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The effect of iron binding on the ability of crocidolite asbestos to catalyze DNA single-strand breaks

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Crocidolite or crocidolite pretreated with desferrioxamine-B (DF crocidolite) was exposed to ferrous chloride solutions to determine whether iron could be bound from solution. Native crocidolite was capable of binding up to 57 nmol Fe²⁺/mg fiber in 60 min, while the DF crocidolite was capable of binding only 5.5 nmol Fe²⁺/mg fiber. The rate of iron binding for the first 5 min of exposure was independent of the concentration of iron in the solution, suggesting that there was a group of rapidly saturable sites, $\sim 1.5 \times 10^{18}$ binding sites/m² crocidolite surface, which were responsible for the immediate binding. This process was followed by a slower binding phase, likely occurring at other sites. Crocidolite and DF crocidolite, with various amounts of iron bound, were assayed for their abilities to catalyze the formation of DNA single-strand breaks (SSBs) in ϕ X174 RFI DNA. Native crocidolite with additional iron bound did not significantly change in its ability to cause DNA SSBs in 15 or 30 min incubations, even though more iron could be mobilized from the iron-treated crocidolite at 4 or 24 h. DF crocidolite, after the addition of iron, had a significantly increased ability to form DNA SSBs. DF crocidolite with 0, 3.0 or 5.5 nmol Fe²⁺/mg catalyzed the formation of DNA SSBs in 21, 42 or 51% of the DNA respectively in the presence of EDTA and ascorbate. Fibers were also incubated in tissue culture medium with or without iron salts. The fibers incubated in the iron-containing medium had an increased ability to form DNA SSBs. These results suggest that fibers such as crocidolite may be capable of binding iron from intracellular sources. This additional iron may be as reactive as the intrinsic iron and may increase the reactive lifetime of the fiber.

Introduction

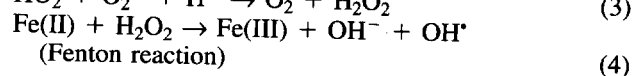
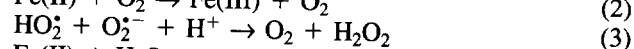
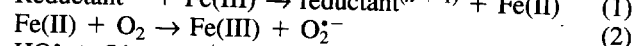
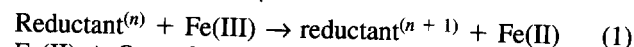
It has been well documented that exposure to crocidolite, the most carcinogenic form of asbestos, increases the risk of mesothelioma of the pleura or peritoneum as well as carcinoma of the lungs, esophagus or stomach (reviewed in 1,2). Results from many studies have indicated that the dimensions of asbestos fibers may be linked to their biological effects after inhalation (3). This hypothesis, however, does not explain the biochemical reactivity of asbestos. The molecular mechanism

for crocidolite carcinogenesis, as well as for fiber carcinogenesis in general, remains obscure.

Recent studies have indicated that the biochemical reactivity and perhaps carcinogenicity of crocidolite may be related to the iron content of the fibers. Iron appears to be involved in several conditions leading to cancer. Persons who suffer from hereditary hemochromatosis (4,5) and porphyria cutanea tarda (6,7), which are iron overload conditions, are at greater risk of developing liver cancer. There is also a correlation between high body stores of iron and an increased risk of developing cancers of all types (8-10).

Crocidolite is a member of the amphibole class of minerals and contains 27% iron by weight (11). Crocidolite is composed of octahedrally coordinated cations sandwiched between two double silicate chains. The oxygen atoms of the silicate chains coordinate both the Si and a variety of other cations, including Mg²⁺, Na⁺, Fe²⁺ and Fe³⁺ (12,13). Minor differences in the overall composition of crocidolite have been reported, depending on the location of mining and on the milling process (14).

Crocidolite has been shown to catalyze the same types of reactions as iron, namely generation of reactive oxygen species (15-17), oxidative damage to DNA (18,19) and lipid peroxidation (20). Lund and Aust have shown that mobilization of iron from asbestos fibers increased the rate at which single-strand breaks (SSBs*) were introduced in DNA (21) and increased crocidolite-dependent oxygen consumption (22). Iron from asbestos has also been reported to be mobilized from crocidolite intracellularly. The amount of iron mobilized into a low molecular weight pool in human lung cells correlated with crocidolite-dependent cytotoxicity (23). Iron which is mobilized from fibers or which is coordinated in accessible sites on the surface of a fiber may be capable of causing damage to biomolecules through the modified iron-catalyzed Haber-Weiss mechanism, shown below (24).



Ascorbate and other cellular reductants potentiate the reactivity of iron. The resulting hydroxyl radicals are known to react with DNA, hydroxylating nucleotide bases and introducing strand breaks (25,26).

The entry and storage of iron in the cell under normal conditions is carefully controlled by the proteins transferrin and ferritin respectively. The inhalation of mineral fibers, which have the capability of binding or releasing iron, may disrupt normal iron metabolism. Fibers which persist in the lung for several decades, such as crocidolite (27), acquire iron on their surfaces. Deposition of large amounts of iron on the surface of a particle leads to the formation of a mature 'ferruginous body', or 'asbestos body' when the core is

*Abbreviations: SSB, single-strand break; desferrioxamine-B, *N*-[5-[3-[5-(aminopentyl)hydroxy-acetamide]pentyl]carbamoyl] propionohydroxamic acid monomethanesulfonate; DF crocidolite, crocidolite which has been incubated for 90 days in desferrioxamine-B to remove iron; ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p*-sulphonic acid; ICP, inductively coupled plasma emission spectrometer; F12-Fe, Ham's F-12 tissue culture medium prepared free of iron salts; F12+Fe, Ham's F-12 tissue culture medium containing 6 μ M FeSO₄.

asbestos, which can be detected even by light microscopy (28–30). The mechanism by which ferruginous bodies are formed is not known. However, it has been proposed that the iron accumulates from macrophages after they phagocytize the fibers and die (29).

Coordination of iron by several crystalline silicates *in vitro* has been demonstrated (31,32) and results from our laboratory with the most carcinogenic mineral fiber erionite are consistent with these reports (33). Ghio *et al.* (31) have shown that crocidolite fibers injected intrapleurally in rats and left for 4 days appeared to acquire iron. They did not determine whether this iron acquisition increased the redox capabilities of the fibers. However, results from our laboratory showed that the iron deposited on mature ferruginous bodies removed from human lungs at autopsy was redox active and was capable of catalyzing the formation of DNA SSBs *in vitro* (34). Since the fibers studied by Ghio *et al.* (31) resided in rats for only 4 days, their observations suggest that iron deposition begins very soon after inhalation of fibers, long before mature ferruginous bodies can be observed. This may represent an early monolayer deposition of iron which may contribute significantly to the biochemical and pathological activities of these fibers.

The present study was undertaken to explore the capabilities of native crocidolite or crocidolite from which some iron had been removed by *N*-[5-[3-[5-(aminopentyl)hydroxy-acetamide]-pentyl]carbamoyl] propionohydroxamic acid monomethanesulfonate (desferrioxamine B) (DF crocidolite) to bind iron from solution. Native crocidolite bound iron from solution more readily than DF crocidolite, but did not show significant changes in its ability to form DNA SSBs, while iron-treated DF crocidolite did. Both native and DF crocidolite incubated in tissue culture medium containing iron showed an increased ability to catalyze the formation of DNA SSBs, under certain conditions. These results suggest that iron can be bound to crocidolite and that additional iron on the fibers increases their potential to catalyze damage to biological molecules.

Materials and methods

Asbestos and reagents

Crocidolite asbestos was obtained from Dr Richard Griesemer (NIEHS/NTP, Research Triangle Park, NC) and contained 26% iron by weight (11). The mean length and diameter of the fibers was 10 and 0.27 μm respectively (11). The density of the fibers was $3.32 \pm 0.08 \text{ g/cm}^3$ and the surface area was $10.1 \text{ m}^2/\text{g}$ (11).

The iron chelator 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p*-sulphonic acid (ferrozine) and ferrous chloride tetrahydrate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Desferoxamine mesylate USP (desferrioxamine-B) was obtained from CIBA (Summit, NJ). Ethidium bromide and the sodium salt of L-ascorbic acid were obtained from Sigma Chemical Co. (St Louis, MO). Ammonium acetate, trichloroacetic acid and NaOH were obtained from EM Science (Cherry Hill, NJ). Nitrogen gas was obtained from Liquid Air (Salt Lake City, UT). Chelex 100 was obtained from Bio-Rad Laboratories (Richmond, CA). Sodium chloride, the disodium salt of EDTA, ferrous sulfate hexahydrate and sodium citrate dihydrate were obtained from Mallinckrodt Inc. (Paris, KY). A custom preparation of Ham's F-12 tissue culture medium, free of added iron salts, was obtained from Gibco BRL (Grand Island, NY). Agarose was obtained from FMC BioProducts (Rockland, ME). Closed-circular, superhelical ϕX174 RFI DNA was obtained from New England Biolabs (Beverly, MA). All remaining reagents were purchased at the highest purity possible.

All NaCl solutions were Chelex-treated before use in the following experiments. DNA was removed from the shipping buffer by ethanol precipitation, as described in Maniatis *et al.* (35), before redissolving in 50 mM NaCl, pH 7.5. Ascorbate solutions were prepared immediately prior to use. All experiments were performed in darkness under red light to prevent the photochemical reduction of iron (36).

Preparation of crocidolite

Native crocidolite was weighed in a hazardous chemicals glovebox and suspended in solution immediately prior to use. Soaked crocidolite was prepared as a control for DF crocidolite by incubation at 1 mg/ml in sodium bicarbonate solution (36 mM) for 90 days. Assays for iron in the supernatant indicated that no iron was leached from the fibers during this period. DF crocidolite was prepared by chelation treatment with desferrioxamine-B (1 mM) in NaCl (50 mM, pH 7.5) for 90 days, as previously described (37). Assays of the supernatant after treatment were performed by USU Analytical Laboratories (Logan, UT) using a Thermojarrell Ash ICAP 9000 inductively coupled plasma emission spectrometer (ICP). ICP analysis of the supernatant of the desferrioxamine-B treatment revealed that $2.66 \pm 1 \text{ p.p.m.}$ silicon was dissolved in the supernatant. The general formula of crocidolite is $[\text{Na}_2\text{Fe}^{(\text{III})}_2\text{Fe}^{(\text{II})}_3\text{Si}_8\text{O}_{22}(\text{OH})_2]$. The amount of silicon in crocidolite comprises 30% of the total weight. The total amount of crocidolite lost to dissolution during incubation was calculated based on the amount of silicon which dissolved. These calculations demonstrated that only 1% of the total mass of crocidolite was dissolved during the treatment.

Iron binding to crocidolite

Ferrous chloride solutions were prepared by dissolution of FeCl_2 into deionized water which had been purged of dissolved oxygen by bubbling with nitrogen gas for 15 min. Solutions were maintained under anaerobic conditions for the duration of the binding assay to reduce the risk of ferrous oxidation and subsequent formation of ferric hydroxides and oxyhydroxides. Crocidolite, soaked crocidolite and DF crocidolite were suspended at 1 mg/ml in FeCl_2 (25, 50 and 100 μM) in the dark. At 5, 15, 30, 60 or 120 min, fiber suspensions were centrifuged at $11\,000\times g$ in a Fisher Scientific 59A microcentrifuge for 3 min to remove the fibers from the supernatant. The supernatant was assayed spectrophotometrically for iron using the total iron assay with ferrozine, as previously described (38). The amount of iron bound to the fibers was calculated as the iron concentration in the supernatant at the time of suspension minus the iron concentration in the supernatant after the indicated time period. Surface coverage was calculated based upon the molar amount of iron bound per milligram crocidolite, knowing the average surface area of the NIEHS sample of crocidolite to be $10.1 \text{ m}^2/\text{g}$ (11). Controls showed that no iron adsorbed to the plastic Eppendorf tubes used for these studies. No iron leached from the crocidolite suspended in deoxygenated water or dilute HCl solutions of the same pH (pH 6.8) as the FeCl_2 solutions during the 1 h incubations.

Determination of iron concentrations

The concentration of ferrous iron in solutions before treatment of fibers was determined by a modification of the total iron assay. Ferrous iron-containing solutions were added directly to ferrozine (70 mM) in the absence of a reductant or any other reagents. The colored ferrous-ferrozine complex was assayed spectrophotometrically as in the total iron assay at 562 nm using a Shimadzu UV160U UV-visible recording spectrophotometer. When assaying for total iron, both $\text{Fe}(\text{II})$ and $\text{Fe}(\text{III})$, in the solution, the total iron assay was performed exactly as described previously (38). The amount of $\text{Fe}(\text{II})$ in solution after loading was always equal to the total amount of iron in the solution, indicating that the iron was not oxidized to the ferric state during the incubation period.

Identification and quantification of ions in solution

Ions released into the supernatant during ferrous ion loading were determined by the USU Analytical Laboratories (Logan, UT) using a Thermojarrell Ash ICAP 9000 ICP. Iron remaining in solution following the binding incubation, as determined by ICP, was in good agreement with the results of the total iron assay.

Induction of DNA single-strand breaks by crocidolite

After crocidolite or DF crocidolite had been incubated in FeCl_2 solutions for 60 min, the fibers were washed five times to remove loosely associated iron. Washing was performed by suspension in distilled-deionized water and centrifugation at $11\,000\times g$ for 3 min. Examination of the washing supernatant showed that no ferrous ions were removed from the fibers during the washing procedure. Fibers were then resuspended in NaCl (50 mM, pH 7.5), adjusted to pH 7.5 and assayed for their ability to catalyze the formation of SSBs in ϕX174 RFI DNA, as described previously (21). Washed fibers (20 μg) were incubated with ϕX174 RFI DNA (0.25 μg). All SSB assays reported here were performed in the presence of 1 mM ascorbate, with or without 1 mM citrate or 1 mM EDTA.

When native or soaked crocidolite were assayed for their ability to induce DNA SSBs in the presence of EDTA, the fibers were incubated with DNA for 15 min in the dark. This length of incubation prevented introduction of DNA SSBs in all of the DNA. All other incubations were for 30 min in the dark. After the incubation, DNA with SSBs was separated from closed-circular, superhelical DNA (RFI) using agarose gel electrophoresis. The amount

of DNA with SSBs was quantified using integrated scanning densitometry as previously described (21) and expressed as percent of untreated control.

Mobilization of iron from crocidolite

Crocidolite treated with iron, as previously described, was suspended at 1 mg/ml in 1 mM citrate or EDTA solutions. The pH of the solutions was adjusted to 7.5 every 15 min for the first 60 min and every hour for the first 8 h. After incubation for 4 or 24 h, fibers were centrifuged at $11\,000\times g$ in a Fisher Scientific 59A microcentrifuge for 3 min. The supernatant was assayed by the total iron assay. The amount of iron mobilized in the indicated time periods is expressed as nmol Fe/mg crocidolite.

Iron binding from tissue culture medium

Two types of tissue culture media were used for these studies, Ham's F-12 containing no Fe_2SO_4 (F12-Fe) or F-12 containing 6 μM Fe_2SO_4 (F12+Fe). The iron-containing F-12 was prepared by adding $\text{Fe}_2\text{SO}_4\cdot 6\text{H}_2\text{O}$ to the powder of the iron-free medium before dissolution. Crocidolite or DF crocidolite was suspended (10 $\mu\text{g}/\text{ml}$) in F12+Fe or F12-Fe for 1 h in the dark with shaking. The fibers were removed from the dilute suspension by centrifugation at $160\,000\times g$ with a Beckman SW 40 Ti rotor. The recovered fibers were resuspended in 50 mM NaCl, pH 7.5, at 1 mg/ml and washed five times as described above (Iron binding to crocidolite) and resuspended in NaCl (50 mM, pH 7.5) for use in the single-strand break assay, as described previously.

Statistical analysis

Results are expressed as the mean \pm SD. The significance of the difference between two means was calculated using Student's *t*-test. Mean values were considered significantly different when $P < 0.05$.

Results

Ability of crocidolite to bind iron

Initial studies were performed using FeCl_3 solutions at pH 4.0. After exposure to crocidolite, assays of the FeCl_3 solutions using the total iron assay with ferrozine indicated that iron was being removed from the solution, suggesting that it was binding to the fibers. However, atomic absorption analysis of the same solutions showed that the iron was still present and was not bound to the fibers (unpublished data). This suggested that the ions were beginning to associate into ferric hydroxide polymers, which have been reported to begin forming immediately in pH 3–10 aqueous solutions (39). The iron in this form was unavailable for chelation and detection using the total iron assay. These ferric hydroxides and oxyhydroxides are likely to precipitate onto the surface of the fibers, but are unlikely to occur intracellularly.

It has been suggested that most intracellular iron exists in the ferrous state, because the cell maintains a reducing environment and because most iron-containing and storage proteins require iron to exist in the reduced state for incorporation (40,41). Therefore, ferrous iron was used for the studies reported here to avoid possible artifacts due to the formation of ferric hydroxide polymers and to more closely reflect intracellular conditions.

Native crocidolite fibers were compared with DF crocidolite and soaked crocidolite for their abilities to bind iron from FeCl_2 solutions. Since iron can be mobilized from crocidolite by cultured human lung cells (23), DF crocidolite may serve as a model for fibers which have existed under similar conditions in the body. As a control for DF crocidolite, native fibers were incubated in aqueous solution for the same period of time in the absence of desferrioxamine-B. As shown in Figure 1, crocidolite, soaked crocidolite and DF crocidolite were capable of acquiring iron from ferrous chloride solutions, but crocidolite and soaked crocidolite always acquired more iron than did DF crocidolite. The soaked fibers demonstrated nearly identical capacities to acquire iron from solution as the native fibers.

When the native or soaked crocidolites were incubated in

anaerobic 25 or 50 μM FeCl_2 , all iron was removed from solution by the crocidolite, so that 25 or 50 nmol Fe^{2+}/mg crocidolite were bound after 1 h incubations (Figure 2). Upon incubation in 100 μM FeCl_2 , ~57% of the iron bound to the native or soaked crocidolite (Figure 1). Native crocidolite fibers incubated for 1 h in aqueous solutions without iron showed no mobilization of iron from the fibers into the solution. DF crocidolite had a significantly diminished ability to bind iron from FeCl_2 solution. Incubation of the DF crocidolite in 25, 50 or 100 μM FeCl_2 for 1 h resulted in binding of only 3.0 ± 0.6 , 4.4 ± 0.9 or 5.5 ± 0.8 nmol Fe^{2+}/mg DF crocidolite respectively (Figure 1).

The average surface area of the crocidolite sample used was $10.1\text{ m}^2/\text{g}$ (11). Assuming an even distribution of bound iron on the surface of the fiber and knowing the average surface area, the surface coverage of ferrous ions bound to the fibers was calculated. The number of ions bound to the fibers incubated in FeCl_2 solutions ranged from 1.8×10^{17} to 3.5×10^{18} iron atoms/ m^2 crocidolite. DF crocidolite incubated in 25 μM

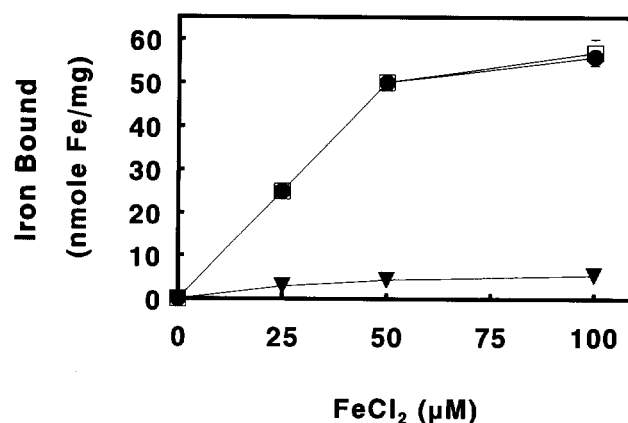


Fig. 1. Ferrous binding by crocidolite from FeCl_2 solutions. Native (\square), soaked (\bullet) or DF crocidolite (\blacktriangledown) was suspended at 1 mg/ml in the indicated concentrations of FeCl_2 solutions, the amount of iron bound in 1 h was determined as described in Materials and methods. Data are presented as the mean \pm SD ($n = 3$). The absence of standard deviation markers indicates that the SD is contained within the symbol.

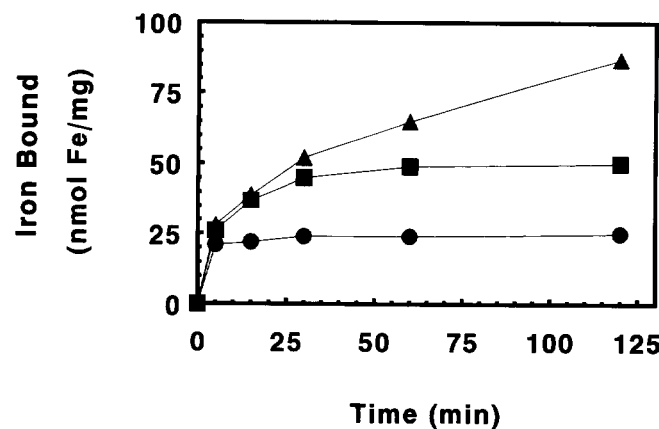


Fig. 2. Kinetics of iron binding to native crocidolite. Native crocidolite was suspended at 1 mg/ml in 25 μM (\bullet), 50 μM (\blacksquare) or 100 μM (\blacktriangle) FeCl_2 solutions. The amount of iron bound to the fibers was determined as described in Materials and methods at the indicated time points. Data are presented as the mean \pm SD ($n = 3$). The absence of standard deviation markers indicates that the SD is contained within the symbol.

FeCl₂ exhibited the lowest surface coverage and native crocidolite which had been incubated in 100 µM FeCl₂ had the greatest surface coverage.

Cations released into the supernatant during the loading process were determined by ICP analysis. During binding of 57 nmol Fe/mg crocidolite, 6.5 ± 1 nmol Mg²⁺, 11.9 ± 6 nmol Ca²⁺, 4.9 ± 6 nmol Na⁺ and 0.66 ± 0.1 nmol Mn²⁺, all ions which are known to be constituents of the crocidolite sample used (11), were released per milligram crocidolite and were observed in the supernatant.

Kinetics of ferrous binding to native crocidolite

To determine the rates at which iron was bound to native crocidolite fibers, the amount of iron bound from FeCl₂ solutions was assayed after 5, 15, 30, 60 or 120 min incubation. As shown in the results in Figure 2, the most rapid binding phase occurred during the initial 5 min at all three concentrations of FeCl₂ solutions. The initial binding rate from the 50 and 100 µM solutions, estimated using the amount of iron bound in the first 5 min, was 5 nmol Fe²⁺/mg crocidolite/min. In the 25 µM solution the initial binding rate was slightly lower, 4.3 nmol Fe²⁺/mg crocidolite/min. The binding rate at all incubation concentrations decreased following the initial 5 min, but iron binding to the crocidolite continued until all of the iron from solution had been bound, except from the 100 µM solution, where 88 nmol Fe bound in 2 h.

Considering the ions bound in the first 5 min to be occupying the sites of highest affinity allowed calculation of the number of binding sites to which ferrous ions are bound most rapidly. These calculations indicate that 1.49×10^{18} such binding sites exist per square meter of crocidolite.

Effect of additional iron on the ability of crocidolite to catalyze the formation of DNA SSBs

The results in Table I show that in the presence of ascorbate, with or without citrate or EDTA, crocidolite or soaked crocidolite with 0, 25 or 57 nmol Fe²⁺/mg additional iron catalyze the introduction of the same amounts of DNA SSBs. Although the total amount of iron bound to DF crocidolite was much lower than the amount bound to the native or soaked crocidolite, the binding resulted in significant increases in fiber reactivity.

The addition of a chelator, citrate or EDTA, increased the strand-break activity of all forms of crocidolite, with EDTA

causing the greatest increase. As before, no significant differences were observed between native crocidolite and soaked crocidolite, and additional iron appeared to have no effect. There was, however, a relationship between the amount of iron added to DF crocidolite and the number of DNA SSBs induced. The addition of 5.5 nmol Fe²⁺/mg to DF crocidolite more than doubled the number of SSBs observed when no iron was added. The DNA SSB activity before and after iron incubation could be completely inhibited by addition of 1 mM desferrioxamine-B to the DNA reaction mixture, indicating that the SSB reactivity was due to iron (unpublished data). Control experiments with native, soaked or DF crocidolites in the absence of chelators and ascorbate showed no detectable SSBs (unpublished data), as has been previously reported for native crocidolite (21).

Effect of additional iron on the amount of iron mobilized from crocidolite

The apparent inability of additional iron on native crocidolite to catalyze greater numbers of DNA SSBs may have been a consequence of the large amount of iron already present on the fibers combined with the sensitivity of the DNA SSB assay. The amount of iron bound to crocidolite in 1 h was relatively small compared with the amount of intrinsic iron and made an insignificant contribution to the concentration of iron mobilized in the DNA SSB assay conducted for 15 or 30 min.

Lund and Aust (21) have previously shown that the amount of iron mobilized from crocidolite by chelators directly correlated with the amount of SSBs introduced in ϕ X174 DNA *in vitro*. To determine the effect that iron acquired by native crocidolite fibers had on the amount of iron that could be mobilized, crocidolite loaded with 57 nmol Fe/mg fibers was incubated in EDTA or citrate solution and the amount of iron mobilized after 4 or 24 h was compared with that mobilized from native fibers which had not been previously loaded with iron. As shown in Table II, more iron was mobilized in 4 or 24 h from fibers which had been treated with iron than from native fibers. Statistically significant increases were seen for both chelators at 4 and 24 h. When native or iron-treated crocidolite were incubated for the same periods of time in the absence of a chelator, no iron was mobilized into solution. Although additional iron bound to native crocidolite did not confer an

Table I. Formation of DNA single-strand breaks by native, soaked and DF crocidolite after ferrous binding

Chelator ^a	Crocidolite		Soaked crocidolite		DF crocidolite	
	Fe ²⁺ bound ^b	%DNA SSB ^c	Fe ²⁺ bound ^b	%DNA SSB ^c	Fe ²⁺ bound ^b	%DNA SSB ^c
None	0	17±3	0	11±3	0	8±4
	25	16±5	25	16±3	3.0	12±3
	57	14±3	56	13±3	5.5	20±3 ^e
Citrate	0	38±1	0	43±3	0	12±7
	25	40±8	25	45±3	3.0	18±7
	57	40±8	56	44±4	5.5	27±7 ^f
EDTA ^d	0	45±5	0	47±3	0	21±3
	25	52±6	25	48±4	3.0	42±2 ^e
	57	52±4	56	51±4	5.5	58±1 ^e

^aIncubation conditions included ascorbate (1 mM), chelator (1 mM) and 0.25 µg ϕ X174 RFI DNA in 50 mM NaCl, pH 7.5.

^bIron bound is expressed as nmol Fe²⁺/mg crocidolite.

^cResults are expressed as the mean±SD (*n* = 3) and are relative to the control, untreated DNA.

^dNative and soaked fibers exposed to EDTA + ascorbate were incubated for 15 min. All other incubations were for 30 min.

^eSignificantly different from corresponding fiber before iron binding (*P* < 0.01).

^fSignificantly different from corresponding fiber before iron binding (*P* < 0.05).

enhanced ability to form DNA SSBs in a short-term 15–30 min exposure to DNA, the additional iron that can be mobilized over longer incubation times would be expected to damage DNA (21).

Effect of incubation in tissue culture medium on DNA SSB formation by crocidolite

In order to assay the ability of crocidolite and DF crocidolite to acquire iron from a complex, more physiologically relevant solution, crocidolite fibers were incubated in F12+Fe or F12-Fe tissue culture media. The reactivity of the fibers incubated in media was assayed by the DNA SSB assay. Since no differences had been observed in previous studies between soaked and native crocidolite fibers, only native and DF crocidolite were used in this study. As shown by the results in Figure 3, both native and DF crocidolite had an enhanced ability to induce DNA SSBs after incubation in an iron-containing tissue culture medium compared with incubation in an iron-free medium. The enhanced reactivity of native crocidolite was only observed in the presence of ascorbate. The increases in DNA SSB activity of native crocidolite after incubation in iron-containing medium were 5, 3 or 7% under assay conditions of ascorbate alone, ascorbate and citrate or ascorbate and EDTA respectively. When DF crocidolite was

incubated in iron-containing medium, the fibers exhibited an increase in DNA SSB activity of 6, 13 or 24% when assayed under the conditions of ascorbate alone, ascorbate and citrate or ascorbate and EDTA respectively. When the assay was performed with or without a chelator in the absence of a reductant, no detectable DNA SSBs were observed (unpublished data). The ability of native crocidolite or DF crocidolite to induce SSBs under all conditions was completely inhibited by addition of desferrioxamine-B to the reaction mixture (unpublished data).

Discussion

Results shown here indicate that native, soaked or DF crocidolites were capable of acquiring additional iron from simple FeCl_2 solutions and native or DF crocidolite from more complex solutions, such as tissue culture media. Iron binding from simple solutions provides a means for studying the kinetics of iron binding. The studies presented here suggest that there are a certain number of saturable sites on the surface of native crocidolite which are filled in the early, most rapid binding phase. These sites are likely to be unoccupied coordination sites or uneven areas in the microtopography of the fiber which appear along cleavage or termination planes in the mineral (42). Other sites which still contain cations are more likely to be involved in the slower phase of iron coordination. Figure 4 shows the M1, M2, M3 and M4 binding sites in the crocidolite crystal structure (43,44). M1, M2 and M3 are generally filled with ions 0.5–0.9 Å in radius, such as Mg^{2+} , Fe^{2+} and Fe^{3+} . Additional ferrous ions (ionic radius 0.76 Å) would be likely to replace Mg^{2+} ions or fill M1, M2 or M3 sites that are not occupied along cleavage planes in the

Table II. Mobilization of iron from iron-treated crocidolite by citrate and EDTA

Time	Iron mobilized (nmol Fe/mg crocidolite) ^a			
	Citrate		EDTA	
	Native	Iron-treated	Native	Iron-treated
4 h	9±0.1	11±0.2 ^b	31±0.4	42±.2 ^b
24 h	24±0.4	38±0.1 ^b	57±0.6	85± 0.1 ^b

^aResults are expressed as the mean±SD ($n = 3$).

^bSignificantly different from fiber before iron treatment ($P < 0.05$).

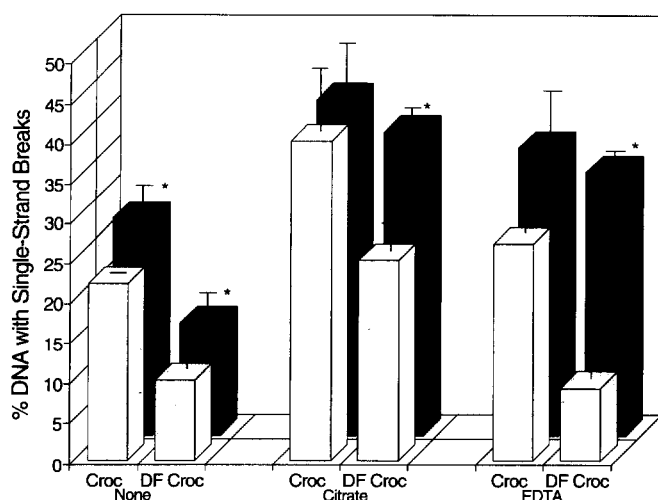


Fig. 3. Effect of incubation in tissue culture media on the ability of native and DF crocidolites to catalyze DNA SSBs. Crocidolite (Croc) or DF crocidolite (DFCroc) was incubated for 1 h in F-12 media (light grey bars) or F12-Fe (black bars). The fibers were subsequently incubated with DNA in the presence of ascorbate and the indicated chelator, as described in Materials and methods. The data are shown as the mean percent DNA with SSBs ± SD ($n = 3$). Bars marked with * indicate the values are significantly different ($P < 0.05$).

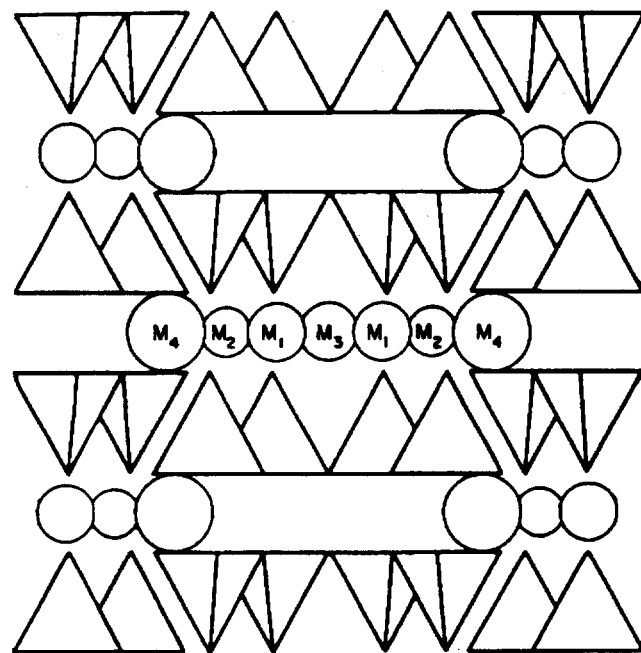


Fig. 4. Structure of crocidolite. This is an idealized diagram of the structure of crocidolite, $[\text{Na}_2\text{Fe}^{(\text{III})}_2\text{Fe}^{(\text{II})}_3\text{Si}_8\text{O}_{22}(\text{OH})_2]$, after that of Earnst (44). Triangles represent the silicon-oxygen tetrahedra which are linked together into infinite one-dimensional chains. Triangles containing vertical lines are the tetrahedra coming out of the plane. Empty triangles are tetrahedra directed into the plane. The circles depict the various cation binding sites (M_1 , M_2 , M_3 and M_4), labeled only in the center of the diagram, which are present between the silicate chains within the crocidolite structure.

mineral fiber. M4 coordination sites are generally occupied by ions 0.7–1.1 Å in radius. Sodium ions are the principal candidates from the idealized structure of crocidolite for the M4 binding site. Ferrous ions are likely to exchange with Na⁺ cations from these M4 coordination sites.

An important effect of iron binding to mineral fibers was that the total iron content increased as the fiber was incubated in iron-containing solutions. Mobilization of iron by citrate or EDTA from iron-treated fibers was significantly greater than from native fibers after 4 or 24 h incubation. However, no increases in the amount of DNA SSBs were observed after 15 or 30 min incubations with DNA for the iron-treated crocidolite compared with native crocidolite. This was probably due to the fact that the native fibers have considerable amounts of iron that can be mobilized to damage DNA, which would make it more difficult to detect a significant increase from the acquired iron mobilized in this short time period.

DF crocidolite exhibited an increased ability to catalyze the formation of DNA SSBs after ferrous ions were bound. This finding is similar to that of Adachi *et al.* (45), who reported that DF crocidolite had an increased ability to form 8-hydroxydeoxyguanosine in the presence of iron-containing solutions. Desferrioxamine-B treatment removed the easily chelated iron from the surface of the fibers, thus greatly reducing their strand break activity. This may have been why it was easier to detect the presence of the iron bound from solution in the subsequent DNA strand break assays than for native crocidolite, which had much higher activity before addition of any iron. During the slow, but observable, dissolution which occurred during chelation treatment, the rapidly filled coordination sites along cleavage planes are likely to have been among the 1% of the fiber which dissolved (42). This may have contributed to the fact that a smaller quantity of iron was bound to the DF crocidolite compared with the amount which bound to native crocidolite incubated in FeCl₂ solutions. However, this iron significantly increased the ability of the DF crocidolite to cause DNA damage, suggesting that small changes in the iron content of fibers may have notable effects on their biochemical reactivity. In the lung, crocidolite which has had iron removed may become re-enriched and regain former or acquire elevated damaging potential.

Desferrioxamine-B has been reported to bind to crocidolite during chelation treatment (46). Binding of desferrioxamine-B may have occurred during the pretreatment used in these studies. The presence of desferrioxamine-B would not have enhanced the DNA SSB activity of the fibers, since ferric iron bound to desferrioxamine-B does not redox cycle to produce reactive oxygen species (41). The increase in SSB activity observed after ferrous ion treatment indicated that additional iron bound to the fiber was readily available for chelation and mobilization by EDTA or citrate during the DNA SSB assay, since the presence of either of these chelators enhanced DNA damage, as has previously been observed for native crocidolite (21).

The observation that both native crocidolite and DF crocidolite were capable of causing more DNA SSBs after incubation in F12+Fe than incubation in F12–Fe strongly suggests that the fibers were able to bind iron from the tissue culture medium, since the singular difference between F12–Fe and F12+Fe was the iron. This suggests that in an aqueous environment like that of the cell, or extracellular space, inhaled durable fibers may also be able to bind iron chelated to organic acids, intermediates in metabolic processes or amino acids,

which can all coordinate iron (47–49). This could potentiate the damaging nature of the fiber, since the fiber may carry the newly acquired iron from cells, such as macrophages, to the target cells for tumor formation. It should also be noted that iron in tissue culture medium can bind to fibers, affecting the reactivity of fibers and possibly results of experiments performed in culture medium.

Results presented here demonstrate that iron can be bound at the same rate to crocidolite fibers in their native, freshly suspended form and after soaking for 90 days in a non-mobilizing, aqueous environment. It is well known that the dissolution kinetics of the silicate structure amphiboles, such as crocidolite, are very slow (42). These results suggest that acute dissolution, which occurs within the first three months after suspension in aqueous solution, has a negligible effect on the reactivity of the intrinsic iron on the fiber. The ability of the fiber to acquire iron from solution and to catalyze the formation of DNA SSBs was also not affected by aqueous suspension, suggesting that no gross structural changes occurred to the fibers as a result of aqueous suspension alone.

The results presented here for Fe(II) binding to crocidolite are consistent with observations by Ghio *et al.* (31,32), who have studied binding of Fe(III) to several silicates, including crocidolite. They observed that the ability of silicates to catalyze lipid peroxidation and DNA damage increased as iron bound to the fibers (31). They concluded that the iron was binding to the silanol groups on the surface of the silicates and proposed that one potential method for determining whether fibers will be health hazards is to analyze for the concentration of silanol groups on their surfaces. The amount of Fe(II) that we observed bound to the high affinity sites on crocidolite, 1.5×10^{18} sites/m², or the total amount bound, 3.5×10^{18} sites/m², is within the range of the number of silanol groups reported to be present on crocidolite, 4.7×10^{18} silanol groups/m² (50). Although this may have some merit in identifying hazardous materials, it is by no means definitive, since the amount of Fe(III) that Ghio *et al.* (32) observed bound to crocidolite in 1 h ranged from 3.0×10^{16} to 2.4×10^{19} atoms/m². In addition, studies in our laboratory have shown that erionite, the most carcinogenic naturally occurring mineral fiber, bound as much or more iron than crocidolite (33), but has been reported to have ~1000-fold less silanol groups (51). Thus, it would appear that identification of the iron binding sites responsible for the fiber reactivity is yet to be determined.

Evidence is accumulating to suggest that iron-catalyzed reactions of asbestos and other mineral fibers may be related to some of their pathological effects. The iron responsible for these effects may be intrinsic to the fiber or acquired, as discussed here. It may be that acquisition of iron will be more important in determining the biochemical reactivity of fibers which reside for decades in the lung. Determining the ability of fibers to bind iron, the biochemical reactivity of the acquired iron and the ease with which acquired iron can be mobilized using assays such as those described here may provide important insights into their pathological activity in laboratory animals and in man.

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