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Iron Mobilization from Asbestos by Chelators and Ascorbic Acid

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The ability of chelators and ascorbic acid to mobilize iron from crocidolite, amosite, medium- and short-fiber chrysotile, and tremolite was investigated. Ferrozine, a strong Fe(II) chelator, mobilized Fe(II) from crocidolite (6.6 nmol/mg asbestos/h) and amosite (0.4 nmol/mg/h) in 50 mM NaCl, pH 7.5. Inclusion of ascorbate increased these rates to 11.4 and 4.9 nmol/mg/h, respectively. Ferrozine mobilized Fe(II) from medium-fiber chrysotile (0.6 nmol/mg/h) only in the presence of ascorbate. Citrate and ADP mobilized iron (ferrous and/or ferric) from crocidolite at rates of 4.2 and 0.3 nmol/mg/h, respectively, which increased to 4.8 and 1.0 nmol/mg/h in the presence of ascorbate. Since ascorbate alone mobilized iron from crocidolite (0.5 nmol/mg/h), the increase appeared to result from additional chelation by ascorbate. Citrate also mobilized iron from amosite (1.4 nmol/mg/h) and medium-fiber chrysotile (1.6 nmol/mg/h). Mobilization of iron from asbestos appeared to be a function not only of the chelator, but also of the surface area, crystalline structure, and iron content of the asbestos. These results suggest that iron can be mobilized from asbestos in the cell by low-molecular-weight chelators. If this occurs, it may have deleterious effects since this could result in deregulation of normal iron metabolism by proteins within the cell resulting in iron-catalyzed oxidation of biomolecules. © 1990 Academic Press, Inc.

Exposure to asbestos fibers results in an increased risk of mesothelioma of the pleura or peritoneum, or carcinoma of the lungs, esophagus, or stomach (1). There is a relationship between the size of the fibers and the carcinogenic potency (2). Investigations in animal model systems have shown that fibers less than 0.25 μm in diameter and longer than 8 μm are the most carcinogenic (3–5). However, this does not explain the molecular mechanism by which asbestos causes cancer. One potentially important observation is that asbestos contains the transition metal iron to levels as high as 36% by weight (6). The amphibolic crystalline form of asbestos (e.g., crocidolite and amosite) consists of parallel chains of silica tetrahedra which are separated by bands of cations, such as Fe(II), Fe(III), Mg, and/or Na (7). The serpentine crystalline form of asbestos (e.g., chrysotile) is comprised of overlapping sheets of silica and brucite [$Mg(OH)_2$], in which the Mg can be replaced by Fe(II) (8, 9). In addition, iron has been shown to contaminate the surface of asbestos. The degree of contamination is dependent upon where the asbestos was mined and the extent to which the asbestos was milled (8).

Iron has been implicated as a causative agent in human cancer. An increased incidence of lung cancer has been observed in iron ore miners (10, 11). Two primary iron overload conditions, hereditary hemochromatosis (12, 13) and porphyria cutanea tarda (14, 15), are associated with an increased risk of liver cancer. Increased body iron stores in individuals without iron overload conditions have been correlated with an increased risk of cancer (16–18).

One way in which iron might cause cancer is by damaging DNA. It is well known that iron catalyzes deleterious oxidation reactions which damage DNA, lipid, and protein (19). Likewise, asbestos is capable of catalyzing the production of reactive oxygen species (20, 21), DNA damage (22–24), and lipid peroxidation (25, 26). Whether the iron remains associated with the asbestos and catalyzes oxidative damage, or must be mobilized in order to catalyze damage is unknown. If the iron remains with the asbestos, the potential for catalysis of DNA damage and lipid peroxidation is limited to the immediate vicinity of the fiber. However, if the iron from asbestos is mobilized, the redox activity of the iron may be altered and the sites for intracellular damage may become unlimited. Iron and other minerals dissociate from asbestos *in vivo* (7, 27, 28), but the chemical nature of the chelates of these minerals is unknown. Moreover, the chemical identity of low-molecular-weight, intracellular iron chelators, in general, is a subject about which

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little is known. It is possible that the iron mobilized from asbestos is bound to proposed physiological low-molecular-weight chelators, such as citrate, acetate, or phosphate compounds (29). The purpose of this investigation was to determine the ability of these chelators to mobilize iron from different forms of asbestos *in vitro*.

It will be shown that a chelator (e.g., citrate or ADP) had to be present to mobilize iron from asbestos. In addition, reduction of the iron did not result in enhanced mobilization by citrate or ADP. Iron mobilization from asbestos was also dependent upon the chelator and the pH of the solvent and dependent upon the surface area, the iron content, and the crystalline structure of the asbestos being studied. These results suggest that iron can be mobilized from asbestos by low-molecular-weight chelators in the cell. This may be deleterious since mobilization of iron by low-molecular-weight chelators rather than proteins represents deregulation of normal iron metabolism.

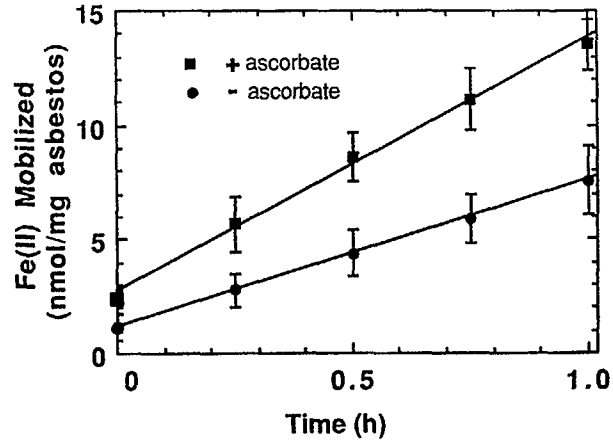

FIG. 1. Fe(II) mobilization by ferrozine from crocidolite. Crocidolite (1 mg/ml) was incubated in 50 mM NaCl, pH 7.5, with 1 mM ferrozine in the presence (■) or absence (●) of 1 mM ascorbate for 1 h as described under *Fe(II) mobilization by ferrozine* in the Materials and Methods section.

MATERIALS AND METHODS

Asbestos and reagents. Samples of crocidolite, amosite, medium- and short-fiber chrysotile, and tremolite were obtained from Dr. Richard Griesemer, NIEHS/NTP (Research Triangle Park, NC).

The sodium salts of L-ascorbic acid and ADP (grade III) and L-histidine were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium citrate was obtained from Mallinckrodt, Inc. (Paris, KY). Ammonium acetate, trichloroacetic acid, and NaOH were obtained from EM Science (Cherry Hill, NJ). Tris 'Baker' and L-tryptophan were from J. T. Baker, Inc. (Phillipsburg, NJ). The iron chelator, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid (ferrozine) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Sodium phosphate (dibasic) and ferric chloride were obtained from Fisher Scientific (Fair Lawn, NJ). Chelex 100 was obtained from Bio-Rad Laboratories (Richmond, CA). Contaminating metals were removed from the NaCl solutions and the distilled–deionized water used for making other solutions by chromatography on Chelex 100.

Fe(II) mobilization by ferrozine. Asbestos (1 mg/ml) was suspended (final volume, 20 ml) and mixed by vortexing for 30 s in 50 mM NaCl, Tris, or sodium phosphate, pH 7.5, containing 1 mM ferrozine. Ascorbate (1 mM) was added to designated samples, and all samples were placed on a wrist-action shaker and kept in the dark for up to 36 h. At regular time intervals 1.5-ml samples were withdrawn and centrifuged at 14,000 rpm in an Eppendorf 5415 centrifuge for 2 min to remove the asbestos. The amount of Fe(II) mobilized as the ferrozine:Fe(II) complex was determined by measuring the absorbance of the supernatant at 562 nm (E_{mM} = 27.9 mM^{-1} cm^{-1} (30)) using a Cary 219 dual beam spectrophotometer. The pH increased in experiments performed in 50 mM NaCl, depending upon the form of asbestos being used. Therefore, the pH was readjusted at regular time intervals throughout the incubation period to prevent alteration in the rates of iron mobilization. The concentrations of iron mobilized by ferrozine were plotted versus time and the slopes of the lines were calculated using computer-assisted linear regression and were reported as nmol Fe(II) mobilized/mg asbestos/h.

Total iron mobilization. Asbestos (1 mg/ml) was incubated in 50 mM NaCl, pH 7.5, as described under *Fe(II) mobilization by ferrozine*, except that ferrozine was replaced with the indicated chelator (1 mM). Samples (1.0 ml) were withdrawn at regular time intervals, centrifuged at 14,000 rpm in an Eppendorf 5415 centrifuge, and the total iron present in the supernatant was determined as described by Brumby and Massey (31) for nonheme iron determination, with the following exception. Ferrozine (0.4%, w/v) was used instead of 1,10-phenanthroline. After a 45-min incubation, the absorbance at 562 nm was measured using a Cary 219 dual beam spectrophotometer. The concentration of iron mobilized and subsequent rate of mobilization were calculated as described in the preceding section.

RESULTS

Effect of chelation on Fe(II) mobilization. As shown in Fig. 1, incubation of crocidolite with ferrozine resulted in Fe(II) mobilization (6.6 nmol/mg/h) and the addition of ascorbate increased the rate 1.7-fold (11.4 nmol/mg/h). Ferrozine was used for the initial experiments because of its high affinity for Fe(II) and the high extinction coefficient of the ferrozine:Fe(II) complex (30). A linear relationship existed between the amount of Fe(II) mobilized by 1 mM ferrozine from crocidolite (1 mg/ml) in 50 mM NaCl, pH 7.5, and the time of incubation (Fig. 1). The rates of Fe(II) mobilization by ferrozine from different forms of asbestos were compared. The results in Table I show that ferrozine mobilized Fe(II) from crocidolite and amosite, but not medium-fiber chrysotile, short-fiber chrysotile, or tremolite. The rates of mobilization were linear for all forms of asbestos during the time periods studied. In the absence of ferrozine, no mobilization of Fe(II) could be detected using the *Total iron mobilization* assay (data not shown).

Effect of pH on Fe(II) mobilization. Since pH is known to influence the redox potential and the solubility of iron, the effect of pH on the rate of Fe(II) mobilization from crocidolite by ferrozine was determined. Crocidolite was selected for this study because it showed the greatest rate of Fe(II) mobilization. The results in Fig. 2 demonstrate an inverse relationship between the rate of Fe(II) mobilization and pH. Since pH had such a dramatic effect on Fe(II) mobilization, iron mobilization was studied in 50 mM Tris or sodium phosphate buffers,

TABLE I

Iron Mobilization from Different Forms of Asbestos

Form of asbestos	Ferrozine (nmol/mg/h)		Citrate (nmol/mg/h)	
	−Ascorbate	+Ascorbate	−Ascorbate	+Ascorbate
Crocidolite [36%][a]	6.6[b] (1.2)[c]	11.4[b] (0.9)	4.2 (0.5)	4.8 (0.9)
Amosite [36%]	0.4	4.9 (0.6)	1.4	ND[d]
Medium-fiber chrysotile [2.9%]	0	0.6	1.6	ND
Short-fiber chrysotile [2.0%]	0	0	0	ND
Tremolite [0.27%]	0	0	0	ND

Note. Asbestos (1 mg/ml) was suspended in 50 mM NaCl, pH 7.5, with the indicated chelator (1 mM) in the presence or absence of 1 mM ascorbate for up to 36 h. A sample was removed at regular time intervals, centrifuged at 14,000 rpm in an Eppendorf 5415 centrifuge, and the amount of iron in the supernatant determined spectrophotometrically using a Cary 219 dual beam spectrophotometer. Ferrozine:Fe(II) was measured directly (562 nm) while citrate:iron was determined as described under Materials and Methods for *Total iron mobilization.* Rates of mobilization were calculated using computer-assisted linear regression of amount of iron mobilized versus time.

[a] Numbers in brackets indicate the percentage iron by weight in asbestos (6).

[b] Data taken from Fig. 1.

[c] Numbers in parentheses represent standard deviations. In the case where no standard deviation is listed, the number is an average of two experiments.

[d] ND, not determined.

pH 7.5, for up to 25 h in the presence or absence of ascorbate. The results in Table II show that Fe(II) mobilization by ferrozine decreased 32% (+ ascorbate) or 79% (− ascorbate) in 50 mM Tris and 80% (+ ascorbate) or 100% (− ascorbate) in 50 mM sodium phosphate, compared with rates obtained in 50 mM NaCl. Tris and phosphate are known to coordinate iron and greatly enhance the oxidation of Fe(II) (32). Because of the profound effects that Tris and phosphate had on Fe(II) mobilization, 50 mM NaCl was used for all subsequent experiments, with careful monitoring and adjusting of pH.

Effect of ascorbic acid on Fe(II) mobilization. The addition of ascorbate (1 mM) to asbestos (1 mg/ml) increased the rate of Fe(II) mobilization by ferrozine, the greatest increase being 12-fold for amosite (Table I). The medium-fiber chrysotile turned increasingly more purple with incubation up to 12 h, indicating that ferrozine was binding iron on the fibers. After 12 h, the purple, ferrozine–Fe(II) complex was released into solution linearly with respect to time. Ferrozine also appeared to bind the Fe(II) present on the short-fiber chrysotile, but none of the Fe(II)–ferrozine complex formed on the fiber was mobilized during the 24-h incubation.

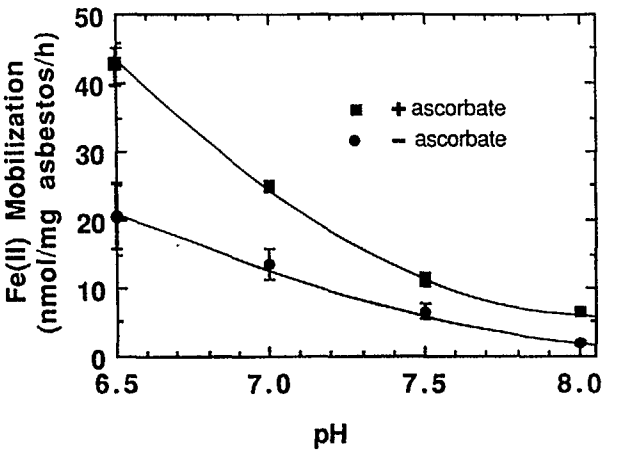

FIG. 2. Effect of pH on Fe(II) mobilization from crocidolite. Crocidolite (1 mg/ml) was incubated in 50 mM NaCl at the indicated pH with 1 mM ferrozine in the presence (■) or absence (●) of 1 mM ascorbate for up to 3 h. The rates of Fe(II) mobilization were determined as described under *Fe(II) mobilization by ferrozine* in the Materials and Methods section.

Chelation of total iron from asbestos and the effect of ascorbic acid. The effects of the potential physiological iron chelators, citrate, ADP, acetate, cysteine, histidine, phosphate, or tryptophan, on iron mobilization from crocidolite were investigated. As shown in Table III, both citrate and ADP mobilized iron from crocidolite during the 36-h incubation period. The addition of ascorbate increased iron mobilization by both citrate and ADP. However, ascorbate alone mobilized iron from crocidolite, suggesting that the increase was an additive effect due to additional mobilization by ascorbate. No iron was mobilized from crocidolite by histidine, tryptophan, acetate, cysteine, or phosphate (data not shown).

TABLE II

Effect of Buffers on Fe(II) Mobilization from Crocidolite Asbestos

Solvent	Rate of Fe(II) mobilization (nmol/mg/h)	
	With ascorbate	Without ascorbate
50 mM NaCl	11.4[a] (0.9)[b]	6.6[a] (1.2)
50 mM Tris	7.7 (0.5)	1.4 (0.3)
50 mM phosphate	2.3	0

Note. Asbestos (1 mg/ml) was incubated in 50 mM NaCl, 50 mM Tris, or 50 mM sodium phosphate, pH 7.5, with 1 mM ferrozine, with or without 1 mM ascorbate, for up to 25 h. Rates were determined as described under Materials and Methods for *Fe(II) mobilization by ferrozine.*

[a] Data taken from Fig. 1.

[b] Numbers in parentheses represent standard deviations. In the case where no standard deviation is listed, the number is an average of two experiments.

TABLE III

Effect of Chelators on the Rate of Iron Mobilization from Crocidolite Asbestos

Chelator	Iron mobilized (nmol/mg/h)	
	−Ascorbate	+Ascorbate
None	0	0.5
Ferrozine	6.6[a] (1.2)[b]	11.4[a] (0.9)
Citrate	4.2[c] (0.5)	4.8[c] (0.9)
ADP	0.3 (<0.1)	1.0
EDTA	30.1	27.3

Note. Asbestos (1 mg/ml) was incubated with the indicated chelator (1 mM) in the presence or absence of 1 mM ascorbate in 50 mM NaCl, pH 7.5, for up to 36 h. Samples were removed at regular time intervals, centrifuged at 14,000 rpm in an Eppendorf 5415 centrifuge, and the amount of iron mobilized determined spectrophotometrically using a Cary dual-beam spectrophotometer. Fe(II) mobilized by ferrozine was measured directly (562 nm). Iron mobilized by all other chelators was determined as described under Materials and Methods for *Total iron mobilization.* The rates of mobilization were calculated using computer-assisted linear regression of amount of iron mobilized versus time.

[a] Data taken from Fig. 1.

[b] Numbers in parentheses indicate standard deviation. In the case where no standard deviation is listed, the number is an average of two experiments.

[c] Data taken from Table I.

However, at 50 mM sodium phosphate, iron was mobilized from crocidolite at a rate of 0.9 nmol/mg/h. The ability of two nonbiological compounds, EDTA and Tris, to mobilize iron from crocidolite was also investigated because of their frequent use in isolation of DNA and because EDTA–Fe(II) is frequently used to induce random strand breaks in DNA. As shown in Table III, EDTA mobilized iron from crocidolite at approximately four times the rate of ferrozine, while Tris was not able to mobilize any iron over a 36-h period at either 1 mM or 50 mM (data not shown). To insure that the iron, chelated and mobilized from asbestos, was remaining in solution, 30 μM $FeCl_3$, chelated with EDTA, ADP, or citrate (1 mM), was incubated under conditions identical to those used for asbestos. Total iron assays done on samples removed over a 72-h period showed no change in the total iron concentration in solution (data not shown). These results showed that the iron mobilized by these chelators was remaining in solution and was not forming insoluble complexes that could not be measured using the total iron assay.

The ability of citrate to mobilize iron from other forms of asbestos was investigated since citrate was the best biologically relevant chelator for mobilizing iron from crocidolite. As shown in Table I, citrate mobilized iron from crocidolite and amosite. Iron was mobilized from medium-fiber chrysotile only during the first 2 h of incubation.

DISCUSSION

The results of the present study demonstrated that an iron chelator, i.e., citrate or ADP, had to be present to mobilize iron from asbestos. This suggests that leaching of iron from asbestos, which has been reported to occur *in vivo* (7, 27, 28, 33, 34), must be the result of chelation. While the chemical identity of the intracellular chelate(s) is (are) unknown, citrate–iron complexes have been identified in the non-transferrin-bound iron fraction of plasma or serum from patients with idiopathic hemochromatosis using NMR spectroscopy (29). Mobilization of iron from asbestos by citrate and ADP may be deleterious to the cell as a result of the loss of iron regulation by specialized proteins. Under normal conditions ferritin and transferrin control the concentrations of and limit the reactions catalyzed by iron. Asbestos may resemble ferritin in that it might be a source of iron for the cell. Iron associated with asbestos can be reduced by ascorbate, but this reduction does not appear to be required for mobilization of iron by citrate or ADP. In contrast, release of iron from ferritin requires both a reducing agent and a chelator (35). Therefore, mobilization of iron from asbestos appears to be a much simpler, less regulated process. Once the iron is mobilized from asbestos by citrate or ADP, the iron is redox active and capable of catalyzing the oxidation of lipids (36) and perhaps other biological molecules, such as DNA, which could be very important for the induction of cancer. In addition, the chelated iron becomes more mobile than that which remains associated with the fibers and could potentially catalyze deleterious oxidation reactions throughout the cell.

In the absence of a chelator, the iron remained with the asbestos. The oxygen-containing silicate structure of the fibers can coordinate the iron which results in asbestos acting as a competing chelator. Carcinogenic fibers which are structurally similar to asbestos, such as glass (37) and rockwool (38), become partially coated with iron, forming ferruginous bodies after two months in the lungs of rodents. These 'pseudoasbestos' bodies may cause tissue damage and cancer by a mechanism similar to that of asbestos. This also suggests that asbestos may chelate intracellular iron as well as release the iron that is resident in the fibers. This would lead to increased cellular damage due to an increase in the concentration of uncontrolled iron.

The rates of Fe(II) mobilization from crocidolite and amosite by ferrozine and ascorbate were significantly greater than the rates seen with medium- and short-fiber chrysotile. The iron content alone of the different fibers did not determine the rate of iron mobilization. However, there did seem to be a relationship between surface area of the fibers and rate of iron mobilization for the amphiboles, crocidolite and amosite. Using the surface areas reported by Campbell *et al.* (6), the ratio of surface area (m^2/g) to rate of mobilization (nmol Fe(II)/mg/h) was calculated. The results are as follows: crocidolite, 0.9; amosite, 0.8; and medium-fiber chrysotile, 42. This was similar to what was observed when the same calculations were done for citrate mobilization of iron from

different forms of asbestos: crocidolite 3.0; amosite, 2.5; and medium-fiber chrysotile, 50. The large differences observed between the amphibole and the serpentine forms suggest that crystalline structure does play an important role when comparing these two types of asbestos. Therefore, mobilization of iron from asbestos appeared to be a function not only of the chelator, but also of the surface area, crystalline structure, and iron content of the asbestos.

The two forms of chrysotile displayed a reluctance to release Fe(II) even after it had been complexed by ferrozine. This suggests that the serpentines examined have a higher affinity for iron than the amphiboles and might show an increased ability to coordinate intracellular iron *in vivo*, thus increasing the toxicity and carcinogenicity of the fiber beyond what its iron content might predict.

Ferrous iron mobilization from crocidolite by ferrozine increased with decreasing pH. This is consistent with what has been observed for mineral leaching from asbestos (9) and decomposition of chrysotile (7). Similar observations have been made for the solubility of ionic iron. However, other factors that should be considered to contribute to mobilization of Fe(II) from asbestos as a function of pH are the reduction potential of asbestos-coordinated iron and the affinity of asbestos for iron.

In conclusion, the results presented here show that a chelator must be present to mobilize iron from asbestos. Physiologically relevant chelators, such as ADP, citrate, and ascorbate, were capable of mobilizing iron. These results suggest that iron can be mobilized from asbestos in the cell by low-molecular-weight chelators. If this occurs, it may have deleterious effects since this could result in deregulation of normal iron metabolism within the cell.

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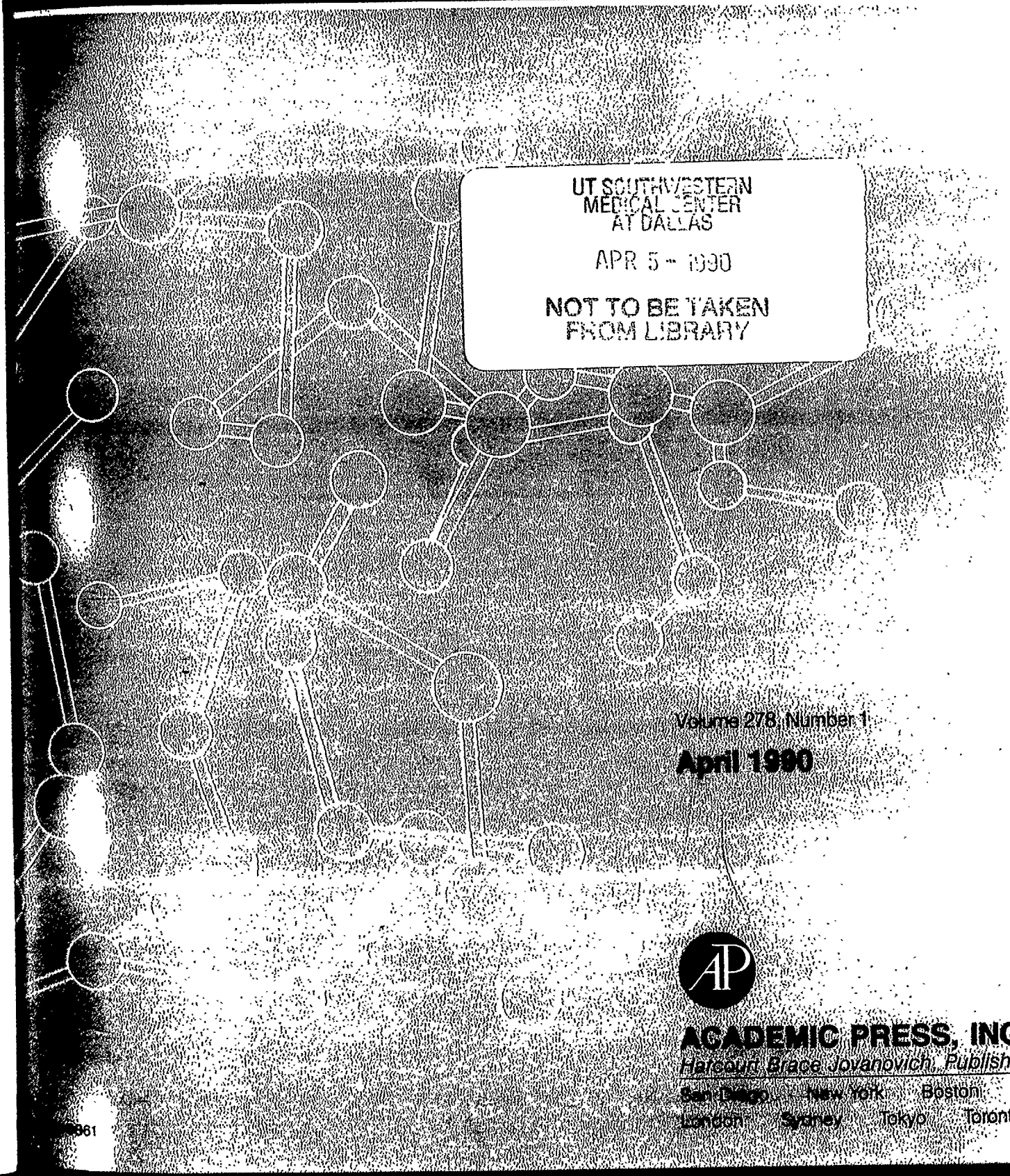

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