature citations are examples of papers that use the given method. [#] *Literature Sources*: 1: Fubini et al. (1995); Shi et al. (2004); 2: LeBel et al. (1992); Rota et al. (1999); 3: Setsukinai et al. (2003); 4:Pou et al. (1993, 1995); Li et al. (1997); 5: Borisenko et al. (2004); 6: Zhou et al. (1997); 7: Mottola et al. (1970); Zhang and Wong (1994); Cohn et al. (2005c); 8: Kosaka et al. (1998); 9: Cooper et al. (1988); Holm et al. (1987); Wilson et al. (2000a); 10: Winterbourn (1987); 11: Casadevall et al. (1984); Yıldız and Demiryürek (1998); Ohyama et al. (2001); Fach et al. (2002); Iwata and Yano (2003)

Electron spin resonance spectroscopy (ESR). Electron spin resonance spectroscopy, often also referred to as electron paramagnetic resonance spectroscopy, is frequently used to determine the products of a spin trap experiment. In ESR spectroscopy, electronic spins are excited by placing the sample in a magnetic field. Unpaired electrons will resonate at specific magnetic field strengths (termed hyperfine splitting-constants) between a state in which their spin is aligned parallel and antiparallel to the external magnetic field. The energy difference associated with the conversion between the two spin states is measured in an ESR experiment and detected as an energy absorbance as a function of magnetic field strength. The absorbance spectrum yields information on the local environment around the unpaired electron. ESR spectra are typically presented as the first derivative of the absorbance vs. magnetic field strength as this conversion improves the apparent spectral resolution and facilitates the interpretation of subtle features in the absorbance spectrum (the same strategy is often used in FTIR spectroscopy; Smith 1996). The ESR technique is both quantitative and qualitative. The signal intensity is related to the number of excited unpaired electrons and identification of radical adducts is determined based on the hyperfine splitting constants. ESR is used to probe unpaired electrons in the solid, liquid and gaseous state. Liquid samples are used in spin trapping experiments. ESR is a well-established technique; the text by Gordy (1980) provides further detail into the theory and practice of the technique. Geochemists may find the introduction by Calas (1988) in one of the earlier Reviews in Mineralogy and Geochemistry volumes useful, although that review is exclusively focused on solid state ESR. An excellent on-line tutorial of the technique is also available (Bruker Biospin 2006).

Spin trapping. In a spin trap experiment, a spin probe molecule is reacted with the sample and the reaction product is analyzed. Typically the reaction products are analyzed using ESR, but some spin probes have been developed that will alter in fluorescence intensity upon reaction with a target radical. Hence, for those probes, the fluorescence intensity of the reaction products is determined. Spin trapping of transient free radicals has been widely used in the biomedical research community, but has seen limited application in the geochemical research community. The purpose of this brief section is to introduce spin trapping techniques as well as potential problems in applying the technique to mineral slurries.

As mentioned above, the goal of a spin trap experiment is to form an adduct that is more stable than the free radical and then measure the concentration of the adduct. A suite of nitrone and nitroso spin traps (e.g., PNB, POBN, DMPO) are routinely used in biomedical research (Khan et al. 2003). These spin traps can be added to cells and have even been injected into mice to detect the presence of superoxide *in vivo* (Dikalova et al. 2001; Takeshita et al. 2004). A successful spin trap experiment requires that the spin trap reacts rapidly and efficiently with the target free radical. A second requirement is that the adduct has a sufficiently long half-life that it remains stable over the course of the ESR measurements. The half-life of the DMPO-OH adduct in aqueous solutions is only on the order of 3 min; the BMPO-OH adduct has a half life approaching 20 min in aqueous solution (Khan et al. 2003). The practical implication is that experiments have to be conducted where there is immediate access to an ESR facility.

One of the challenges faced in spin trap experiments is the level of specificity that can be achieved. One of the most commonly used spin traps, DMPO, reacts with hydroxyl radical, superoxide, and dissolved ferric iron. The reaction of DMPO with superoxide and hydroxyl radical leads to two different adducts, DMPO-OOH and DMPO-OH, respectively. However, the DMPO-OOH adduct spontaneously decays to DMPO-OH on a timescale that makes it impossible to measure the DMPO-OOH adduct. This limitation can be circumvented by a standard method to determine if the signal is derived from superoxide or hydroxyl radical. This involves conducting a series of ESR experiments as shown in Figure 5 taken from (Shi et al. 2005). The top spectrum of Figure 5 is a characteristic 4-line ESR spectrum of the DMPO-OH adduct. This spectrum could have originated from either the interaction of the superoxide

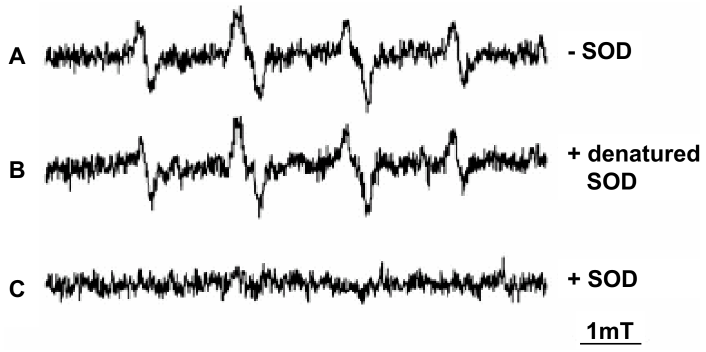


Figure 5. 1,6-Benzo(a)pyrene quinone (BPQ) induced superoxide radical formation in human mammary epithelial cell line, MFC-10A. Spectrum A: BMPO (50 mM) was mixed with 1E6 cells, 100 μL cell medium, and 10 μM BPQ was added to start reaction. Spectrum B: same as spectrum A but with pre-added heat denaturated SOD (400 U/mL). Spectrum C: same as spectrum A with pre-added SOD (400 U/mL). [Reprinted from Shi et al., *Archives of Biochemistry and Biophysics*, Vol. 437, p. 59-68. Copyright (2005),with permission from Elsevier.]

radical or the hydroxyl radical with the probe. However, addition of the enzyme superoxide dismutase (SOD) shows that superoxide is responsible for the 4-line DMPO-OH spectrum and not hydroxyl radical. A control experiment with denatured SOD is necessary to rule out that the addition of SOD itself would alter the spectrum. SOD is inactivated (denatured) by heating.

In the case of studies involving iron-bearing minerals, it is important to note that the presence of ferric iron can induce the formation of a DMPO-OH adduct in the absence $^{\bullet}$OH (Makino et al. 1990). The interaction between ferric iron and DMPO presents a problem for studies with iron-containing minerals. However, there is a standard protocol to verify the presence of radicals. In this protocol another reactant, which acts as a *radical scavenger* (e.g., ethanol, mannitol, dimethyl sulfoxide), is added to compete with DMPO for reaction with $^{\bullet}$OH. Addition of radical scavengers is often used to verify the presence of a particular radical species and to determine the contribution of the radical species when non-radical reactions will lead to the same spectrum (Rice-Evans et al. 1991). When ethanol is used as a radical scavenger, its reaction with $^{\bullet}$OH forms the α-hydroxyethyl radical that adds to DMPO resulting in the DMPO-CH(CH$_3$)OH adduct (Makino et al. 1990). In the absence of $^{\bullet}$OH, but presence of ferric iron (or other nucleophiles), ethanol will add to DMPO with its oxygen and not carbon moiety. This then results in the formation of a DMPO-OCH$_2$CH$_3$ adduct (Makino et al. 1990). The DMPO-CH(CH$_3$)OH and DMPO-OCH$_2$CH$_3$ adducts have different ESR spectra, see Figure 6.

Fluorogenic spin trap methods are relatively new (Blough and Simpson 1988; Pou et al. 1993; Li et al. 1997; Zhou et al. 1997; Setsukinai et al. 2003; Borisenko et al. 2004) and have only recently been applied to the study of soil slurries by Blough and coworkers (Petigara et al. 2002). These reagents are relatively stable free radicals themselves, which form spin-paired adducts with more highly reactive radicals. The emission from the fluorophore in these reagents is quenched by the delocalized unpaired spin in the unreacted probe, but in the spin-paired products the quantum yield becomes significant. The major advantage of these spin traps is that they do not require an ESR facility to quantify the adduct. A relatively simple and inexpensive fluorometer can be used to determine the adduct concentration. This simplicity also has the advantage that fluorogenic spin trap experiments can be conducted in anaerobic chambers and in the field. Fluorogenic spin traps can also be used to image the spatial distribution of the radical formation within cells or tissue (Pou et al. 1995). It should be pointed out, however, that in many cases, ESR spectra are specific to certain radicals, while fluorogenic spin traps do not

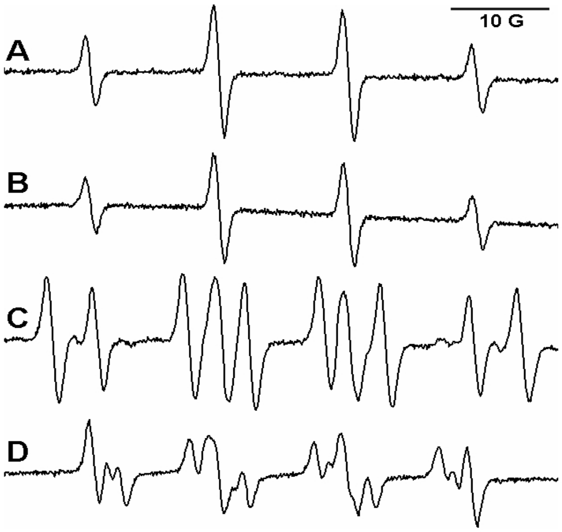


Figure 6. ESR spin-trapping with 100 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO): (A) Fenton reaction (120 μM H_2O_2 + 25 μM Fe(II)), (B) 100 μM Fe(III), (C) Fenton + 33% EtOH, (D) 500 μM Fe(III) + 33% EtOH. The experiments were conducted by mixing the reactants and immediately injection into a liquid flow-through cell at room temperature (24 ± 2 °C) with the following ESR settings: magnetic field of 3470 ± 100 G, microwave power of 20 mW, modulation frequency of 100 kHz and amplitude of 1 G, receiver gain of 2×10^5, time constant of 0.64 s, and scan time of 2 min 47 s.

provide a spectrum—only a change in fluorescence. A fluorogenic spin trap for hydroxyl radical will show an increase in fluorescence as it reacts with the radical, but it will not generate the diagnostic ESR spectrum. The use of some fluorogenic spin traps is complicated by the fact that they can react with a number of radicals with a considerable range of efficiency in formation of the spin-paired fluorescent product; hence, it is important to develop a panel of assays.

Our group has been using fluorogenic spin traps to study the formation of radicals within mineral slurries and coal slurries as well as in cell cultures exposed to mineral particles, see Table 4. All the methods listed in Table 4 were designed to be used in biomedical studies and not to detect radicals in mineral or coal slurries. We have, however, found that the methods listed in Table 4 can be used to determine radicals in mineral/coal slurries and or cell cultures exposed to minerals. In all these experiments, a reactant probe is added to the slurry or culture at the start of the experiment. In experiments with mineral slurries, aliquots are withdrawn periodically and the fluorescence of the spin trap is determined after filtration. Calibration curves for hydroxyl radical can be created using the Fenton reaction or reaction of horseradish peroxidase (HRP) with hydrogen peroxide. For some probes, the addition of HRP to H_2O_2 in the presence of a fluorogenic spin trap results in conversion of H_2O_2 to water and oxidation of the spin trap (Eqn. 12).

$$\text{probe} + H_2O_2 \rightarrow \text{oxidized probe} + H_2O \qquad (12)$$

Thus, measured fluorescence can be related to a known equivalent hydrogen peroxide concentration. In experiments performed to evaluate mineral particle generation of ROS in cell cultures, the fluorescence of the probe in the presence of cells exposed to minerals is compared to the fluorescence of the probe in the presence of cells that are untreated. These measurements can be efficiently performed directly on the reaction mixtures of particles, cells, and probe using a well plate reader.

By combining fluorogenic spin traps with fluorogenic assays for hydrogen peroxide, it is possible to rapidly determine hydrogen peroxide and hydroxyl radical formation in mineral slurries. The most commonly used fluorogenic probe is DCFH which will react with a broad spectrum of ROS (Rota et al. 1999). DCFH is widely used in ROS detection in cell cultures. A critical step in its use in cell cultures is the esterification of DCFH, which generates a product which is highly cell-permeable and ensures uptake of DCFH into the cells. Within the cell, nonspecific esterases in the cytosol of viable cells regenerates the DCFH. Most commercially available DCFH reagent preparations are designed for use in cell cultures and, therefore, in the ester form. Hence, when DCFH is used with cell-free mineral slurries, it is important to revert the ester form to DCFH (LeBel et al. 1992). The DCHF protocol does require daily preparation of the reagent and its storage under an inert gas, which makes it cumbersome. Amplex UltraRed, a H_2O_2 specific assay, is a better suited for work with mineral slurries. In brief, the Amplex UltraRed reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in a 1:1 stoichiometry in the presence of horseradish peroxidase (HRP) to produce highly fluorescent Resorufin ($C_{12}H_6NNaO_3$). Compared to the most widely used probe for H_2O_2, DCFH, Amplex UltraRed is 5 to 20 times as sensitive, and Resorufin is more stable to chemical and photo-induced oxidation (Zhou et al. 1997). Amplex UltraRed has been used to measure H_2O_2 production in cells exposed to asbestos (Xu et al. 2002), cancer cells (Wagner et al. 2005), plant cells (Janisch and Schempp 2004), and brain mitochondia (Gyulkhandanyan and Pennefather 2004).

ROS analysis in cell cultures. ROS analysis in cell cultures presents additional challenges. The initial products generated by reactions of minerals with water and oxygen—observed in studies of mineral slurries—may themselves serve as substrates for further reactions catalyzed by enzymes present in the cellular environment. The products formed by such reactions may themselves be highly reactive and longer lived than such highly reactive species as hydroxyl radicals. These secondary species may then go on to modify proteins, lipids, and nucleic acids within cells, triggering a range of pathologic consequences. An appreciation of the potential consequences of reactivity of various mineral dusts and particulates therefore requires a panel of assays to detect formation of initial mineral-derived reactive species as well as secondary reactive species, which may be generated by enzymatic conversions of the initial reactive species within live cells. For example, hydroxyl radicals, which may be formed directly at the mineral surface, may trigger formation of secondary species such as superoxide ions, hypochlorite ions, and hydrogen peroxide by serving as substrates for secondary reactions mediated by cellular components. Once such secondary species are formed, further reactions initiated by the response of specialized inflammatory cells to modifications of their constituents produced by primary and secondary reactive oxygen species may lead to generation of yet additional highly chemically reactive species, such as hypochlorite ions, which are typically formed only when such cells have been stimulated by exogenous "foreign" materials. Therefore, it would be desirable to use a range of assays that are not only sensitive and specific, but, which can also detect secondary and tertiary products and be compatible with the cells and enzymes involved in generating such species.

In addition to probes that detect hydroxyl radicals via direct reaction, such as APF, other fluorogenic spin traps are available that are capable of detecting a number of free radical species, including hydroxyl radicals and can be used as long as a suitable molecule is also present to serve as a mediator. These fluorogenic spin traps are far more sensitive detectors of free radicals than the "parent" spin traps themselves because of the inherently lower limits of detection by fluorescence spectroscopy than electron spin resonance spectroscopy. Proxyl fluorescamine (PF) was developed by Blough's laboratory (Blough and Simpson 1988) to serve as a fluorogenic derivative of the proxyl spin trap: the fluorophore is quenched in the presence of the nitroxide with its delocalized unpaired electron but emits with a high quantum yield when the nitroxide is converted to the corresponding spin-paired hydroxylamine by alkyl free radicals. In the presence of hydroxyl radicals, however, PF fails to undergo direct reaction. Blough and coworkers (Pou et al. 1993; Li et al. 1997) have overcome this lack of reactivity by adding dimethylsulfoxide (DMSO) (< 1 M, which is compatible with normal cell functions; 5% DMSO = 705 mM), to a mixture of a ferrous salt and a source of hydrogen peroxide, components which generate hydroxyl radicals via the Fenton reaction. Under these conditions, the hydroxyl radicals react with the DMSO to form methyl radicals, which then react with the PF to yield the fluorescent hydroxylamine. Blough et al. have gone on to show that PF can also be used as a spin trap for hydroxyl radicals generated intracellularly by metabolism of a quinone-based drug by a mouse epidermal cell line. As an alternative reagent, Borisenko et al. (2004) have employed TEMPO-acridine (TEMPO-Ac) as a fluorogenic spin trap. Conversion of this nitroxide to a fluorescent product, acridine-piperidine, cannot be effectively achieved except by thiyl radicals, formed under physiologically relevant conditions within cells from the free radical scavenger, glutathione, under oxidizing conditions. Borisenko et al. added phenol and hydrogen peroxide to cells to "commandeer" their intracellular myeloperoxidase. TEMPO-Ac in the presence of exogenous glutathione and phenol can also be used to detect hydroxyl radicals formed from mineral suspensions. Furthermore, TEMPO-Ac along with low concentrations of phenol can be used as an intracellular probe to detect secondary formation of thiyl free radicals from the endogenous glutathione within MonoMac 6 cells and human neutrophils which have been allowed to phagocytose (i.e., engulf) mineral particles.

Using chemiluminescence with luminol or lucigenin, particle-induced ROS have been detected in cell cultures. The probes become luminescent when valence electrons are promoted to higher energy states by an oxidation reaction. This luminescence is detected with a luminometer. Lucigenin and luminol have been used to detect particle-induced superoxide and hydrogen peroxide (Casadevall et al. 1984; Fach et al. 2002; Iwata and Yano 2003; Ohyama et al. 2001) and luminol has been shown to react with hydroxyl radicals (Yıldız and Demiryürek 1998).

PART II: MECHANISMS OF MINERAL-INDUCED ROS FORMATION

In Part II each of the five mechanisms listed in the beginning of the chapter will be discussed in detail. Specific examples from studies—mostly *in vitro*—conducted by our group as well as by others are used to illustrate these mechanisms.

Homogeneous catalysis of ROS (trans)formation by dissolved metal ions (mechanism 1)

Minerals may promote the formation or transformation of ROS by (partially) dissolving into body fluids. The dissolution of minerals is covered in the chapter by Plumlee et al. (2006) in this volume. Important concepts such as biodurability and bioavailability are introduced and discussed in depth in that chapter. In addition the chapter by Reeder et al. (2006) discusses the importance of metal speciation and the techniques that can be used to determine metal composition and speciation in Earth materials and the changes these materials undergo when exposed to body fluids. There is also a vast body of literature on the thermodynamics and kinetics of the dissolution of minerals in aqueous fluids ranging from distilled water, to seawater, to hydrothermal water in the geochemical literature (Lasaga 1998). However, in the context of ROS formation, it is important to consider the release of metals and metalloids as minerals are reacting with biofluids. For this chapter, we will only consider the interaction with lung fluid and the more acid (pH ~ 4.5) environment encountered in phagocytes. Inhalation of mineral dust may provoke lung cells and can lead to an array of lung diseases as well as exacerbating other diseases as mentioned in the introduction. ROS species play an important role in the development of these diseases.

The role of metals in the formation and transformation of ROS has been extensively studied in the biomedical community (see review by Halliwell and Gutteridge 1990). Figure 7 adapted from Kawanishi et al. (2002) shows, for example, how metals and metalloids may

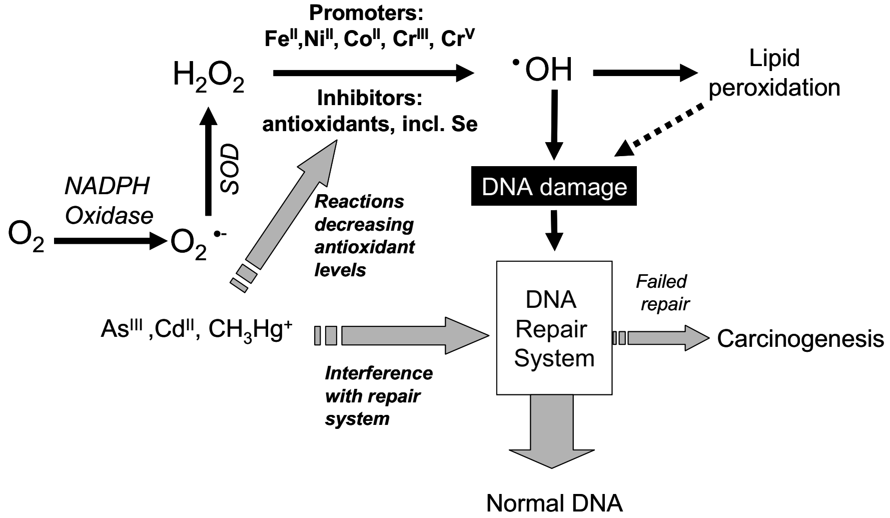


Figure 7. Role of metals and metalloids in promotion of carcinogenesis. Figure adapted from Kawanishi et al. (2002).

play a role in DNA damage, which can lead to cancer. It is important to stress that dissolved metals/metalloids may either promote DNA damage by catalyzing the formation of hydroxyl radical or by interfering with DNA repair, which is an important defense strategy against mutations. It has been estimated that mammalian cells experience as many as 10,000 "hits" by ROS per day (Ames 1987). Hence, partial impairment of the repair system can shift the balance between DNA damage and repair, thereby increasing the risk for cancer.

Iron is the most abundant transition metal in the body and its role in ROS transformation reactions has received the most attention (Toyokuni 1996; Welch et al. 2002; Valko et al. 2005), although other metals, such as copper, nickel, cobalt, chromium, and vanadium, have also been implicated in the formation and transformation of ROS (Shi and Dalal 1992; Shi et al. 1992; Stohs and Bagchi 1995; Lloyd and Phillips 1999; Strlič et al. 2003; Valko et al. 2005). Below we will focus mainly on reactions involving dissolved iron. The effect of chelation on ROS formation is also discussed as this is an important factor in ROS formation and DNA damage (Lloyd and Phillips 1999). Iron is an essential element; however, iron can also promote deleterious reactions involving ROS (Morris et al. 1995; Welch et al. 2002). Within the body, iron speciation is tightly controlled (Harrison and Arosio 1996). Exposure to iron-bearing minerals can lead to an increase in labile or free iron, which can catalyze several of the one-electron steps in the molecular oxygen reduction chain. The kinetics of the reduction of molecular oxygen in stepwise reactions with dissolved iron has been studied not only because of the biomedical relevance (Graf et al. 1984), but also because these reactions are important in redox cycling in natural waters (Emmenegger 1998) and in remediation processes that rely on the formation of ROS species to degrade organic pollutants, such as solvents (Watts et al. 1997; Watts and Teel 2005) and dyes (Ma et al. 2005). The iron-catalyzed reduction of hydrogen peroxide to hydroxyl radical and hydroxide is perhaps of greatest physiological significance. As discussed in Part I, free ferrous iron as well as some ferrous iron complexes can promote this reaction. However, complexing ferrous iron with DFO inhibits the Fenton reaction.

While the overall reaction rate between ferrous iron and oxygen has received considerable attention in the geochemical community, the role and formation of superoxide, hydrogen peroxide, and hydroxyl radical as reaction intermediates has received less attention in this

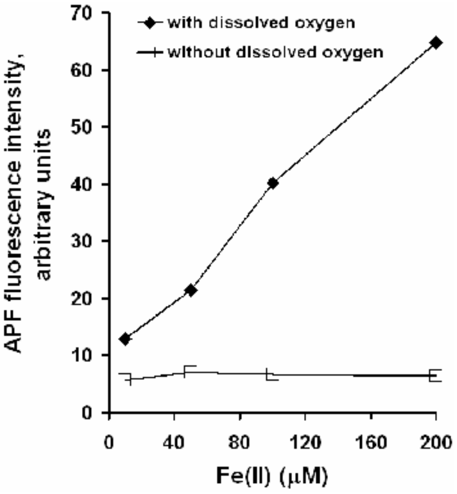


Figure 8. Hydroxyl radical formation in Fe(II) solutions in the presence and absence of dissolved oxygen. Fe(II) solutions were incubated with 10 μM APF at pH 7.4 for 24 h in the dark at room temperature (24 ± 2 °C) either at atmospheric levels of O_2 or in an anaerobic glove box followed by fluorescence readings.

community. However, recent studies that have shown the presence of ROS in natural waters (Cooper and Zika 1983; Holm et al. 1987; Willey et al. 1996; Herut et al. 1998; Price et al. 1998; Goldstone and Voelker 2000; Voelker et al. 2000; Wilson et al. 2000a,b; Kieber et al. 2001; Goldstone et al. 2002; Scott et al. 2003; Hakkinen et al. 2004; Peake and Mosley 2004; Vione et al. 2006) and their role in the degradation of organic pollutants (EPA 1998; Watts et al. 1999b; Petigara et al. 2002; Kwan and Voelker 2002, 2003, 2004) have drawn attention the importance of ROS in the environment. Figure 8 shows the formation of hydroxyl radical in a ferrous solution when exposed to air, while the radical is not formed if the solution is kept strictly anaerobic. The aerobic solution was saturated with air, while the anaerobic solution was purged with nitrogen and kept in an anaerobic chamber with an atmosphere of 5% H_2 and 95% N_2. While Figure 8 shows the formation of hydroxyl radical, Figure 9 shows the effect on the stability of RNA. The experiments summarized in Figure 9 were conducted with aerated solutions. It is thought that the presence of dissolved iron leads to the formation of hydroxyl radical, which is capable of degrading RNA. Hydroxyl radical will rapidly degrade nucleic acids by hydrogen abstraction or addition to bases (Tullius and Dombroski 1986; Heilek and Noller 1996; Brenowitz et al. 2002; Tullius and Greenbaum 2005).

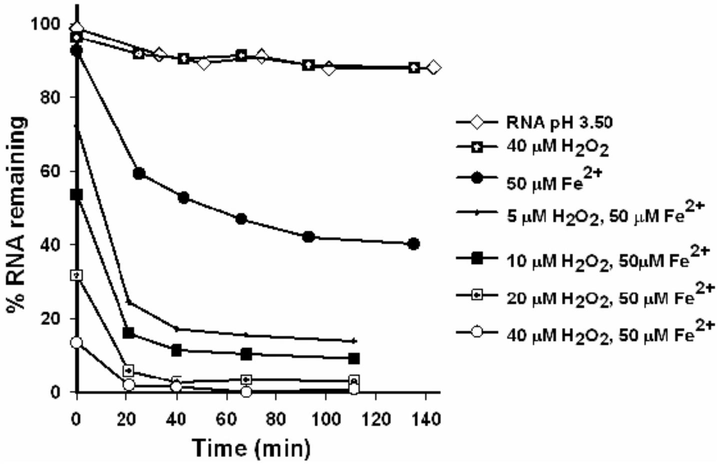


Figure 9. Yeast RNA degradation in the presence of H_2O_2, Fe(II), and Fenton reagents. 1.5 mg/L RNA was exposed to the various reagents at room temperature (24 ± 2 °C) in the presence of atmospheric levels of dissolve oxygen. RNA was quantified using the fluorescent dye RiboGreen (Invitrogen).

Other reducible transition metals may also promote the Fenton reaction, although there are fewer studies that have addressed the kinetics and mechanisms of the non-iron Fenton metals. In a recent comprehensive review of the role of metal ions, iron, cobalt, copper, and vanadium are listed as “Fenton” metals (Valko et al. 2005). In addition, there is ongoing discussion whether nickel is a Fenton metal. Shi et al. (1993a) have shown that both trivalent and pentavalent chromium ion form hydroxyl radical when exposed to hydrogen peroxide. The mechanism involving Cr(III) is uncertain. There is good evidence, however, that hexavalent Cr is reduced in the cell to Cr(III) via an intermediate Cr(V) state. ESR studies indicate that the Cr(V) species is oxidized to Cr(VI) while hydrogen peroxide is reduced to hydroxyl radical and hydroxide. A comparative study by Strlič et al. (2003) suggests that Cr(III) is a more efficient Fenton catalyst than ferrous iron is. There is no consensus on whether Ni(II) is a Fenton metal. Strlič et al. (2003) also found that Ni(II) will only promote the Fenton reaction above pH 7.5. Complexation of Ni(II) may enhance its reactivity. Similar to iron, complexation of Ni(II) with a ligand that forms a strong complex with Ni(III) stabilizes Ni(III) within the water stability field and makes it possible for nickel to cycle between the two oxidation states (Shi et al. 1993b; Van den Broeke et al. 1998). In other words, Ni^{3+}(aq) is not stable in water, but chelation is expected to stabilize this unusual valence state and allow the Ni(II) complex to promote the Fenton reaction. A similar argument has been made for the reactivity of cobalt (Leonard et al. 2004). Chromium and nickel are of great interest because both metals are known or suspected carcinogens, yet they are common in metal contaminants and some ultramafic rock types and soils developed on these types of rocks are naturally rich in these metals (Som and Joshi 2002).

Mineral surface-promoted formation of ROS by catalysis of O_2 reduction (mechanism 2)

While in the preceding mechanism the mineral played a rather passive role, reactions that take place at the mineral surface in the mechanism are considered in this section. Within the geochemical community there is a growing appreciation for the catalytic role mineral surfaces can play in electron transfer (redox) reactions (Schoonen and Strongin 2005). In fact, one of the best studied redox reactions is the surface-mediated oxygenation of transition metals, such as iron. It has been shown that the reaction between ferrous iron and molecular oxygen is much faster when the ferrous iron is sorbed onto a substrate, particularly a ferric-oxide surface (Wehrli et al. 1989; Wehrli 1990). An in-depth review of this topic has been presented recently in the context of environmental chemistry (Schoonen and Strongin 2005). A second type of mineral-promoted O_2 reduction reactions are reactions in which the mineral itself is oxidized. In this section, these two types of mechanisms are briefly reviewed.

Reactions with sorbates. The rate of the reaction between molecular oxygen and dissolved ferrous iron [$Fe(H_2O)_6^{2+}$] is about six orders of magnitude slower than the same reaction with the ferrous iron sorbed onto goethite (FeOOH). The reason for this increase in rate is that coordination of ferrous iron with hydroxyl groups derived from the surface effectively shifts the redox potential of the ferrous iron couple downward, making it a better electron donor. For example, the redox potential for a bidentate surface complex of Fe(II) onto goethite has been estimated to lie at 360 mV (NHE) (Wehrli 1990), while the standard potential for the $Fe(H_2O)_6^{2+}/Fe(H_2O)_6^{3+}$ redox couple lies at 770 mV (NHE) (Bard et al. 1985). Similarly, ferrous iron sorbed on silica has an estimated redox potential of between 230 mV (Strathmann and Stone 2003) and 550 mV (Buerge and Hug 1999). By comparison, ferrous iron as a structural component of silicate minerals has an estimated redox potential ranging from 330 to 520 mV (NHE). It should be emphasized that the redox couples involving sorbed or structural ferrous iron are difficult to measure. However, there is a growing body of literature that supports the notion that sorbed or structural ferrous iron are far better electron donors than free ferrous iron. In essence, the formation of bonds with a mineral surface or the coordination to other components within a mineral tunes the ferrous/ferric couple to different redox levels, not unlike ligands can tune this redox couple, see Figure 10.

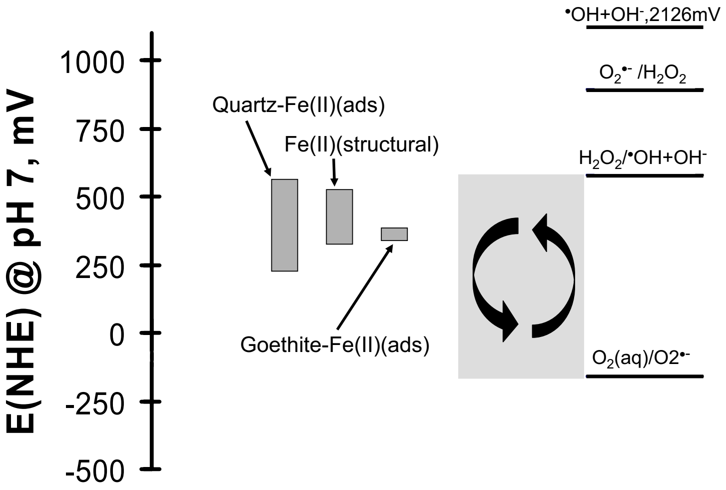


Figure 10. Estimated redox couples for adsorbed and structural Fe(II)/Fe(III) in mineral systems. Diagram based on compilation by Schoonen and Strongin (2005).

While most of the geochemical literature and environmental science literature has been focused on sorbed or structural ferrous iron, there are some reports that the oxidation reaction of other metals is similarly affected. Oxidation of free dissolved Mn(II) with molecular oxygen as electron acceptor is extremely slow (Davies and Morgan 1989; Martin 2005). However, Mn(II) sorbed onto alumina, quartz, and goethite reacts rapidly (Davies and Morgan 1989). The implication is that manganese taken up in a cell as free dissolved Mn(II) may not promote ROS formation, but Mn(II) sorbed onto a mineral might be able to promote ROS formation or transformation. The effect of sorption has also been studied on the reduction of Cr(VI) (Deng and Stone 1996). Hexavalent chromium is reduced by low molecular weight carboxylic acids. The presence of goethite, alumina, and titanium dioxide catalyze these reduction reactions. Hence, it is possible that inhalation of dust containing hexavalent chromium may promote the reduction of this carcinogenic metal and make it available for the (heterogeneous) catalysis of the Fenton reaction.

Recent, unpublished work by Levangie in our group shows that iron sorbed onto quartz promotes the decomposition of RNA. In this study, the rate of RNA decomposition was measured for (1) a pure, hydrothermally-synthesized quartz; (2) a RNA solution without any solids or trace elements; (3) ferrous iron sorbed onto quartz, and (4) an equivalent amount of dissolved ferrous iron or more without quartz. The methods used in the work by Levangie have been reported elsewhere (Cohn et al. 2005b). As seen in Figure 11, RNA decomposes most rapidly in the experiment with ferrous iron sorbed onto quartz compared to uncomplexed ferrous iron. The batch experiments with RNA solution alone and transition metal-free quartz + RNA are identical; hence, pure synthetic quartz that has not undergone any mechanical stress does not induce RNA degradation. This simple study corroborates the notion that redox metals sorbed onto inhaled particles may be important reactants that may lead to lung malignancies.

Oxidation of ferrous-iron containing minerals. The oxidation of ferrous-iron containing minerals by oxygenated water has been extensively studied by geochemists and environmental scientists. Perhaps the best studied reaction is the oxidation of pyrite, FeS_2. Pyrite is the most abundant metal sulfide on Earth and it is found in almost all anaerobic environments (Rickard et al. 1995; Schoonen 2004). It is a common mineral component of coal and accounts, together with its dimorph marcasite, for half or more of the sulfur content in coal (Calkins 1994). The

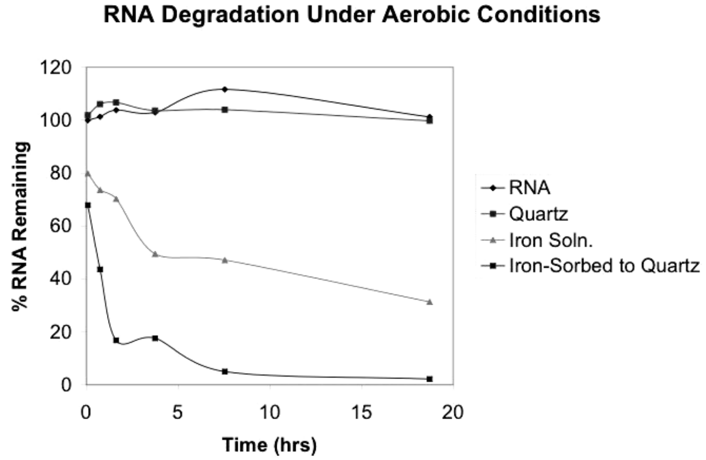


Figure 11. Degradation of yeast RNA exposed to quartz with or without adsorbed iron. RNA degradation under aerobic conditions in the presence of pure synthetic quartz, iron sorbed to the surface of pure synthetic quartz, and dissolved iron (779 μM). Note that the iron-sorbed quartz was prepared by exposing 1 g of pure synthetic quartz to a solution of 40mL of water with 50 mg of Mohr's salt added under anaerobic conditions. The dissolved iron concentration in the slurry with iron-sorbed quartz was 251 μM. Bath experiments were carried out buy mixing 2.64 mg/L RNA with 1.56g/L quartz and iron-sorbed quartz. Samples were filtered and the RNA was quantified using the fluorescent dye, RiboGreen.

oxidation of pyrite by molecular oxygen is a key reaction in the development of acid mine drainage, which is a widespread environmental problem in coal mining and in base-metal mining districts around the world (Banks et al. 1997). The reaction with molecular oxygen leads to the formation of hydrogen peroxide (Ahlberg and Broo 1996; Cohn et al. 2005c) and hydroxyl radical in solution (Cohn et al. 2006). A critical step is the interaction of molecular oxygen with the pyrite surface (Moses et al. 1987; Borda et al. 2004; Usher et al. 2004). Theoretical calculations as well as electrochemical studies have shown that molecular oxygen binds to an exposed ferrous iron (Biegler et al. 1975; Rosso et al. 1999). Hence, the interaction between molecular oxygen and the pyrite surface may be represented by the following reactions.

$$\mathrm{Fe^{II}(pyrite)} + \mathrm{O_2} \rightarrow \mathrm{Fe^{III}(pyrite)} + (\mathrm{O_2}^{\bullet})^- \qquad (12)$$

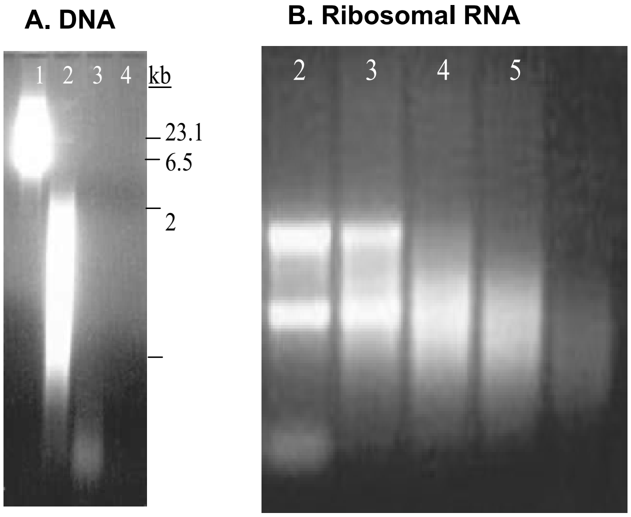
$$\mathrm{Fe^{II}(pyrite)} + (\mathrm{O_2}^{\bullet})^- + 2\mathrm{H}^+ \rightarrow \mathrm{Fe^{III}(pyrite)} + \mathrm{H_2O_2} \qquad (13)$$

On the pyrite surface these two one-electron transfer reactions may be effectively accomplished by one two-electron transfer reaction. A simultaneous two-electron step bypasses the thermodynamically unfavorable initial one-electron step from molecular oxygen to superoxide, see Figure 3. In addition, the ferric iron species formed on pyrite may readily be reduced by the pyrite substrate, which is a semiconductor (Xu and Schoonen 2000). This reduction would make it possible to rapidly cycle the surface-bound iron between the two oxidation states with molecular oxygen as the terminal electron acceptor (Moses et al. 1987; Eggleston et al. 1996). Considering that hydroxyl radical-induced degradation of nucleic acids is well-documented, we propose that the mechanism that leads to nucleic acid degradation in the presence of pyrite is by Fenton-generated hydroxyl radicals.

The reaction mechanism for the formation of hydrogen peroxide in pyrite suspensions is clearly a surface mediated process. Ahlberg and Broo (1996), using electrochemical techniques, showed that pretreatment of the surface changes the concentration of hydrogen peroxide found in solution. Cohn et al. (2005c) showed that addition of an excess of EDTA stabilizes hydrogen peroxide formed in pyrite slurries and that the concentration is proportional to the amount of pyrite surface area in the slurry. Cohn speculated that the excess EDTA interfered with the Fenton reaction, which decomposes hydrogen peroxide. This notion is corroborated by an additional study in which the formation of hydroxyl radical was demonstrated by ESR spin trapping (Cohn et al. 2005a).

The effect of the formation of ROS in pyrite slurries on nucleic acids was investigated using yeast RNA, ribosomal RNA, plasmid DNA, and adenine. The results with yeast RNA, ribosomal RNA, and plasmid DNA have been reported in a recent publication (Cohn et al. 2006). Figure 12 shows the effect of the presence of pyrite on plasmid DNA and ribosomal RNA. In these experiments the progress of the nucleic acid through the agarose gel is greatly influenced by changes to the nucleic acid strand lengths. It is clear from the gel that the presence of pyrite leads to strand breakage. It has been proposed that the reaction between hydroxyl radical and the bases in nucleic acids constitutes an important pathway in the degradation of the nucleic acids. To evaluate this we conducted several proof-of-concept experiments with adenine. As shown in Figure 13, adenine is slowly decomposed in pyrite slurries. The decomposition is inhibited by either adding EDTA, ethanol (a radical scavenger), or the enzyme catalase. Catalase decomposes hydrogen peroxide and prevents the transformation of this ROS to hydroxyl radical.

Within the biomedical community the reaction of iron associated with asbestos has received considerable attention. It has been proposed that the presence and coordination of iron is an important cofactor in the toxicity of asbestos (Fubini et al. 2001a; Martra et al. 2003). Similarly, it has been suggested that structural iron in asbestos exposed to the surface is important in the production of asbestos-induced ROS (Hardy and Aust 1995; Gazzano et


A. DNA

B. Ribosomal RNA

Figure 12. Nucleic acid degradation in pyrite-water suspensions. (A): 100 mg/L plasmid DNA exposed to pyrite (~1-50 mm in size, ~1 m^2/g BET surface area) at different particle loadings: lane 1) nothing added, 2) 6 g/L pyrite, 3) 64 g/L pyrite, 4) 640 g/L pyrite. The suspensions were vortexed for 4 h and filtered samples were observed on a 3% agarose gel. The negatively-charged RNA samples were loaded at the top of the agarose gels. When a voltage is applied across the gel, the RNA strands travel downward with the smaller strands moving faster than larger strands. DNA strand-lengths are given on the right side of the gel. (B) 100 mg/L ribosomal RNA exposed to 100 g/L pyrite. Samples were taken at various times after mixing the RNA with pyrite: 1) nothing added, 2) pyrite mixed with RNA for 0.5 min, 3) 10 min, 4) 30 min, 5) 1.5 h. The two bright fluorescent bands from the RNA are due to 28S and 18S ribosomal moieties.

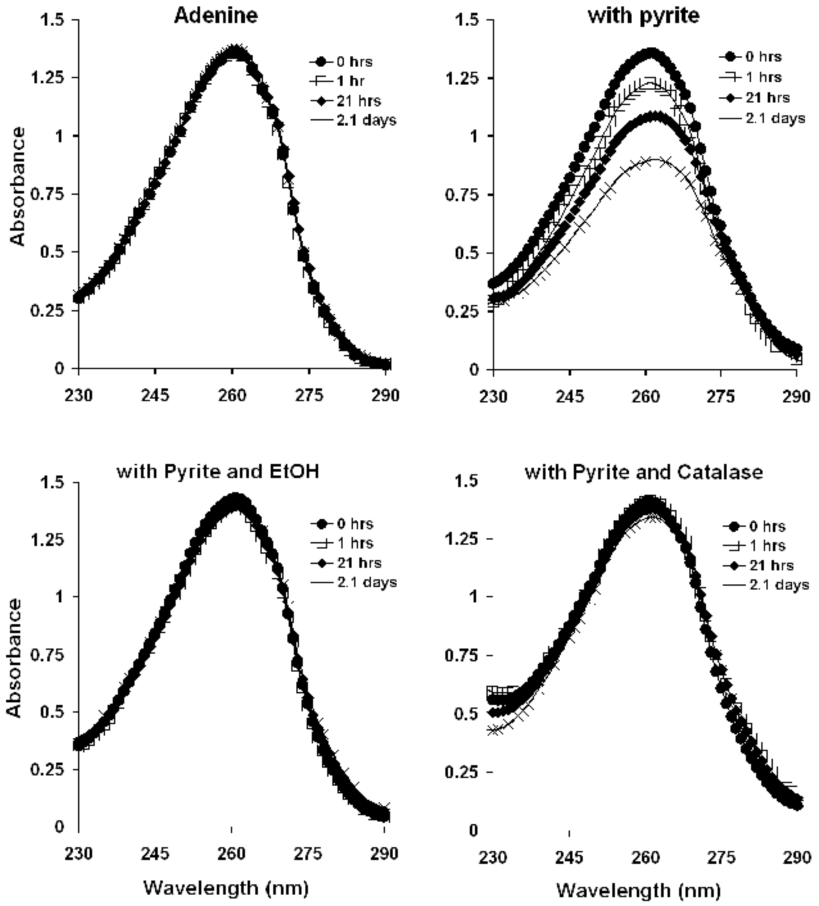


Figure 13. Adenine exposed to pyrite suspensions. 100 μM adenine was exposed to 10 g/L pyrite particles (~1-50 μm in size, ~1 m^2/g BET surface area) with nothing added, 50% EtOH, or 64 kUnits catalase. Samples were filtered and wavelength scans were recorded.

al. 2005). This last notion is corroborated by studies on the ROS formation and cytotoxicity of asbestos fibers that have been exposed to siderophores or have undergone iron leaching (Martino et al. 2003, 2004; Martra et al. 2003; Daghino et al. 2005; Favero-Longo et al. 2005) (Fig. 14). One important difference between pyrite and asbestos-form minerals is that the latter are insulators. Hence, their reactive capacity is limited to the surface-exposed iron (and possibly other redox metals).

Hetland et al. (2001) evaluated whether the inflammatory response after exposure to different quarry stones was induced by the soluble fraction or the mineral solids. They showed that the upregulation of a cytokine (IL-8) in A549 lung cells is more pronounced when exposed to a slurry of solid quarry stone particles as opposed to just the leachate of these solids. In their system, both metal sorbates and structural metals may have contributed to the formation of ROS. One of the quarry stones that Hetland and coworkers studied was a basalt. Our group has shown that a principal component of basalt, forsterite (Mg olivine), forms ROS and degrades yeast RNA (Cohn et al. 2005b).

In summary, this second mechanism has only been investigated on a limited basis. It is to be expected that many minerals may promote ROS formation either via reaction of the molecular oxygen with sorbed metals or by a reaction with structural metals. In a recent study we report on a first set of minerals we have evaluated for their ability to form ROS and degrade yeast RNA (Cohn et al. 2005b). The results are summarized in Figure 15.

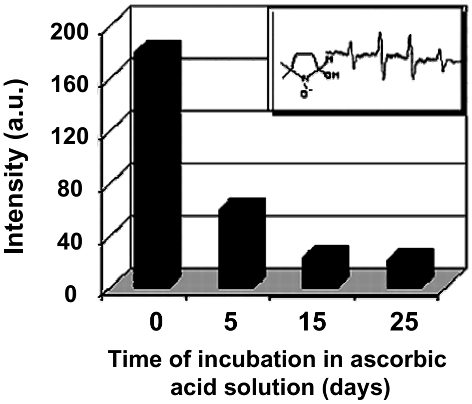


Figure 14. Free radical release from aqueous suspensions of crocidolite fibers. Integrated intensity of the EPR spectra of the [DMPO-OH] adduct produced by suspending in the aqueous solution of H_2O_2 and DMPO the original crocidolite fibers and fibers incubated for 5, 10, and 25 days in aqueous solution of ascorbic acid. Inset: scheme of the [DMPO-OH] adduct and the corresponding EPR spectrum. [Used by permission Amercian Chemical Society, from Martra et al. (2003) *Chem. Res. Tox.* 16, Figure 4, 328-335].

Mineral defect-driven formation of ROS (mechanism 3)

Stress induces defects in minerals, adding to intrinsic defects that any mineral contains (Borg and Dienes 1992). In the context of human health, the most common form of stress-induced defects is related to grinding of minerals. Grinding is a common step in the processing of Earth materials and frequently leads to an occupational exposure. Exposure to olivine dust by workers in the glass blowing industry prompted Victor M. Goldschmidt, often referred to as the father of modern Geochemistry (Mason 1992), to contribute to an *in vivo* study on the effect of olivine dust in rats (King et al. 1945). While the objective of grinding in mining operations is typically to decrease grain size and break waste minerals from ore minerals or coal, grinding

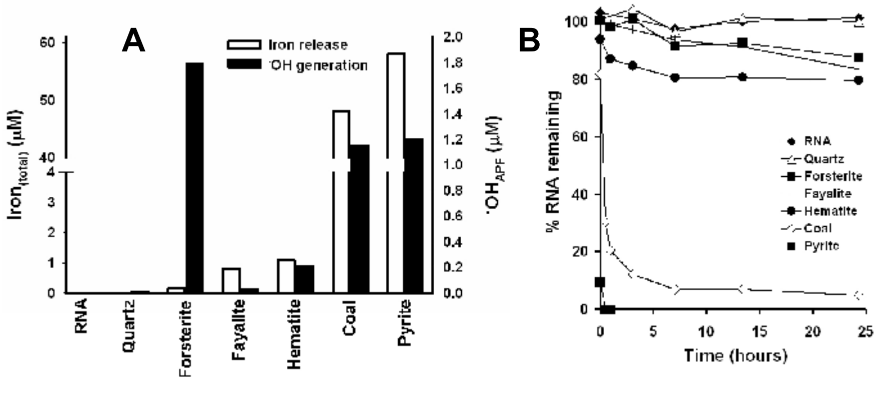


Figure 15. Iron release, hydroxyl radical generation, and RNA degradation induced by several common minerals. Minerals are all from Wards except for the coal (no. 2685b, ~2-3% pyrite), which is from the National Institute for Standards and Technology. The minerals were crushed (63-38 μm) in an agate mill ~50 days before their use in experiments. (A) Iron release measured with ferrozine and hydroxyl radicals quantified using APF after 24 h exposures in opaque centrifuge tubes. (B) 1.5 mg/L yeast RNA exposed to 0.5 m^2/L mineral suspensions at room temperature (24 ± 2 °C). RNA was quantified from filtered samples using RiboGreen (Invitrogen). Data is from Cohn et al. (2006).

does more than simply increase the specific surface area of an Earth material. Grinding can change the physical and chemical properties of minerals, including the mineral composition, defect density, lattice strain, and transition-metal spin state (Balaz 2003). Not unexpectedly, there are active research programs in hydrometallurgy and mining engineering to exploit these so called *mechanochemical* reactions to improve mineral separation (Vandeventer et al. 1993; Kirillova et al. 2000; Peng et al. 2003; Yigit and Ozkan 2004) and benefaction (Achimovicova and Balaz 2005), leaching (Hu et al. 2003; Hu et al. 2004; Achimovicova and Balaz 2005) and bioleaching (Balaz et al. 1994; Balaz 2003), as well as to synthesize nanomaterials (Balaz et al. 2003, 2004).

Within the medical community, quartz has received the most attention. In particular Bice Fubini's group in Turin has been leading the way in advancing our understanding of the role of surface defects in the formation of ROS (Fenoglio et al. 2000a,b; 2003; Fubini 1998; Fubini et al. 1987, 1990, 1999, 2001a,b, 2004; Fubini and Hubbard 2003; Cakmak et al. 2004;). As shown in Figure 16, grinding of quartz leads to several types of surface defects resulting from homolytic or heterolytic cleavage of Si-O bonds. These defects can react with water and oxygen to form ROS (Fubini and Hubbard 2003). Recent theoretical calculations by Narayanasamy and Kubicki (2005) suggest that there will always be a small pool of surface radicals on a quartz surface. This work also indicates that the reaction between water and a surface Si-O$^{\bullet}$ radical, producing a hydroxyl radical, is thermodynamically favorable. The presence and reactivity of the surface defects (in essence, surface-bound radicals) has been studied extensively by Fubini's group using solid state ESR and spin trapping among other techniques. These surface-bound radicals then react with target molecules to produce an array of soluble radicals. As may be expected, the reactivity of freshly ground quartz drops off as the material is exposed to humid air. Hence, not surprisingly the toxicity of freshly ground dust in animal studies is higher than for aged materials for a review, see Fubini and Hubbard (2003). Other Earth materials that have been investigated in the context of grinding-induced radical formation are metal oxides (Costa et al. 1989b; Fubini et al. 1995; Lison et al. 1997), sulfides (Costa et al. 1989a), asbestos (Fubini et al. 1995), and zeolites (Fubini et al. 1995).

A fundamental issue that has not been resolved is the distinction between contributions of surface defects and bulk defects to the overall reactivity. It is expected that the contribution of bulk defects is minimal for insulator materials. In other words, the reactivity of insulator materials is expected to be dominated, if not solely determined, by the surface defect density. By contrast, it is expected that grinding of metallic materials and semiconducting materials leads to defects in the bulk that can migrate to the surface and contribute to their stress-induced reactivity. The difference in stress-induced reactivity between these two endmember models is

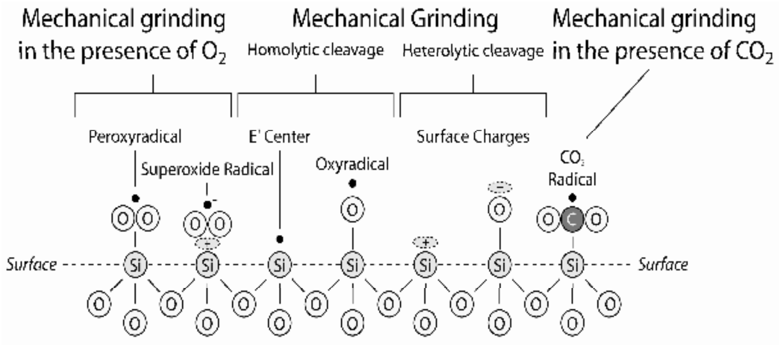


Figure 16. Reactive functionalities at the quartz surface and their formation mechanisms. After Fubini et al. (2003), courtesy of Joel Hurowitz, Stony Brook University.

schematically illustrated in Figure 17. The shape of curve b is notional; the key attribute is that the surface normalized reactivity of the material is dependent on the grinding time. Building on our earlier work on the use of yeast RNA as a probe of mineral-induced hydroxyl formation, we have used the rate of RNA decomposition to evaluate how grinding affects the reactivity of minerals. The rates of RNA decomposition are normalized with respect to surface-area based on BET measurement. For pyrite these experiments show that the surface normalized rate data are dependent on grinding time (Fig. 18), which is indicative of a major contribution of bulk defect. By contrast, the results with quartz indicate that the reactivity appears to be dominated by contributions from the surface (Fig. 18). Note also that quartz is far less reactive than pyrite (the rates of quartz were multiplied by a factor of 1000 in Fig. 18).

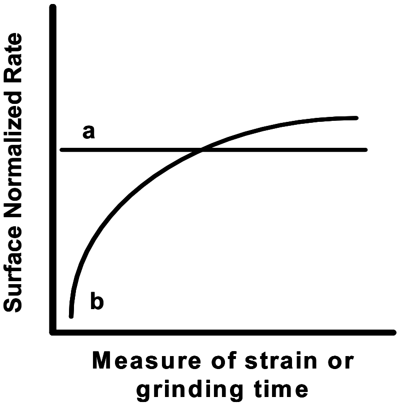


Figure 17. Schematic diagram illustrating two endmember conditions. Curve a represents a material in which only surface defects contribute to the reactivity, curve b is a material with a considerable contribution of the bulk to the reactivity.

Cellular particle-induced formation of ROS (mechanism 4)

The fourth mechanism to be considered is the cellular response to the presence of particles. We purposefully use the term particle because we consider here the non-specific reaction of the immune system to the presence of an otherwise inert solid. In reality, particulate matter may very well promote ROS formation or transformation directly, but those mechanisms are discussed separately (see text on mechanism 2). The cellular response to a provocation brought on by inhaling particulates has been extensively studied (Driscoll et al. 1997; Schins 2002) and it is beyond the scope of this chapter to review the subject. For the purpose of this chapter, we will outline some of the key cellular processes that can contribute to ROS production triggered

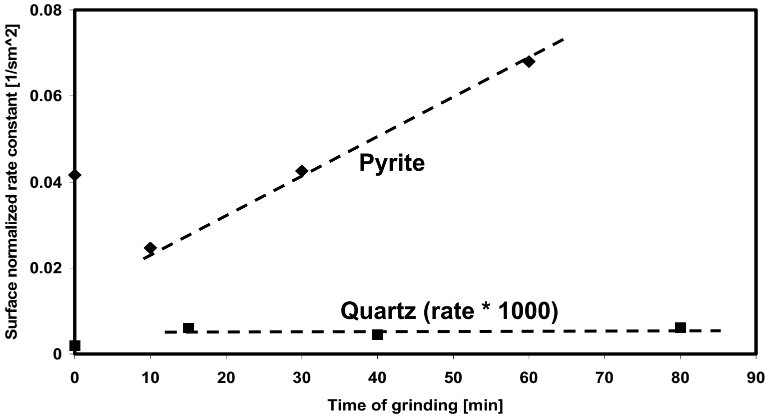


Figure 18. Surface-normalized rates of yeast RNA decomposition by ground pyrite and ground quartz . Note that the rates for quartz were multiplied by a factor of 1000. Data points for unground materials are not taken into account for regression as these points are subject to large uncertainty in BET measurements. Data suggest that bulk defects migrate to the surface of pyrite, while reactivity of quartz is limited to surface defects.

by the mere fact that particles have been inhaled. A general introduction to the topic of pulmonary physiology and some of the processes briefly described in this section is provided by Levitzky (2003)

In the mammalian lung (Fig. 19), gas exchange is facilitated by an alveolar complex that combines a huge surface area with a thin alveolar capillary membrane. Such a delicate structure would be vulnerable to injury from inhaled particulates (organic and inorganic) which gain entry to lungs as a consequence of the critical need to exchange air continuously between the alveolar space and the external atmosphere. There are, however, mechanisms of defense against this inadvertent deposition of particles in the alveolar space. First, the angular configuration of the upper extrapulmonary airway and the branching structure of lower airways (the "tracheobronchial tree") cause particles to deposit on the walls of these airways through inertial impaction. After deposition in airways proximal to the terminal airspaces, the particles become engulfed in mucus and are in turn transported proximally by the movement of cilia to the pharynx and then swallowed, a process called mucociliary clearance. In addition, some particles, especially those that cause irritation, can be expelled by cough clearance. In contrast, if an insoluble particle deposits in the airspaces distal to the ciliated airways, it will be ingested by alveolar macrophages, which will in due course migrate into the ciliated tracheobronchial tree or deliver the particle to regional lymph nodes. This process is called alveolar clearance.

The process of phagocytosis is designed to protect the delicate alveolar tissue from noxious particles, including infectious agents. Sometimes, however, the process of phagocytosis can itself be more injurious to the lung than the continued presence of free exogenous particles in the alveolar space; many investigators have studied this phenomenon. There is evidence that if a macrophage is overloaded with an inert particle, it will secrete inflammatory mediators. In addition if the particles are very small (ultrafine with a diameter less than 0.1 μm) more inflammation is generated compared to an equivalent mass of larger particles, possibly because the ultrafine particles have more surface area. Surface activity, surface charge and surface smoothness are other potential variables. The presence of transition metals is also believed to contribute to inflammatory potential of ingested particles. Inflammatory cells such as macrophages may exhibit greater inflammatory responses to ingested particulates if they

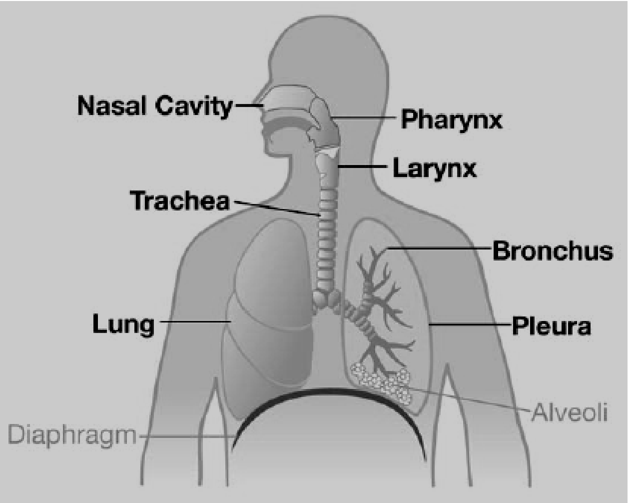


Figure 19. Schematic diagram of human lung. Source National Institute of Health. *http://www.nlm.nih.gov/medlineplus/lungsandbreathing.html.*

have been primed by an earlier different proinflammatory stimulus such as lipopolysaccharide, a bacterial product. While this is well recognized *in vitro* there are some intriguing clinical and population health correlations. For example the acute death rates in urban areas increase in response to subtle increase in airborne particulate material with most of the additional deaths occurring in people with chronic cardiopulmonary disease. Similar observations of increased mortality and morbidity associated with certain forms of industrial exposure to inhaled particulates, as may be encountered in mining, combined with chronic exposure to cigarette smoke are also consistent with the concept of a "two-hit" process of activation of the inflammatory response with attendant negative impact on health.

At the molecular level, phagocytosis is a complex process, phagocytes, such as macrophages and neutrophils, engulf and destroy invading bacteria. A coating process *with biomolecules*, opsonization, is required for the attack on cellular invaders, which have a negative surface, just like the phagocytes. In addition to alleviating electrostatic repulsion, opsonization leaves the surface covered with molecular "ligands" that can be engaged by the engulfing cell in a process of receptor-mediated phagocytosis. As shown in a study with albumin-coated fluorescent latex particles, reaction of the albumin-coated particles with an anti-albumin antibody followed by deposition of these opsonized particles in the lung caused pulmonary inflammation in hamsters, but the uncoated particles showed little or no response (Kobzik et al. 1993). The implication is that opsonization followed by binding of antibody-coated particles to immunoglobulin receptors in the lung can transform an inert material into a stimulant which is recognized by the lung as pro-inflammatory.

Once the alveolar phagocytes become activated, they start to release signaling compounds (e.g., cytokines, transcription factors, and enzymes) as well as ROS and RNS to counteract the provocation. The production of one subclass of signaling compounds, exemplified by the so-called chemokine Interleukin-8 (IL-8), has the effect of recruiting additional inflammatory cells. While pathogens are typically efficiently engulfed and decomposed by the battery of ROS, RNS, acids, and enzymes that are unleashed within the phagocytes, particles, particularly fibrous particulates, can lead to frustrated attempts at engulfment and disposal. Macrophages are 12 to 18 μm in size. Hence, any particles that approach this length may thwart total engulfment (Fig. 20). This process of "frustrated phagocytosis," a phenomenon seen in both macrophages and neutrophils, is known to induce the production of ROS (Vallyathan et al. 1998). As a result, exposure to even an inert particle may lead to chronic upregulation of various signaling compounds and elevated levels of ROS and RNS (Fubini and Hubbard 2003). Conversely, some particles that show ROS formation *in vitro* may be readily cleared by destruction within the phagosome or migration of the cell

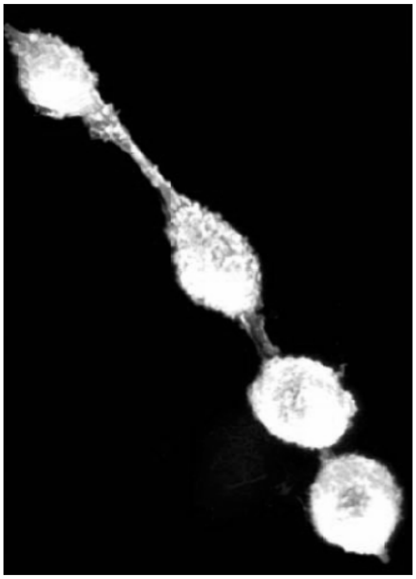


Figure 20. Example of frustrated phagocytosis induced in a Rat AM cell culture by exposure to glass fibers with a length of 17 micron or more. [Used with permission by Dr. Vincent Castranova, National Institute for Occupational Health and Safety, Morgantown, WV. URL: *http://www.epa.gov/oswer/asbestos_ws/docs/castranova.pdf.*]

containing the internalized particles towards the gastrointestinal tract where it is digested; such mechanisms of clearance do not necessarily present a health problem.

The cellular response to the inhalation of "inert" particles is confounded by the state of the cells. It has been shown that pre-existing pulmonary inflammation unrelated to an acute particle exposure event may lead to a stronger proinflammatory response (Stringer and Kobzik 1998; Imrich et al. 2000). In order words, one or more previous stimuli to inflammatory cells in the lung can amplify the ROS formation associated with a subsequent exposure of those cells to particulates. The modulation of the response to a provocation involves a complex system of receptors and cell signaling molecules (Kobzik et al. 1993; Stringer and Kobzik 1998; Imrich et al. 2000). The details of the interactions between minerals and various cells components, cell signaling molecules, and receptors are not well understood.

The effect of inhalation of inert particles has been extensively studied to unravel the cellular response without the confounding factors related to particle reactivity. In many of these studies, cells are exposed to inert polystyrene beads (Palecanda and Kobzik 2000) or TiO_2 (Driscoll et al. 1991; Stringer and Kobzik 1998). In Figure 21, we show the results of a comparative study conducted by our group in which the formation of ROS was detected as conversion of DCFH to its fluorescent product DCF in cells exposed to polystyrene beads, TiO_2, SiO_2, and pyrite. Out of these particles, pyrite shows the highest DCF production. All other particles show no difference from exposure to beads. While TiO_2 is typically thought of to be inert, a recent *in vitro* study in which co-cultures of alveolar macrophages and lung epithelial cells were used showed that TiO_2 does trigger a cellular response in the co-culture, but not in separate alveolar macrophages or lung epithelial cells (Tao and Kobzik 2002). It is thought that the synergistic effect in the co-cultures is the result of cell signaling between the two types of cells (Driscoll et al. 1996; Tao and Kobzik 2002) and may be a more realistic *in*

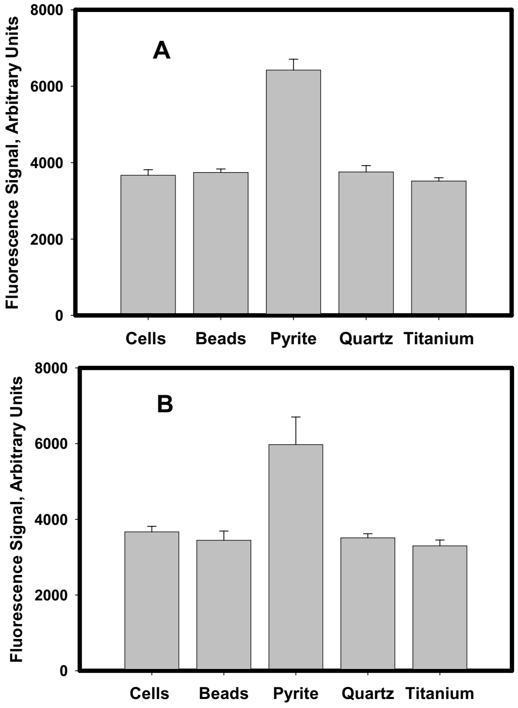


Figure 21. Formation of secondary ROS in MonoMac cells, based on conversion of DCFH to DCF. Panel A (top) MonoMac cells exposed to 400 microgram of mineral per mL of cell culture volume, except for column labeled cells, which contained no minerals. Panel B (bottom), same as Panel A except cells are pretreated and provoked by exposure to Lipid Poly Saccharide (LPS). [Figure based on a presentation by Daccueil F, Fijman A, Roemer EJ, Simon SR (2005) Measures of production of oxidant species in vitro and in cells: assessment for evaluation of possible health risks. Meeting of the Society for In Vitro Biology, Baltimore, MD.]

vitro model of *in vivo* conditions. Huang and coworkers provide more background on mineral-induced cell signaling and cytokine upregulation (Huang et al. 2006).

Minerals as carriers of ROS stimulants (mechanism 5)

The final mechanism in this chapter considers the role of minerals as carriers for compounds that stimulate ROS formation or transformation. In this mechanism the mineral provides a substrate for a stimulant and may promote its transport and residence in the lung. The co-exposures of cigarette smoke and minerals (mostly asbestos) (Valavanidis et al. 1996), diesel exhaust and aerosols (Nikula et al. 1997; Murphy et al. 1998; Garcon et al. 2001; Dybdahl et al. 2003, 2004; Li et al. 2003; Sorensen et al. 2003; Muller et al. 2004; Avogbe et al. 2005), and endotoxins (Imrich et al. 2000) and aerosols (Monn and Becker 1999; Heinrich et al. 2003; Kleinman et al. 2003; Schins et al. 2004) have received considerable attention. The interactions between the mineral substrate and the ROS stimulant often lead to ROS production over and above those seen in the exposures to the isolated components. These augmented responses are partly a reflection of the simultaneous activation of inflammatory cells by two discrete stimuli but are also considered to arise from the physical association of the two stimuli with each other as they trigger the cells.

As an example of augmented stimulation by two activating materials which are in direct association, the response to co-exposure to cigarette smoke and asbestos may be considered. It is well-documented that the co-exposure of tobacco smoke and asbestos leads to an increase in free radicals (Valavanidis et al. 1996; Kamp et al. 1998; Jung et al. 2000; Churg 2003) and other measures of inflammation and cell injury over either smoke or asbestos alone. The synergistic effect between cigarette smoke and asbestos may be caused by several factors. First, the sorption of smoke-derived compounds onto the mineral surface changes its surface properties. It has been shown that the sorption of smoke-derived compounds onto asbestos leads to an increased adhesion onto epithelial cells, thereby increasing the net uptake. The morphology of the mineral particle may lead to low clearance rates, which increases the residence time of the stimulant in the sites where inflammatory cells are most abundant. *In vivo* studies show an increased penetration of asbestos fibers into the walls of the airways. The higher uptake, penetration, and longer residence time of the ROS stimulant *in situ*ations of co-exposure to these two materials eventually leads to an exaggerated fibrotic response, which impairs lung function. Churg (2003) showed that addition of ROS scavengers can lead to a decrease of adhesion and uptake of these materials. This observation suggests that redox reactions may modify the chemical functionality of the adsorbate-substrate system. An ESR study showed that iron derived from asbestos reacts with hydrogen peroxide derived from tobacco-derived tar (Valavanidis et al. 1996).

The interaction of exogenous ROS stimulants and mineral surfaces is an area in which geochemists are poised to make a contribution. Interaction of PAH and other suspected ROS stimulants with mineral surfaces is an active research area in the geosciences and environmental sciences (Gustafsson et al. 1996; Arey 2000; Krauss et al. 2000; Kubicki 2000; Van Metre et al. 2000; Zhu et al. 2004). However, the fate of these composite particles has not been explored by these communities in the presence of inflammatory cells or even cell-free lung fluids or proxies that mimic some of the complexity encountered as composite particles are inhaled.

PART III: SILICOSIS AS AN EXAMPLE OF A MINERAL-INDUCED DISEASE

Silicosis is a form of lung disease resulting from occupational exposure to silica dust over a period of years. Silicosis causes slowly progressive fibrosis of the lungs but the degree of impairment of lung function in workers exposed to silica varies from minimal to respiratory failure. This range of disease severity reflects variations in the composition of silica dust as well as differences in the inflammatory responses of individuals exposed to similar levels of silica.

Silicosis is often associated with occupations, such as mining, masonary, construction, glass and ceramic fabrication, and stone carving, in which workers are exposed to silica dust over long periods of time. Silicosis is, in some cases, an irreversible disease. Even after the exposure ceases the disease may progress. Deposition of silica particles in the lungs leads to chronic inflammation and induces the growth of nodules, which may coalesce into fibrotic growths. Ultimately, such growths in the lungs contribute to respiratory stress and in some cases respiratory failure (Rimal et al. 2005). It has been estimated that about 250 deaths/year are related to silica exposure (Castranova and Vallyathan 2000). While occupational exposures have been well documented, silicosis may also occur in household activity as shown in an example from Saudi Arabia where an Afghani woman was diagnosed with the disease. In that case the exposure was related to cleaning duties as a child that included the daily cleaning of the walls and floor of a mud hut with a hard brush (Safa and Machado 2003). Whether smoking and silicosis are positively correlated remains uncertain (Hessel et al. 2003); however, smokers diagnosed with silicosis have a higher risk of developing lung cancer (Kurihara and Wada 2004). Silicosis may also contribute to immune system deficits. For example, mine workers are often at increased risk for tuberculosis (Davies 2001; Rimal et al. 2005).

The mechanism by which silica exposure leads to silicosis remains an area of active research(Castranova and Vallyathan 2000; Elias et al. 2000; Donaldson et al. 2001; Rimal et al. 2005). As summarized by Castranova and Vallyathan (2000), it is not clear what causes the disease. It has been suggested that silanol groups on silica surfaces lead to a strong interaction with biological membranes, possibly leading to cell damage. Another explanation is that crystalline silica interacts strongly with scavenger receptors on macrophages, thereby stimulating a cellular release of ROS/RNS. Finally, it has been noted that radicals on the silica surface react with water to yield ROS, which may induce a cascade of cell signaling events leading to inflammation. The ability of the solid to generate ROS and trigger cell signaling to induce inflammation has perhaps received most attention, although a combination of mechanisms cannot be ruled out. In the remainder of this section, factors that are of interest to geochemists in relation to silicosis will be briefly reviewed.

Mineralogy

The mineralogical form of the silica exposure is thought to be an important factor in the development of silicosis. In the context of silicosis, the word silica is used to mean SiO_2 polymorphs as well as silicates. One of the most widely used test materials is a quartz sand from a Tertiary formation in Germany (DQ-12 quartz). This sand has been described as 87 to 89% crystalline quartz, some amorphous silica, and a small fraction of kaolinite (Adamis et al. 2000; Donaldson et al. 2001). A chemical analysis of the DQ12 quartz sand indicates that the sand is a very pure quartz sand (Donaldson et al. 2001), with 98.51 wt% SiO_2, less than 0.05 wt% Fe_2O_3, and 0.1 wt% Al_2O_3. In studies that compare the toxicity of various forms of silica, crystalline quartz often emerges as one of the most toxic forms of silica. For example, an *in vivo* and *in vitro* comparison of quartz, diatomaceaous Earth, mordenite and clinoptilolite showed marked difference in toxicity (Adamis et al. 2000). As part of this study the materials were instilled in rats and the response to the instillation was studied over a period of a year. Quartz induced acute, subacute and chronic inflammation and fibrosis in rats. Diatomaceous Earth showed acute and subacute inflammation, but the effects moderated after 60 days. Mordenite showed signs of inflammation, which has been attributed to the fibrous morphology of this zeolite. By contrast, clinoptilolite was inert. Other studies have compared different types of crystalline quartz. Fubini and coworkers showed that different commercial quartz powders have very different biological reactivity *in vivo* and *in vitro* (Bruch et al. 2004; Fubini et al. 2004; Seiler et al. 2004). Donaldson et al. (2001) pointed out that DQ-12 quartz is far more inflammatory than quartz collected at actual worksites. Cazmak et al. (2004) on the other hand found that DQ-12 and several commercial quartz powders elicited about the same response in A549 epithelial cells. Schwarze et al. (2002) studied the inflammatory response of various quarry stones by intratracheal instillation of ground rock samples in rats. They found that quartz content and metal release to solution were poor predictors of inflammatory response. A mylonite quarry stone generated the most pronounced response.

Surface chemistry

The role of surface chemistry in the toxicity of quartz has been well established (Fubini 1998). There are two lines of evidence that corroborate the notion that the surface chemistry of quartz is an important factor in its toxicity. On one hand there are several studies that show that treatment of the surface with a sorbate that also scavenges radicals depresses the toxicity of quartz in *in vivo* studies (Duffin et al. 2001; Albrecht et al. 2004, 2005; Stone et al. 2004). Figure 22 shows that rats instilled with uncoated quartz have elevated hydrogen peroxide concentrations in their alveolar solution and significantly higher levels of MPO, the enzyme that produces HClO (Fig. 23). Fubini attributed the difference in biological activity of four different commercial quartz flowers to their difference in surface composition (Fubini et al. 2004). The other line of evidence is based on studies that evaluated the state of the surface in relation to its capacity to produce ROS and RNS, which is an important step in the development of the disease. Fubini and coworkers showed, using solid state ESR, that freshly

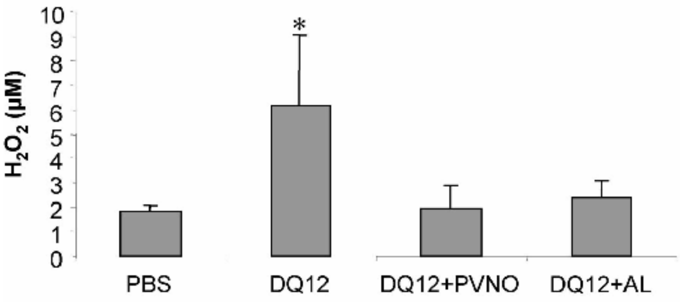


Figure 22. H_2O_2 generation in the rat lung. H_2O_2 levels were analyzed spectrophotometrically in the BALF obtained from rats exposed to non-coated DQ12 or DQ12 coated with AL or PVNO (7 days after i.t. instillation). Data are expressed as mean ± SD (n = 5). *p < 0.01 vs. PBS (ANOVA, Tukey). [Permission under Creative Commons Attribution License. Original citation Albrecht et al. (2005) Respiratory Research 6:129. *http://respiratory-research.com/content/6/1/129*]

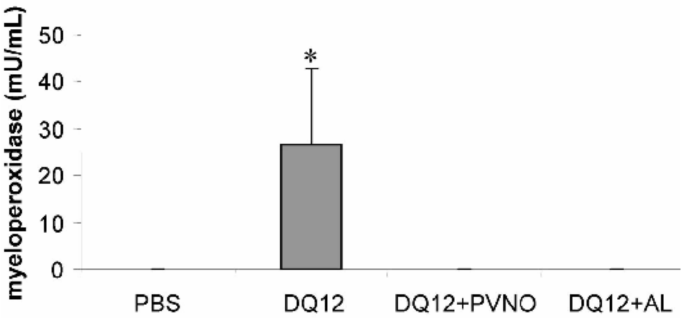


Figure 23. Myeloperoxidase activity in BALF of rat lungs 7 days following i.t. instillation of 2 mg DQ12 or DQ12 coated with either AL or PVNO. Data are expressed as mean ± SD (n = 5). *p < 0.01 vs. PBS (ANOVA, Tukey). [Permission under Creative Commons Attribution License. Original citation Albrecht et al. (2005) Respiratory Research 6:129. *http://respiratory-research.com/content/6/1/129*]

fractured quartz has a higher surface concentration of radicals. These surface radicals react with water to produce hydroxyl radicals (Vallyathan et al. 1998). Reactions with NO may yield peroxynitrite. The reactivity imparted by the mechanical stress dissipates over the course of days when the material is left to react with ambient air (Fubini et al. 1990). Vallyathan et al. (1995) instilled freshly ground quartz and aged quartz powder in rats and found that the freshly ground quartz induced a far more pronounced inflammatory response. Heating of a fibrous crystobalite to 1300 °C inactivated an otherwise cytoxic material by removing surface radicals and increasing its hydrophobicity (Fubini et al. 1999).

Metal impurities and Co-exposures

While pure quartz has been shown to induce an inflammatory reaction in rats, the effect is enhanced by the presence of iron (Castranova et al. 1997) and coal dust (Donaldson et al. 2001; Stone et al. 2004). Iron is a common natural trace constituent in quartz. In addition, grinding of quartz is likely to introduce iron, which increases its reactivity (Fenoglio et al. 2001, 2003). The augmentation of the inflammatory response, although not uniform (Donaldson et al. 2001), is likely due to the fact that ferrous/ferric iron can act as a Fenton element. In our group we have shown that iron sorbed onto pure quartz can drastically accelerate the degradation of RNA, which presumably requires the formation of hydroxyl radical (Fig. 11). Coal contains quartz and silicates as mineral components. The prevalence of silicosis among coal miners has been linked to the co-exposure to these minerals and coal dust (Borm 2002). Pyrite is another common mineral component in coal. Its presence in coal may be a factor in silicosis as pyrite is know to spontaneously generate hydroxyl radicals (Cohn et al. 2006).

PART IV: CONCLUDING REMARKS

There is growing *in vitro* and *in vivo* evidence that minerals such as asbestos, quartz, pyrite, and forsterite can induce the formation of ROS. The development and adaptation of ROS assays for use in mineral suspensions allows for broad testing of Earth materials. By further developing acellular assays it may become possible to rapidly assess the ROS-formation potential for Earth materials. These results can then be combined with assays of further ROS generation in the presence of cells as well as other biomedical *in vitro* tests. In aggregate these *in vitro* results can provide useful guidance for subsequent *in vivo* tests (Fubini 1996), which are the preferred basis for a toxicological assessment (Bernstein et al. 2005).

Although some of the mechanisms by which minerals induce ROS in aqueous solutions have been resolved, much remains largely unknown. However, surface chemistry appears to play an important role as it is thought to influence adhesion, interaction with receptors, sorption of ROS stimulants, and the direct formation of ROS. Geochemists are in a unique position to contribute to a better fundamental understanding of the role of minerals in diseases such as silicosis and asbestosis. Experimental geochemists can contribute by synthesizing materials for proof-of-concept studies, characterize synthetic as well as natural materials and surfaces, and induce mechanical stress in controlled ways. Theoretical geochemists can team up with their biochemical colleagues to explore the interaction of mineral surfaces with receptor molecules as well as ROS stimulants, such as PAH and components of cigarette smoke, to better understand cellular ROS formation. The first step is to form close collaborations among the relevant disciplines, start cross-disciplinary discussions, and develop common objectives. It is our hope that this chapter is helpful in starting this process.

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