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Inhalation studies on the effects of tremolite and brucite dust in rats

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Samples of commercially used asbestos, especially chrysotile, are frequently contaminated by small amounts of other fibrous minerals. Among these are tremolite and brucite although pure tremolite is also produced commercially in relatively small quantities. In order to determine how harmful commercially exploited tremolite might be in comparison with other asbestos types and to explore the possibility that small amounts of tremolite and brucite as contaminants could significantly affect the pathogenicity of industrially used chrysotile, long-term animal inhalation and injection studies using rats were undertaken with what were considered to be mineralogically pure samples of these minerals. Rats treated with tremolite developed very high levels of pulmonary fibrosis as well as 16 carcinomas and two mesotheliomas in a group of 39 animals. Tremolite thus proved to be the most dangerous mineral that we have studied. Animals treated with 'brucite' developed moderate levels of pulmonary fibrosis and two carcinomas. Both tremolite and brucite produced mesotheliomas in >90% of animals following i.p. injection. However, it was found that the supposedly pure brucite in fact contained 10% chrysotile, a level of contamination that could well have been responsible for the pathological changes found in both inhalation and intraperitoneal injection studies. The greatest care should be exercised by industry in handling tremolite or materials contaminated with it.

Introduction

The fibrogenic and carcinogenic potential of the main commercially used varieties of asbestos have been well documented (1). Recently, however, attention has been drawn to the fact that asbestos ore bodies, particularly those of chrysotile, are not mineralogically pure. Mixed with the asbestos are other minerals, some of them fibrous, which remain as contaminants in the final asbestos products supplied to industry from the mines. Two such minerals are brucite and tremolite. Brucite, $Mg(OH)_2$, is quite distinct from all the asbestos varieties in that it does not contain silicon but it is commonly found in chrysotile and other serpentines since it often forms in similar geological conditions. It can occur in the form of solid fibres, as tubular crystals, or in large foliated masses. Tremolite, $Ca_2Mg_5Si_8O_{22}(OH)_2$, is a commonly occurring amphibole mineral which is only rarely found as a pure fibrous asbestiform variety in quantities large enough for commercial exploitation. It is found more frequently in both the asbestiform and non-fibrous states as a contaminant in other materials such as talc, chrysotile and vermiculite (2). The identification sample of tremolite as asbestiform depends among other upon its length and diameter distributions. Campbell (. Campbell *et al.* (4) have shown that asbestiform varie minerals have significantly longer and finer size distrib than their non-asbestiform equivalents.

Environmental exposure to tremolite in Turkey ha. implicated in the development of human bronchial carc and mesotheliomas (5). It has also been reported that the of chrysotile miners can contain as much tremolite at aut chrysotile even though only a small percentage of tremol present in the original ore body (6,7). Mesothelior workers exposed to chrysotile are extremely rare and been disputed whether or not chrysotile alone can produc tumours in humans. A few well-documented cases of thelioma in chrysotile workers do exist, where expos crocidolite or amosite appears to have been excluded b it would appear that tremolite rather than chrysotile itsel be the cause. Experimental *in vitro* and *in vivo* injection so far undertaken (8) confirm that tremolite fibres . carcinogenic in experimental animals. So far there epidemiological evidence to suggest that brucite is imp in human disease but animal injection studies by Pott (9,10) show that brucite fibres can produce mesothelic injected into experimental animals.

At the IOM in Edinburgh we have used both inhalati injection studies to compare the biological effects of a r of samples of asbestos and other mineral fibres. The s studies were undertaken in order to compare the h potential of tremolite and brucite to that of these other materials and to obtain more information on the likely i ance of their presence as contaminants in other products commercially used talc and chrysotile.

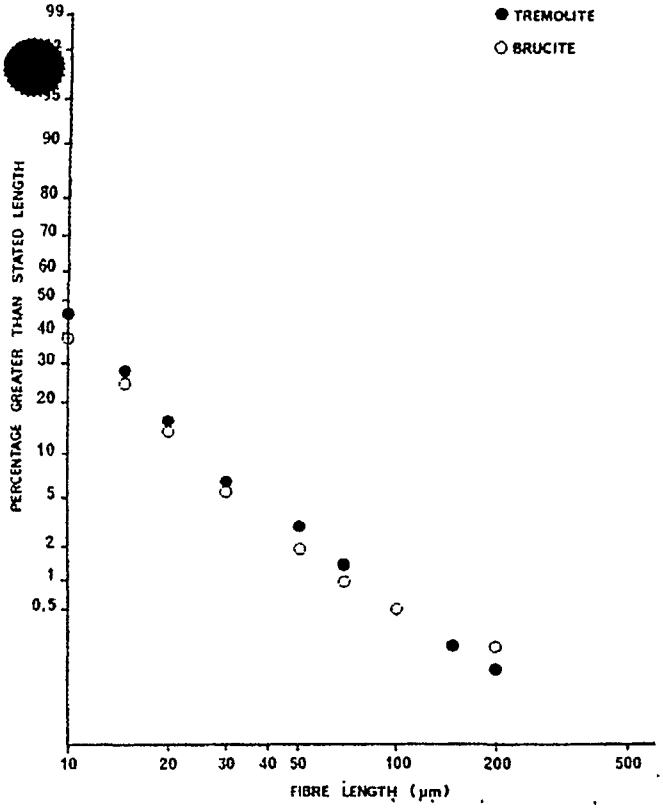
Materials and methods

Mineralogy

The sample of 'tremolite' used in this study was a commercial mate Korea. It contained ~95% by mass of pure fibrous tremolite wh chemically and structurally consistent with published data as confi scanning electron microscopy (SEM)* and X-ray diffraction (XRD) However, a few XRD peaks were present which could not be account tremolite. Some of these impurities had a layer structure with a 7 3.6 A spacing typical of minerals such as kaolinite, serpentine, or ch The presence of talc was also suspected. The tremolite itself was ch pure, being composed almost entirely of calcium, magnesium and silic minor amounts of iron, well within the limits of the composition permitted for the mineral.

The brucite used in this study was chemically and structurally consis published data. The specimen was 85–90% by mass of pure bruc firmed by SEM and XRD analysis. In addition to brucite, however, material contained Pyraurite ($Mg_6Fe_2CO_3(OH)_{16}4H_2O$). This app mottled brown segments on a proportion of the brucite fibres under th microscope. The Pyraurite probably comprised 5–10% of the bulk Chrysotile was also present as confirmed by XRD. Since, however, t of analysis gives poor quantitative results for chrysotile when small are mixed with other minerals, the bulk sample was treated with glac acid to remove the brucite and the chrysotile content was estimate

*Abbreviations: SEM, scanning electron microscopy; XRD, X-ray diffraction.



1. Length distributions of fibres of tremolite and brucite >5 μm in length obtained by phase contrast light microscopy.

analysis. This technique showed that chrysotile comprised ~10% of the 'brucite' sample.

Dust generation and monitoring

The planned dust concentrations was 10 mg/m³ of respirable dust. The dust clouds were generated using a Timbrell dust generator and inhalation chambers as described by Timbrell *et al.* (11) but modified as described by Beckett (12). Selecting the airborne dust by a cyclone system prior to injecting it into the chamber airstream ensured a high proportion of respirable dust in the clouds. The dust generators were able to produce suitable clouds of tremolite dust from the bulk sample as it was received. Generation of a respirable dust cloud from the brucite sample required some preliminary breakage and dispersion by passing it through square-toothed intermeshed steel rollers prior to loading into the dust generator.

The mass concentration of dust in the chambers was measured daily by sampling throughout the 7 h of exposure using both an open filter holder facing vertically downwards and a Casella MRE113A dust sampling instrument. The former sampler monitored the total dust concentration and the latter monitored the respirable dust mass concentration. The target mean respirable dust concentration of 10 mg/m³ was achieved by adjusting the equipment in response to each daily measurement.

The fibre number concentration was estimated from short period membrane filter samples taken on ~100 separate days. The number concentration for these samples was taken as being proportional to the mass concentration for that day, thus giving a mass/number ratio which was then averaged over 100 days. The membrane filter samples were examined by phase-contrast optical microscopy, following the normal practice for occupational hygiene measurements (13). This routine technique defines fibres to be included in the count by criteria of size; length >5 microns, diameter <3 microns, and aspect ratio >3:1.

The size distribution of fibres was examined by both phase-contrast optical microscopy and SEM. The optical studies were performed by a single observer, but all SEM studies were undertaken by two observers. Samples for electron microscopy were collected on Nucleopore filters. A portion of each filter was subsequently attached to an electron microscope stub with a conductive adhesive (carbon paste) and sputter coated with a thin layer of gold. The samples were examined by SEM (Cambridge Instruments S600) at 10 000x magnification and measurements taken on the length and diameter of those fibres with aspect ratio >3:1, length >0.4 microns and diameter >3 microns. The minimum length required arises from the combination of the aspect ratio definition and the resolution limit, which for the SEM corresponds to a fibre of ~0.1 micron diameter. Some fibres of <0.1 micron diameter were detected and reported and measured as closer to 0.05 microns but generally fibre diameters were measured to the nearest 0.1 micron.

Animal inhalation studies

For the inhalation studies groups of 48 SPF male Wistar rats of the AF/Han strain were exposed to either tremolite or brucite dust for 7 h each day, 5 days a week, for a total of 224 days, during a period of 12 months. The animals were 10 weeks old at the start of dusting. A batch of 36 undusted animals was maintained within the same unit as controls during the same overall time period.

Four animals from each group were killed at the end of the 12 months dusting period and four more were killed 6 months later. The remaining animals were left for their full life span except that the study was terminated when the number of survivors in one group dropped to six. Estimations of early pathological lesions were limited to the small groups of animals from the first two killing dates. However, for the more advanced lesions occurring in the oldest animals, it was decided to include all those dying within 2 months of the final killing date. In practice this produced groups of 12 animals for each dust treatment.

Tissue used for histological examination was fixed with 10% formol saline solution and embedded in paraffin wax. Lungs were fixed by inflation at a standard pressure of 30 cm of fixative. Subsequently the tracheas were ligated and the lungs excised and immersed in fixative. Sections were cut in the coronal plane at 1 mm intervals and were stained by either haematoxylin and eosin, Van Geison's method for collagen or Gordon and Sweet's stain for reticulin.

Measurement of pulmonary fibrosis was undertaken by similar methods to those previously published by Davis *et al* (14) except that an electronic image analyser (Graphic Information Systems Limited, GDS1) was available for use in conjunction with the light microscope. Single lung sections were examined with the section selected to contain the maximum area of lung parenchyma. As previously described, interstitial fibrosis was estimated using a 2x microscope objective lens and is expressed as a percentage of total lung-tissue area. Peribronchiolar lesions are more numerous and smaller and so the lung tissue was scanned with an eye-piece graticule covering an area of 2.9¹ mm and divided into 100 squares. A 4x objective lens was used. Peribronchiolar lesions were recorded as a percentage of squares containing lesions of this type. Small areas of irregular alveolar wall damage were measured by the same techniques. However, since these lesions were difficult in all cases to distinguish from fragments of peribronchiolar fibrosis included in the sections an overall measurement was undertaken initially to include both lesions, followed by a second one involving only definite peribronchiolar fibrosis. An estimate of lung tissue with irregular alveolar wall damage was obtained by subtraction.

Lung dust content

Lung dust estimations were performed on animals from the first two killing dates. Only the left lung was used so that the right lung was available for histological studies. Dust retained in the lungs was recovered by a low-temperature plasma ashing process using a Nanotech P100 apparatus. Studies in this laboratory have shown that the dust content ratio between left and right lungs following experimental inhalation of fibrous dusts such as asbestos in rats is 0.6:1 and this correction factor was therefore used to estimate the total pulmonary dust burden of each animal.

The tremolite residues were washed in 0.2 m HCl at room temperature before estimations of the amounts of retained fibre were made using the i.r. spectrophotometer techniques described by Middleton *et al.* (15). Since brucite is known to be highly soluble in acids, the brucite residues were washed only with distilled water initially. However, because the original dust cloud had contained a significant proportion of chrysotile asbestos, the analysis of these lung dust residues was repeated after washing with glacial acetic acid.

Animal injection studies

In addition to the inhalation studies, the ability of the tremolite and brucite to produce mesotheliomas was examined using the i.p. injection assay. A dose of 25 mg of dust suspended in 2 ml of Dulbecco's phosphate buffered saline was injected under ether anaesthetic into the peritoneal cavities of two groups of 32 rats of the AF/Han strain. The dust was collected from the animal inhalation chamber by an elutriation process and represented the respirable fraction of the dust cloud (16).

Results

Dust characterisation

The planned average mass dust concentration of both tremolite and brucite was achieved during the dusting period with >40% of the daily dust concentrations within 3 mg/m³ of the target concentration.

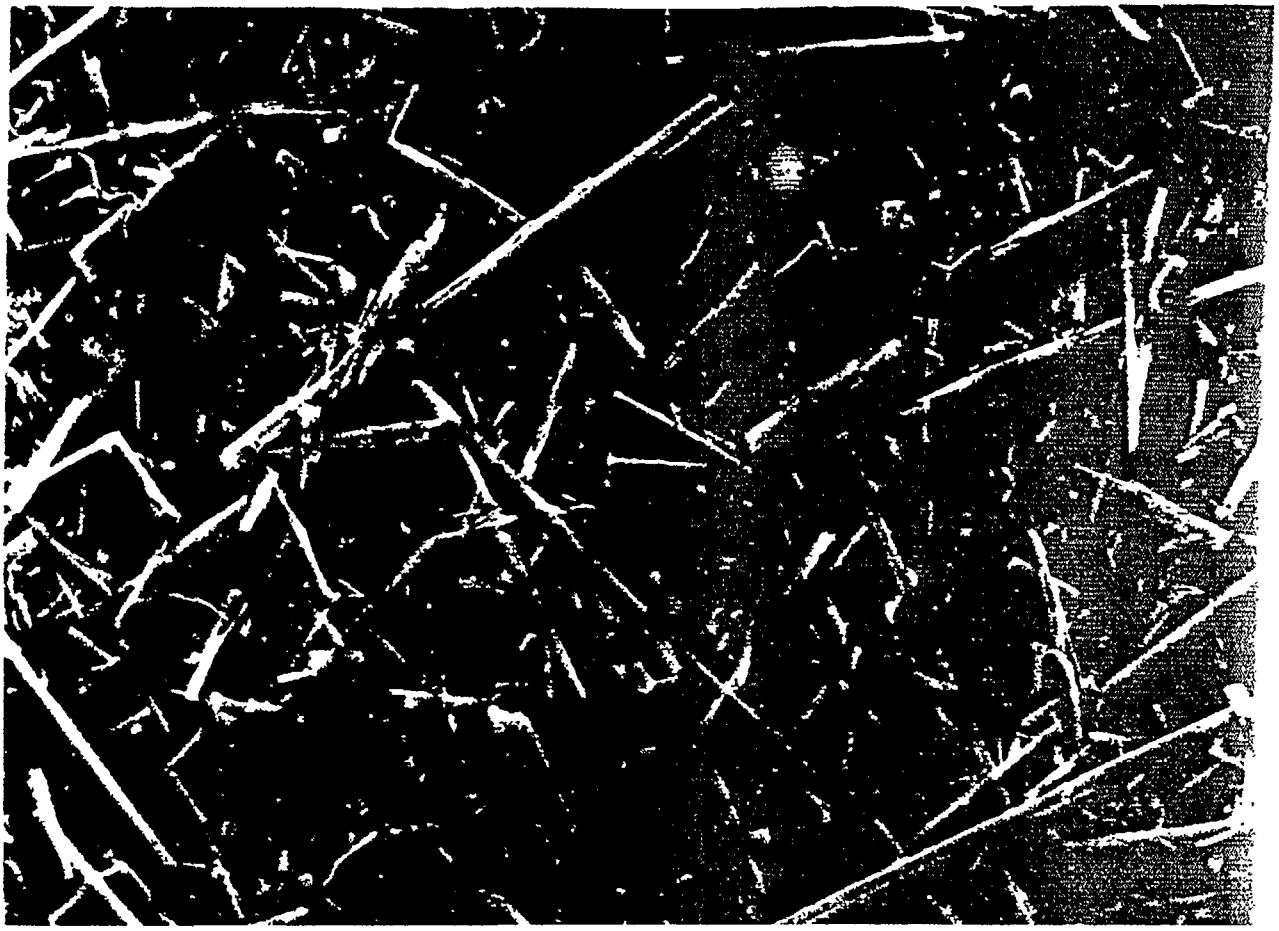


Fig. 2. SEM of the tremolite dust used in the inhalation and injection studies. While the tremolite fibres are of uniform type the brucite is a mixture of relatively thick straight brucite fibres and thin curly ones which are chrysotile asbestos. (10 000x magnification)

The fibre number concentrations of the materials as determined by phase-contrast optical microscopy for fibres longer than 5 microns was 1600/ml for tremolite and 230/ml for brucite. This substantial difference in fibre number for equal mass concentrations of respirable dust was partly due to the large amount of non-fibrous dust in the brucite cloud as well as to differences in the fibre dimensions. The length distribution of 1000 fibres longer than 5 microns, as determined by one observer using phase-contrast optical microscopy, is shown in Figure 1. The tremolite sample contained more fibres between 10 μm and 100 μm in length but the differences were small. Typical SEM photographs of the tremolite and brucite dusts are illustrated in Figures 2 and 3. The tremolite is seen to consist almost entirely of straight fibres of similar type, many of them extremely thin. The brucite sample, however, is more complex. Apart from particulate material the bulk of the sample is made up of straight fibres with a relatively large diameter. These are identifiable by EDXA analysis as brucite. There are also large numbers of very thin fibres, the longest of which are distinctly curly. EDXA analysis showed that these are chrysotile. Analysis by i.r. subsequent to washing with glacial acetic acid showed that the proportion of chrysotile was ~ 10% by mass. Almost all the fibres seen with the resolution of the light microscope counts would have been brucite but the majority counted by SEM were probably chrysotile.

The cumulative fibre size distributions obtained from the SEM at 10 000x magnification are produced in Figures 4 . These showed that the tremolite sample contained a g proportion of long fibres than the brucite sample, and th tremolite fibres on average were also thinner unless they extremely short. The distributions were obtained b observer using the SEM, although a second observer conf that the tremolite samples tended to be longer and thinne the brucite. However, there were differences betwee absolute fibre size estimates obtained by the two obse confirming the recent observations of Cherrie *et al.* (17). it was found that inter-observer differences were a source of variation in fibre size estimations obtained Cambridge Instruments S600 SEM. In the same study, shown that any single observer tended to produce cons fibre size estimates from repeated assessments, and fc reason the data presented in Figures 4 and 5 were cons appropriate to illustrate the relative differences in the si the tremolite and brucite dust samples.

Histopathological findings

Both groups of dusted animals developed the same patte pathological change previously reported in similar studies the Institute but to varying degrees. At the end of the 12- dusting period the main lesions present were deposits of g lation tissue around the terminal and respiratory bronc (Figure 6). This granulation tissue consisted mainly of m

Fig. 3. SEM of the brucite dust used in the inhalation and injection studies. While the tremolite fibres are of uniform type the brucite is a mixture of relatively thick straight brucite fibres and thin curly ones which are chrysotile asbestos. (10 000x magnification)

phages and fibroblasts but a few foreign giant cells were also present. The deposits of granulation tissue tended to be both larger and more frequent in animals treated with tremolite than in those that had inhaled brucite. When the degree of involvement of the lung parenchyma was assessed (Table I), the difference between the two dust treatments at both 12 and 18 months after the start of dusting was statistically significant ($p<0.01$). During this period, the lesions did not increase in size and frequency and indeed in the tremolite-treated animals the 18-month group produced lower figures than those found at 12 months. With the small groups of animals examined, however, this reduction was not statistically significant. After 18 months from the start of dusting widespread interstitial fibrosis developed and this tended to obscure the earlier fibrotic deposits. For this reason, estimations of peribronchiolar fibrosis were limited to the first two killing dates. At 12 months after the start of dusting there was marked reticulin staining in the peribronchiolar deposits although relatively little collagen [illegible] be demonstrated by Van Geison's stain. As the animals aged, however, collagen staining became progressively more marked. Within the areas of peribronchiolar fibrosis, many tremolite fibres were clearly visible with the light microscope. Brucite fibres could not be seen, however, at any stage.

In addition to the peribronchiolar lesions, macrophages containing dust were visible in most alveoli at the end of the dusting period although they were more frequent close to the terminal bronchioles. In addition, however, there were small irregular patches of alveolar wall damage and thickening (Figure 6). It is possible that these small areas of damage represent precursors of the more widespread and distinct areas of interstitial fibrosis that developed in the older animals although this has not yet been proved. As shown in Table I, however, the extent of this type of pathological change in the individuals of any group of animals bears a close relationship to the amount of peribronchiolar fibrosis present. In the present study, animals treated with tremolite had significantly more of this alveolar damage than those treated with brucite ($p<0.01$).

From about 18 months onwards, areas of lung tissue in some animals showed a progressive thickening of alveolar septa. In its earliest form this thickening was caused almost entirely by hyperplasia of alveolar lining cells but later there was considerable deposition of reticulin and eventually collagen in the septal walls (Figure 7). Dust deposits were frequently visible among the fibrous tissues in the thickened septa in animals treated with tremolite dust. As shown in Table I, areas of interstitial fibrosis became more widespread in both treatment groups as the animals aged but far more was found in the tremolite-treated group than in the animals that had inhaled brucite ($p<0.01$). In some areas the interstitial fibrotic element of these lesions remained predominant throughout the study but in others the hyperplasia of alveolar epithelial cells became progressively more marked to produce a pattern of adenomatosis. Some

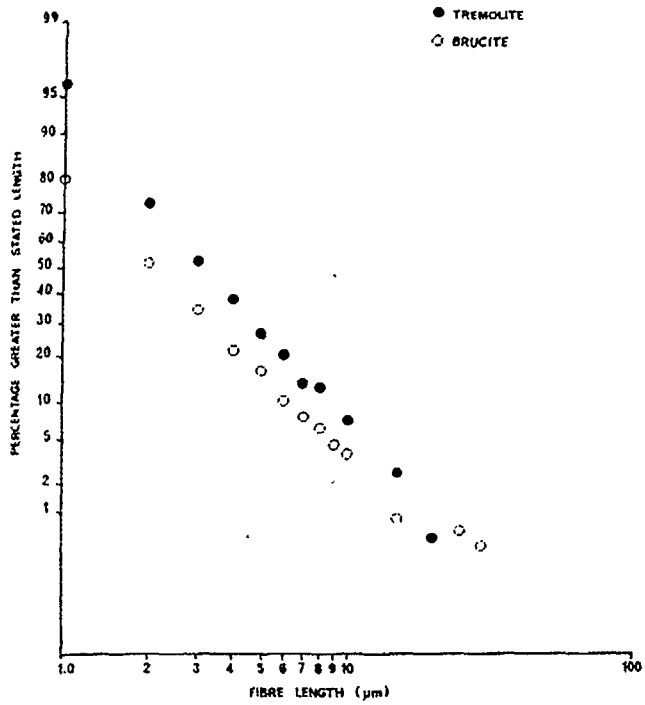


Fig. 4. Length distributions of fibres of tremolite and brucite >0.4 μm in length obtained by SEM at a magnification of 10 000x.

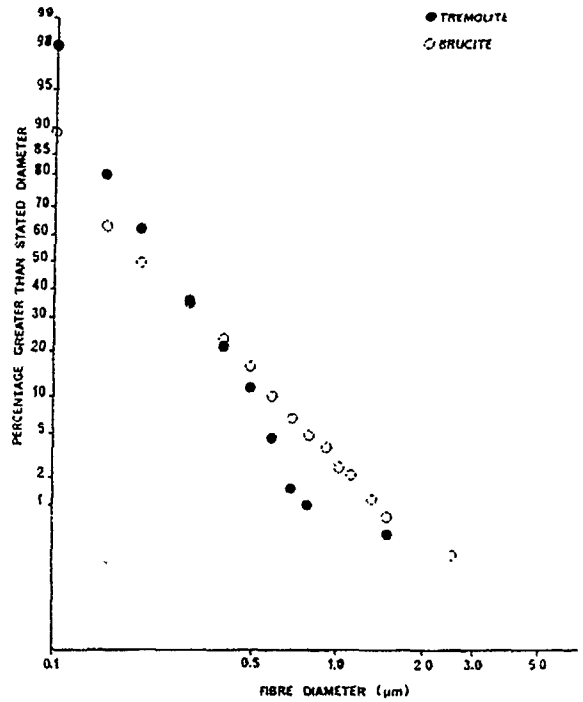


Fig. 5. Diameter distributions of fibres of tremolite and brucite >0.4 μm length obtained by SEM at a magnification of 10 000x.

definite adenomas could be seen to have developed from the central regions of these areas and it is likely that this was also the site of origin of some carcinomas although by the time most of these were discovered they were too widespread to be certain.

Two pulmonary adenomas were found in the group of rats treated with tremolite and three in the brucite group. As shown in Table II, however, a total of 16 carcinomas and 2 mesotheliomas developed in the tremolite group while only 2 carcinomas occurred with brucite. This difference in the number of malignant tumours is statistically significant ($p<0.001$). No pulmonary tumours were found in the lungs of control animals. The numbers of tumours occurring in other tissue sites for the three groups is illustrated in Table III. While the control group produced the highest numbers of both benign and malignant tumours these numbers were not significantly higher than in the groups treated with either tremolite or brucite.

Lung dust burden

The retained dust burden of tremolite in the lungs of a group of four rats killed at the end of the dusting period showed a mean value of 10 800 μg. After a further 6 months the mean value for the second group of four rats to be killed was 6210 μg. The extracted dust samples for the four brucite-treated animals killed at the end of the dusting period were destroyed during analysis. In the group killed 6 months after the end of dusting, the mean lung dust content was 64 μg when measured using the i.r. absorbance figures for brucite. However, since it was kno[illegible]hat the original brucite dust clouds had been contaminated with ~10% of chrysotile, the lung residues were treated with glacial acetic acid to dissolve brucite and then re-analysed using the i.r. absorbance figures for chrysotile. No significant loss of lung dust mass was found and it was concluded that 6 months after the end of dusting almost all the remaining lung dust was chrysotile.

Injection studies

In the i.p. injection studies a very high proportion of me theliomas was produced by both tremolite and brucite. In tremolite study 27 animals out of 29 available for considera (93%) developed tumours and in the brucite study 27 anir out of 28 (96%): However, tremolite dust induced m theliomas faster than brucite. The mean survival time animals with tumours was 352 days in animals injected tremolite and 418 for animals injected with brucite.

Discussion

The present study has shown that both the tremolite and bru dusts used were fibrogenic and carcinogenic to rats. While amounts of fibrosis and the number of pulmonary tumours duced by brucite were relatively low, tremolite prodt significantly more of these lesions than UICC chrys ($p<0.01$) which previously had been the most carcinogenic treated by our Unit (using the same exposure facilities) (14 these previous studies all amphibole dusts tested had sh only low levels of pathogenicity. The reasons for the activity in tremolite, which is also an amphibole are, there of great interest. Stanton *et al.* (18,19) suggested tha carcinogenicity of mineral fibre samples was most cl related to the number of fibres present >8 microns in length <1.5 microns in diameter. The fibre sizing of tremolite ur taken by SEM in the present study indicated that the propo of fibres >5 μm and >10 μm in length was approxim double that found for either UICC crocidolite or amosit Davis *et al.* (14) although the tremolite figures were simil those reported for a factory amosite sample in 1980 (20). tremolite fibres were, however, rather thinner than the am and crocidolite samples examined and the number of f >5 microns in length and visible by phase-contrast light m scopy was more than double that found with any of these.

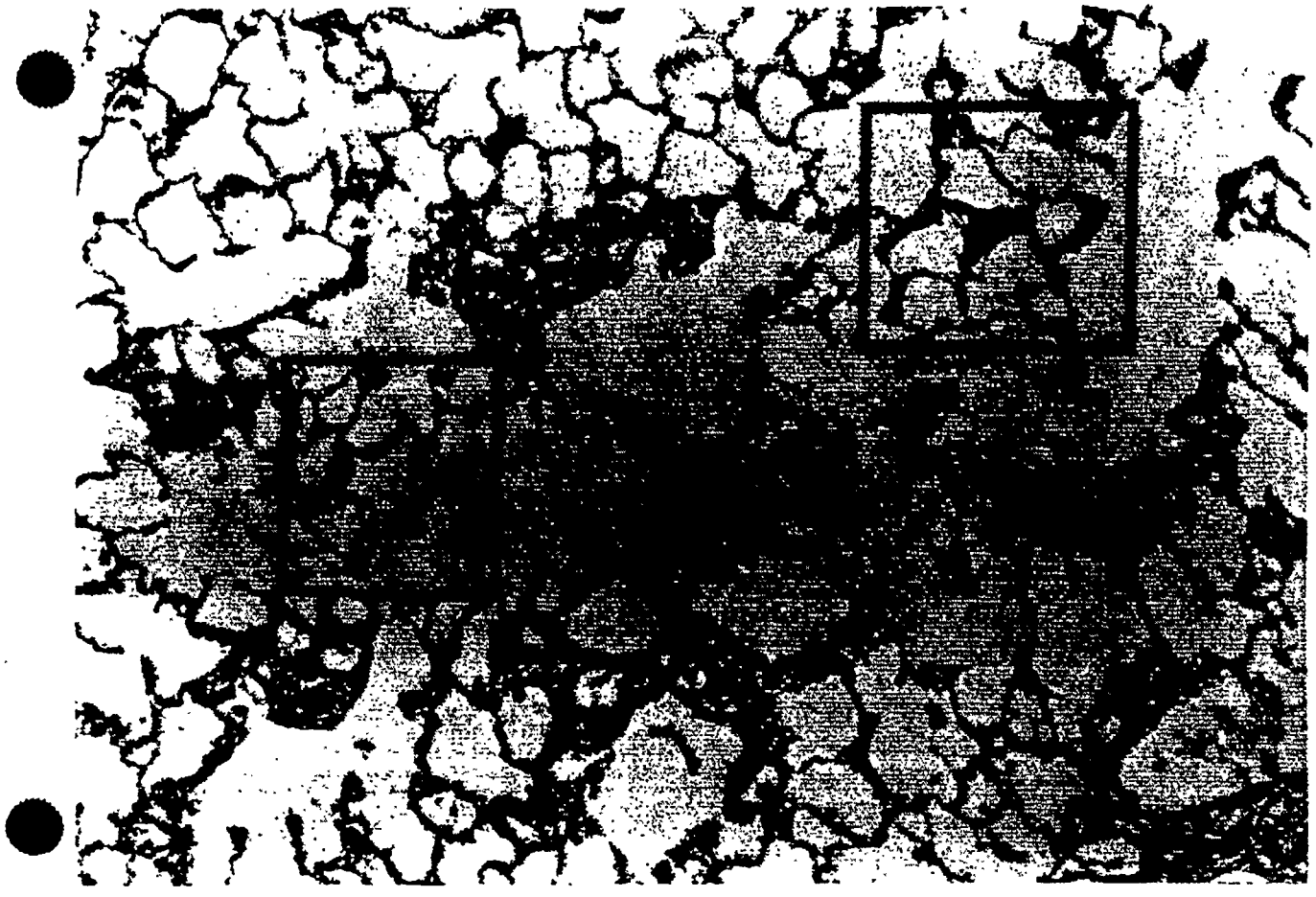


Fig. 6. A light microscope section of lung tissue from a rat that had inhaled tremolite dust for 12 months. One respiratory bronchiole is surrounded by masses of fibrous tissue (F). In addition, the alveoli in two areas (outlined) show irregular thickening. (200x magnification)

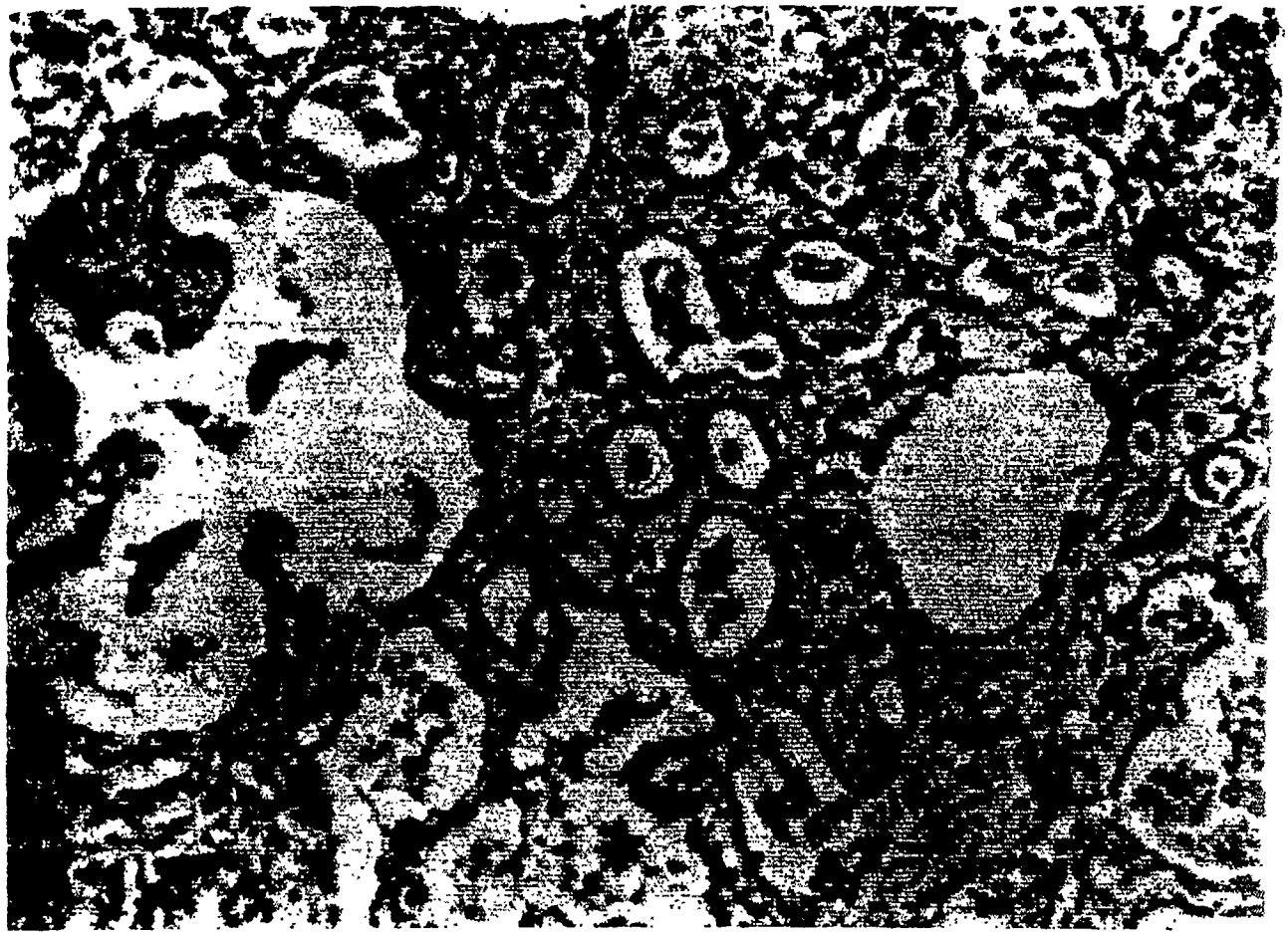
Table I. Levels of pulmonary fibrosis and irregular alveolar wall thickening produced by tremolite and brucite dusts

	Tremolite			Brucite		
Time after start of exposure (months)	12	18	27–29	12	18	27–29
Peribronchiolar fibrosis	23.0 (21.4–24.2)[b]	13.4 (9.7–18.9)	–	1.6 (0.5–2.7)	1.7 (1.0–2.9)	–
Irregular alveolar wall[a] thickening	35.2 (27.7–41.0)	27.7 (20.8–35.4)	–	5.8 (3.1–7.5)	7.6 (3.6–9.9)	–
Interstitial fibrosis	0	3.0 (0–5.6)	14.5 (3.8–26.9)	0.8 (0–3.3)	0	2.9 (0.2–8.9)
Number of rats examined	3	4	12	4	4	12

[a]Estimates obtained by subtraction (see text).
[b]Figures in brackets are standard deviations.

mass of tremolite retained in the rat lungs was almost identical to that previously found with other amphibole dusts at both 12 and 18 months after the st[illegible] f dusting so that the number of tremolite fibres present in th[illegible] ng tissue would have been correspondingly high. Stanton's work was undertaken using the technique of intrapleural implantation and the injection studies reported in the present paper have confirmed that tremolite is very highly carcinogenic in this type of experiment. There is, however, no reason why the same criteria should not apply to the lung parenchyma and the carcinogenic potential of the tremolite dust used in our inhalation study is likely to have been due to its high content of long thin fibres. These studies would suggest that tremolite samples containing thin fibres should be treated with appropriate caution if used by industry. In addition, samples of chrysotile and talc contaminated with tremolite are more likely to be more harmful than uncontaminated material.

The results of the brucite studies indicate more complex patterns. The bulk brucite sample used for this work was considered to be as pure as can be obtained. However, both the



masses

Fig. 7. A light microscope section of lung tissue from a rat treated with tremolite that died 29 months after the start of dusting. The normal alveolar struc has been replaced by spaces lined by cuboidal epithelium which are separated by deposits of interstitial fibrosis. (400x magnification)

Table II. Pulmonary tumours produced by tremolite and brucite clouds

	Tremolite	Brucite	Control
Number of rats examined	39	38	36
Adenomas	2	3	0
Total carcinomas	16	2	0
Adenocarcinomas	8	1	0
Squamous carcinomas	8	1	0
Mesotheliomas	2	0	0

Table III. Sites of tumours other than lung

	Tremolite		Brucite		Co
Number of rats examined	39		38		
Organ system	B[a]	M[b]	B	M	B
Digestive/peritoneal	1	2	1	–	3
Urinogenital	–	1	–	–	1
Endocrine	4	1	4	1	2
Musculo, skeletal and integumentary	2	3	–	4	3
Reticuloendothelial/vascular	–	1	–	1	2
Totals	7	8	5	6	11

[a]B = benign.
[b]M = malignant.

original sample and the respirable dust cloud generated from this material were found to contain ~10% of chrysotile by weight. Since most of the 'respirable' brucite fibres in the dust cloud were of much larger diameter than the chrysotile present, chrysotile is likely to have formed >10% of the total fibre number and may even have been more numerous than brucite. Only very small amounts of mineral remained in the rat lungs 6 months after the end of dusting and analysis indicated that most of this was chrysotile. It is likely, therefore, that this residual burden of chrysotile was responsible for the observed lung damage, with brucite producing little effect. In the present inhalation study, the rats were effectively exposed to a 'brucite' dust cloud containing a chrysotile mass concentration of slightly more than 1 mg/m^3. They developed similar numbers of pulmonary tumours and similar levels of fibrosis to animals previously exposed at this Institute to a dust cloud of Rhodesian chrysotile at a dust concentration of 2 mg/m^3. Similarly in the injection studies the rats injected with 25 dust effectively received doses of ~2.5 mg of chry Mesotheliomas were produced in similar numbers to previously reported at a dose level of 2.5 mg of Rho chrysotile in dose response studies, reported by Bolton (21).

In conclusion, these studies suggest that pure tremoli any materials contaminated with it should be treated

appropriate care by industry. In addition, the idea that the pathogenicity of the 'brucite' sample examined could be due to its chrysotile content highlights the problem of attributing observed pathological effects to individual components of a mixed dust sample.

Acknowledgement

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