Iron in Asbestos Chemistry and Carcinogenicity

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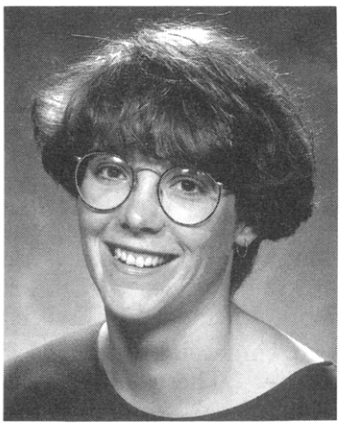
Contents



Jeanne A. Hardy was raised in Wyoming and began doing research on the reactions of iron and asbestos during her sophomore year at Utah State University. She spent a summer as a UCLA-AWU undergraduate fellow studying the effects of ionizing radiation on wound healing where she realized that her genuine interests were in biochemistry. Jeanne received her B.S. in Chemistry and M.S. in Biochemistry concurrently from Utah State in 1994. She is currently working toward a Ph.D. in Biochemistry and Molecular Biology at the University of California—Berkeley.

Ann E. Aust was born in Texas and graduated with a B.S. in Biophysical Science from the University of Houston in 1970. She attained her Ph.D. in Biochemistry in 1975 working on yeast pyruvate kinase with Dr. Clarence H. Suelter at Michigan State University. Ann spent a year at the Ruakura Agricultural Research Station in Hamilton, New Zealand, studying the hormonal events leading to parturition in cows. In 1977 she began postdoctoral work with Drs. Veronica Maher and Justin McCormick at Michigan State University. It was here that she became interested in the molecular mechanisms by which mutagenic carcinogens cause cancer. In 1982 Ann became the Director of Genetic Toxicology at Parke-Davis Pharmaceutical Research Division in Ann Arbor, MI. In 1987 she moved to Utah State University, Department of Chemistry and Biochemistry, where she began to study the role of iron in asbestos-induced cancer. She is currently an Assistant Professor.

I. Introduction

Asbestos has been widely used for the manufacture of many products because of its remarkable durability, tensile strength, flame retarding capacity, and slow dissolution properties.[1] In the late 1950s, reports of lung cancer in asbestos miners and millers appeared. Since that time it has become well established that asbestos causes pulmonary interstitial fibrosis, mesothelioma of the pleura, pericardium, and peritoneum,[2] and carcinoma of the lungs, esophagus, and stomach.[3–7] Because of these documented carcinogenic effects in humans, the use of asbestos for most applications was banned in the United States almost two decades ago, restricting exposure in the workplace. It has been estimated that over

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11 million people in the United States alone have had occupational exposure to asbestos between 1940 and 1979, of which 2000 die from mesothelioma each year.[8] Since these cancers do not appear until 20 or more years after the first exposure to asbestos, the health effects of these occupational exposures may be seen for some years to come. Concern about exposure to ambient levels of asbestos in structures built before the ban and in the air and water surrounding mining operations continues. However, the health consequences of these lower-level exposures are the subject of much debate.[9]

Since the first reports of lung tumors in asbestos miners, there has been an intensive research effort to understand the mechanism by which asbestos causes cancer. Although fiber dimension and durability have been shown to be important determinants of the carcinogenic potential, the molecular mechanism by which asbestos causes cancer remains to be elucidated. Thus, the manufacture of replacement materials may produce fibers which are as carcinogenic as, or more carcinogenic than asbestos itself.

Asbestos is a commercial term used for a group of crystalline silicates. There are two major subdivisions of asbestos minerals, amphibole and serpentine. Crocidolite and amosite, members of the amphibole family, contain high levels of iron as a normal constituent of the crystal structure[10] and are carcinogenic in laboratory animals and man. Chrysotile contains much less iron, present as a substitution for Mg in the crystal structure or as iron oxide contaminants, and is a member of the serpentine, meaning lizardlike, asbestos mineral family.[10] Chrysotile is at least as carcinogenic in laboratory animals as the amphiboles.[11] Whether or not it induces mesothelioma in man is the subject of debate.[12] This disparity between carcinogenicity in animals and man may result from differences in durability between the amphiboles and chrysotile.[13]

Erionite is a naturally occurring member of the zeolite family of mineral fibers and is not classified as an asbestos mineral. Erionite is not used commercially. It was first shown to be carcinogenic when Turkish villagers, living in caves carved from an outcropping of the mineral, showed a 100-fold increased incidence of mesothelioma.[14,15] Erionite normally contains low amounts of iron, but induces more mesothelioma in the pleura or peritoneum than any form of asbestos both in man[14,16] and in laboratory animals.[17–24] The mechanism by which this mineral causes cancer is also not understood. Erionite, like other zeolites, has the ability to undergo cation exchange. This cation exchange capability is one of the reasons why zeolites are used in industry as solid catalysts for many types of reactions, including oxidation–reduction reactions. The unusual chemical properties of the highly carcinogenic mineral, erionite, led to its inclusion in this review for comparison with the carcinogenic forms of asbestos.

Evidence is accumulating to suggest that chemical reactions catalyzed by asbestos and other mineral fibers may be responsible for their pathological effects. Asbestos is known to catalyze many of the same reactions that iron does, such as lipid peroxidation, DNA strand breaks, formation of oxidized nucleotide bases, and oxidative damage to protein. This review will address the role of iron in the chemical and physiological reactions attributed to asbestos and will enumerate several lines of evidence which point to the involvement of iron from asbestos in the causation of disease.

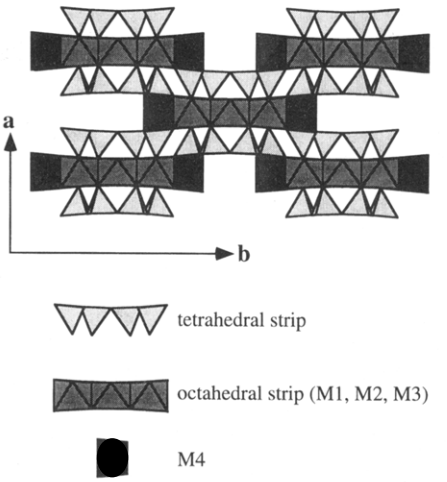

Figure 1. The idealized structure of the amphiboles viewed down the *c*-axis. The triangles represent silicon–oxygen tetrahedra. The octahedral cation sites (M1, M2, M3) are generally filled with Mg^{2+}, Fe^{2+}, and Fe^{3+}, while the M4 sites are generally filled with Na^{1+}. Structural diagram courtesy of G. D. Gutherie, Jr. (Los Alamos National Laboratory, Los Alamos, NM).

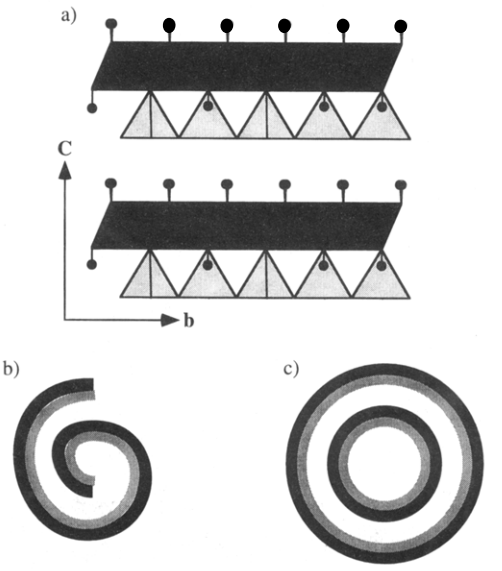

Figure 2. The idealized structure of chrysotile: (a) The lizardite structure viewed down the *a*-axis (the triangles represent the silicon–oxygen tetrahedra and the darker layers, the $Mg(OH)_2$ octahedral sheet); (b) the chrysotile structure based on rolled lizardite layers (darker band is the octahedral sheet); and (c) the chrysotile structure based on concentric lizardite layers. Structural diagram courtesy of G. D. Gutherie, Jr. (Los Alamos National Laboratory, Los Alamos, NM).

II. Structure of Asbestos and Erionite

Figures 1–3 show the structures of crocidolite, amosite, chrysotile, and erionite. The amphibole minerals, crocidolite and amosite (Figure 1), are composed of octahedrally coordinated cations, including iron, sandwiched between two double silicate

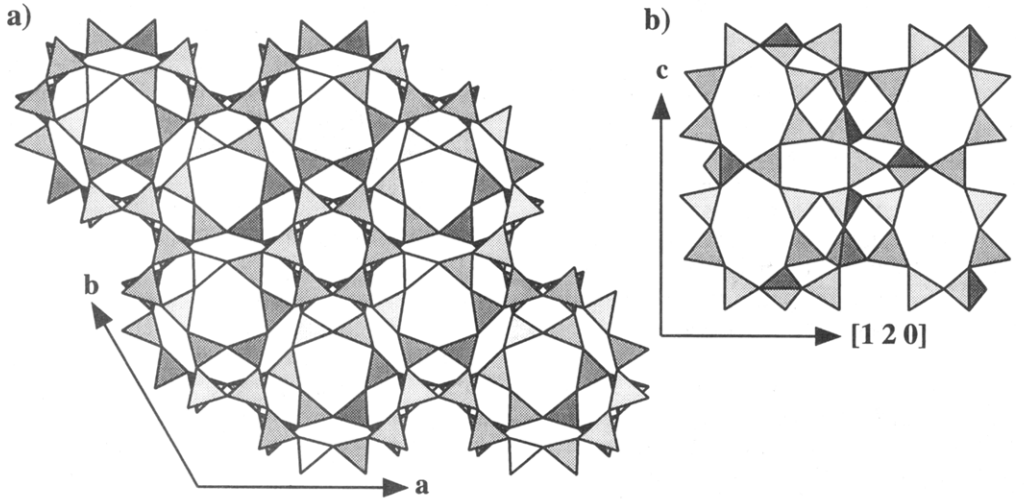

Figure 3. The idealized erionite structure viewed down the *c*-axis (a), which is the axis along the fiber length, and the *a*-axis (b). Shaded triangles are drawn as going into the plane of the paper while white triangles are extending outward from the plane of the paper. The white areas represent the openings through which cations can migrate. (Reprinted from ref 183. Copyright 1993 Mineralogical Society of America.)

Table 1. Physical Properties of Crocidolite, Amosite, Chrysotile, and Erionite

mineral fiber	surface area (m^2/g)[a]	iron content (% by weight)[b]	surface silanol groups (groups/nm^2)[c]	density (g/cm^3)[a]
crocidolite $Na_2Fe^{III}_2(Fe^{II},Mg)_3Si_8O_{22}(OH)_2$	2–15	27.3	4.7 ± 0.6	3.2–3.3
amosite $(Fe^{II},Mg)_7Si_8O_{22}(OH)_2$	1–6	28.5	7.6 ± 1.8	3.1–3.3
chrysotile $Mg_3[Si_2O_5](OH_4)$	10–27	0.7	1.8 ± 0.1	2.4–2.6
erionite $NaK_2MgCa_{1.5}(Al_8Si_{28}O_{72})\cdot 28H_2O$	354 ± 9[d]	ND[e]	0.0042–0.039[f]	2.28[d]

[a] Reference 33. [b] Campbell, W. J.; Huggins, C. W.; Wylie, A. G. *Chemical and Physical Characterization of Amosite, Chrysotile, Crocidolite, and Nonfibrous Tremolite for Oral Ingestion Studies*; National Institute of Environmental Health Sciences, Bureau of Mines Report of Investigations 8452, 1980. [c] Reference 25. [d] Johnson, N. R.; Hoover, M. D.; Thomassen, D. G.; Cheng, Y. S.; Dalley, A.; Brooks, A. L. *Am. J. Ind. Med.* **1992**, *21*, 807–823. [e] ND, not detectable. [f] Reference 26.

chains.[10] The oxygen atoms of the silicate chains coordinate both the Si and a variety of other cations. Chrysotile (Figure 2) is a cylindrical lattice of lizardite resulting from an inherent misfit between the $Mg(OH)_2$ octahedral sheet and the Si tetrahedral sheet.[10] In chrysotile, the magnesium ions can be substituted with iron, which accounts for the iron content of the pure mineral. The layers comprising chrysotile coil together to form scroll-like tubes with the $Mg(OH)_2$ surface facing outward. Small fibrils of chrysotile aggregate together into bundles which are known to break down and disseminate in aqueous suspension.

Erionite, like all zeolites, is an aluminosilicate composed of silicon–oxygen tetrahedra which associate into six-membered rings to form channels and cages, as shown in Figure 3. Occasional substitution of Al atoms for Si gives rise to an overall negative charge on the lattice, which allows countercations to associate with the mineral. Cations can migrate into the 4.3 Å openings of the network of cages. This open cagelike structure gives erionite a surface area that is up to 50 times greater than the surface areas of asbestos fibers.

III. Physical Properties of Asbestos Involved in Carcinogenesis

Several of the characteristics and the empirical formulas of carcinogenic mineral fibers discussed in this review are compiled in Table 1. Many of the reactions that will be discussed in this review have been reported to be strongly influenced by the surface area and interactions of iron with surface silanol groups of the various mineral fibers. The surface coverage of silanol groups, as listed in Table 1, is by far the greatest in the asbestos minerals. Amosite has the greatest number of terminal OH groups, followed by crocidolite, chrysotile, and finally, erionite.[25,26] When comparing the surface area, erionite has the greatest by far, 10–100 times larger than the asbestos fibers. Finally, the intrinsic iron content is greatest in the amphibole forms of asbestos, crocidolite and amosite. The iron in amosite is exclusively Fe(II) while crocidolite contains Fe(II) and Fe(III).

Two properties of the asbestos fibers appear to affect their carcinogenicity: size and durability. The first, and perhaps most familiar, is fiber size, or what has been termed "aspect ratio". For some years Stanton's hypothesis, that fiber size is the determining factor in fiber carcinogenicity, was widely accepted.[27] A correlation has been reported between the induction of pleural mesothelioma in rats and the number of long, spindle-shaped fibers with diameter ≤ 0.25 μm and length ≥ 8 μm residing in the lungs.[28] This subject has been previously reviewed and will not be discussed at length here.[29] However, aspect

ratio does appear to be important because only respirable, durable fibers which are not small enough to be cleared from the lung will remain for periods of time sufficient to cause disease. Goodglick and Kane[30] observed that when clearance of fibers was prohibited by repeated administration, short fibers produced the same cytotoxicity response as long fibers. Notions about the aspect ratio of fibers cannot fully account for the chemistry of, nor the biological reactions to, mineral fibers. Recently the limitations of the utility of the Stanton hypothesis in accessing the carcinogenic potential of mineral fibers have been discussed.[31,32]

A second property that appears to influence carcinogenesis is fiber durability. Generally, the longer the fiber resides in the lung, the more likely it is to be carcinogenic. Amphibole fibers remain in the lung for the lifetime of the individual. Chrysotile has historically been used in 90% of the applications of asbestos,[33] but is much less carcinogenic in man than crocidolite or amosite. This may be due to the more rapid dissolution kinetics of the serpentine minerals compared with the amphiboles. Although few studies have been performed to quantify the dissolution of mineral fibers, it is generally believed that chrysotile dissolves at a significantly greater rate than the amphiboles. Hume and Rimstidt[34] incubated chrysotile in aqueous solutions similar to human lung fluids and observed rapid dissolution of the fibers. In comparing chrysotile, crocidolite, and amosite, fibers were incubated in a severe environment of 4 M HCl and refluxed to encourage dissolution. Sixty percent of the chrysotile was lost in 30 min while only 6% of crocidolite or 8% of amosite dissolved in the same period of time. After 8 h of this treatment, 8.5% of the crocidolite fibers had been dissolved, while 30% of the amosite fibers dissolved.[35] This difference in dissolution between the amphibole and serpentine minerals may help to explain why the amphiboles are highly carcinogenic in both laboratory animals and man, while chrysotile can be equally carcinogenic in laboratory animals, but less carcinogenic in man.[13] Studies on the dissolution of mineral fibers have been reviewed by Morgan and Holmes.[36]

IV. Reactions Catalyzed by Iron

Iron is by far the most abundant transition metal in the body. The average male has approximately 4 g of iron in his body while the average female body contains 3 g. The amount of iron is greater than the combined amounts of zinc, copper, molybdenum, cobalt, and all other trace metals in the body.[27] Hundreds of proteins are known to contain iron atoms, including proteins which transport oxygen, allow the synthesis of proteins and nucleic acids, produce energy through oxidative phosphorylation, and facilitate a multitude of other reactions for growth and reproduction. Although iron is essential for all life, if uncontrolled, it has the potential to catalyze the oxidation of DNA, lipid, and protein. Living organisms have evolved proteins to transport and store iron in an unreactive form until it is needed.[38] When iron is absorbed from the diet, it is bound to transferrin for transport in the blood. Transferrin receptors are synthesized and incorporated into the plasma membrane for binding and endocytosis of transferrin in cells which need iron. In a process that is not completely understood, iron is passed from transferrin to ferritin, where it is stored until needed. The reduction potential of these protein complexes with ferric iron is too negative to be reduced by typical intracellular reductants such as ascorbate, cysteine, or glutathione, which probably explains the stability of these protein–iron complexes *in vivo*.[39]

There are diseases where iron may be observed bound to compounds other than protein. Hemochromatosis is a genetic disease which results in the abnormal accumulation of iron to high levels in the body. Under these circumstances, citrate-chelated iron has been observed in the blood.[40,41] Patients with hemochromatosis[42,43] or another iron overload disease, porphyria cutanea tarda,[40,45] are more likely than persons without these conditions to develop liver cancer. The unusual appearance of citrate-chelated iron in the blood of patients with iron overload conditions may be involved in the symptoms observed in these individuals through the iron-catalyzed generation of oxygen radicals.[40,46,47] More recently it has been noted that high levels of iron in the body are associated with an increase in all types of cancers.[48–50]

A. Generation of Reactive Oxygen Species

Reactions of many biomolecules with molecular oxygen to generate reactive oxygen radicals do not occur because most organic molecules exist in the singlet spin state while O_2 exists in the triplet spin state. Reactions of a triplet with a singlet molecule are formally forbidden and will generally be slower than 10^{-5} M^{-1} s^{-1}. However, transition metals like iron can bridge this kinetic restriction by reducing O_2 to form radical species that are capable of reacting with organic molecules.[39] Other transition metals with a free coordination site could catalyze the reduction of O_2 or H_2O_2. Although the exposure to other transition metals is generally low, they are capable of catalyzing deleterious reactions when introduced into biological systems.[51] Since iron exists at the highest concentration of any transition metal in most living organisms, it is thought to be responsible for most of the abnormal oxygen radical production observed.

Many mechanisms have been proposed as the potential reactions of iron to generate reactive oxygen species. One of these is shown in the following series of reactions of iron which together lead to the generation of the hydroxyl radical.[52] These reactions are the modified, iron-catalyzed Haber–Weiss reactions.

$$\text{reductant}^{(n)} + \text{Fe(III)} \rightarrow \text{reductant}^{(n+1)} + \text{Fe(II)} \quad (1)$$

$$\text{Fe(II)} + O_2 \rightarrow \text{Fe(III)} + O_2^{\bullet -} \quad (2)$$

$$HO_2^{\bullet} + O_2^{\bullet -} + H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$\text{Fe(II)} + H_2O_2 \rightarrow \text{Fe(III)} + OH^- + {}^{\bullet}OH \quad \text{(Fenton Reaction)} \quad (4)$$

In addition to generation of $^{\bullet}OH$, the ferryl iron

species, $Fe^{IV}=O$, and $Fe^{II}-Fe^{III}-O_2$ complex have been proposed to be involved in reactions catalyzed by iron. Of all of the reactive species generated by iron, the •OH radical is the only species that has been extensively studied, probably because methods are more readily available for its detection and studying its participation in reactions. Although the other iron species may be participating in damage induced by mineral fibers, much of the work that has been done has focused on the involvement of the •OH. Thus, a more thorough discussion of what factors might be expected to affect the production of •OH will aid in understanding experimental results to be reviewed here.

Hydrogen peroxide, which reacts very sluggishly with biomolecules, and $O_2^{\bullet-}$, which reacts with second-order rate constants ranging from 2.3×10^4 to 2×10^9 M^{-1} s^{-1}, are not considered to be damaging species in biological systems.[53] The •OH reacts with most biomolecules with second-order rate constants ranging from 2×10^8 to 3.6×10^{10} M^{-1} s^{-1} which are considered diffusion-controlled reaction rates.[53] Thus, it is the production of •OH or a similarly reactive species that is generally considered to be dangerous to the cell.[54] Therefore, the important intracellular reactions are those that generate Fe(II) and H_2O_2. Ferrous iron may already be present or may be generated by the reduction of Fe(III) by ascorbate, cysteine, glutathione, or other cellular reductants. Activated macrophages can produce $O_2^{\bullet-}$ which is also capable of reducing iron, under unusual conditions.[39,55] Hydrogen peroxide may come from an exogenous source, such as activated macrophages, or be generated by the spontaneous or enzyme-catalyzed dismutation of $O_2^{\bullet-}$. Iron is a catalyst in these reactions and will continue to redox cycle, producing •OH as long as there is sufficient reductant and either O_2 or H_2O_2. Redox cycling of iron may lead to significant damage to biomolecules in a manner similar to X-rays or γ-rays, where the •OH is thought to be responsible for the induction of cancer.[56]

Pryor[57] has suggested that the •OH is the predominant damaging species *in vivo* because the elements which generate the •OH, such as iron, are often capable of binding to the DNA itself and allow •OH production to occur in the immediate vicinity of the DNA. For radicals to damage DNA they must not only be thermodynamically favored to do so, but must also have the kinetic energy to reach the site of damage and to react.[57] Other radicals, like carbon-centered radicals, are known to react readily with nucleic acids *in vitro*, but excreted methyl radical adducts are observed with a much lower frequency than •OH adducts, although their predicted reactivities are similar. Pryor suggests that the most electrophilic radical that DNA is generally exposed to is the •OH. The combination of these three characteristics: high electrophilicity, high thermokinetic reactivity, and a mechanism for production near DNA, make the •OH the only radical which generally damages DNA bases.[57]

The reactivity of iron is highly dependent upon its electronic environment. For example, iron bound to low-molecular-weight chelators like citrate, adenosine diphosphate (ADP), or ethylenediaminetetraacetic acid (EDTA) is redox active.[39,46,58] Graf *et al.*[55] have shown that coordination of iron by these chelators allows water, or other small molecules like O_2, to occupy an available coordination site. This allows the iron chelates to reduce O_2 and generate highly reactive species.[55] In contrast, when all of the iron coordination sites are tightly bound by the chelator, excluding other molecules, the complex will be redox inactive, as occurs with *N'*-[5-[[4-[[5-(acetylhydroxyamino)pentyl]amino]-1,4-dioxobutyl]hydroxyamino]pentyl]-*N*-(5-aminopentyl)-*N*-hydroxybutanediamide (desferrioxamine B)[55] and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p*-sulfonic acid (ferrozine) which stabilize the Fe(III) and Fe(II) forms, respectively. Inhibition of chemical reactions or biological effects by desferrioxamine B has been extensively used to determine whether reactions are catalyzed by iron. Although desferrioxamine B is generally considered to bind Fe(III) preferentially [log *K* of 31] over Fe(II) [log *K* of ~7], it is known to coordinate both oxidation states.[53,59,60]

The location of iron within the cell also influences potential reaction, since the •OH generated by the iron-catalyzed reactions is diffusion limited in its reaction kinetics. Thus, iron must be within 10 Å of the target molecule in the cell for damage to occur. This restriction will become very critical when asbestos-catalyzed reactions are discussed.

B. Oxidation of Biological Molecules

Since the short-lived •OH cannot be detected directly, electron paramagnetic resonance spectroscopy (EPR) is used to detect the formation of a more stable radical species resulting from reaction of spin-trapping chemicals with •OH. To determine whether •OH is responsible for the oxidation of biological molecules, antioxidant enzymes, such as superoxide dismutase (SOD), which catalyzes the dismutation of $2O_2^{\bullet-}$ to H_2O_2 and O_2, or catalase, which causes the decomposition of H_2O_2 to H_2O and O_2, are often used. By reexamining reactions 1–4 discussed previously, one can see that introduction of SOD into a reaction system which is generating •OH should, if anything, increase the rate of formation by increasing the rate of generation of H_2O_2 (reaction 3). In contrast, catalase should inhibit the formation of •OH by removing H_2O_2 and inhibiting reaction 4. Further evidence for the participation of •OH in reactions is to determine whether radical scavengers, such as 5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO), salicylate, mannitol, ethanol, dimethyl sulfoxide, or dimethylthiourea, inhibit the formation of products being monitored. All of these methods are fairly easily used for *in vitro* reactions, but become much more difficult to implement and interpret *in vivo* or in cultured cells.

1. DNA Damage

Iron appears to induce the same types of damage to DNA that γ irradiation does through the generation of the •OH.[61] Iron-catalyzed reactions with DNA result in damage to all four bases and the deoxyribose, which is manifest as strand breaks to DNA. To quantify and identify the many products produced,

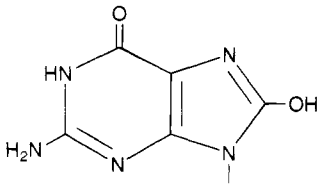

Figure 4. 8-Hydroxuguanine.

Dizdaraglu *et al.*[62] have used gas chromatography/mass spectroscopy with selected-ion monitoring. Seventeen different modified base products have been reported using this technique.[56] The predominant product observed is 8-hydroxy-2′-deoxyguanosine (8-OHdG),[62] the structure of which is shown in Figure 4. They detected an enhancement in the amount of adducts from EDTA:Fe(III) in the presence of H_2O_2 and/or ascorbate. The production of hydroxylated bases was inhibited by the addition of antioxidant enzymes, radical scavengers, or the inactivating chelator, desferrioxamine B,[63] suggesting that iron-catalyzed Haber–Weiss chemistry may be responsible for the production of the adducts observed. They later showed that the chelator nitrilotriacetate (NTA) produced by far the greatest amount of these base products in the presence of H_2O_2.[47] This observation is of particular interest because NTA-chelated iron is a known carcinogen causing acute nephrotoxicity, renal carcinoma,[64–66] and lipid peroxidation.[67,68] Mammalian chromatin was also susceptible to oxidative damage by iron. This effect was enhanced by addition of ascorbate, and the investigator concluded that the damage was due to the •OH formed by redox active iron.[62]

Floyd *et al.*[69] have developed a more rapid and sensitive means of detecting this oxidized base product using reversed phase HPLC with electrochemical detection. Because of the preponderance of this base product and the ease and sensitivity of the HPLC assay, most laboratories investigating the participation of iron-catalyzed oxygen radicals in DNA damage are currently using this technique. Many of the observations which have been made on the redox chemistry of iron chelates, both *in vitro* and *in vivo*, have been recently reviewed.[51]

The relationship between the formation of modified DNA bases and the induction of cancer is not clear. Although 8-OHdG may not be directly responsible for carcinogenicity, its presence at elevated levels is a very good indication that abnormal oxidative reactions are occurring. Floyd[70] has outlined what he considered to be a direct correlation between the presence of 8-OHdG and conditions leading to carcinogenesis. He enumerated the following four lines of evidence supporting his proposal. First, ionizing radiation is known to cause cancer and induces 8-OHdG. Second, the strong oxidant $KBrO_3$ causes kidney tumors which contain elevated levels of 8-OHdG. NaClO or $NaClO_2$, which are equally strong oxidants, do not cause tumors or an increase in 8-OHdG in treated tissue. Third, treatment of rats with the Fe:NTA complex is carcinogenic in kidney and causes an increase in 8-OHdG in kidney DNA. The complex Na:NTA is not carcinogenic and does not cause elevated levels of 8-OHdG. Fourth, a liver carcinogen, 2-nitropropane, is associated with increased levels of 8-OHdG, whereas the isomer 1-nitropropane, which is not carcinogenic, does not cause any increase in 8-OHdG formation upon administration. Floyd[70] noted that these conditions reflected oxidative stress, and under carcinogenic conditions not involved in oxidative stress, the correlation was not observed. Iron is known to generate many products which are associated with oxidative stress, but 8-OHdG may be a valuable indicator of the carcinogenic potential of various iron-containing compounds.

Iron-catalyzed oxygen radical production is also known to introduce strand breaks into DNA.[61,71] DNA strand break assays can be highly sensitive to the reactions of iron when closed-circular, superhelical DNA is used as the target. DNA strand breaks have also been reported to occur in the presence of various chelates of iron in isolated cellular DNA of prokaryotic[52,72] and eukaryotic chromosomal DNA.[73] The specificity and location at which the damage to the DNA occurs appears to depend on the chelator to which iron is bound.[72,73]

The relationship between the types of DNA damage observed after exposure to iron and mutation are largely unknown. The presence of 8-OHdG in DNA has been shown to lead to misincorporation of bases during replication of DNA *in vitro*.[74] Loeb *et al.*[75] observed mutations in ϕX174 DNA after exposure to iron and transfection into bacterial spheroplasts. The investigators concluded that the types of mutations observed were typical of those observed after reactions with oxygen radicals. The pattern of mutations was nonrandom suggesting that these mutations occurred at specified locations.[76] A thorough review on metals in carcinogenesis has recently been published.[77]

2. Lipid Peroxidation

The role of iron in lipid peroxidation has been extensively studied, and the identity of the oxygen species responsible remains controversial. A reactive oxygen species other than •OH appears to be responsible for lipid peroxidation because catalase, superoxide dismutase, or mannitol did not inhibit iron-dependent lipid peroxidation. Several investigators have suggested that both Fe(II) and Fe(III) and O_2 are required for lipid peroxidation to occur.[78–81] The maximal rate of lipid peroxidation was observed when Fe(II) and Fe(III) were available at a 1:1 ratio.[78–81] It has been proposed that a complex between Fe(II), Fe(III), and O_2 may be involved,[81] but no definitive evidence for this complex exists at this time.

Some of the products of lipid peroxidation, 4-hydroxynonenal, 4-hydroxyhexenal, and malonaldehyde, shown in Figure 5 have been extensively studied and are generally considered to be dangerous because of their long lifetimes and ability to traverse the cell. Their reactivity with biomolecules, such as proteins and nucleic acid bases, may be involved in the initiation of cancer in the same way that damage to these biomolecules by radicals may be involved. This is the subject of a recent, comprehensive review.[82]

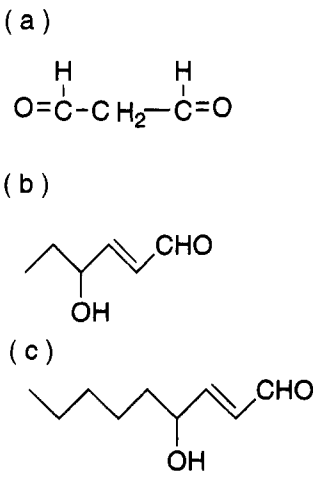

Figure 5. Malonaldehyde (a), 4-hydroxyhexenal (b), and 4-hydroxynonenal (c).

3. Protein Oxidation

Iron has also been implicated in the oxidation of proteins. Oxidation of proteins is thought to be significant in aging,[83] but its role in the development of cancer is not clear. Oxidation of proteins, like glutathione or glutathione peroxidase, may lead to a compromised antioxidant defense system, and the oxidation of DNA repair enzymes at a critical time may allow mutations to occur. Both Fe(II) and Fe(III), as well as iron-containing proteins and hemoglobin degradation products, have been reported to cause oxidation of individual amino acids as a result of iron-catalyzed reactions.[84] Although the reactive oxygen species responsible for this damage have not been confirmed, a complex model for the involvement of both Fe(II) and Fe(III) and O_2 has been proposed in the oxidation of proteins.[83,85]

C. Iron-Induced Carcinogenesis

Iron may cause cancer as a result of oxygen radical-induced damage to DNA. There is increasing evidence that iron from sources other than asbestos can increase the risk of cancer in humans.[86-88] In rodents, the evidence is even more convincing. Iron–NTA has been shown to induce renal adenocarcinoma[89] and irondextran to induce sarcomas at the site of injection.[90] An even more significant observation was that intraperitoneal injections of iron saccharate in rats caused mesothelioma,[89] the same type of rare tumor induced by asbestos. In all of these experiments with rodents, it was convincingly demonstrated that iron was required for the carcinogenic response.

V. Reactions Catalyzed by Asbestos

The surface character of mineral fibers determines their chemical reactivity, their ability to bind and release elements, and their surface charge. These surfaces are not inert, but are dynamic and highly interactive with their environment. Fiber surfaces can be modified simply by milling, suspension in aqueous solution, or changes in temperature or pressure. The surface composition can be modified as a result of binding or releasing of atoms or molecules. The type of binding, adsorption in a monolayer or nucleation and formation of a three-dimensional structure, is extremely important in determining chemical reactivity and binding and desorption kinetics. The surfaces can also have lateral heterogeneity, which plays an important role in mineral surface chemistry. Surface heterogeneity can arise during adsorption or desorption of elements.

Mineral surfaces are complex not only in terms of atomic structure and composition, but also in terms of microtopography.[91] It is clear that there are "active sites" on the surfaces which will facilitate adsorption or desorption. "Active sites" often occur where surfaces are rough or the microtopography is uneven. The atoms that make up the top of the rough edges or terraces have unsaturated coordination sites and therefore are capable of undergoing reactions.

Studies to understand the chemical reactivity, the ability to bind and release elements, and the surface microtopography of mineral fibers that pose health problems have increased in the past 10 years. In this section, we will summarize what has been learned about these unique mineral fibers.

A. Surface Chemistry

In order for any chemical reaction to be catalyzed by solid fibers, *in vitro* or *in vivo*, interactions between the surface of the fiber and the environment must occur. Fubini *et al.*[92] and Mollo *et al.*[93] stress that the reactivity of fibers *in vivo* will be controlled by the surface chemistry of the individual fibers. Some of the surface chemical functionalities which are present on the surface of fibers are H-bonding sites, dangling bonds, poorly coordinated metal ions, charges which occur due to lattice vacancies and defects, unoccupied cation coordination sites, and Lewis acids or bases.[94]

1. Reactive Sites

Hochella has pointed out that, unlike the figures which are commonly drawn of mineral fibers (see Figures 1–3), the surfaces of most mineral fibers are not atomically flat, but contain atoms above the plane of the mineral and holes which were previously occupied.[91] The microtopography of the surface of even a very flat mineral might appear uneven on an atomic scale (Figure 6). This type of occurrence is magnified when minerals are weathered or milled, and mineral fibers develop additional areas of non-ideal microtopography. Fractures occur along cleavage planes to create the edges of minerals which are often considered to be the areas of greatest deviation from the idealized structure. These portions of the mineral are where dangling bonds, silanol groups, and unoccupied cation coordination sites may exist. Many of the reactions catalyzed by asbestos and erionite fibers may be strongly influenced by the surface functional groups made accessible both by the native structure of the mineral and by mechanical processes like milling or weathering, generating nonideal surfaces which may be the areas of the greatest reactivity.[91]

Responses of silicate surfaces to grinding have been compiled by Fubini *et al.*[95] Homolytic and heterolytic cleavage of bonds leads to structures like those in

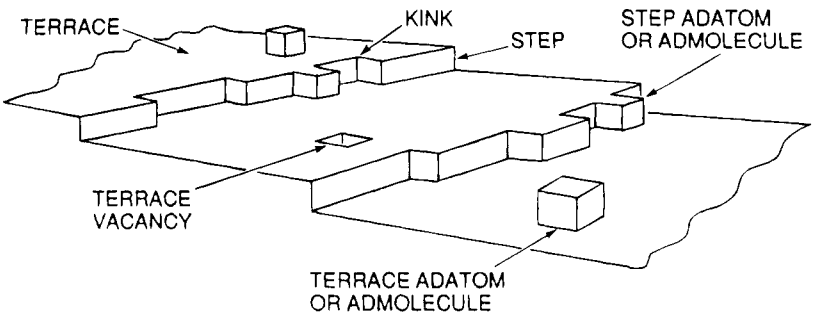

Figure 6. The microtopography of solid surfaces depicting the various deviations from ideal structure. (Reprinted from ref 91. Copyright 1993 Mineralogical Society of America.)

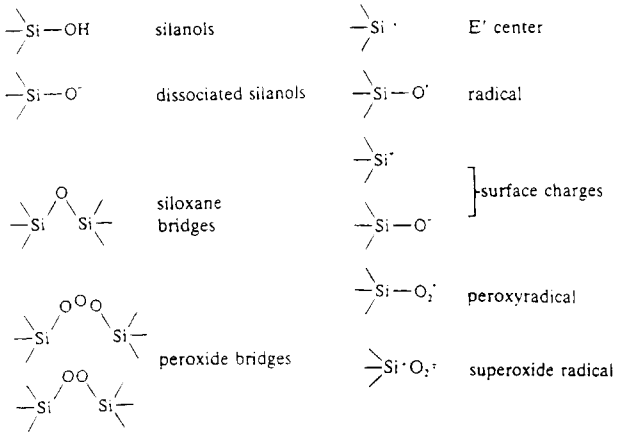

Figure 7. Observed reactive groups on the surface of silicate minerals observed after grinding, heating, or other physical or chemical manipulations.

Figure 7: distorted siloxane bridges, peroxide bridges, silica radicals, Si^+ or $Si-O^-$ surface charges, $Si-O_2^{\bullet}$ peroxyradical, and $Si^+O_2^{\bullet -}$ superoxide radical which have been observed on silicate surfaces after mechanical grinding.[95] These surface functional groups may play a role in freshly ground silicates, but are rapidly lost by heating, which converts silanols (hydrophilic surfaces) to siloxanes (hydrophobic surfaces). These surface reactive groups would be dissipated shortly after inhalation of fibers into the moist environment of the lung. While these groups may be involved in acute biological effects, the long-term contribution of these surface reactive groups to carcinogenicity is questionable.[92]

2. Redox Active Iron

The long-term reactivity of the surface of asbestos fibers may be largely governed by the amount of iron which is coordinated to the surface and the redox activity of that iron. Shen *et al.*[96] have developed a novel way to measure the reactive iron on the surface of iron-containing mineral fibers. This electrochemical method utilizes soluble mediators to carry electrons between the solid electrodes and the solid sample, which is contained within a thin-cell of 25 μm thickness. Freshly suspended crocidolite and amosite were examined using this mediated, thin-layer cell, coulometric method. The total amount of redox active iron on the surface of crocidolite was 4.3 $\pm$ 0.7 nmol of Fe/mg, whereas amosite contained 3.3 $\pm$ 0.7 nmol of Fe/mg. The total amount of redox active iron on the surface of the fibers remained constant after repeated oxidation and reduction cycles. The surface coverage of iron on the two minerals was of course dependent on the surface area of the mineral fibers themselves. Crocidolite held 4.3 $\times$ 10^{-11} mol of Fe/cm^2 and amosite had 9.5 $\times$ 10^{-11} mol of Fe/cm^2. Seventy-six percent of the redox active surface iron on crocidolite was in the Fe(II) form while only 25% of that on amosite was in the reduced form.[96]

These results are in agreement with previous findings on the oxidation state of mobilized iron from crocidolite using an iron chelator, ferrozine, in the presence or absence of a reductant ascorbate.[97] This study demonstrated that 66% of the iron mobilized from crocidolite was in the Fe(II) state, while only 10% of iron mobilized from amosite was reduced. More iron was mobilized than the amount of surface reactive iron determined by the electrochemical method, but the ratio between the oxidation states was similar.[96,97] In the mobilization experiments, it is likely that the greater quantity of iron observed may have come from the outer layers rather than exclusively from the surface *per se*. An important outcome of the electrochemical experiments was that they showed that the iron can be repeatedly oxidized and reduced. This may be very important in the reducing environment of the cell where O_2 is also present. Many of the investigators studying iron associated with mineral fibers are most concerned about the Fe(II) content. This may be inconsequential compared with the total iron present, if the iron can redox cycle.

3. Hydroxyl Radical Generation

Weitzman and Graceffa[98] were the first to actually study the surface reactivity of fibers. They observed that chrysotile, crocidolite, or amosite, suspended in aqueous solution with H_2O_2, generated $^{\bullet}OH$, observed by EPR after spin trapping with DMPO. The addition of desferrioxamine B to the reaction mixture inhibited the $^{\bullet}OH$-DMPO adduct signal, indicating that the reactivity was due to iron on the various fiber surfaces.[98]

The spin adducts observed in these experiments were in an environment free of iron chelators, suggesting that the observations made were the result of surface iron. The work to be discussed now reported studying surface reactivity. However, the experiments were performed in the presence of buffers, such as phosphate or Tris, which are capable of mobilizing iron from asbestos into solution.[52]

Pezerat[99] has proposed that there are two types of reactive oxygen species generated by iron which are

responsible for the fact that some fibers induce predominantly bronchial carcinoma while others induce predominantly mesothelioma. He suggests that A*, a strongly oxidizing species capable of hydrogen atom abstraction, e.g. •OH or $Fe^{V}=O$, is capable of direct attack on the genome and is not affected by the antioxidant enzymes SOD or catalase. The second P* group of species is comprised of all other types of oxidizing species and is, he proposes, responsible for activities such as lipid peroxidation, the products of which he proposes lead to mesothelioma. Erionite, for example, was not capable of generating A* species and erionite is very efficient at inducing mesothelioma, but only rarely causes lung cancer. The more reactive A* species was proposed to be responsible for causing bronchogenic carcinoma.[99] DMPO was employed to assay mineral fibers of the same vitreous structure and closely related chemical composition, but variable amounts of Fe(II), for their ability to form •OH radical adducts for EPR analysis, and a linear correlation was observed between the oxidizing surface activity and the Fe(II) content of the fiber.[100] In these studies, reactions of the A* and P* sites may have been enhanced due to mobilization of iron from the fibers, since phosphate buffer was used in the reaction mixtures.

Zalma *et al.*[101] have also suggested that the preliminary step in the generation of reduced oxygen species occurs when H_2O binds to a Fe(II) on the surface of a fiber. In this model, attack of an O_2 results in oxidation of the iron with binding of hydroxide while H^+ and $O_2^{\bullet-}$ are released.[101] A scheme such as this would require subsequent interaction of more Fe(II) to generate the further reduced oxygen species they have observed. The details of this mechanism come from a previous study which noted that coordination by H_2O allowed reduction of O_2 to $O_2^{\bullet-}$ only after the H_2O was displaced by O_2 in the proper coordination site.[55]

A comparison of the ability of a fiber to generate •OH and the formation of 8-OHdG also yielded a very strong correlation.[102] Grinding was used to increase the oxidizing potential of minerals. After grinding, amosite or crocidolite had a 145- or 125-fold increase in ability to generate DMPO adducts and a 53- or 22-fold increase in ability to catalyze the formation of 8-OHdG, respectively. Erionite was only slightly capable of generating DMPO adducts before or after grinding, and there was a meager 5-fold increase in the ability of ground erionite to generate 8-OHdG. This was interpreted as evidence for the P* species before grinding and A* species in crocidolite and amosite after grinding.[102] Grinding appears to expose more new surfaces with Fe(II), since reducing agents were not used in these experiments. An alternative explanation for the apparent discrepancy between the biochemical inactivity of erionite *in vitro* and its potent carcinogenic activity *in vivo* will be discussed at some length in the section on iron binding to erionite.

In work by Fubini *et al.*,[92] fibers were treated with ferrozine, a chelator with a high affinity for Fe(II), or with desferrioxamine B, which is a chelator for Fe(III) predominantly. In either case, treatment with

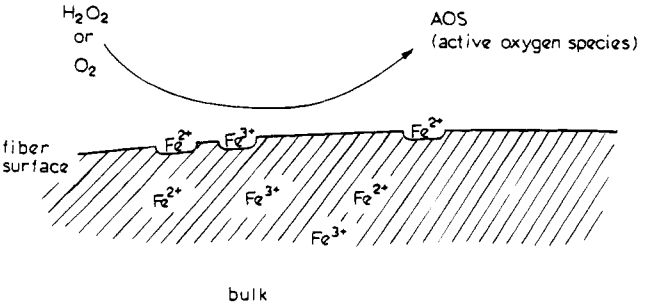

Figure 8. The surface reactivity scheme proposed by Fubini and associates depicting the occurrence of both Fe(II) and Fe(III) on the surface and in the interior of the mineral fibers.

the chelator resulted in a loss of free radical release which was detected using EPR with the spin trap DMPO. The investigators proposed that both Fe(II) and Fe(III) species are required for the generation of free radicals through the reduction of O_2. A proposal of the required conformation of Fe(II) and Fe(III) on the surface of fibers as described by this research is shown in Figure 8. This figure depicts how both oxidation states of iron are present in and on the surfaces of fibers and are able to react with small molecules like O_2 and H_2O_2 to generate activated oxygen species. Desferrioxamine B has been shown to bind to crocidolite.[103] The investigators suggested that the loss of activity observed after chelator treatment may have been a result of the chelators obstructing the normal interactions with the surface of the mineral fiber, preventing the Fenton reaction.[93] Another reason for loss of reactivity may be explained on the basis of removal of iron from the catalytic sites on the fibers. This will be discussed in more depth in the section on mobilization of iron.

Ghio *et al.*[25] examined the role of surface iron in the ability of crocidolite, suspended in phosphate buffer with H_2O_2 and ascorbate, to catalyze the formation of •OH. The formation of •OH was assessed by measuring the formation of thiobarbituric acid reactive products from DNA and lipid and by measuring the hydroxylation of salicylate. These investigators defined surface iron to be the iron which was mobilized in 30 min into a solution containing citrate, bicarbonate, and dithionite at 70 °C. Crocidolite, pretreated with increasing concentrations of the iron-chelator desferrioxamine B, became less capable of generating •OH. They concluded that the amount of •OH produced was proportional to the amount of Fe(III) on the surface of the fibers.[25] Although these results appear to differ from those of Fournier *et al.*,[100] who observed that Fe(II) rather than Fe(III) was related to the ability of fibers to generate •OH, the methodology used in the work by Ghio *et al.*[25] may provide an explanation. The electrochemistry studies[96] and the reports by Fubini *et al.*,[92] which were discussed previously, both observed Fe(II), as well as Fe(III), on the surface of crocidolite fibers. Therefore, there may be two reasons for the apparent conflict. First, an inherent assumption in the experiments of Ghio *et al.*[25] was that desferrioxamine B only binds Fe(III). However, desferrioxamine B is known to bind both Fe(II) and Fe(III),[53,59,60] so both ions may have been bound. If surface Fe(II) was also

Table 2. Effect of Iron Chelators on Reactivity of Iron in Solution and on Crocidolite

chelator	stability constant[a] (log K_a) Fe(II)	Fe(III)	rate of iron mobilization from crocidolite (nmol/mg per h)	crocidolite-dependent DNA SSBs[d] (percent)[e]	Fe-dependent •OH formation[b] (nmols HCHO/30 min)[f]	Fe-dependent 8-OHdG formation[c] (per 10^5 dG)[g]
desferrioxamine B		30.7	12.4 ± 1.7[h]	ND[i]	ND	
EDTA	14.3	25.0	30.1[j]	29 ± 10	16.1 ± 0.3	1.43 ± 0.31
NTA	8.3	15.9	29.2[k]	61 ± 3	23.8 ± 0.3	13.40 ± 0.03
citrate	4.4[l]		4.2 ± 0.5[j]	2 ± 3		0.71 ± 0.07

[a] Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1974; Vol. 1. [b] Incubated in solutions of 50 μM $FeCl_3$ and 500 μM chelator. [c] Incubated in solutions of 25 μM $FeCl_3$, 2.8 M H_2O_2, and 100 μM chelator. [d] Expressed as the percent of closed-circular DNA with SSBs after incubation with 1 mg/mL crocidolite and 1 mM chelator in the absence of a reducing agent. [e] Reference 46. [f] Reference 55. [g] Reference 47. [h] Werner, A. J.; Hochella, M.; Hardy, J. A.; Aust, A. E.; Rimstidt, J. D. *Am. Mineral.* **1995**, in press. [i] ND, not detectable. [j] Reference 52. [k] Dawson, R. M. C.; Elliott, C. C.; Elliot, W. H.; Jones, K. M. *Data for Biochemical Research*, 3rd ed.; Oxford University Press: New York, 1986; pp 400–413.

removed, the reactivity of the fibers would have been reduced. Second, the reducing agent ascorbate was used in the experiments measuring thiobarbituric acid reactive products or salicylate hydroxylation products, which makes it difficult to differentiate between Fe(II) and Fe(III) reactivity. Despite the difficulties in interpretation, a relationship between the total surface iron and the ability to generate the •OH was observed.

In order for chemical reactions to occur between a solid, like a fiber, and an aqueous environment, such as exists in the cell, surface interactions must take place. Many of the results discussed here have suggested the transfer of an electron from iron on the surface of asbestos to O_2 to generate reactive oxygen species. Generation of •OH on the surface of asbestos is only important in reactions with biomolecules when the asbestos fiber is within approximately 10 Å of the target biomolecule because of the diffusion-controlled reaction kinetics of •OH. Therefore, DNA damage from surface generation of •OH is only possible when the fiber is in the nucleus of the cell. However, fibers are seldom observed in the nucleus. Unscheduled DNA synthesis[104] and DNA single-strand breaks (SSBs)[105] have been observed in asbestos-treated, cultured cells when no fibers were observed in the nucleus. Therefore, the surface of fibers may be more important in regulating the binding and release of other molecules, like iron, than in generating reactive oxygen species directly.

B. Iron Mobilization

Phagocytosis of asbestos fibers may constitute an uncontrolled entry of iron into the cell, since the fibers have bypassed control by the protein transferrin. If iron can be mobilized from the fibers by low-molecular weight chelators, such as citrate, the redox activity might be altered, and the chelate complex could diffuse throughout the cell and have the potential of catalyzing the formation of •OH to damage DNA.

Mobilization of iron into solution has often been detected using the chelator ferrozine which forms a colored complex of high extinction coefficient with Fe(II).[106] Mobilization of Fe(II) by ferrozine can be determined directly by quantifying the amount of ferrozine–Fe(II) complex formed. With other chelators which do not form complexes with high extinction coefficients, asbestos is incubated in solutions containing the chelator for varying periods of time, the asbestos is removed, and the iron mobilized by the chelator is quantified using a total iron assay with ferrozine.[52] Other chelators that have been used by investigators to detect and quantify iron mobilization are desferrioxamine B, which forms a colored complex with Fe(III), and EDTA, NTA, and citrate, which bind both oxidation states of iron. Several investigators have used chelators such as these to determine the rates of iron mobilization from various forms of asbestos and other minerals and to determine the factors which affect mobilization.

1. Factors Influencing Mobilization

Aust and co-workers[46,52,97] have studied mobilization of iron from crocidolite, amosite, chrysotile, and erionite *in vitro*. These studies have revealed that mobilization requires the presence of a chelator at physiological pH.[52] This suggests that leaching of iron from asbestos, which has been reported *in vivo*[107–111] must be the result of chelation. They have also found that the rate of mobilization depends upon the chelator being used.[52] Table 2 lists some of the chelators which have been used for mobilization studies and some of the experiments examining the catalytic activities of crocidolite fibers. In reviewing this table, it is clear that the stability constants of the chelators do not correlate well with the rate of mobilization of iron from the fibers. Other factors influencing mobilization may be the geometry and size of the chelator and the complimentarity of its coordination of iron with that of the fiber.

When using these chelators to study the effects of mobilization on reactivity of iron, one must also take into account the effect of the chelator on the reactivity of iron. This is best illustrated by examining the reactivity of iron chelates in solution without the added complication of mobilization. The ability of iron chelates to produce reactive oxygen species, detected as •OH formation or 8-OHdG, does not correlate with the stability constants of the complexes (Table 2). Thus, in examining the ability of crocidolite to induce DNA SSBs in the presence of various chelators, the results reflect differences between the chelators in their abilities to mobilize iron and to catalyze the formation of oxygen radical species. With desferrioxamine B, the coordination complex formed with Fe(III) made the reduction of iron very difficult,[55] and the desferrioxamine B complex was unable to catalyze the formation of DNA SSBs, although iron was mobilized from the fibers. In the

case of EDTA or NTA, iron was mobilized at approximately the same rate from crocidolite, yet NTA had the greater ability to catalyze the formation of DNA SSBs (Table 2). This correlated with the greater ability of NTA chelates to facilitate the formation of reactive oxygen species, followed by EDTA and citrate. These experiments stress that the reactivity of iron mobilized *in vivo* from asbestos fibers will depend upon the chelator involved. They also suggest that mobilized iron, rather than surface complexed iron was responsible for the DNA SSBs observed. Experiments have been done to address this issue in more detail and will be discussed in the following sections.

When comparing mobilization of iron from different mineral fibers, factors which may be important are the crystalline structure, the surface area, and the iron content of the fibers. Crocidolite and amosite have very similar iron contents. When mobilization rates with either ferrozine or citrate were compared, iron was mobilized more rapidly from crocidolite than amosite on a weight basis.[52] However, when corrections were made for the difference in surface areas, the rates were approximately the same for these two amphiboles.[52] Very little iron was mobilized from long or short fiber chrysotile[52] and no iron could be mobilized from erionite.[112] For the iron-containing fibers, the amount of iron mobilized was consistent with the observed carcinogenicities of the various fibers and was representative of the iron content.

The most carcinogenic forms of asbestos can reside in the lungs for decades. Therefore, it may be possible that iron would be removed from these fibers over long periods of time. To determine the effect of long-term removal of iron from crocidolite or amosite, fibers were incubated in aqueous solutions of desferrioxamine B for varying times up to 15 days.[113] The rate of iron mobilization decreased with time, and after 15 days, totals of 215 or 70 nmol of Fe/mg fiber were removed from crocidolite or amosite, respectively. As more iron was removed from the crocidolite or amosite fibers by desferrioxamine B during this time, less iron was available for subsequent mobilization by citrate or EDTA, and fewer DNA SSBs were observed.[113] This is similar to the observation of Ghio *et al.*,[25] discussed in an earlier section, that short-term desferrioxamine B treatment of crocidolite resulted in a decrease in oxygen radical formation. Longer term incubations (90 days) showed that iron could still be mobilized from both forms of asbestos by desferrioxamine B, even after that prolonged period of time.[113] If iron is responsible for the cytotoxicity of fibers, these results would predict that desferrioxamine B treatment would reduce, but not eliminate the toxicity of these fibers to cultured cells. This is consistent with what has been reported for several different cell types.[30,114-120]

The effect of iron mobilization on the structure of asbestos has been studied by Mollo *et al.*[93] Figure 9 shows high-resolution transmission electron micrographs of crocidolite and amosite fibers, which have been incubated either in water or in desferrioxamine B to remove iron. The outer layer of the desferrioxamine B-treated fibers had become amorphous, probably due to removal of cations. However, the overall silicate structure appeared to be intact. This suggests that as iron is mobilized, cation binding sites are vacated, which may allow subsequent occupation by cations from solution.

Other factors which have been shown to influence mobilization of iron from asbestos *in vitro* are temperature, pH, and time in aqueous suspension. Mossop[121] has shown that the initial rate of iron mobilization from crocidolite by EDTA was diminished 30% after 30 min incubation in aqueous suspension at room temperature or by 85% after 30 min of autoclaving at 125 °C in aqueous suspension. Autoclaving the dry fibers had no significant effect on the rate of iron mobilization from the fibers. This could have important implications, especially for *in vitro* biochemical studies, where suspensions of fibers are allowed to sit for some time before use, or studies in cultured cells, where autoclaved fibers are often used.

The pH of incubation also appears to be important in iron mobilization from fibers.[52,113] The rate of iron mobilization from crocidolite or amosite by desferrioxamine B was consistently greater at pH 5 than at pH 7.5.[113] The investigators concluded that this may be relevant to physiological conditions, since fibers are often observed in phagosomes of cells where the pH can be 5.[113]

The oxidation state of the iron on the surface of fibers might also be a determining factor in the specificity or rate at which iron is mobilized. This did not appear to be the case for crocidolite when incubated with the chelators citrate, ADP or EDTA in the presence of the reductant ascorbate. No change in the rate of total iron mobilization was observed.[53] EDTA, NTA, and citrate appeared to be capable of mobilizing both oxidation states of iron from crocidolite to catalyze damage to DNA in the absence of a reductant or H_2O_2.[46] In fact, 42% or 19% of the iron mobilized by NTA or citrate, respectively, under anaerobic conditions was in the Fe(II) state.[46] Since ferrozine and radical scavengers, mannitol, salicylate, or DMPO, inhibited DNA SSB formation under these conditions, it was concluded that the Fe(II) that was being mobilized by the chelators was reacting with O_2 to produce $^{\bullet}OH$ or a similarly reactive species to damage DNA.

In conducting iron mobilization studies, the importance of not using buffers[52] and not exposing the solutions to cool-white fluorescent light[71] has been emphasized. Buffers, such as Tris and phosphate, are known to bind iron[122] and were found to inhibit mobilization of iron by ferrozine.[52] These investigators used 50 mM NaCl solutions where the pH had been carefully adjusted to 7.5 for their experiments. In addition, fluorescent light was found to potentiate the reduction of iron by the chelators citrate and NTA.[71] This can lead to overestimation of the amount of Fe(II) being mobilized and can also greatly affect other assays, such as the formation of DNA SSBs or $^{\bullet}OH$ spin trapping with DMPO. In these photocatalyzed reactions, NTA and citrate were ultimately degraded and could no longer chelate the iron. This could have an impact on longer term experiments.

A strong synergism has been found to exist between cigarette smoking and asbestos exposure in the

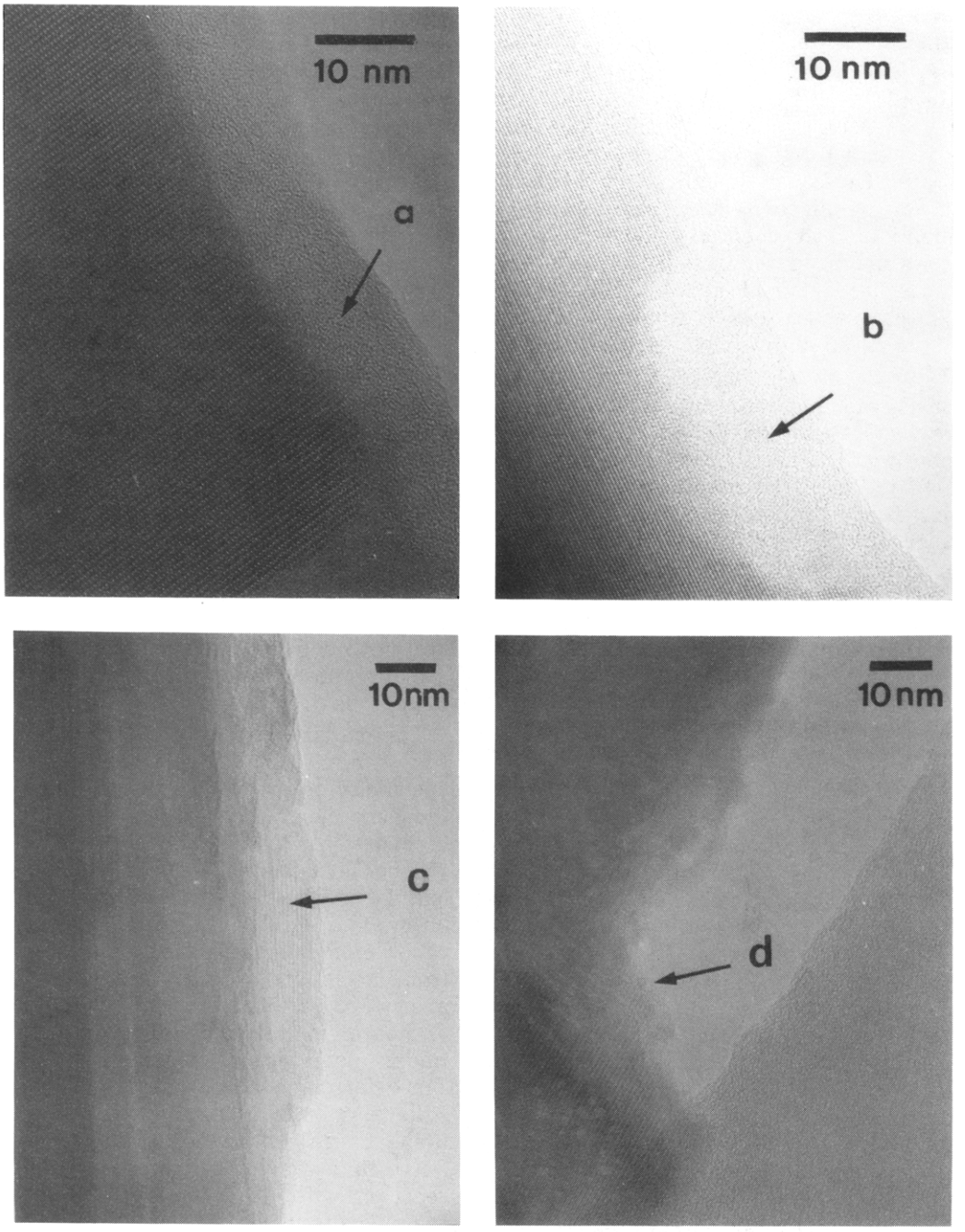

Figure 9. Crocidolite after incubation in water (a) and desferrioxamine B (b) and amosite after incubation in water (c) and desferrioxamine B (d). (Reprinted from ref 93. Copyright 1994 Springer Verlag, Heidelberg.)

development of bronchial carcinoma.[123,124] In investigating a chemical mechanism for this synergism, Qian and Eaton have observed that organic acids found in cigarette smoke were able to chelate and mobilize iron from asbestos.[125,126] They identified the organic acids responsible for the mobilization as stearic and palmitic acid. Iron associated with these acids could be translocated into intact red blood cells and remained associated with the cells even after washing with desferrioxamine B.[125] These observations may explain much of the synergism of the two known carcinogens. The effect of smoking on asbestos carcinogenicity has been extensively reviewed.[123]

2. Oxygen Consumption and Hydroxyl Radical Formation

A way of comparing the reactivity of surface iron on fibers with iron mobilized into solution is to measure the amount of O_2 consumed under a variety of conditions, using an O_2-sensitive electrode. To determine whether mobilization of iron from crocidolite enhanced its reactivity, crocidolite fibers were incubated with the chelators citrate, NTA or EDTA for varying periods of time to allow iron mobilization.[97,127] The ability of the fiber suspension to catalyze O_2 consumption in the presence of ascorbate[97] or cysteine[127] was compared with that of the solution from which the fibers had been removed. In every case the fiber-free solution, containing the mobilized iron, had the same activity as the fiber-containing solution. Aust and Lund[127] have observed a strong linear correlation between the amount of iron mobilized by EDTA from crocidolite and the amount of $^{\bullet}OH$ radicals produced in the presence of cysteine and H_2O_2, as detected by EPR using the spin

trap DMPO. It should be noted that no O_2 consumption or •OH formation was observed in the absence of chelators. Taken together, these results suggest that iron mobilized from crocidolite by citrate, NTA, or EDTA was ultimately responsible for reactions with O_2 that led to •OH formation.

Gulumian and van Wyk[128] have made similar observations, reporting that the ability of several types of asbestos fibers to consume O_2 in the presence of H_2O_2 and a spin trap and generate reduced oxygen species on the surface of the fiber decreased across the series of fiber samples: crocidolite > amosite > chrysotile. This series also is representative of the amount of iron contained in the various fibers (see Table 1) and the intensities of the corresponding EPR spectra of the fibers in the presence of H_2O_2 and the spin trap DMPO.[128] The investigators did not specify whether iron was mobilized during the 24 h incubation time used.

3. DNA Strand Breaks

Mobilization of iron has also been shown to have a strong correlation with the amount of crocidolite-dependent DNA SSBs observed. Lund and Aust[46] investigated the ability of crocidolite to catalyze the formation of SSBs in ϕX174 RFI DNA in the presence of various chelators and reductants. By conducting experiments in a similar manner to those described for O_2 consumption, a correlation was found between the amount of iron mobilized and the ability of crocidolite to cause the formation of DNA SSBs, suggesting that the formation of SSBs was strictly due to the mobilized rather than surface iron. They also observed an increased ability to form SSBs in the presence of a reducing agent which promoted redox cycling of the catalytically active iron.[46] Table 3 shows the percent of DNA with SSBs formed after incubation of ϕX174 RFI DNA with various mineral fibers in the presence of ascorbate with or without the chelators citrate or EDTA. Crocidolite and amosite were more active in catalyzing the formation of SSBs than either chrysotile or erionite. When a chelator was added to amosite, the result was similar to that for crocidolite, and an enhancement in the amount of SSBs were observed when a chelator facilitated mobilization. Because erionite has no native iron associated with the fibers, the addition of a chelator did not enhance the ability of the fibers to damage DNA. In a subsequent section the effect of binding of iron to fibers on the abilities of fibers to generate DNA SSBs will be discussed.

DNA SSBs have also been observed in cells after treatment with crocidolite.[105,129] Libbus *et al.*[105] utilized the method of nick translation to assess the amount of DNA SSBs induced by crocidolite. They observed a dose-dependent response which reached a maximum 24 h after treatment. They also observed the same amount of SSBs in cells where no fibers were visible. The investigators proposed that oxygen radicals were responsible for this. However, this would only be possible if a transition metal, such as iron, were also available. These observations would be consistent with iron mobilization from the crocidolite. Interestingly, in these experiments, riebeckite, which is a nonfibrous, noncarcinogenic form of crocidolite, was about one-third as capable of inducing DNA SSBs as crocidolite.

Table 3. Percent DNA with Single-Strand Breaks Introduced by Various Mineral Fibers[a]

fiber	none	citrate	EDTA	ref
amosite	26 ± 8	52[b]	96[b]	46
chrysotile	7 ± 2			46
crocidolite	19 ± 4	29 ± 4	87 ± 3	46
Fe(II)-loaded crocidolite[c]	16 ± 5	40 ± 8	104[d]	157
DF crocidolite	8 ± 4	12 ± 7	21 ± 3	157
Fe(II)-loaded DF crocidolite[e]	20 ± 3	27 ± 7	58 ± 1	157
erionite	ND[f]	ND	ND	152
Fe(II)-loaded erionite[c]	28 ± 4	114[d]	72[d]	152
Fe(III)-loaded erionite[c]	16 ± 3	56 ± 5	96[d]	152

[a] The percentage of closed-circular superhelical DNA with single-strand breaks introduced in the presence of ascorbate with or without citrate or EDTA in 30 min. [b] Aust, unpublished data. [c] Loaded with 24 nmol of Fe/mg of fiber. [d] Corrected to reflect a 30-min incubation with DNA. Actual experiments were for 5 (erionite) or 15 (crocidolite) min. [e] Loaded with 5.5 nmol of Fe/mg of fiber. [f] ND, not detectable.

Turver and Brown[129] used identification of S_1 endonuclease sensitive sites as a method of detection of DNA damage induced by crocidolite. They also observed a dose-dependent increase in the amount of DNA damage caused by crocidolite. This activity was significantly attenuated by the addition of desferrioxamine B to the cells,[129] suggesting that induction of DNA damage may have been due to iron from the fiber. The authors did not address the question of whether iron mobilization was important in these observations. However, results to be discussed on cellular mobilization of iron from crocidolite may have some bearing on the results discussed here.

4. DNA Oxidation

Mobilization of iron from crocidolite, amosite and chrysotile has been shown to increase asbestos-dependent formation of the oxidized nucleoside 8-OHdG.[130] The formation of this oxidized base product has been shown to cause AT to GC base pair transition[74] and has been observed under conditions producing oxidative stress that lead to cancer, as previously discussed.[131] Adichi *et al.*[130] have demonstrated that the addition of H_2O_2 to a mixture of calf thymus DNA and asbestos consistently increased the level of 8-OHdG formed. The addition of the chelator EDTA to the reaction mixture including H_2O_2 increased the levels of 8-OHdG formed by 3.2-fold for Rhodesian chrysotile, 3.8-fold for Canadian chrysotile, 2.3-fold for crocidolite, and 2.7-fold for amosite, over the levels observed without EDTA. The levels of 8-OHdG were 2-fold higher for the amphibole minerals than for either variety of chrysotile in the presence of EDTA,[130] which is consistent with a greater rate of iron mobilization from crocidolite or amosite than from chrysotile. In the presence of phosphate buffer, which is known to chelate iron, chrysotile has also been shown to generate low levels of 8-OHdG from deoxyguanosine.[132]

An increase in the amount of intracellular 8-OHdG has also been reported after crocidolite treatment of cultured cells. Takeuchi *et al.*[133] observed an increase in the levels of 8-OHdG in DNA isolated from human promyelocytic leukemia cells (HL60) which were treated with crocidolite. The investigators demon-

strated that the 8-OHdG observed was not due to the DNA isolation procedure, but was due to the 24-h crocidolite treatment. The amount of 8-OHdG increased with the time of incubation. Extracellular treatment with SOD and/or catalase did not inhibit 8-OHdG formation,[133] suggesting that the formation was due to intracellular generation of a reactive oxygen species, such as the •OH radical. Also in these studies the investigators did not determine whether iron mobilization was involved. Once again, intracellular iron mobilization from crocidolite may have some bearing on these observations.

5. Intracellular Iron Mobilization

Iron and other ions, specifically magnesium, have been observed to be leached from chrysotile asbestos *in vivo*.[107-111] The amount of iron leached *in vivo* was small and not quantified. Iron has also been reported to be mobilized from asbestos fibers by cells in culture. Chao *et al.*[134] observed that when human lung cells (A549) were treated with neutron-activated crocidolite containing ^{55}Fe, iron was mobilized intracellularly and was found associated with proteins and chelators of molecular weight <10 kD. There was a linear relationship between the amount of iron in the <10 kD, or low-molecular-weight pool, and the cytotoxicity, suggesting that the iron in this fraction was indeed damaging and may have been responsible for the toxic effect of crocidolite. The rate of iron mobilization in A549 cells was comparable to the rate of iron mobilization from crocidolite by citrate *in vitro* at pH 7.5.[134] This finding suggests that iron can be removed from the fibers by intracellular chelators and that this intracellular chelator(s) may be similar in size and mobilizing ability to citrate.

The redox activity of iron in a low-molecular-weight pool in biological systems has been the subject of intense speculation, since iron has been observed chelated to low-molecular-weight chelators only in disease states, such as hemochromatosis. Citrate-chelated iron is known to cause damage to biomolecules[47,52] and has been observed in the blood of hemochromatosis patients.[40] The observation that iron is mobilized from crocidolite into a low-molecular-weight pool may prove to be very important in understanding how iron from mineral fibers becomes involved in the development of disease states. The reactivity of the iron mobilized from crocidolite may be similar to iron in other pathological conditions, since it was directly related to the cytotoxicity of the fibers.

C. Iron Binding

Mineral fibers not only can liberate iron, but also can acquire iron, under certain conditions. Because of the number and damaging potential of the reactions catalyzed by iron, additional reactive iron on fibers is likely to potentiate the dangerous nature of the fiber.

1. Ferruginous Bodies

Respirable, durable fibers are known to acquire iron on their surfaces during residence in the lung. These coated fibers are known as ferruginous bodies,[135] or asbestos bodies if the core is asbestos.[138] Crocidolite, amosite, chrysotile, and erionite are all known to form ferruginous bodies after long-term residence *in vivo*. Ferruginous bodies were first observed in 1906.[137] Not all inhaled mineral fibers form ferruginous bodies. However, the chemical properties of fibers that determine whether they become iron-coated are not known.[138]

Chronic inflammation, which occurs after inhalation of mineral fibers, is frequently accompanied by systemic changes in iron metabolism. There is a depression of serum transferrin, while the intracellular iron level in inflammatory macrophages increases. A possible explanation for this is that inflammatory macrophages appear to have a greater rate of iron uptake[139] and slower release[140] than resident macrophages. Macrophages are the primary scavengers of effete erythrocytes. This pathway has been estimated by some investigators to represent 80% of iron turnover.[141] Macrophages have been proposed to be the source of the iron in ferruginous body formation, and there may be good reason to suspect an involvement of macrophages in deposition of iron on phagocytized fibers which have a high affinity for iron.

There are two observations which indicate that macrophages accumulate iron during the inflammatory response following exposure to mineral fibers. First, the iron content in the lungs of workers chronically exposed to mineral dusts greatly increases after the influx of macrophages.[142] Second, macrophages, taken from the peritoneum of mice injected with crocidolite, accumulate iron.[143] Although the optimal time for the formation of ferruginous bodies in humans is not known, it is known that individuals vary in their ability to coat fibers. This may be due to variation in the total body burden of iron.

The formation of the asbestos bodies may begin by the deposition of monolayers of iron in high affinity sites followed by nucleation and formation of a three-dimensional aggregate or precipitate of iron on the surface. The intracellular source of iron that has been proposed is ferritin or hemosiderin, which is oxidized ferritin from lysosomes.[138] Suzuki and Churg[144] have suggested that the ferruginous body formations are composed of iron, protein and probably other material. It has also been demonstrated that a layer of mucopolysaccharide appears to be associated with ferruginous bodies.[145] Figure 10 shows a scanning electron micrograph of macrophages and neutrophils in the early stages of engulfing an amosite fiber. Mature ferruginous bodies form over decades of exposure in the lung.

It has been reported that the iron on ferruginous bodies is crystalline in nature. However, the mechanism by which iron binds to ferruginous bodies or the exact source of the iron is not well understood. Several studies have demonstrated adsorption of iron onto silicates[146,147] and binding of iron onto silanol groups on the surface of particles.[148,149] The size of ferruginous bodies varies based upon the size of the original fiber population. Koerten *et al.*[150] have observed that when crocidolite was added to cultures of macrophages, only fibers which were too large to

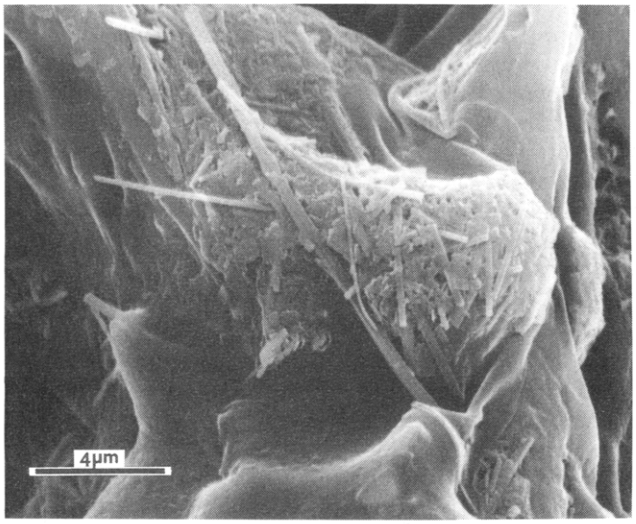

Figure 10. Scanning electron micrograph of early stages of amosite asbestos phagocytosis by guinea pig alveolar macrophages and neutrophils. Photo courtesy of M. G. Williams and R. F. Dodson (Department of Cell Biology and Environmental Sciences, University of Texas Health Center at Tyler, TX).

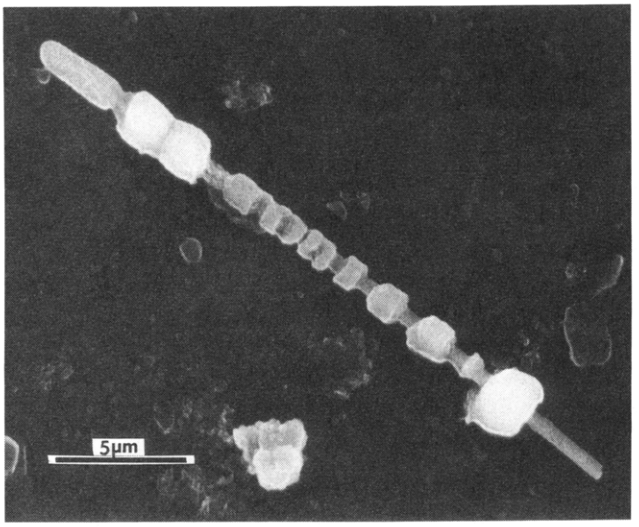

Figure 11. Scanning electron micrograph of a mature amosite asbestos body isolated from the lungs of a deceased shipyard worker. Photo courtesy of M. G. Williams and R. F. Dodson (Department of Cell Biology and Environmental Sciences, University of Texas Health Center at Tyler, TX).

be completely phagocytized were coated to form asbestos bodies. This may be the result of macrophage death after attempting to phagocytize fibers that are too large. Thus, formation of asbestos bodies on fibers less than 25 μm in length was not observed. After 4 weeks in culture, the large fibers were beginning to be coated, but the thickness and the segmentation of the added material became more prominent after longer times of exposure.[150] A scanning electron micrograph of an amosite core ferruginous body, which was removed from a human lung at autopsy, showed that the iron coating was not homogeneous but formed thicker plaques at the ends and at intervals along the length of the fiber. The iron coat was approximately 0.9−1.7 μm thick. A scanning electron micrograph of a mature asbestos body with an amosite core, isolated from the lung of a deceased shipyard worker, is shown in Figure 11. Note the segmented nature of the coating. This is the same type of asbestos body used by Lund *et al.*[151] in the following study to determine the reactivity of the iron on asbestos bodies.

The iron on the surface of asbestos bodies with amosite cores has been shown to be catalytically active and capable of causing the formation of SSBs in ϕX174 RFI DNA.[151] This reactivity appeared to be due to iron on the ferruginous body structure, since the ability of the fibers to form SSBs was enhanced by the addition of EDTA (SSBs in 77% of DNA) or citrate (SSBs in 21% of DNA) in the presence of a reductant and inhibited by the addition of desferrioxamine B to the reaction mixture.[151] An equal number of native amosite fibers of similar length were unable to catalyze formation of detectable amounts of DNA SSBs under the same conditions because of the low number of fibers used. Previous to these studies, it was generally accepted that the coating of fibers to form ferruginous bodies was a protective mechanism. However, because the deposited iron appears to be redox active, it may actually contribute to the catalytic potential of the fibers.[151]

2. Erionite

Several *in vitro* and *in vivo* studies have been undertaken to determine the role of iron acquisition on reactivity of mineral fibers. Eborn and Aust[152] found that native erionite was incapable of catalyzing damage to DNA *in vitro*, even in the presence of a reductant and an iron chelator, suggesting that the fiber is unable to generate reduced oxygen species. This chemical inactivity is consistent with the previous report of Zalma *et al.*[101] who demonstrated that no $^{\bullet}$OH−DMPO adducts were observed in the presence of erionite.

When erionite was incubated in solutions of 25−500 μM ferrous of ferric ions, the erionite fibers were able to remove iron from solution.[152] Erionite was capable of binding 176 nmol of Fe(II)/mg, or 239 nmol of Fe(III)/mg from 500 μM ferrous or ferric chloride solutions. Ferrous ions appeared to bind through a process of ion exchange while ferric ions may bind through a precipitation or crystallization process,[153,154] which is consistent with what is understood about the activity of ferric ions in solution.[155] After ferrous or ferric binding, the erionite fibers acquired the ability to catalyze the formation of DNA SSBs *in vitro* in the presence of a reductant and/or chelator. The amount of SSBs was directly proportional to the amount of iron mobilized when a chelator was present.[152] Erionite, with only 24 nmol of Fe(III)/mg erionite was able to catalyze the formation of DNA SSBs in nearly 100% of the DNA in the presence of EDTA and ascorbate during a 30-min incubation. This is indeed striking since crocidolite, which contains approximately 4.8 μmol of iron/mg (200 times that on erionite) was capable of catalyzing the formation of DNA SSBs in only 87% of the DNA under the same conditions[46] (Table 3).

Other investigators have also observed increases in the ability of a synthetic Y zeolite[94] or erionite[156] to catalyze the formation of $^{\bullet}$OH after loading with Fe(II). Because of the enormous surface area of erionite, it is possible that under iron-binding condi-

tions, large amounts of iron could be added to the fiber. The apparent discrepancy between the biochemical inactivity of erionite *in vitro* and its potent carcinogenic activity *in vivo* may be explained by acquisition of iron in the lung after inhalation.[152]

3. Crocidolite, Amosite, and Chrysotile

The ability of crocidolite to bind iron from solution has also been examined. Hardy and Aust[157] reported that crocidolite bound 57 nmol of Fe(II)/mg from $FeCl_2$ solutions at pH 7.0, which increased the iron available for mobilization by either EDTA or citrate. DF crocidolite had a diminished, but detectable, ability to bind ferrous ion from solution. Binding of ferrous ion enhanced the ability of these fibers to form DNA SSBs[157] (Table 3). Treatment of iron-containing mineral fibers with chelators, such as desferrioxamine B, has been proposed as a cure for the pathological effects of the fibers. On the basis of these findings, it appears that this treatment is not adequate to reverse the catalytic capabilities of the fibers if they are later exposed to a source of chelatable iron.

Crocidolite and DF crocidolite were also examined for their ability to form SSBs after incubation in iron-free or iron-containing tissue culture medium.[117] Both types of fibers were more capable of inducing DNA SSBs after preincubation in an iron-containing medium than after preincubation in an identical, but iron-free medium. Fibers were also more toxic to human lung A549 cells cultured in iron-containing medium than to cells cultured in the same, but iron-free medium. A correlation between the crocidolite-dependent cytotoxicity in cells cultured in various media and the amount of DNA SSBs *in vitro* produced by crocidolite preincubated in the corresponding medium was reported.[117] The investigators concluded that because crocidolite was capable of acquiring iron from a complex solution with many chelators present, it is plausible that fibers may bind iron from similar low-molecular-weight chelates intracellularly. It also appears that iron bound in this manner has a biological effect, since fibers were more toxic in A549 cells after exposure to iron from the medium.

Ghio *et al.*[158] have also been active in investigating the role of iron acquisition on the reactivity of mineral fibers. They observed that crocidolite, as well as three other silicates, silica, kaolinite, and talc, were able to bind all of Fe(III) from solutions ranging in concentrations from 1 μM to 1 mM.[158] The fibers apparently bound all of the iron from the solutions, 1 to 1000 nmol of Fe(III)/mg of fiber. Following binding of iron, an increase in the formation of thiobarbituric acid reactive products was observed over control fibers exposed to solutions without iron. Crocidolite showed the greatest increase in reactivity of all the fibers examined, and desferrioxamine B treatment after iron binding consistently decreased the reactivity of the fibers. Iron-treated fibers were also more active in stimulating release of leukotriene B_4, an indicator of the inflammatory response, in rat alveolar macrophages than wetted or desferrioxamine B-treated fibers. Ghio *et al.*[158] also injected crocidolite, silica, kaolinite, or talc into the pleural cavities of rats. The fibers that were recovered 4 days later had increased amounts of chelatable iron on their surfaces. The investigators concluded from all of this work that iron was bound to fibers from both inorganic and biological sources and proposed that the iron was bound to silanol groups on the surface. The addition of iron appeared to be responsible for increased abilities to generate oxidants and was proposed to be responsible for the induction of biological activities.

In a later study, Ghio *et al.*[159] compared crocidolite, amosite, and chrysotile for their ability to bind iron from solution. By using the same iron treatment techniques with 1 mM solutions as were discussed above, crocidolite was reported to bind approximately 300 nmol of Fe(III)/mg, amosite 280 nmol of Fe(III)/mg, and chrysotile 175 nmol of Fe(III)/mg.[159] This conflicts with the previous report by these investigators that incubation of crocidolite in 1 mM solutions resulted in 1000 nmol of Fe(III)/mg.[158] This discrepancy was not discussed, but may reflect the difficulties that can be encountered in handling $FeCl_3$ solutions or may be due to the manner in which binding was being assessed. In any case, the amount of DNA SSBs increased after iron binding by approximately 19% for crocidolite, 13% for amosite and 4% for chrysotile. A similar increase in the generation of oxidants, as measured by the thiobarbituric acid reactive products assay, was observed. After intrapleural injection of fiber suspensions, 240, 135, or 25 nmol of Fe/mg were observed on crocidolite, amosite, or chrysotile, respectively.[159] It is likely that additional iron from intracellular sources would demonstrate the same types of effects on induction of DNA SSBs as the iron from inorganic sources, but this was not investigated. As before, the investigators proposed that the silanol groups on the surface of a mineral fiber may be responsible for binding cations from solution. The investigators proposed that one potential method for determining whether the fibers made to replace asbestos will be dangerous may be to analyze for the concentration of silanol groups on the surface.[159] Although this may have some merit in identifying potentially hazardous materials, it is by no means definitive, since erionite, the most carcinogenic mineral fiber, has approximately 1000-fold less silanol groups, but binds as much or more iron as crocidolite (see Tables 1 and 3).

VI. Fiber Inactivation

The unique blend of physical properties possessed by asbestos fibers, coupled with the high demand for such materials in a variety of applications, has stimulated several investigators from around the world to modify fibers in hopes of rendering them biologically inactive.

A. Ferric Oxide Coating

Gulumian *et al.*[160] and Hearne *et al.*[161] have been active in studying modifications of crocidolite which may render the fibers less biologically active. These investigators used a method of coating asbestos fibers with ferric oxide (Fe_2O_3) which was developed by

Flowers.[162] The effects of this treatment on the bulk and surface of crocidolite fibers were studied in detail.[161] Mössbauer studies revealed that ferrous ions were oxidized to ferric ions in specific sites in the mineral fiber and that no changes in the structure occurred during the treatment. Furthermore, the surface concentration of ferric ions increased. The investigators interpreted the chemical formula of the surface complex to be $Fe(H_2O)_6$. This treatment rendered the fibers less capable of releasing ferrous ions to ferrozine and less capable of catalyzing the formation of •OH in the presence of H_2O_2, detected by EPR spin trapping with DMPO.[160] Since ferrous ions were still available for mobilization and because the detoxified fibers remained capable of generating reduced oxygen species, it appears that this type of bulk and surface modification was not sufficient to completely inactivate the fibers, rendering them safe for human exposure.

In an interesting reverse study, the activation of crocidolite fibers was conducted using H_2 gas as a reductant for crocidolite iron.[163] This treatment increased the amount of Fe(II) by reduction of Fe(III). The activated fibers released more Fe(II) to ferrozine chelation and were able to generate more •OH[163] than unactivated fibers. The converted Fe(II) was stable for at least 3 months. While the reducing conditions used to activate these fibers were much different than those that would be encountered in the cell, these studies still point out that iron on fibers might be reactivated by conversion from ferric to ferrous in the presence of cellular reductants. This raises serious questions about the use of iron to prevent the pathological effects of fibers.

B. Polymer Coating

Brown *et al.*[164] were successful in binding C_8 or C_{18} polymers to the surface of amosite by refluxing the fibers in octyldimethylchlorosilane (C_8) or octadecyldimethylchlorosilane (C_{18}) in toluene for 6 h. The modified fibers were less able to associate with cultured V79 cells and were less toxic to these cells than control fibers. The investigators proposed that fibers modified in this way might produce less damage *in vivo*. However, when the same fibers were intrapleurally injected into rats, the native, C_8-modified, and C_{18}-modified fibers were all carcinogenic.[164] Although the C_{18}-modified fibers were less carcinogenic than the native fibers, the investigators concluded that this method of inactivation was not viable because it did not render the fibers completely safe for human exposure.

C. Chelation Treatment

Chao and Aust[113] explored the potential of long-term mobilization as a method of inactivating fibers. After removal of only 4.5% or 1.2% of the total iron from crocidolite or amosite, respectively, the fibers were no longer capable of causing DNA SSBs in the presence of ascorbate and EDTA in a 30 minute incubation. This finding suggests that only a small percentage of the iron on crocidolite and amosite is available for chelation and mobilization away from the fiber by one specific chelator in a 15-day treatment. Nevertheless, subsequent treatment of the same fibers with the same or another chelator resulted in further mobilization of iron from the fibers.[113] The investigators concluded that although the removal of iron decreased the activity of the fibers, it would not completely inhibit the activity in a biological system with longer exposure times. Perhaps an even greater hazard would come if the fibers from which iron was removed subsequently bound iron, as was described previously.[157] This would reconstitute their damaging capabilities.

In summary, attempts to modify asbestos to render the fibers safe for use and human exposure have been rather unsuccessful. Several of the modifications have lowered the ability of the modified fibers to catalyze reactions *in vitro*, specifically generation of •OH, formation of DNA SSBs, or association with cells. None of the modifications attempted, to date, have been capable of completely inhibiting catalytic reactivity. The findings of Brown *et al.*[164] suggest that even when a demonstrable decrease in an *in vitro* parameter is observed after modification of fibers, a concomitant decrease in carcinogenicity may not be observed. Due to the durability of asbestos fibers in the lung, it is likely that inactivation of fibers by modification might change their properties rendering them less useful.

VII. Physiological Effects

It is difficult to know which of the many carcinogenicity tests *in vitro* and in animals are the most important in predicting human carcinogenicity. Johnson[165] suggested that animal inhalation models are relevant for identifying hazardous fibrous materials in the absence of epidemiological data, since use of intrapleural or intraperitoneal installations can give false positive results. However, when two parameters correlate well, it suggests that perhaps what is observed in one is reflected in the other. Maples and Johnson[166] demonstrated a correlation between the tumor incidence after intrapleural administration of erionite, crocidolite, amosite, or chrysotile in rats with the ability of these fibers to generate •OH *in vitro* in the presence of H_2O_2 ($r^2 = 0.896$). There also appeared to be a strong correlation between the mortality rate for mesothelioma in humans and the ability of these fibers to produce •OH ($r^2 = 0.990$). No correlation was observed between the ability of fibers to cause tumors after intraperitoneal administration in rats and their ability to generate •OH.[166] This is in agreement with previous reports by Carthew *et al.*[18] who observed a similar correlation between the carcinogenicities of mineral fibers in humans and the relative carcinogenicities of mineral fibers administered intrapleurally in rats, but not with mineral fibers administered intraperitoneally in rats. They found that, as in human exposure, intrapleural administration of erionite was much more carcinogenic than crocidolite, which was more carcinogenic than chrysotile.[18]

The abilities of crocidolite, amosite, and chrysotile to generate •OH as in the Maples and Johnson study[166] seems to generally correlate with both the iron content and the amount of iron mobilized from these fibers, as demonstrated by other investiga-

tors.[46] However, the generation of •OH by erionite observed by Maples and Johnson[166] is not easily explained, since erionite contains little or no iron. Other investigators have consistently found erionite unable to generate •OH,[152] even after grinding.[156] One possible explanation for the observed generation of •OH by erionite may be that a catalytic amount of iron was introduced to the fibers as a contaminant from solutions used in these studies. Even a small amount of iron bound to zeolites is very reactive, as previously discussed.[94,152,154] This study, combined with information from other studies,[52] suggests that both the amount of iron on the fiber, and the amount of iron which can be mobilized from the fiber may be related to the rate of human mortality from mesothelioma through the iron-catalyzed generation of reduced oxygen species, specifically the •OH.

A. Participation of Iron

The role of iron has been extensively studied using desferrioxamine B in a number of different physiological reactions. When fibers were pretreated with desferrioxamine B, the fibers were less able to form malonaldehyde-like products[118] or to cause lipid peroxidation *in vitro*.[167,168] The cytotoxicity of desferrioxamine B-pretreated crocidolite was lower than untreated crocidolite in human lung cells,[114,117] macrophages,[30,115,116,119] red blood cells,[118] fibroblasts,[119] and endothelial cells.[120] Kamp *et al.*[169] treated human pulmonary epithelial cells with amosite asbestos in the presence of polymorphonuclear leukocytes and saw a decrease in cytotoxicity with the addition of desferrioxamine B. These studies utilizing desferrioxamine B seem to indicate that a number of varied reactions associated with exposure of cultured cells to asbestos fibers are catalyzed by iron.

Active oxygen species generated by iron on amosite have been reported to be involved in the uptake of the fibers by rat tracheal epithelial cells.[170] Evidence for this hypothesis is that increasing concentrations of desferrioxamine B were progressively more capable of preventing fiber uptake at concentrations of 10 μM to 1 mM. The antioxidant scavenger enzymes superoxide dismutase and catalase were also increasingly effective at inhibiting fiber penetration as the concentration increased from 325 to 1300 U/mL catalase or 150−1200 U/mL superoxide dismutase in 1 or 3 days. The inactive enzymes were not capable of inhibiting fiber uptake in the same manner as the active enzyme, suggesting that the normal activity of the enzymes was required.[170] The authors did not conclude which kind of cellular damage was required for amosite uptake, but suggest that since catalase, superoxide dismutase, and desferrioxamine B were all able to independently inhibit fiber uptake, H_2O_2, $O_2^{\bullet-}$, and iron are all involved in the mechanism of uptake.

B. Antioxidant Proteins

Reactions catalyzed by iron are known to generate strong oxidants. When a cell experiences conditions where the levels of these species are elevated, the condition is commonly referred to as "oxidative stress". Many of the complex physiological reactions to oxidative stress have previously been reviewed[123] and will not be the focus here. A few of the most recent and important findings regarding asbestos-induced oxidative stress will be included because they suggest that oxygen radicals are being generated in the lungs of asbestos-exposed animals or in treated cells.

The synthesis of a variety of proteins, e.g. glutathione, glutathione peroxidase, catalase, and SOD, can be increased in response to oxidative stress conditions. Glutathione is thought to be an important intracellular antioxidant protein. Glutathione peroxidase eliminates organic peroxides and H_2O_2 while catalase catalyzes the decomposition of H_2O_2 to H_2O and O_2. Superoxide dismutase accelerates the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2.

Asbestos has been shown to induce synthesis of several proteins which play pivotal roles in the cellular defense against oxygen radicals.[171,172] Janssen *et al.*[172] observed that after inhalation of asbestos by rats, steady-state levels of mRNA for two forms of superoxide dismutase and glutathione peroxidase were elevated relative to untreated controls. The overall enzyme activities of catalase, glutathione peroxidase, and both forms of superoxide dismutase also increased in the exposed lung cells after asbestos exposure.[172] Holley *et al.*[171] have also shown that treatment with crocidolite increases the levels of mRNA for Mn superoxide dismutase. A dose-dependent increase in glutathione has been observed after administration of crocidolite to pulmonary alveolar macrophages *in vitro*.[173] The induction of antioxidant proteins suggests that the same types of cellular signals that are associated with oxidative stress, e.g. generation of reactive oxygen species, are associated with the reactions catalyzed by asbestos.

VIII. Mutations and Cancer

Carcinogenesis appears to be a multistep process that has been divided into two stages, initiation and promotion. The initiation phase is believed to be the introduction of a heritable genetic change (mutation) resulting from carcinogen-induced DNA damage. With the discovery of oncogenes, which are activated by mutations to cause cancer, and tumor suppressor genes, which are inactivated by mutations leading to cancer, some of the genetic targets for this damage have been identified. Initiation is followed by the promotion phase in which the initiated cell proliferates, undergoing further changes that result in the malignant phenotype. Studying the initiation phase of cancer, a variety of assays have been developed to assess the mutagenicity of carcinogens. Among these is the well-known Ames assay with bacteria. Surprisingly, asbestos is relatively inactive in almost all mutation assays. This may not be so difficult to understand if iron is responsible for the DNA damage induced by asbestos.

It appears from work with cultured cells that asbestos must be phagocytized in order to exert its toxic effects.[174] For mammalian cells, phagocytosis of high iron content asbestos fibers would represent an uncontrolled entry of iron into the cells. Since phagocytosis is not possible for bacteria, the fibers would never enter the cells where they might be able

to catalyze the formation of reactive oxygen species. Thus, no mutations would be observed when the bacteria were incubated with the fibers. But fibers are phagocytized by mammalian cells. Why then are the fibers not causing mutations? The answer appears to lie in the type of DNA damage induced by the fibers.

It is well-known that asbestos causes chromosome aberrations, both structural and numerical, in mammalian cells.[175] This chromosomal damage may be the basis for asbestos-induced transformation of Syrian hamster embryo cells.[176] While it has been proposed that chromosome aberrations are the result of direct fiber interaction with DNA or spindle proteins,[177] it may be that some, or perhaps all, of the damage is the result of iron-catalyzed oxygen radical attack on DNA, which has been shown to occur in cultured cells.[129] This is very consistent with what is observed for ionizing radiation which generates •OH. Interestingly, it is also somewhat difficult to observe the induction of mutations by ionizing radiation. Special bacterial strains were developed to be used in the Ames assay for the purpose of detecting oxygen radical damage.[178] Until recently, no such strains were available in mammalian cells. With the development of the A_L hamster–human hybrid cell line, Hei *et al.*[179] have been able to observe mutations by ionizing radiation and by asbestos at the a_1 locus of human chromosome 11 in the A_L cell line. Hei *et al.*[180] also observed that reactive oxygen species induce mostly deletion mutations in mammalian cells. This work suggests that asbestos damages DNA to cause deletion mutations via iron-catalyzed generation of oxygen radicals. The induction of deletion mutations can be very toxic to the affected cell, and this probably explains why asbestos-induced mutations have been rarely observed except in the A_L hybrid cell line.

Current understanding of the initiation and promotion events leading to cancer reveals that the expression of oncogenes and tumor suppressor genes is important in regulation of cellular function. The protooncogenes c-*fos* and c-*jun* encode a family of AP-1 transcription factors that form homodimeric and heterodimeric protein complexes. These AP-1 transcription factors bind to specific regulatory sequences in DNA to control the transition from G_1 to S phase in the cell cycle. In rat pleural mesothelial cells and hamster tracheal epithelial cells c-*fos* and c-*jun* have been shown to be persistently induced by treatment with asbestos.[181] The mRNA synthesis for these oncogenes was induced in a dose-dependent manner and persisted for at least 24 hours. Crocidolite was more active than chrysotile in inducing mRNA synthesis for these two genes in mesothelial cells. This emphasizes the relevance of this work, since crocidolite is known to induce mesothelioma more easily than chrysotile. In the tracheal epithelial cells, c-*jun* was induced by crocidolite or chrysotile, but c-*fos* was not. The protein transcription factor AP-1 was also persistently observed. Treatment of either cell line with polystyrene beads or riebeckite did not induce c-*fos* or c-*jun*, suggesting that the response was specific to fibrous asbestos minerals and was not stimulated by the presence of a solid body alone. Induction of these proteins could lead to chronic stimulation of cell proliferation.[77] Currently, this study provides some of the molecular understanding of the consequences of asbestos exposure that may cause cancer.

IX. Future Directions

Findings compiled in this review support the view that intrinsic or acquired iron is responsible for the biochemical properties of some of the most carcinogenic minerals. Many studies have shown a direct relationship between the biochemical reactivity of fibers and the iron content of those fibers. Iron acquisition was the key factor in converting erionite from a biochemically unreactive form to a highly reactive form. This may explain the apparent discrepancy between the *in vitro* inactivity of erionite and its highly carcinogenic activity *in vivo*. The binding and mobilization of iron appear to be key factors in the regulation of reactivity of fibers both *in vitro* and *in vivo*. Several of the physiological responses to asbestos have been decreased by the use of desferrioxamine B, suggesting that these intracellular responses to asbestos are mediated or generated by iron. Ultimately, the fundamental differences between durable mineral fibers in their abilities to bind and later release iron may explain why some are more toxic and carcinogenic than others.

Significant advances have been made in the field of mineral surface science due to the development of X-ray photoelectron spectroscopy, Auger electron spectroscopy combined with scanning electron microscopy, and scanning force microscopy. Thus, the surface requirements for iron binding and effects of mobilization will undoubtedly be investigated and compared among the carcinogenic fibers to determine what factors are necessary for reactivity of the fibers. Studies such as these will aid in the design of man-made mineral fiber replacements for asbestos that are safe for human exposure.

Many investigators have studied the ability of asbestos to catalyze damage through the iron-mediated generation of reactive oxygen species. This appears to be one of the most important chemical reactivities catalyzed by asbestos fibers in relation to the long-term effect of cancer. Work summarized here showed a strong correlation between the ability of fibers to generate reduced oxygen species and the mortality rate from mesothelioma in humans. Since these reactions require iron to be very near the target for damage, mobilization of iron from the fibers may be a key step, and studies were discussed which provide evidence that iron can be mobilized *in vitro* and in cultured cells.

Intermediate reactive factors produced by iron-catalyzed reactions, which have not been extensively studied with relation to mineral fibers, are the reactive aldehydes produced from lipid peroxidation. These species are longer lived and more mobile than some of the oxygen radical species discussed and may be involved in mediating biological response. Another reactive oxygen species which may be involved in mediating biological response is nitric oxide. Nitric oxide is released from activated macrophages and macrophages treated with asbestos[182] and may

be released from other types of cells under conditions of oxidative stress. Nitric oxide can participate in many reactions with iron and with reduced oxygen species produced by iron-catalyzed reactions. Thus, this will likely be a research area that will be developed in the coming years.

Future studies will address the exact interaction of iron and other components of mineral fibers with various oncogenes and tumor suppressor genes. Early studies on the protooncogenes c-*fos* and c-*jun* suggest that indeed asbestos-induced carcinogenicity may be regulated by a complex pathway of signals which are influenced and perhaps even directed by asbestos. Studies like those on induction of c-*fos* and c-*jun* are critical to understanding cellular response. As the interaction between biological molecules and asbestos fibers becomes more clear, possible treatments for people who have already been exposed to asbestos and will suffer from asbestos-induced cancer may be feasible.

X. Abbreviations

DNA SSB	DNA single-strand break
NTA	nitrilotriacetate
DMPO	5,5′-dimethyl-1-pyrroline *N*-oxide
EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance spectroscopy
ferrozine	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*-sulfonic acid
desferrioxamine B	*N*′-[5-[[4-[[5-(acetylhydroxyamino)pentyl]amino]-1,4-dioxobutyl]hydroxyamino]pentyl]-*N*-(5-aminopentyl)-*N*-hydroxybutanediamide
DF crocidolite	crocidolite treated with desferrioxamine B for 90 days to remove iron
8-OHdG	8-hydroxy-2′-deoxyguanosine

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