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FREE RADICAL GENERATION AT THE SOLID/LIQUID INTERFACE IN IRON CONTAINING MINERALS

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The potential for free radical release has been measured by means of the spin trapping technique on three kinds of iron containing particulate: two asbestos fibers (chrysotile and crocidolite); an iron-exchanged zeolite and two iron oxides (magnetite and haematite). DMPO (5,5′-dimethyl-1-pirroline-N-oxide), used as spin trap in aqueous suspensions of the solids, reveals the presence of the hydroxyl and carboxylate radicals giving rise respectively to the two adducts $[\text{DMPO-OH}]^{\cdot}$ and $[\text{DMPO-CO}_2]^{\cdot}$, each characterized by a well-defined EPR spectrum. Two target molecules have been considered: the formate ion to evidence potential for hydrogen abstraction in any biological compartment and hydrogen peroxide, always present in the phagosome during phagocytosis. The kinetics of decomposition of hydrogen peroxide has also been measured on all solids. Ferrozine and desferrioxamine, specific chelators of Fe(II) and Fe(III) respectively, have been used to remove selectively iron ions. Iron is implicated in free radical release but the amount of iron at the surface is unrelated to the amount of radicals formed. Only few surface ions in a particular redox and coordination state are active. Three different kinds of sites have been evidenced: one acting as H abstractor, the other as a heterogeneous catalyst for hydroxyl radical release, the third one related to catalysis of hydrogen peroxide disproportionation. In both mechanisms of free radical release, the Fe-exchanged zeolite mimics the behaviour of asbestos whereas the two oxides are mostly inert. Conversely magnetite turns out to be an excellent catalyst for hydrogen peroxide disproportionation while haematite is inactive also in this reaction. The results agree with the implication of a radicalic mechanism in the *in vitro* DNA damage and in the *in vivo* toxicity of asbestos.

KEY WORDS: active oxygen species, hydroxyl radical, carboxylate radical, spin trapping, asbestos, iron, iron chelators, hydrogen peroxide, magnetite, haematite, Fe-zeolite.

INTRODUCTION

Iron is the most abundant d-block element in the human body. If small traces escape control of the body defences, however, iron may result toxic because of its activity in redox cycling and free radical release.[1-3] When iron containing minerals happen to be in intimate contact with body fluids, iron ions may be either released or become active at the solid/liquid interface, with adverse reactions from cells and tissues.[4] Very often iron is present in the inhaled inorganic particulate and may contribute or be the direct cause of the pathogenicity of the inhaled dust.[5,6]

The role of iron derived free radicals in asbestos-induced diseases has been reviewed by Kamp *et al.*[7] Many *in vivo* and *in vitro* investigations are reported pointing to the crucial role played by active oxygen species (AOS) and iron in

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pulmonary damage following asbestos exposure.[8-12] AOS and iron are implied in the proposed pathogenic mechanisms[8-9] and AOS scavengers decrease the damage caused by asbestos to cells cultures.[10-11] *In vivo* tests have also revealed that AOS mediate fiber uptake.[13]

Several cell-free tests have been performed in order to evidence whether the mineral particle *per se* is capable of free radical release.[14-17] In earlier work Weitzmann and Graceffa[14] reported that asbestos catalyze the formation of hydroxyl radicals from hydrogen peroxide. Desferrioxamine, a strong iron chelator, inhibited such a reaction and, when administered *in vivo*, decreased asbestos toxicity.[18] This effect was interpreted as a strong adsorption of the molecule at the surface. The radical reactivity in their case was strictly related to the presence of hydrogen peroxide. It would be thus confined, *in vivo*, to the phagocyting medium, where H_2O_2 is present.

It was subsequently reported that even in the absence of H_2O_2 some radicals are produced by asbestos.[15,19] Moreover, this reactivity was not confined to fibrous materials; a large variety of iron minerals – all originating from mines where an excess of lung cancer was found – were also active;[20] the inactive ones either did not contain Fe(II) or were covered by a passivation sheet.

Iron may be either reactive at the solid surface or brought into the solution in a biological medium. Aust and Lund have concentrated their work on the iron which may be mobilized from asbestos, pointing out that iron bound to low molecular weight chelators may reach the target cell, and particularly DNA, more easily than iron retained on asbestos.[21-24] Considerable amounts of iron were extracted from crocidolite and other asbestos by endogenous chelators and it was clearly demonstrated that DNA was damaged by this mobilized iron.[21,25]

To a massive work on the biological and biochemical side, however, does not correspond an equivalent effort on the physico-chemical one, in spite of the fact that it is the **surface chemistry** that governs the primary reactivity of a particle.

More information is needed on the chemical nature of the sites active at the asbestos surface and to their modifications *in vivo* following release of radicals and ions. Bearing in mind that all fiber-related diseases (malignant mesothelioma, bronchogenic carcinoma and asbestosis) are long term ones, the molecular mechanism proposed should either trigger a cascade series of reactions or provide a long lasting release of toxic molecules likely *via* a catalytic process.

In this view there are still some open questions on the role of surface chemistry in asbestos-mediated free radical release:

- is **all iron** at the fiber surface active in free radical release?
- which is the **oxidation** and **coordination state** of iron at the active site?
- can the fiber surface act as a **heterogeneous catalyst** for radical production *in vivo*?
- how **do chelators modify radical release**? Iron depletion, strong adsorption of the chelator, variations in the redox potential of iron?

In order to give some answers to the above questions we have started a systematic investigation on the surface reactivity of some typical asbestos, namely serpentine **chrysotile** (by far the most widespread and used) where Fe(II) ions substitute for Mg(II) in the brucitic ($Mg(OH)_2$) layers and **crocidolite**, the so called "blue asbestos" from South Africa, whose amphibole structure imparts a sharp needle-like form to its fibers. Both Fe(II) and Fe(III) are stoichiometric components of the mineral. The present paper reports data on their radical release potential and its modification following mechanical fracture and prolonged incubation in chelators. The results are compared with those obtained on well defined iron-containing materials, namely a Fe(II)-exchanged Y zeolite and two iron oxides (haematite (Fe_2O_3) and magnetite (Fe_3O_4) exhibiting respectively Fe(III) and Fe(II) + Fe(III) in a crystalline oxidic structure.

Two target molecules have been considered for free radical release:

1) the formate ion (HCO_2^-), which is transformed into the $CO_2^{-\bullet}$ radical, via hydrogen abstraction upon contact with the solid. This reaction is representative of H-abstraction from other molecules, with functional groups similar to the formate one, taking place at the solid-liquid interface in various biological compartments.[5,7,15]
2) H_2O_2, originating the $^{\bullet}OH$ radical via a Fenton-type mechanism, which epitomizes a reactivity confined to a biological compartment where H_2O_2 may be present; typically, in pulmonary induced diseases, the phagolysosome in phagocyting alveolar macrophages.[7,14]

The chelators used were desferrioxamine and ferrozine, both successfully employed to remove Fe(III) and Fe(II) respectively[21–25] and EDTA, which on some asbestos enhanced and on other ones depressed radical release.[14]

The overall surface reactivity of the original and modified dusts was monitored by their activity in catalyzing the decomposition of hydrogen peroxide.

EXPERIMENTAL

Materials

Solids

1) Asbestos: Canadian Chrysotile (B) [$Mg_3Si_2O_5(OH)_4$], serpentine structure, and Crocidolite [$(M^+)_2(M^+, M^{2+}, M^{3+})_5(Si, Al, Fe^{3+})_8O_{22}(OH)_2$], amphibole structure, were both from UICC (Union Internationale Contre le Cancer).
2) The NaY zeolite sample, which exhibits large cavities (connected by channels with a diameter of 7Å) where counterions are accessible to the spin trap adopted, was a gift from Dr Fajula (Montpellier, France). Ionic Fe exchange was performed by soaking and stirring 1 g of the zeolite in a 0.05 M solution of $FeSO_4$. A particular care was given to avoid all factors facilitating the spontaneous oxidation of Fe (II) to Fe (III) in air. Argon was bubbled through the solution and the pH was kept at 4.5 (acetate buffer). Washed and dried samples were stored in inert atmosphere. The total amount of exchanged iron, as measured by atomic absorption spectroscopy was about 7.10^{-4} moles per gram of zeolite. A direct evaluation of the amounts in each oxidation state is not straightforward. Indirect evidence from other techniques (e.g. diffuse reflectance spectroscopy) indicated that the lower valency state prevailed (unpublished results).
3) Oxides: haematite, Fe_2O_3 from Merck and magnetite, Fe_3O_4 from BDH were employed. The surface areas of all samples (Table 1), have been measured with the BET method (Nitrogen adsorption at 77K) by means of Quantasorb (Quantachrome).

TABLE 1
Characteristics of the solids and relevant release of iron in chelators

solids	specific surface m^2/g	iron content % w/w	iron removal (III) with Deferoxamine μmol/g	iron removal (II) with Ferrozine μmol/g	iron removal with EDTA μmol/g
chrysotile	27	3	50	2.5	38
crocidolite	8	36	108	10	42
FeY zeolite	*	5	720	110	–
hematite	3	70	16	0	–
magnetite	21	72	50	2	–

*Zeolite exhibits an extremely high internal surface

Chemicals
Spin trap: DMPO (5,5′-dimethyl-1-pirroline-N-oxide), from Fluka, was purified by filtration through activated charcoal according to the method proposed by Buettner and Oberley[26]

Target molecules: sodium formate was from Sigma and hydrogen peroxide (30%) from Prolabo. Phosphate buffer: mono and disodium phosphate were from Merck.

Chelators: Deferoxamine mesylate USP (desferioxamine B); Ferrozine 3-(2-pyridil)-5,6-diphenil-1,2, 4-triazine-p, p′-disulfonic acid; disodium EDTA, were from Sigma. Titration of H_2O_2: peroxidase and leuco crystal violet, from Sigma, acetate buffer (acetic acid and sodium acetate), from Fluka.

METHODS

EPR spectroscopy

A Varian E109 EPR spectrometer working in X band (9–9.5 GHz) with a double resonant cavity was employed. For the evaluation of the number of spin DPPH (purified diphenylpicrylhydrazyl from Sigma containing about 0.95 unpaired spins per molecule) was employed.

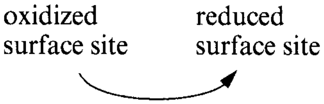
Spin trapping

The technique was substantially the one described by Zalma *et al.*[15,19,27] for aqueous suspensions of particulate materials. To detect the formation of radical species in aqueous suspensions of the materials, the spin trapping agent 5,5′-dimethyl-1- pyrroline-N-oxide (DMPO) was employed, which in an aqueous medium gives stable radical adducts, allowing a quantitative evaluation of free radical release. The pH in all experiments was kept at 7.4 (phosphate buffer). The intensity of the EPR signal measures the number of radicals trapped, thus relatable to those formed in the solution. Two target molecules are used: formate ion (F) and hydrogen peroxide (P). They respectively give rise to the DMPO-$CO_2^{-\circ}$ and to the DMPO-OH° adduct, each characterized by a typical EPR spectrum (fig.1 and 2). These two tests will be referred to as F-C (for carboxylate radical) and P-OH (for hydroxyl radical) respectively.

A F-C test, which indicates the abstraction from the formate ion of a hydrogen atom, with formation of the carboxylate radical is performed as follows. 0.5 ml of aqueous phosphate buffer (0.5 M HPO_4^{2-} and 0.5 M in $H_2PO_4^-$, pH = 7.4), 1 ml of DMPO (0.05 M) in distilled water free from iron and copper (chelex 100) and purified by filtering trough activated charcoal and 1 ml of $HCOO^-$ are put in contact with 45 mg of the solid sample. The suspension is then incubated at 37°C and shaken in a dark reactor.

Aliquots of the suspension are withdrawn at 25′ and 55′ then filtered through porosity filters. The liquid is introduced into a flat cell, appropriate for aqueous solutions, and the corresponding EPR spectrum recorded at room temperature some five minutes after withdrawal of the aliquots.

The reactions taking place may be summarized as follows:



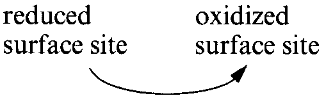
$$HCO_2^- \xrightarrow{\textit{solid surface}} CO_2^{-\circ}$$

$$DMPO + CO_2^{-\circ} \longrightarrow [DMPO\text{-}CO_2^-]^{\circ}$$

This kind of reactivity is not confined to the formate ion. A similar behaviour has been found with ethanol and with some peptides (unpublished results). In these cases, however, the yield in free radicals was lower than with formate. This is likely due to the fact that on the latter the negative charge favours the homolytic rupture of the C-H bond, by respect to the same bond in other molecules. Formate is thus the most appropriate target molecule for quantitative evaluations and comparisons.

In a P-OH test, which allows the detection of hydroxyl radicals from hydrogen peroxide, the sequence of operations is similar to that for the F-C test, but, because of the lower stability of the DMPO adduct, the times of incubation before withdrawal of the supernatant aliquots are shorter. 0.5 ml of H_2O_2 (0.5 M in phosphate buffer) and 1 ml of DMPO (0.05 M) are added to 45 mg of the solid sample. The OH formation is monitored by recording at 10 min and 30 min minutes the EPR spectrum of the $[DMPO\text{-}OH]^{\circ}$ adduct.

The reactions taking place may be summarized as follows:



$$H{-}O{-}O{-}H \xrightarrow{\textit{solid surface}} OH^- + {}^{\circ}OH$$

$$DMPO + {}^{\circ}OH \longrightarrow [DMPO\text{-}OH]^{\circ}$$

unless, instead of a Fenton mechanism, a homolytic rupture of the H_2O_2 molecule takes place.

Blanks are made by operating in the same way except that no solid particulate is introduced into the solution. With H_2O_2 the blanks nearly always exhibit weak spectra, due to the reaction of H_2O_2 *per se* with some impurities, while the blank for the F-C test does not show any (or minimal) radical formation. Because of an intrinsic variability from one DMPO preparation to another, blank tests were performed in each set of experiments.

In some cases the formate ion was also used as a secondary trap for the hydroxyl radical originated in a test with H_2O_2. Following the reaction which takes place

$$HCO_2^- + {}^{\bullet}OH \longrightarrow CO_2^{-\bullet} + H_2O$$

the test will be referred to as PF-C (Peroxide/Formate-Carboxylate). This test may monitor, in some cases, more than one radical mechanism. It is however much more sensitive than the P-OH and thus more apt to quantitative comparisons.

In order to establish whether radicals are produced via a catalytic mechanism, the same sample was used in subsequent tests of the same type, each time after thorough washing in metal-free distilled water and drying (catalytic tests). The same procedure was adopted for F-C tests following P-OH and viceversa.

The intensity of the spectra is reported in arbitrary units (a.u.) where each unit corresponds to about 1.10^{15} spin per ml of solution and is referred to the same amount of solid employed in the experiment (45 mg).

Because of the intrinsic variability of the spin trapping measurements each test was reproduced at least three times. The intensity of the spectra, averaged over all the experiments performed, is reported in histograms.

Iron Mobilization

50 mg of the solid sample were suspended in 5 ml of a 100 mM solution of the chelating agent. Ferrozine was dissolved in phosphate buffer (pH = 7.4).

The suspension was incubated at 37°C and shaken in a dark reactor for three days. Mobilization of Fe^{3+} (desferrioxamine) and Fe^{2+} (ferrozine) was evaluated by visible spectroscopy at 428 nm (complex Fe^{3+}-Desferioxamine) and at 560 nm (complex Fe^{2+}-Ferrozine). Mobilization by EDTA was measured by means of atomic absorption spectroscopy.

Kinetics of Decomposition of H_2O_2

1 ml of hydrogen peroxide 0.1 M and 30 ml of distilled water were added to 50 mg of the sample and then incubated at 37 and shaken in a dark reactor.

Aliquots of the suspension (100 μl) were withdrawn at 10 min, 30 min, 60 min, 120′, 240′, 300′ and then filtered through 20 nm porosity filters.

The residual amounts of hydrogen peroxide in solution was determined by an enzymatic assay whereby peroxidase catalyzes the oxidation of a substrate (leuco crystal violet) by H_2O_2, following a method described by Mottola *et al.*[28]

RESULTS

Free Radical Release from Asbestos

The activity in radical release of the two asbestos as received (a, b) and after a mild grinding in a mortar (a′, b′) are reported in fig. 1. Section A illustrates the results in the F-C test (formate as target molecule) and section B in the P-OH one (hydrogen peroxide as target molecule). In the upper parts the typical spectra of the two adducts

$[\mathrm{DMPO\text{-}CO_2}]^{\bullet}$ $[\mathrm{DMPO\text{-}OH}]^{\bullet}$ are reported. As the stability of the two radicals $^{\bullet}OH$ and $CO_2^{-\bullet}$ are different, quantitative comparison between sections A and B is meaningless. In contrast, as the tests have been performed accurately in the same conditions, quantitative comparisons with the blank and between the various experiments can be made within each section.

The main results are:

- the two asbestos exhibit a similar behavior in both tests
- grinding creates new sites for the reaction with formate while no significant variations are found with hydrogen peroxide.

In order to investigate the catalytic nature of the surface active sites, each test has been performed subsequently on the same sample. The results are reported in fig.2, where the activity of the fresh samples (I) is compared with what found with samples just washed and dried after the first test (II). Data on both asbestos clearly show the following:

- the activity in the P-OH and in the PF-C tests (i.e. towards H_2O_2) does not vary significantly in subsequent assays, thus the process leading to $^{\bullet}OH$ production from hydrogen peroxide is basically a catalytic reaction.
- at the opposite, the sites for the F-C test are consumed in the first contact and the fiber is subsequently totally inactive towards formate.

Figure 3 reports the results obtained when a test is made on samples previously submitted to the other one, i.e. experiments designed to evidence whether after reaction with formate the surface is still active towards hydrogen peroxide and vice versa. Results on both asbestos indicate that:

- after a F-C test is performed the surface is still nearly equally active in the P-OH one (figure 3, B, B′)
- a P-OH test reduces (but does not eliminate) the activity of the fiber in the F-C one (figure 3, A, A′).

Effect of chelators on the fibers

In order to evidence the chemical nature of the active surface sites, the fibers were modified by prolonged incubation in various chelators. It has been largely reported by Aust and associates that incubation with chelators mobilize iron from asbestos.[21–25] No data are available on the modifications brought about by the chelating agents on the surface when iron is removed. Aim of the present work was a selective removal of Fe(II) or Fe(III), in order to establish which is the role of the two iron oxidation states in the surface reactivity. Ferrozine, a bidentate N donor chelator, is selective for Fe(II) and was found to bind to Fe(II) on crocidolite asbestos.[21,29] Desferrioxamine, the hexadentate chelator with six oxygen atoms as donors, specifically binds Fe(III).[25] EDTA is expected to bind both.

The amount of iron removed during the incubation in the various chelators is reported in Table 1. The amount of iron mobilized by desferrioxamine – likely Fe(III) – is nearly tenfold what removed by ferrozine – likely Fe(II) – and close to that removed by EDTA, which may virtually bind Fe in both oxidation states. Consistent amounts of iron (about 50% of that on fresh samples) was still removed by ferrozine after incubation in desferroxiamine and vice versa. Iron still available for the other chelator somehow confirms the specificity of the two molecules in removing respectively Fe(II) and Fe(III).

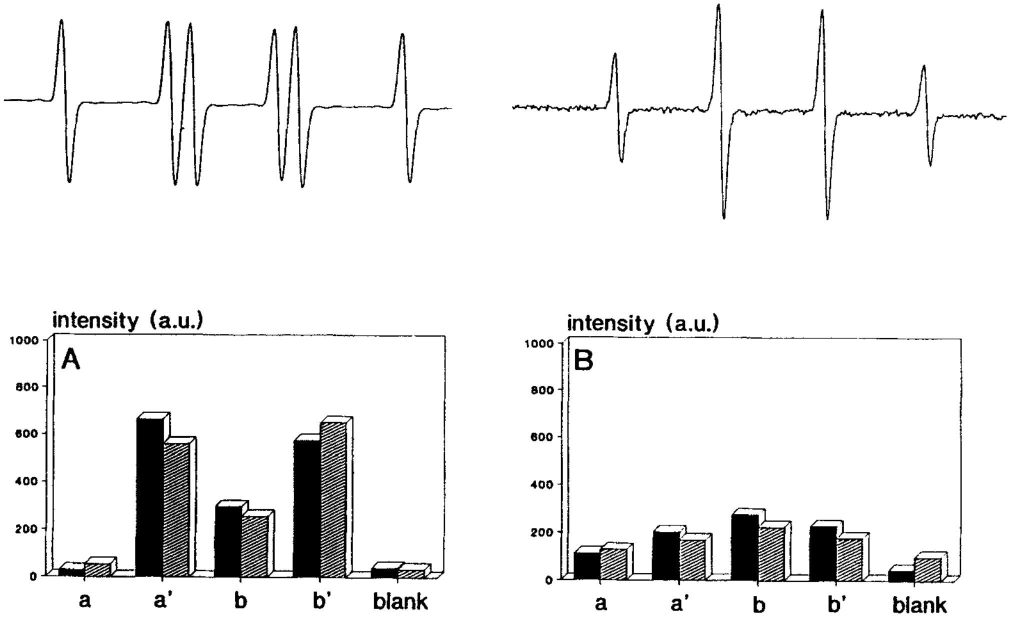


FIGURE 1 Radical release from suspensions of crocidolite (a) and chrysotile (b) as received and after grinding (a′, b′). Upper part: typical EPR spectrum of the DMPO adduct, lower part: intensity of the spectra averaged over several measurements; the two histograms for each sample refer to the measures at subsequent times. Section A: F-C test, formate ion as target molecule, DMPO-$CO_2^{-\bullet}$ adduct, spectra recorded after 30 min and 60 min (full and dashed histograms respectively). Section B: P-OH test, hydrogen peroxide as target molecule, DMPO-$OH^{\bullet}$ adduct, spectra recorded after 10 min and 30 min (full and dashed histograms respectively). Blank: intensity of the spectra recorded in the absence of the solids (averaged over several independent measurements)

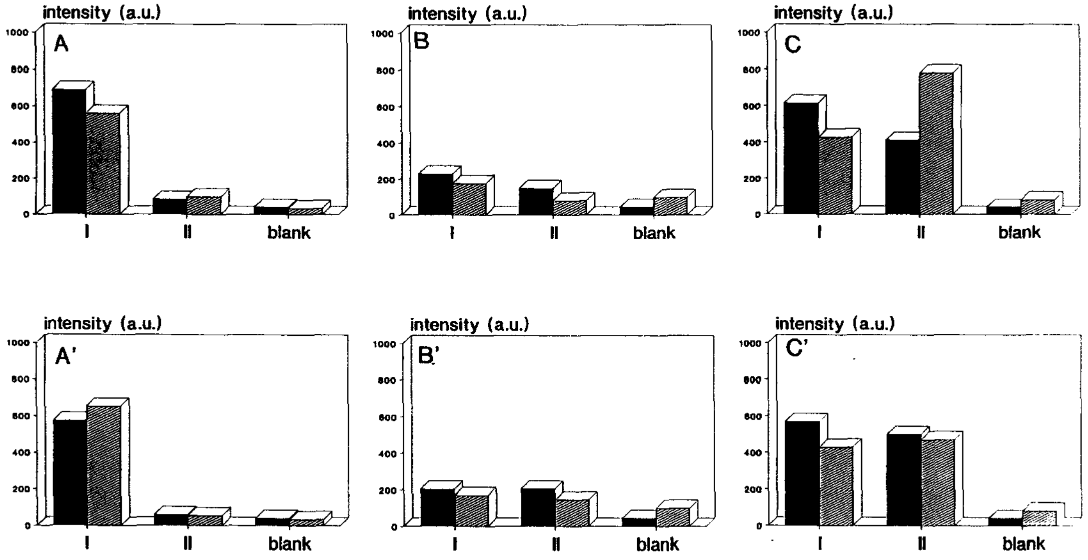


FIGURE 2 Catalytic test: the same test was performed subsequently on the same sample, the intensity of the EPR spectra in the first (I) and in the second (II) measurement are compared. A,B,C: crocidolite. A′,B′,C′: chrysotile. A,A′: F-C test, formate ion as target molecule, DMPO-$CO_2^{-\cdot}$ adduct, spectra recorded after 30 min and 60 min (full and dashed histograms respectively). B,B′: P-OH test, hydrogen peroxide as target molecule, DMPO-$OH^{\cdot}$ adduct, spectra recorded after 10 min and 30 min (full and dashed histograms respectively). C,C′: PF-C test, hydrogen peroxide as target molecule, formate ion as secondary trap DMPO-$CO_2^{-\cdot}$ adduct, spectra recorded after 30 min and 60 min (full and dashed histograms respectively). Blank: intensity of the spectra recorded in the absence of the solids (averaged over several independent measurements).

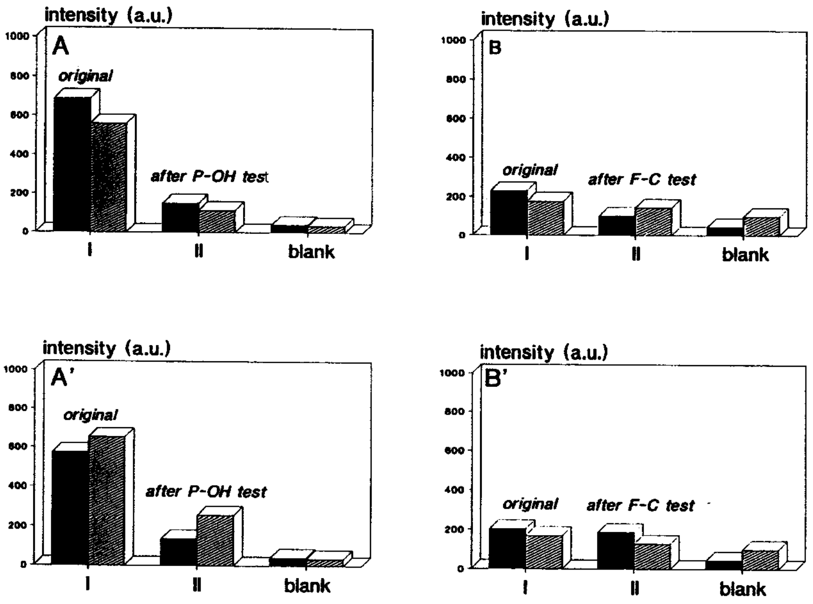


FIGURE 3 Consumption of the active sites by the tests performed. A test is made on a sample previously submitted to the other one. A,B: crocidolite, A′,B′: chrysotile. A,A′: F-C test, formate ion as target molecule, DMPO-CO$_2^{-\bullet}$ adduct, spectra recorded after 30 min and 60 min (full and dashed histograms respectively). I, fresh sample; II sample already submitted to a P-OH test. B,B′: P-OH test, hydrogen peroxide as target molecule, DMPO-OH$^{\bullet}$ adduct, spectra recorded after 10 min and 30 min (full and dashed histograms respectively). I, fresh sample; II sample already submitted to a F-C test. Blank: intensity of the spectra recorded in the absence of the solids (averaged over several independent measurements)

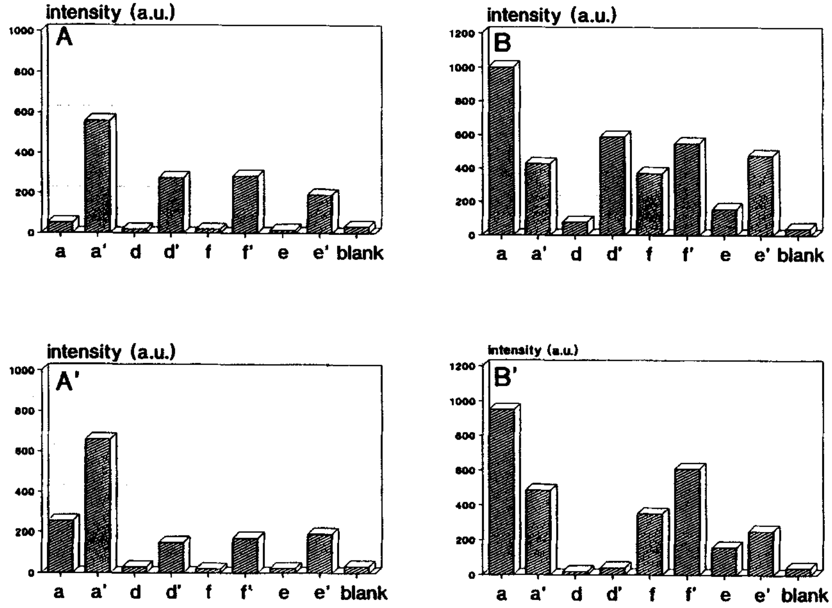


FIGURE 4 Free radicals release from asbestos. Effect of incubation in chelators on the potential for radical release. A,B: crocidolite, A′, B′: chrysotile. A,A′: F-C test, formate ion as target molecule, DMPO-CO_2^- adduct, spectra recorded at 60 min. Fresh sample, a; ground sample, a′; after incubation in desferrioxamine, d; EDTA, e; ferrozine, f; ground after incubation in the respective chelators, d′, e′, f′. B,B′: P-FC test, hydrogen peroxide as target molecule, DMPO-CO_2^- adduct, spectra recorded at 30 min. Fresh sample, a; ground sample, a′; after incubation in desferrioxamine, d; EDTA, e; ferrozine, f; ground after incubation in the respective chelators, d′, e′, f′. Blank: intensity of the spectra recorded in the absence of the solids (averaged over several independent measurements)

In order to evidence variations in free radical release potential following selective removal of iron, the spin trapping tests have been performed on samples thoroughly washed and dried after incubation in chelators (figure 4). The activity towards hydrogen peroxide is here represented by the PF-C tests (both formate and hydrogen peroxide introduced in the suspension) being the most sensitive, thus most appropriate for comparisons.

Incubation in chelating solutions deeply affects the capability of releasing free radicals on both asbestos. In particular:

- All chelators destroy or reduce the reactivity in the F-C test, which is only partially restored by grinding.
- Desferrioxamine destroys also the reactivity in the test with hydrogen peroxide, whereas ferrozine does not. EDTA is somehow intermediate between the two.

The amounts of iron removed by chelators exceed what would be expected to be strictly at the surface. X-rays diffractograms showed no detectable changes in the crystalline structure following incubation while the EPR spectra of the fibers as received, after grinding and after incubation in chelators (not reported) indicated some variations in the Fe(II)/Fe(III) ratio in the outmost layers. This is in agreement with what reported by us in a previous paper on the modifications in the micromorphology of the fibers caused by iron chelators; HRTEM pictures revealed, in fact, erosions in the external lattice layers, likely due to depletion of iron and other cations[30]

Non Fibrous Iron Containing Minerals

a) Iron oxides

In spite of the large amounts of iron contained, both oxides showed a very low activity in free radical release. Hematite is always at the level of the blank; magnetite, only when ground, exhibits a small activity, definitely lower than that of the crocidolite one. Haematite suspensions do not show any trace of radical release, not even the usual amounts obtained in the blank tests. In this respect it appears to act even as a quencher of any radical formed.

b) Iron in zeolite

Iron in the Fe-exchanged Y zeolite is the easiest to mobilize (Table 1), being all at the surface and not fixed into a crystal lattice as in asbestos or in oxides. Free radicals are released from its suspensions with both target molecules (figure 5). It is noteworthy that the production of carboxylate radicals with formate (figure 5, section A, a) is much higher than with asbestos (more than twice what found on the freshly ground fibres, figure 1 A, a′b′), whereas the reactivity towards hydrogen peroxide (figure 5, section B, a) is of the same order of magnitude. The two chelators, ferrozine and desferrioxamine, fully inhibited hydrogen abstraction in the F-C test (figure 5, section A, d, f). As to the P-OH test, desferrioxamine decreased (figure 5B, d), but ferrozine even increased the $^{\cdot}$OH production (figure 5B, f). The release of $CO_2^{-\cdot}$ was, also in this case, a non catalytic reaction while that of $^{\cdot}$OH was a catalytic one (figure 5, compare A, b to B, b).

The zeolite was also submitted to a thorough oxidation (by means of concentrated 1% hydrogen peroxide) in order to eliminate via oxidation as much as possible Fe(II) from the surface. The results (figure 5, A, B, h) are opposite in the two tests: all reactivity towards formate was eliminated but the release of hydroxyl radicals was even enhanced.

Decomposition of Hydrogen Peroxide

Iron containing minerals are expected to act as heterogeneous catalysts for the decomposition of hydrogen peroxide into oxygen and water. The simplest way to assess this property is to measure the overall reactivity towards aqueous solutions of H_2O_2 from hydrogen peroxide consumption or oxygen development. The kinetics of such a reaction, monitored from the variation of the H_2O_2 concentration in solution, is reported, for all the solids examined in this study, in figure 6. The heat of reaction measured calorimetrically (data not reported) confirmed that the reaction taking place in the experimental conditions adopted was the simple disproportionation of H_2O_2.

Figure 6 clearly indicates the following:

- the various solids exhibit quite a different behaviour one from the other.
- the catalytic activity does not parallel the release of $^{\bullet}OH$ nor $CO_2^{-\bullet}$.
- the catalytic activity is not relatable to the mere iron content of the solids, nor to the iron concentration at the surface estimated on the basis of the surface areas reported in Table 1.

It is particularly noteworthy that of the two oxides one, magnetite, is the most efficient catalyst, whereas hematite is fully inactive. The Fe exchanged zeolite, in spite of the widespread use of metal dispersed into zeolites in various catalytic processes, is moderately active. It does in fact catalyze the reaction up to a full decomposition, but at a much lower rate than magnetite and even than asbestos. Crocidolite and chrysotile exhibit a similar trend, in spite of the higher amount of iron present in the former by respect to the latter. (Table 1). This may be partly because chrysotile has a lower Fe content, but a higher specific surface than crocidolite. Nevertheless they are far less active than magnetite, with exhibits a much lower specific surface.

It clearly appears that several factors – such as crystal faces exposed, Fe coordination at the surface, perfection of the microcrystals, arrangements of Fe ions at the surface – govern the catalytic activity, beyond the mere iron amounts exposed at the surface.

DISCUSSION

The behaviour of the examined mineral dusts in free radical release, hydrogen peroxide disproportionation and iron mobilization by chelators indicates that iron gives rise to a variety of surface arrangements, which act as reactive or catalytic sites. The presence and abundance of the various sites vary from one solid to another.

Aqueous suspensions of iron containing minerals in finely divided form turn out to be a relevant source of free radicals. While the crucial role played by surface iron ions is confirmed by the inhibition by iron chelators, the extent of radicals formed is remarkably unrelated to the iron amount actually present at or near the surface. This is in agreement with what found in homogeneous systems where to an increase in iron concentrations did not correspond a parallel increase in the free radicals produced.[31] Thus not all iron is active but only some **peculiar arrangements of iron**

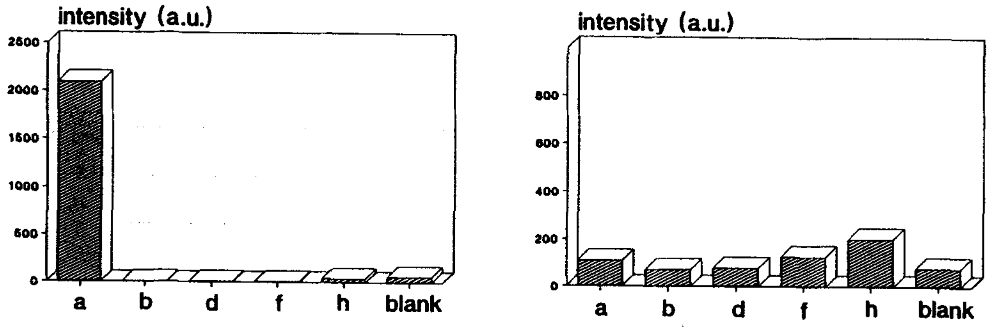


FIGURE 5 Free radicals release from Fe exchanged Y Zeolite. Section A: F-C test, formate ion as target molecule, DMPO-CO$_2^{-\cdot}$adduct, spectra recorded after 60 min. Section B: P-OH test, hydrogen peroxide as target molecule, DMPO-OH$^{\cdot}$ adduct, spectra recorded after 10 min. Blank: intensity of the spectra recorded in the absence of the solids (averaged over several independent measurements). Fresh sample, a; "catalytic" test, b; after incubation in desferrioxamine, d; ferrozine, f; treated with an aqueous solution of hydrogen peroxide 0.1%, h.

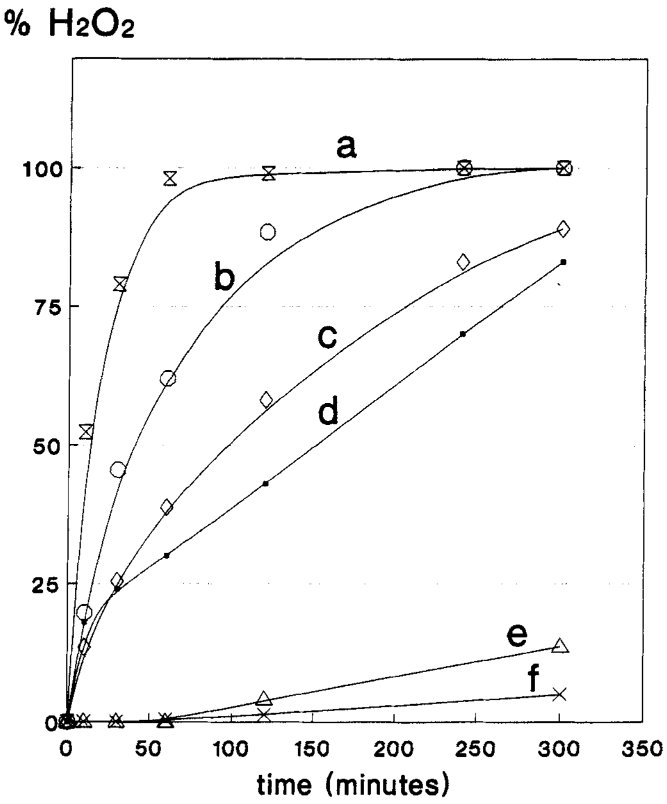


FIGURE 6 Catalytic decomposition of hydrogen peroxide. Percentage of H_2O_2 decomposed as a function of time: a, magnetite; b, FeY-zeolite; c, chrysotile; d, crocidolite; e, haematite; f, blank,

at the surface are responsible for radical production. This also agrees with what found by us and other authors on free radical release from glass fibers,[32–34] where to a relatively low iron concentration in the solid corresponded a substantial amount of free radicals produced.

More than one radical mechanism takes place, so that at least three different kinds of active sites have to be involved respectively for:

- H-abstraction from formate ions
- the catalytic release of $^{\bullet}OH$ from hydrogen peroxide
- the catalytic decomposition of hydrogen peroxide

Mechanisms of Radical Release

Hydrogen abstraction

The sites releasing hydroxyl radicals in the presence of hydrogen peroxide via a Fenton-like mechanism are clearly different from those capable of H abstraction from formate, for the following reasons.

The hydroxyl radical production:

- is not enhanced by grinding, i.e. does not require fresh surfaces (fig.1)
- is a catalytic reaction (fig.2)
- does not interfere with the activity towards formate (fig.3)
- is not affected by selective removal of Fe(II) (chelation with ferrozine, fig.4)

To the opposite, the capability of H abstraction:

- requires (crocidolite) or is enhanced by (chrysotile) freshly created surfaces (fig.1); takes place on Fe(II) rich exposed iron clusters (Fe-Y zeolite) (fig.5)
- is not a catalytic reaction and once reacted the active sites are inactivated (fig.2)
- removal of either Fe(II) or Fe(III) fully inhibits the reaction (fig.4).

The former reaction appears to require only the presence of iron (III), while the latter, as previously reported,[5,15,20] requires the presence of iron (II), typically occurring on freshly ground materials, where the slow oxidation from Fe(II) to Fe(III), brought about by exposure to the atmosphere, is not yet accomplished. It is noteworthy, however, that removal of Fe(III) ions by desferoxamine, which still leaves some Fe(II) on the solid (subsequently removable by ferrozine), inhibits the H-abstraction reaction. As the solids were thoroughly washed after incubation, no desferrioxamine should be left at the surface, with the only exception of molecules strongly chemisorbed on it. Any of the reactions between radicals and desferrioxamine, reported to occur in aqueous solution,[35–37] would take place in our system at the solid-liquid interface. This points to a model where iron in both oxidation states is required to trigger the reaction.

As there is no correspondence between iron content and free radical release, only a particular arrangement of the iron ions is responsible for the hydrogen abstraction reaction. The abstraction of hydrogen involves a reduction on the surface site which is likely to involve a cation-anion couple:

$$\mathrm{Fe}^{n+}\text{–}\mathrm{O}^{2-} + \mathrm{HCO_2}^{-} \longrightarrow \mathrm{Fe}^{(n-1)+}\,\mathrm{OH}^{-} + \mathrm{CO_2}^{-\bullet}$$

where iron acts as electron acceptor and the reaction extinguishes the reactivity of the site, which can be regenerated only by a successive oxidation.

Simple Fe^{3+} ions do not react in a similar way. Haematite and ferrozine-treated asbestos and zeolite – all supposed to exhibit only Fe(III) – are in fact fully inactive. Incubation in hydrogen peroxide, which causes full oxidation of exchanged ions, also inactivates the zeolite.

Two hypotheses may be envisaged. The active site may consist either of interacting couples of Fe(II)-Fe(III) or of iron ions in higher oxidation states (e.g. IV)

A surface Fe^{2+}-O^{2-}-Fe^{3+} arrangement somehow recalls the requirement of both redox states in aqueous solutions to initiate lipid peroxidation brought about by hydrogen peroxide.[38–40] Similarly, the abstraction of a hydrogen atom from the formate ion, which in a complex biological medium may well be the first event of a chain of reactions, will only occur in the presence of interacting Fe(II)-Fe(III) suitably located at the surface.

Elimination of Fe(II) by ageing, oxidation or selective extraction destroys the active site.

An alternative hypothesis is that some surface Fe(II) may be converted to Fe(IV) by atmospheric oxygen, with formation of the ferryl ion, FeO^{2+}, which was found to abstract a hydrogen atom from ethanol in homogeneous media.[31] Iron in the highest oxidation states (e.g. Fe $=$ O^{2+} or μ-oxo) was also invoked to explain the radical reactivity of suspensions of a large amount of minerals.[20]

Reduction of the ferryl ion would bring about oxy/hydroxy formation which are known to be very stable in iron compounds. This hypothesis is consistent with the high reactivity found on freshly created surfaces of asbestos and with the Fe-exchanged zeolite, where a relatively large amount of Fe(II) ions would be present which could easily be converted into the ferryl ones. In this second hypothesis, however, it is not clear why also Fe(III) should be required for H abstraction. Furthermore it is not clear how chelators would modify the ferryl ion. It has however to be pointed out that chelators, by affecting the redox potential,[41,42] may well favour redox reactions at the surface while removing the ions from the mineral. Under these circumstances the consequent modifications in the arrangement of iron ions, including the possible reduction of Fe from its highest oxidation state, would inactivate the surface site and hence blunt the activity in free radical release.

A direct evidence of iron in an oxidation state higher than Fe(III) at the solid surface has not been given so far. The second hypothesis therefore is just a speculative one.

Catalytic release of hydroxyl radicals

The generation of $^{\bullet}OH$ from Fe(II) and H_2O_2 via the Fenton reaction is a well-documented process both in homogeneous systems[1,2] and at the solid – liquid interface 7, 14 32:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

In the presence of superoxide anions ferric ions can be reduced back to ferrous establishing a cycle by which iron catalyzes the conversion of hydrogen peroxide into hydroxyl radicals, the so called iron catalyzed Haber-Weiss reaction:

$$O_2^{-\bullet} + H_2O_2 \xrightarrow{\text{iron}} {}^{\bullet}OH + OH^{-} + O_2$$

Several workers have implicated iron in asbestos in these reactions.[7] Zalma *et al.*[15] hypothesized a direct electron transfer from asbestos to oxygen to generate superoxide anions.

The present data seem to suggest that hydroxyl radicals are released also in the absence of Fe(II) because ferrozine, which selectively binds Fe(II), does not affect the free radical release potential. At the opposite, desferoxamine that would be expected to remove only Fe(III) fully inhibits the reactivity. This may be achieved either by complete depletion of any accessible iron (including Fe(II), which may be easily oxidized in the presence of desferoxamine) or by strong adsorption onto the surface that would hinder accessibility to surface ions.

The mere fact that even on the fully oxidized zeolite the reaction still takes place confirms that Fe(III) is sufficient to initiate it. This rules out a simple Fenton reaction and suggests a mechanism by which Fe(III) catalyzes the homolytic rupture of the oxygen-oxygen bond in hydrogen peroxide. Beside oxidation state, however, also the coordination state of the ion has to be taken into account. It is well known in fact in homogeneous systems that some chelators enhance and some depress Fenton activity.[42,43] This is related to the inner and outer sphere of the complex and to the availability of at least one iron coordination site open or occupied by a readily dissociable ligand such as water.[43] Iron at the surface is poorly coordinated by lattice anions, with at least one coordination site unsaturated, thus particularly apt to act as the catalytic site. The catalytic activity will be inhibited at the solid surface either by removal of the ion or by the saturation of its coordinative site by a strong ligand such as desferrioxamine. This should apply to Fenton activity but also to other catalytic processes.

A Fenton/Haber-Weiss mechanism, however, may be operating under particular circumstances. It definitely requires Fe(II) and redox cycling but even very small amounts of Fe(II), originated by superoxide anions (from H_2O_2) or by rearrangement at the surface of the two redox states, could be sufficient to initiate the reaction cycle. When iron is present at the solid liquid interface, a spontaneous redistribution of the two redox states is likely to be established.

Under such circumstances the cycle would generate the hydroxyl radical release.

Catalytic Activity towards H_2O_2

The capability of catalyzing H_2O_2 disproportionation does not parallel free radical release. Magnetite, in fact, which is very poorly reactive in both mechanisms of free radical release turns out to be the most efficient one in H_2O_2 catalytic decomposition. FeY zeolite, the most active in H abstraction, does indeed catalyze H_2O_2 decomposition, but at a lower rate. Aged crocidolite catalyzes hydrogen peroxide decomposition, but grinding is required for a consistent hydrogen abstraction; the $^{\circ}OH$ production with hydrogen peroxide is also rather low.

The activities of the various solids towards hydrogen peroxide cannot either be related merely to the actual amounts of iron at the surface (Table 1), but appear determined by various factors likely including redox potential and coordination state of the iron ion, both dependent upon location of the ion into the crystalline structure.

Haematite, one of the richer in iron, is fully inactive. This reveals how the redox potential of an ion at a crystal face is determined by the surrounding moieties (O atoms in the present case) and by the crystal structure. Although one of the richer in iron this oxide is so stable and unreactive that even the kinetics of decomposition of hydrogen peroxide, which is catalyzed by a large variety of metal ions, is virtually unaffected by the presence of it.

The proposal of a correct reaction mechanism for the heterogeneous catalysis of this reaction is beyond the scope of this paper. The reaction may be the final product of a cascade reactions initiated by the formation of hydroxyl radicals, but may also take place via different paths. As there is evidence that the catalytic potential is unrelated to free radical release, a non-radicalic mechanism is suggested.

Effect of Chelators

Upon contact with the surface, chelators either extract an exposed ion from the lattice into the solution, or become irreversibly bound at the surface, preferentially at the site where the iron ion is located. The first mechanism is often followed by a substantial ion mobility,[30] which accounts for the mobilization of more ions than what originally present at the surface. The surface reactivity is affected by both mechanisms. Abstraction of the ions destroys active sites. Chelates adsorbed at the surface ions modify their redox potential and hence their propensity for catalytic activity and free radical production. The inhibition in hydroxyl radical release from asbestos by desferroxamine was assigned by Weitzman and Graceffa to adsorption of the molecule. Their hypothesis may not be ruled out, but the substantial amount of iron mobilized[24,25,30] suggests that the mere depletion may account for inactivation.

Iron content of the solid by no means parallels what removed by each chelator (Table 1). Iron in hematite, strongly held in the lattice, is not removable, opposite to Fe in the exchanged Y zeolite, which, being coordinated at the surface, is easily removed. Both micromorphology of the solid particles (form, lattice energy, crystal faces exposed), and state of iron at the surface (location, coordination, oxidation) determine the propensity to be mobilized by chelators.

It is noteworthy that grinding always increases ion removal at a much larger extent than what expected from the induced increments in surface area (to be published). The reason for the substantial inactivity of the iron oxides, compared to asbestos or Fe-Y zeolite is due both to the fact that all surface iron is oxidized or to their high degree of crystallinity. In order to react or be extracted by chelators the iron ions have to be exposed, poorly coordinated and in a somewhat disordered array. This is likely the case of the exchanged zeolite or of the surface originated on asbestos upon grinding.

CONCLUSIONS

Free radicals are frequently involved in the mechanism of toxicity of water soluble chemicals of various origin[44,45]

In some minerals iron triggers various kinds of radical reactions that may be considered as one of the possible causes of the pathogenic response to the inhalation of mineral dusts and fibers. Damage to DNA has been proved so far in several kind of test.[17,22,24,25,46] This does not mean that all iron containing minerals are toxic, accordingly with recent findings from *in vivo* tests that several iron oxides are non carcinogenic.[47] In fact radical release is not related to iron abundance but to the redox and coordination state of the ions at the surface. The excess of lung cancer found among haematite mines miners may be either due to other factors[48] or to the comminution state of the inhaled dust.

Two different mechanisms of free radical release at least have been evidenced: $H^{\cdot}$ abstraction, which will take place ubiquitously *in vivo* and $^{\cdot}OH$ production, which appears confined to the biological compartments where hydrogen peroxide is produced, e.g. phagocyting cells. Asbestos fibers in vivo elicit recruitment of phagocyting cells (mostly alveolar macrophages) with consequent frustrated phagocytosis as a consequence of their fibrous habit. The biological compartment where the reaction takes place may thus also provide the long lasting pathogenic response. For instance, if the process is mediated by alveolar or interstitial macrophages, through a continuous recruitment and failed phagocytosis, the particle will experience cyclic variations of pH and of the oxidizing power of the milieu, by which active sites are regenerated *in vivo*. Under these circumstances redox cycling of iron ions at the interface and ion release may constitute a continuous source of free radicals.

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