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# Comparisons of the pathogenicity of long and short fibres of chrysotile asbestos in rats

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**Summary.** Long-term inhalation and intraperitoneal injection studies were undertaken with laboratory rats treated with a specially prepared short-fibre sample of Canadian chrysotile asbestos. This was compared, at an equal mass dose, to dust generated from the same chrysotile batch so as to contain the highest possible number of long fibres. The long-fibre cloud contained roughly five times more fibres $>5\mu m$ in length as seen by phase contrast optical microscopy (PCOM). For increasing lengths, the ratio between the dust clouds increased progressively, reaching over 80:1 for fibres $>30\mu m$ in length. Rats treated with long-fibre chrysotile developed six times more advanced interstitial fibrosis (asbestosis) than animals treated with short-fibre chrysotile and three times more pulmonary tumours. At the end of the 12-month dusting period, three times more short chrysotile than long had been retained in the rat lung tissues. During the following 6 months, however, the short-fibre chrysotile was removed from the lungs much more rapidly than the long. Following intraperitoneal injection at a mass dose of 25mg of dust, both long and short chrysotile produced mesotheliomas in more than 90% of rats. At a dose level of 2.5mg of dust, the short-fibre chrysotile produced mesotheliomas in only one-third as many rats as the long-fibre dust which still produced mesotheliomas in more than 90% of animals injected. At a dose level of 0.25mg of dust, the short-fibre chrysotile produced no mesotheliomas while the long-fibre chrysotile still produced these tumours in 66% of rats. In the two highest doses, where short-fibre chrysotile produced mesotheliomas, the mean tumour induction period was significantly longer than for tumours produced by long chrysotile.

Keywords: asbestos, dimensions, carcinogenicity

Since the dangers of industrial exposure to asbestos were first realized much experimental work has been undertaken to determine which parameters of any dust cloud are most important in disease production. One of the earliest factors to be examined was fibre length and King *et al.* (1946) administered chrysotile samples cut on a special microtome to rabbits by intratracheal injection. Animals injected with dust of fibre length approximately either 15 or 2.5 $\mu$m developed nodular peribronchial fibrosis with some

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progressive fibrosis of the alveolar walls, but these changes were much more marked in the animals receiving the long-fibre dust. Similar intratracheal injection studies, using guinea-pigs, were reported by Vorwald *et al.* (1951). This group injected 'long' and 'short' samples of the main asbestos varieties, chrysotile, amosite and crocidolite as well as anthophyllite, tremolite and brucite. Only the short-fibre samples of chrysotile and amosite would now be considered really short with almost all fibres $<3\mu m$ in length and while these produced little tissue reaction, long-fibre preparations produced distinct pulmonary fibrosis. Klosterkotter (1968) extended these studies by using both the intratracheal and intraperitoneal injection of chrysotile and crocidolite ground to an average fibre length of $<5\mu m$. They found that these samples produced little or no fibrosis in either site. In contrast, longer fibres of the same asbestos types resulted in considerable fibrosis in both areas. Almost identical results were obtained by Hilscher *et al.* (1970) using very similar techniques. Davis (1972) administered many finely ground mineral samples to mice by intraperitoneal injection. Included in this series of dusts were a normal long-fibre chrysotile sample and two short-fibre chrysotile preparations. These were synthetic chrysotile with a maximum length of $1\mu m$ and chrysotile fragmented by ultrasonic treatment in liquid until all fibres were below $1\ \mu m$ in length. The short-fibre samples produced almost no tissue reaction while the long-fibre chrysotile produced massive peritoneal fibrosis. Wright & Kuschner (1977) injected samples of crocidolite, synthetic fluoramphibole and glass fibre intratracheally into guinea-pigs. For each mineral there was a long-fibre preparation with up to 80% of fibres $>10\mu m$ in length and a short-fibre sample with few fibres $>5\mu m$ in length. All the long-fibre samples produced widespread pulmonary fibrosis while the short-fibre materials produced little tissue reaction.

Stanton & Wrench (1972) demonstrated that, in addition to its effect on fibrosis, fibre length was important in carcinogenesis with the production of mesotheliomas. Carefully sized samples of a number of mineral fibre types were administered to rats by pleural implantation and it was found that mesothelioma production was related to the number of long, thin fibres implanted. Also in 1972, Smith *et al.* reported that hamsters given intrapleural injections of crocidolite ground to a fibre length $<1\mu m$ did not develop mesotheliomas while those injected with a long-fibre preparation of the same material did. Stanton's work was further developed (Stanton *et al.* 1977, 1981) and it was determined that the most dangerous fibres were those $>8\mu m$ in length and $<1.5\mu m$ in diameter. Similar findings were reported by Pott & Friedrichs (1972) and Pott *et al.* (1976) and the work of this group has been continued until the present time with many further publications (e.g. Pott (1978, 1983), Pott & Ziem (1983)).

Fibre length also appears to be important if dusts are inhaled and Vorwald *et al.* (1951) reported that guinea-pigs, rats and mice treated for up to two years with ball-milled chrysotile developed no appreciable pulmonary reaction and no suggestion of asbestosis. Complete fibre sizing of the dust clouds was not undertaken but it was reported that while 90% of the dust was non-fibrous, one-third of the fibres present were $>10\mu m$ in length. These amounted to a concentration of approximately 25 fibres/ml.

The importance of long fibres in the production of pulmonary fibrosis following inhalation was further emphasized by Timbrell & Skidmore (1968) and Webster (1970). The former authors exposed rats and guinea-pigs to long and short fibres of amosite and obtained a much greater reaction with the long-fibre sample. Webster treated baboons with a finely ground crocidolite dust with a fibre length below $5\mu m$ and obtained only a macrophage reaction in the lungs.

Until recently inhalation studies with short-fibre samples of asbestos had not been continued for sufficient time to explore the production of pulmonary tumours and

some cases the dust clouds had been insufficiently categorized regarding the numbers of fibres in any size range for definite conclusions to be drawn. Davis *et al.* (1986*b*) reported life-time inhalation studies in rats undertaken with a specially prepared short-fibre sample of amosite with almost all fibres $< 5\mu m$ in length. The effects of this dust were compared to those of a long-fibre dust cloud generated from raw commercial amosite. While the long-fibre dust produced widespread pulmonary fibrosis and pulmonary tumours or mesotheliomas developed in one-third of the animals, neither pulmonary tumours nor pulmonary fibrosis developed in animals exposed to the short-fibre dust.

Long-term inhalation studies with a short-fibre chrysotile preparation produced by ball-milling were reported by Platek *et al.* (1985). These workers examined rats for up to 24 months after the start of dust exposure and monkeys for up to 28 months and reported that the short-fibre chrysotile produced 'no compound related lesions including fibrosis'. The dose level used in these studies was, however, only 1mg/m$^3$, which is lower than used by most workers examining fibre pathogenicity, and it has also been suggested by Lancer *et al.* (1978) that short-fibre chrysotile produced by ball-milling is subject to a level of crystal damage. This is sufficient to make results difficult to interpret in relation to hazards resulting from short fibres produced during the manufacture of asbestos products or during the subsequent usage of these materials.

Jolicoeur *et al.* (1981) reported on a method of obtaining short-fibre samples of chrysotile by a sedimentation technique which has overcome these difficulties. This preparation contained no fibres $> 8\mu m$ in length and has been used in a number of short-term studies to examine the early pathogenicity of the short chrysotile fibres. This short-fibre chrysotile preparation retained the haemolytic activity normally associated with chrysotile asbestos (Pele & Calvert 1983) and was toxic to pulmonary macrophages in short-term *in vitro* culture (Nadeau *et al.* 1986). Lemaire (1985) examined cell recruitment into rat lungs following intratracheal injection of the short-fibre chrysotile preparation and UICC chrysotile (B). While the UICC material produced a prolonged response with increased numbers of neutrophils in lavage fluid for up to 14 days following injection, the short-fibre chrysotile produce only an insignificant increase in neutrophils for 1 day following injection. Both chrysotile samples produced increased numbers of pulmonary macrophages which continued for up to 14 days, but the short-fibre dust produced a much lower level of response. In a separate publication Lemaire *et al.* (1985) reported on changes in pulmonary morphology produced in rats following the intratracheal injection of the two chrysotile samples. While the UICC dust produced fibrosis around distorted and obstructed airways, the short-fibre dust, although causing an accummulation of inflammatory cells, produced no fibrosis at all.

While these studies have suggested that the harmful potential of very short chrysotile is much less than that of chrysotile dust containing long fibres, intratracheal injection is an abnormal technique which bypasses the normal pulmonary clearance mechanisms. In order to investigate the fibrogenic and carcinogenic potential of any fibre sample in normal situations, inhalation studies are necessary.

This paper reports long-term inhalation and injection studies in rats treated either with dust generated from a specially prepared short-fibre sample of chrysotile or dust from the same type of raw chrysotile generated in such a way as to contain a large proportion of long fibres.

## Materials and methods

The short-fibre chrysotile sample used in these studies was supplied by the Institute for Research and Development of Asbestos (IRDA) and was prepared by the differential sedimentation techniques reported by Jolicoeur *et al.* (1981). Chemical, X-ray diffrac-

tion and differential thermal analyses have confirmed that the chrysotile structure was substantially unchanged by the sedimentation process. The long-fibre dust cloud was generated directly from the same batch of chrysotile (grade 4-T-30) that had been used to prepare the short-fibre sample.

The dust clouds were generated using a Timbrell fibrous dust dispenser (Timbrell *et al.* 1970) modified as described by Beckett (1975), and a fluidized bed aerosol (TSI 3400) for respectively the original long-fibre and short-fibre chrysotile respectively. The fluidized bed aerosol generator was needed to break up particle aggregates in the short-fibre material and to produce a cloud consisting mainly of separate fibres. The electrostatic charge carried on the airborne dust was reduced by exposure to ionizing radiation from $\beta$-sources. The purpose of reducing the charge was to avoid differences in charge level which might have arisen from using different types of dust dispenser (Johnston *et al.* 1987). The level of electrostatic charge can substantially enhance the deposition of chrysotile fibres (Jones *et al.* 1983).

The respirable dust mass concentrations were measured daily using the Casella MRE 113A with samples taken for the full 7 h of dust exposure and on each of the 224 days of dusting. The total dust concentrations were measured using open-face 50mm-diameter filter holders facing downwards with 7h-samples collected daily on ten days at a flow rate of 2 l/minute. The fibre number concentrations were assessed from membrane filter snatch (or very short period) samples collected using 25mm-diameter Gelman heads (open-faced filters) on 85 separate days. The samples were examined using phase contrast microscopy (at 600 × magnification) and counts made of fibres with length $>5\mu$m, aspect ratio greater than 3 : 1 and diameter $<3\mu$m. The counting procedures are essentially the same as those used for assessing samples from occupational environments (AIA 1979, HSE 1984).

The fibre size distributions were assessed also from snatch samples collected on membrane filters and nuclepore filters for optical and scanning electron microscopy respectively. Phase contrast microscopy was used to produce length distributions for the fibres which constituted the number concentration, i.e. those fibres longer than $5\mu$m, etc. The scanning electron microscope (SEM) was used to produce size distributions for fibres longer than $0.4\mu$m, with measurements taken from the video screen at 10000 × magnification. The minimum length corresponds to a detection limit of $0.15\mu$m for the diameters of fibres with an aspect ratio of approximately 3 : 1.

Samples of long and short-fibre chrysotile used in the intraperitoneal injection studies were collected directly from airborne material in the inhalation chambers by an elutriation process chosen to select the respirable fraction of the dust cloud (Bolton *et al.* 1982). For counting and sizing purposes, weighed amounts of dust were resuspended in known volumes of 35% ethanol in distilled water. These suspensions were treated with a brief period of ultrasonication to disperse fibre clumps before filtering on nuclepore filters. The filters were air dried under controlled conditions before being coated with gold for examination by scanning electron microscopy (SEM) at a magnification of 10 000 ×.

In the inhalation studies two groups of 48 rats of the AF/HAN strain were exposed to clouds of either long or short-fibre chrysotile at a dose level of 10mg/m$^3$ of respirable dust for 5 days each week during a period of 1 year. The animals were aged 3 months at the start of the study. Following the cessation of dusting, four animals from each group were killed to examine early histological changes in the lung tissue and to determine the pulmonary content of retained chrysotile. Four more animals from each group were killed 6 months after the end of dusting but the remainder were allowed to live out their full life span until the experiment was terminated when the number of survivors in one group dropped to six. This point was reached at 31 months after the start of dusting when the surviving rats were aged 34 months.

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For the injection studies six groups of 24 rats were given a single intraperitoneal injection of either long or short chrysotile at dose levels of 25, 2.5 or 0.25mg. For injection the animals were anaesthetized with ether and the dust was suspended in 1ml of Dulbecco's phosphate buffered saline. All animals in the injection studies were allowed to live out almost all of their full life span and were killed when moribund.

Forty-eight rats of the same age as the test groups were maintained in the experimental unit for their full life span as controls. Control animals were kept under identical conditions to the experimental groups except that they were maintained in normal animal holding rooms while animals used in the inhalation studies were in the inhalation chambers. Animals were killed when they showed signs of ill-health until the study was terminated when the seven surviving controls had reached an age of 3 years.

The autopsy schedule for animals in the inhalation studies and the control groups included the macroscopic examination of all major organ systems for signs of abnormality, and particular attention was paid to the visceral and parietal pleural surfaces. Any abnormalities found were taken for histological examination. In addition representative material was routinely taken from the following organs for histological examination whether pathological change was evident or not: lungs, mediastinal and hilar lymph nodes, liver, spleen, pancreas, kidney, gastro-intestinal tract, mesenteric lymph nodes, adrenal glands, testis and brain. Tissue for histological examination was fixed in 10% formal saline and embedded in paraffin wax. The lungs were fixed by instillation prior to excision from the thoracic cavity. Sections of all tissues were routinely stained with H&E. Van Giesen's collagen stain and Gordon and Sweet's reticulin stain were used for the study of pulmonary fibrosis. Serial sections were cut from the lungs of all animals autopsied with groups of sections being mounted at approximately 1mm-intervals throughout the block. This procedure provided material for detailed examination from six to eight levels of each lung.

Measurement of pulmonary fibrosis was undertaken by similar methods to those previously described by Davis *et al.* (1978) except that an electronic image analyser (Graphic Information Systems Limited, GDS1) was available for use in conjunction with the light microscope (Davis *et al.* 1985). Single lung sections were examined and the sections selected to contain the maximum area of lung parenchyma. As previously described, interstitial fibrosis was estimated using a ×2 microscope objective lens and expressed as a percentage of total lung tissue. Peribronchiolar lesions are more numerous and smaller and so the lung tissue was scanned with an eyepiece graticule covering the tissue area of 2.92mm² and divided into 100 squares. A ×4 objective lens was used. Peribronchiolar lesions were recorded as the percentage of squares containing lesions of this type.

The amount of chrysotile retained in the left lung was assayed for rats killed at the end of dust exposure and 6 months later. The right lung from these animals was prepared for histology. The lung burdens of chrysotile were recovered by ashing the lungs in oxygen plasma in a low-temperature asher (Nanotech P100), washing the residue with distilled water and recovering on a filter. The residue material was formed into a potassium bromide disc and the chrysotile content determined by infrared spectrophotometry (Dodgson & Whittaker 1973). Comparisons of the dust contents of left and right lungs have indicated that the ratio of the contents is 0.6:1 and this ratio was used to estimate the total lung burdens.

For statistical analysis the survival functions for each experimental group were estimated using the product-limit method (Kaplan & Meier 1958). The survival curves for the different series were tested for homogeneity using the Geralized Wilcoxon (Breslow) statistic in the statistical package BMDP (Dixon *et al.* 1983). In order to compare the overall mortality of the two groups of ani-

mals, the sacrifice of an animal as part of a planned kill was treated as a censoring event, all other deaths were treated as responses. Incidence of tumours in particular sites was also compared by estimating survival functions. All deaths of animals without tumours in the site in question were recorded as censoring events, whilst tumours in the specific site were treated as responses irrespective of whether the death was planned or unplanned. From the intraperitoneal injection studies, the number of tumours recorded as well as the survival times of the animals were combined using Cox's method to estimate the relative hazard of the different asbestos types at all dose levels (Cox 1972, Kalbfleisch & Prentice 1980).

Comparisons of levels of pulmonary fibrosis and lung dust burdens were made using the generalized linear models facilities in the statistical package GENSTAT (Alvey *et al.* 1977). The proportion of lung area with interstitial fibrosis was logarithmically transformed before analysis using conventional analysis of variance methods. The proportion of the points on the lung with peribronchial fibrosis was analysed by logistic regression techniques. Lung dust burdens were compared by analysis of variance.

## Results

The target mean respirable dust concentrations of 10mg/m³ were achieved. The total dust mass concentrations and fibre number concentrations corresponding to the respirable dust concentration of 10mg/m³ are shown in Table 1.

PCOM examination of samples from the two dust clouds confirmed that the 'long-fibre' dust cloud did contain many more fibres of all sizes above 5$\mu$m than the 'short-fibre' cloud. However, the short-fibre material used had obviously contained more long fibres than the original preparations reported on by Jolicoeur *et al.* (1981) when it was suggested that the sedimentation technique produced chrysotile samples with all fibres $<8\mu$m in length. In the present study the ratio of fibre number concentration between the long and short-fibre dust clouds was 5:1 for all fibres longer than 5$\mu$m rising to 20:1 for fibres longer than 20$\mu$m and 80:1 for fibres longer than 30$\mu$m. The fibre-length distributions obtained by PCOM for the long and short-fibre chrysotile clouds are illustrated in Fig. 1 together with recently obtained figures for UICC chrysotile 'A' which are included for comparison. The fibre-length and diameter distributions obtained by SEM for the three chrysotile varieties are illustrated in Figs 2 and 3. These sizings show that the fibre-length distributions of the long-fibre chrysotile cloud generated from bulk 4-T-30 material and used in the present study were very close to dust

Table 1. Airborne dust concentrations

| Type of measurement | Short-fibre chrysotile | Long-fibre chrysotile | UICC chrysotile |
|---|---|---|---|
| Mass of respirable dust mg/m³ (by Casella MRE 113A) | 10.0 | 10·0 | 9·9 |
| Mass of total dust mg/m³ (by downward-facing open 50mm-filter sampling at 2 l/min) | 13.9 | 11.81 | 11.0 |
| Fibre number obtained by PCOM fibres/ml for fibres longer ($\mu$m) than | | | |
| 5 | 1170 | 5510 | 2560 |
| 10 | 330 | 1930 | |
| 20 | 33 | 670 | |
| 30 | 4 | 320 | |

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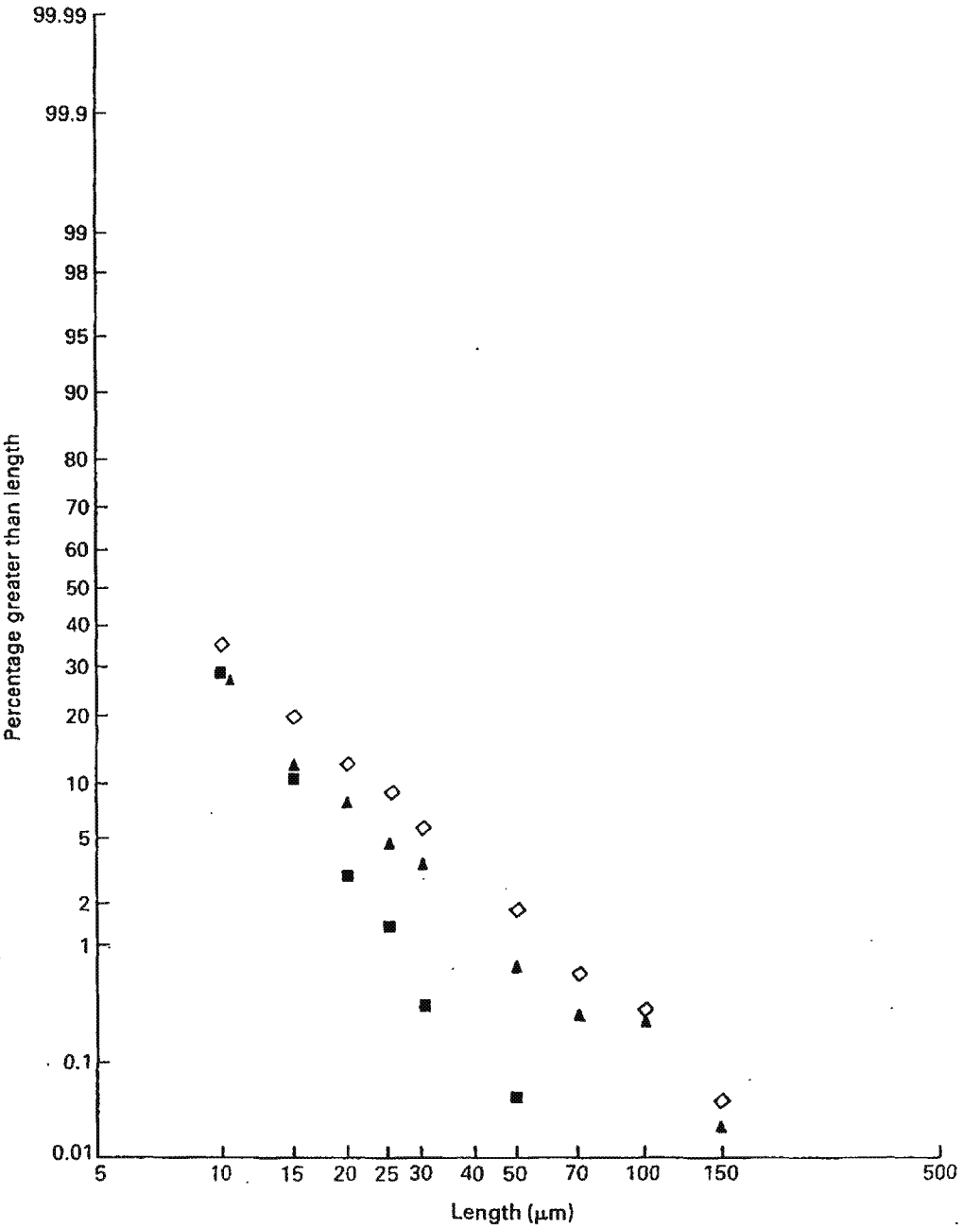


Fig. 1. Fibre length distributions of long and short-fibre chrysotile dust clouds as well as UICC chrysotile 'A'. Fibres sized by phase contrast optical microscopy at a magnification of ×600. Number sized in parentheses; ■, short fibre (2250); ◊, long fibre (2245); ▲, UICCA (4476).

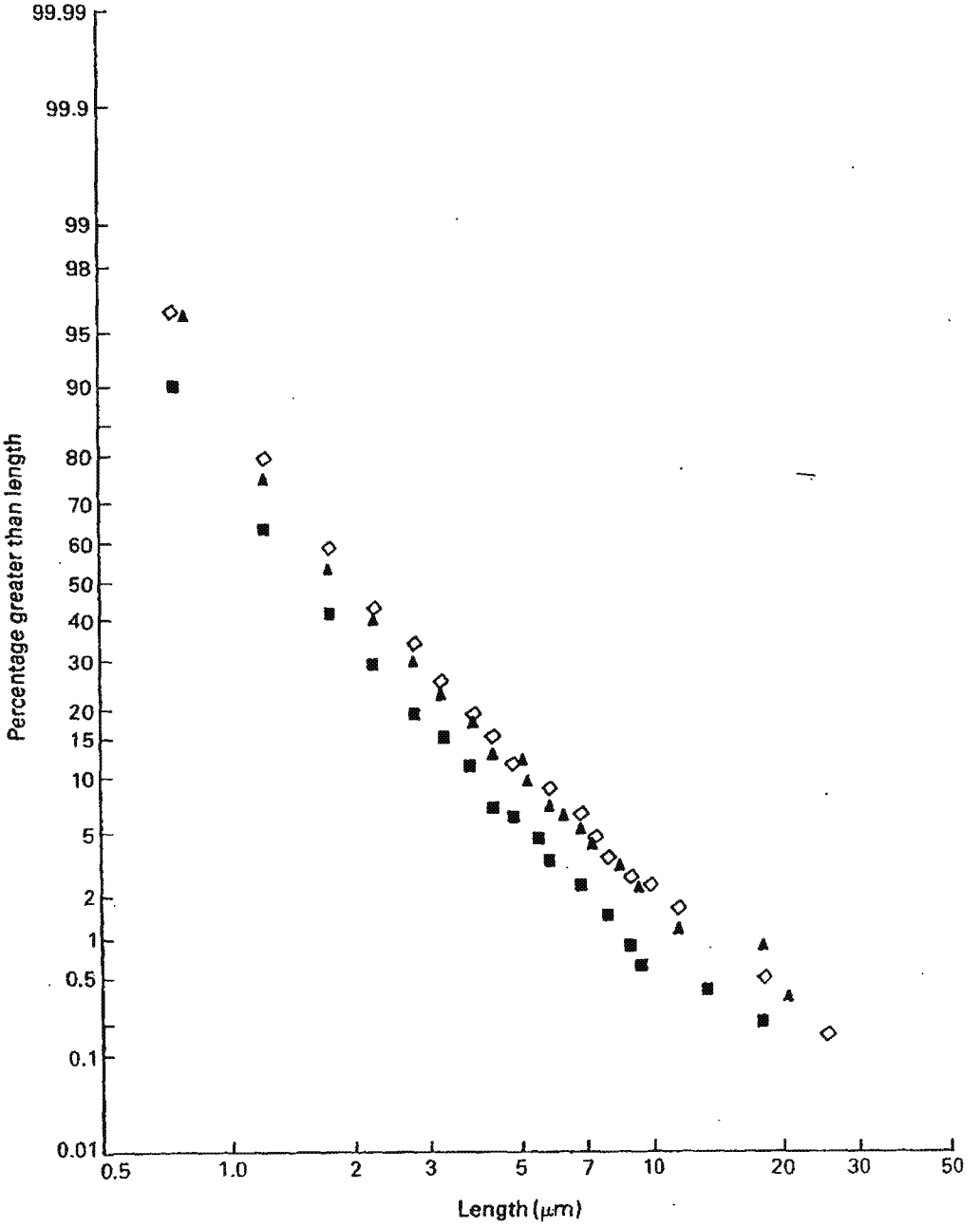


**Fig. 2.** Fibre length distributions of long and short-fibre (longer than 0.4μm) chrysotile dust clouds as well as UICC chrysotile 'A'. Fibres sized by scanning election microscopy at a ×10 000. Number sized in parentheses: ■, short fibre (500); ◊, long fibre (600); ▲, UICCA (600).

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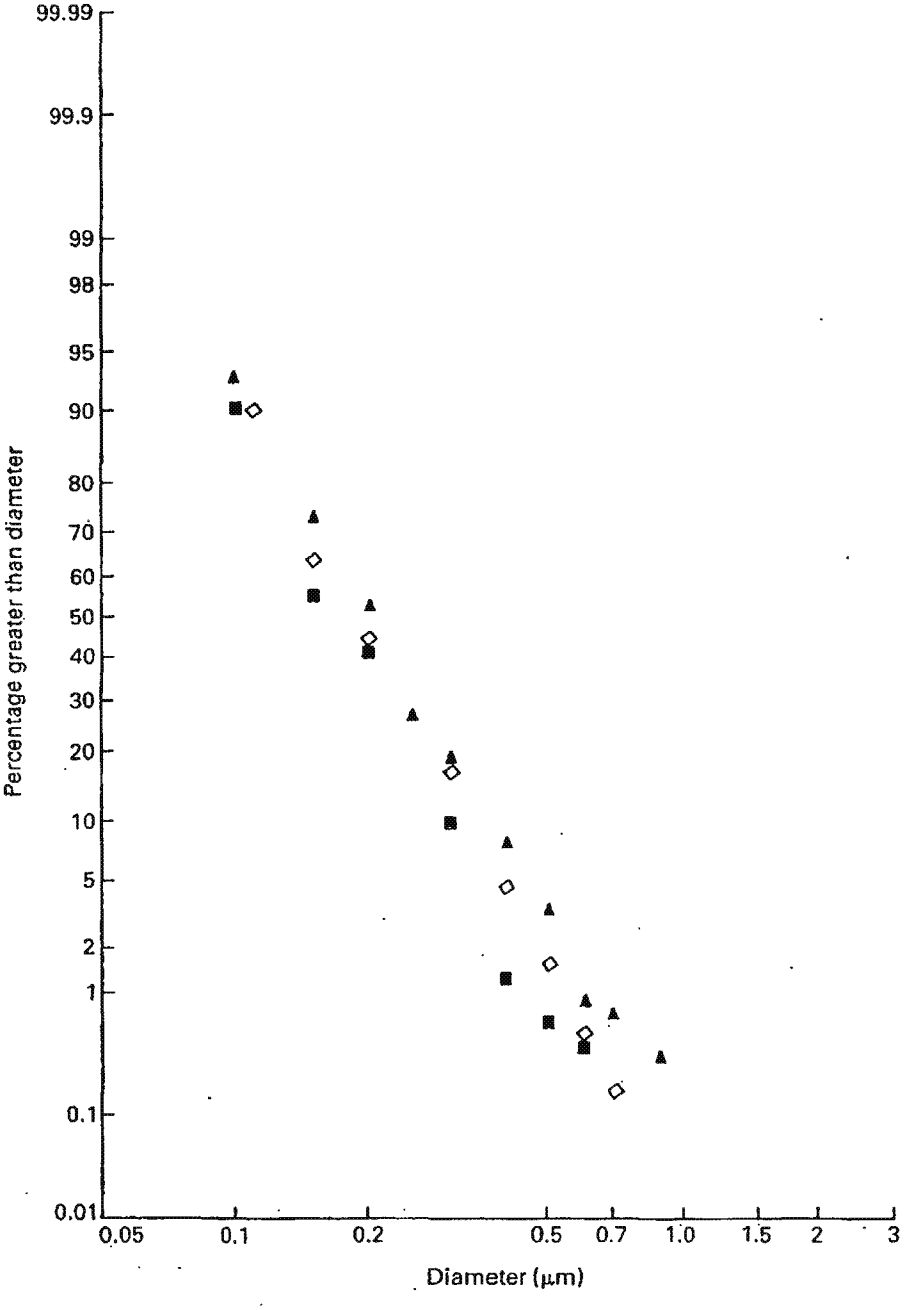


Fig. 3. Fibre diameter distributions of long and short-fibre (longer than 0.4$\mu$m) chrysotile dust clouds as well as UICC chrysotile 'A'. Fibres sized by scanning electron microscopy at a magnification of × 10 000. Number sized in parentheses: ■, short fibre (500); ◊, long fibre (600); ▲, UICCA (600).

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clouds recently generated in our laboratory from UICC chrysotile (Davis *et al.* 1988).

The fibre diameters of the long-fibre material were, however, somewhat lower than those of the UICC chrysotile. Probably because of this diameter difference the number of fibres > 5$\mu$m in length counted by PCOM was higher for the long-fibre dust clouds than for clouds of UICC chrysotile of the same respirable dust mass.

The survival of animals treated with both long and short-fibre chrysotile and animals in the control group was extremely good with the majority reaching an age of more than 800 days. There were no significant differences between the survival of the two test groups treated with the long and short chrysotile preparations but the control animals did survive longer on average ($P=0.002$).

Both groups of rats dusted with the chrysotile preparations developed the same pattern of pathological change previously reported in similar studies from this Institute (Davis *et al.* 1978, 1985, 1986*a–c*). At the end of the 12-month dusting period the main lesions present were deposits of granulation tissue around the terminal and respiratory bronchioles (Fig. 4). This granulation tissue consisted mainly of macrophages and fibroblasts but foreign-body giant cells were also present. At 12 months after the start of dusting there was marked reticulin staining in the peribronchiolar deposits although relatively little collagen could be demonstrated by Van Giesen's stain. With increasing time after dust exposure, however, collagen staining became progressively more marked and in old animals the lesions consisted of mainly acellular fibrous tissue. Both

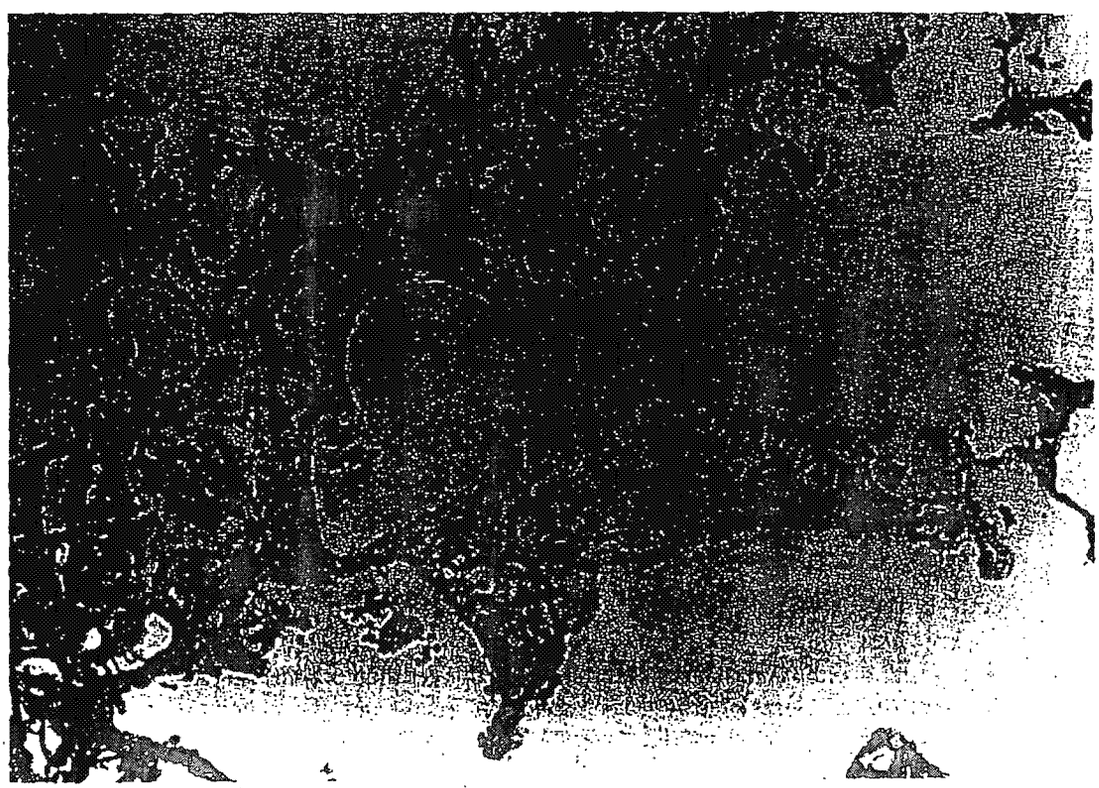

Fig. 4. An area of peribronchiolar fibrosis from the lungs of a rat after 12 months exposure to a dust cloud of 'long' chrysotile. × 350.

Table 2. L

| Time after of expos (months | Number of examine | Peribronch fibrosis | Interstitial fibrosis |
|---|---|---|---|

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Table 2. Levels of pulmonary fibrosis produced by long and short-fibre chrysotile dusts

| | Chrysotile dust | | | | | | |
|---|---|---|---|---|---|---|---|
| | Long chrysotile | | | Short chrysotile | | | Control group |
| Time after start of exposure (months) | 12 | 18 | 28–30 | 12 | 18 | 28–30 | 33 |
| Number of rats examined | 4 | 4 | 11 | 4 | 4 | 10 | 23 |
| Peribronchiolar fibrosis | 14.0 (9.4–18.2) | 8.9 (6.8–11.2) | — | 3.9 (2.9–5.7) | 2.1 (1.7–2.3) | — | 0 |
| Interstitial fibrosis | 0 | 0 | 12.6 (6.4–23.9) | 0.2 (0–0.9) | 0 | 2.4 (0.3–6.7) | 0.1 (0–1.9) |

The figures in brackets are ranges.

Fig. 5. An area of early interstitial fibrosis from the lungs of a rat 12 months after the termination of exposure to a dust cloud of long-fibre chrysotile. The alveolar walls are thickened and the epithelial lining appears to consist almost entirely of rounded type II pneumocytes. ×350.

treatment groups showed deposits of this peribronchiolar fibrosis around many of the smallest airways at 12 months (Table 2) but levels in the animals treated with long-fibre chrysotile were significantly higher than for the group treated with the short-fibre material ($P<0.001$). By 18 months both treatment groups showed a reduction in peribronchiolar fibrosis and taken overall, this reduction was also significant ($P<0.001$). After 18 months from the start of dusting, widespread alveolar interstitial fibrosis developed and this tended to obscure many of the earlier fibrotic deposits. For this reason, estimations of peribronchiolar fibrosis were limited to the first two killing dates.

From about 18 months onwards areas of lung tissue in some animals showed a progressive thickening of alveolar septa. This thickening of alveolar walls in response to treatment with asbestos was described in detail by Davis *et al.* (1986*c*). In its earliest form it was caused almost entirely by hyperplasia of alveolar lining cells (Fig. 5) but later there was considerable deposition of reticulin and eventually collagen in the septal walls. As shown in Table 2, areas of alveolar interstitial fibrosis became more widespread in both treatment groups with increasing time after the end of the dusting period. Mean areas of interstitial fibrosis found in those animals that survived to within 2 months of the final killing date were 12.6% for the group treated with long-fibre chrysotile (11 rats) and 2.4% for those treated with short-fibre chrysotile (10 rats). This difference is significant ($P<0.0001$). In many areas of lung tissue the interstitial fibrotic element of

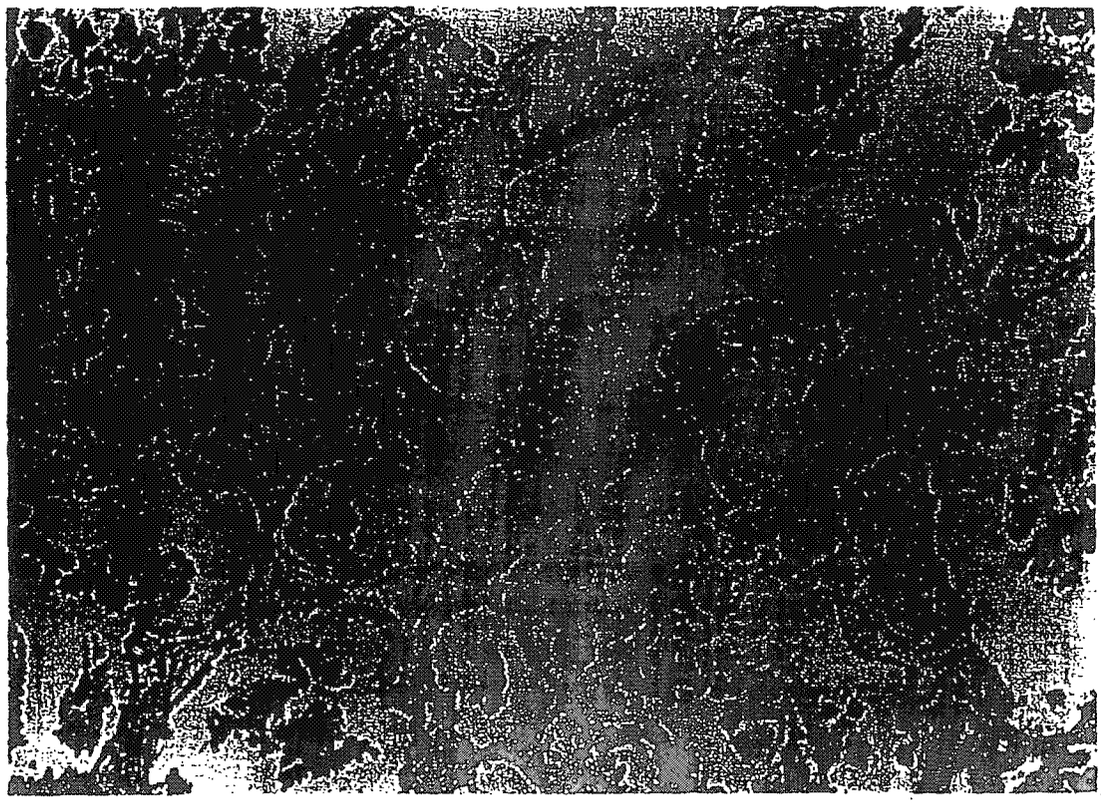

Fig. 6. An area of advanced interstitial fibrosis from the lungs of a rat 17 months after the termination of exposure to a dust cloud of 'long' fibre chrysotile. The airspaces which in many cases are enlarged and no longer correspond to the original alveoli are lined with rounded type II pneumocytes. The airspace walls are greatly thickened with reticulin and collagen which can be demonstrated by special stains. ×350.

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these lesions remained predominant throughout the study (Fig. 6) but in others the hyperplasia of alveolar epithelial cells became progressively more marked to produce a pattern of adenomatosis (Fig. 7). Some 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. All animals developing pulmonary neoplasms had significant levels of interstitial fibrosis or adenomatosis in addition.

Rats from both the groups treated with either long-fibre or short-fibre chrysotile developed some pulmonary neoplasms. However, while animals treated with the long-fibre preparation developed eight adenomas, eleven carcinomas and two pleural mesotheliomas, animals treated with short-fibre chrysotile developed only one adenoma and six carcinomas (Table 3). These overall tumour numbers are significantly different ($P<0.002$). The figures used in Table 3 represent the most serious pulmonary tumour present in any one rat. Two animals treated with the long-fibre chrysotile dust had benign pulmonary adenomas as well as a pulmonary carcinoma.

While only two pleural mesotheliomas developed in the present study, many of those rats examined in advanced age that had been treated with either the long or the short-fibre chrysotile preparation showed areas of vesicular pleural metaplasia. This type of lesion was also found in previous long-term inhalation studies with different asbestos preparations (Davis *et al.* 1986*a*). Areas of metaplasia consisted of loose,

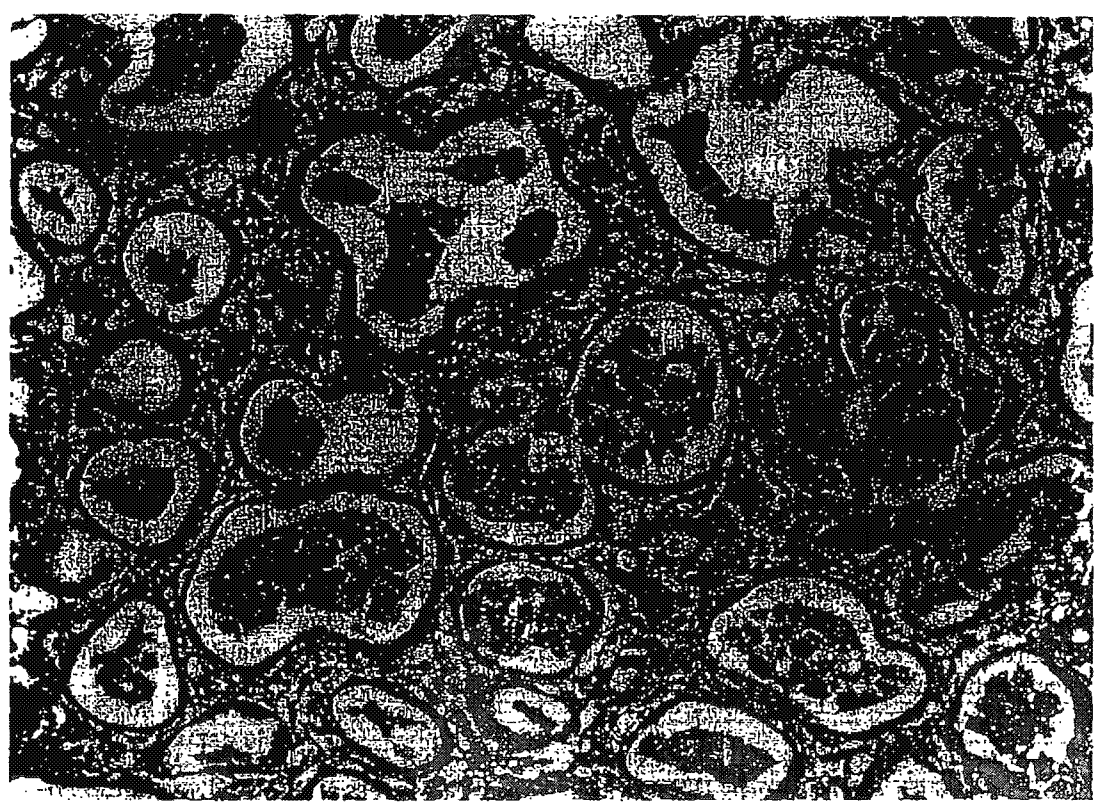

Fig. 7. An area of advanced interstitial fibrosis from the lungs of a 33-month-old rat treated with long-fibre chrysotile. The epithelial lining of the airspaces has become so prominent that the pattern is one of adenomatosis. ×350.

Table 3. Pulmonary tumours and mesotheliomas found in animals treated with long and short-fibre samples of chrysotile asbestos and in a control group of rats

| | Long chrysotile | Short chrysotile | Controls |
|---|---|---|---|
| Number of rats | 40 | 40 | 47 |
| Tumour type | | | |
| Adenomas | 8 | 1 | 1 |
| Adenocarcinomas | 6 | 6 | 1 |
| Squamous carcinomas | 5 | 0 | 0 |
| Histiocytomas | 1 | 0 | 0 |
| Pleural mesotheliomas | 2 | 0 | 0 |
| Peritoneal mesotheliomas | 1 | 1 | 0 |
| Totals | 23 | 8 | 2 |

Fig. 8. Vesicular hyperplasia of the visceral pleural surface of a rat lung 18 months after the end of exposure to 'long' fibre chrysotile. Many tissue spaces have been formed in a matrix of loose connective tissue and these are lined with flattened cells of mesothelial type. × 350

fibrous tissue containing large vesicular spaces lined with flattened cells (Fig. 8) and previous TEM studies had shown that these cells were of mesothelial type. Occasionally the walls between vesicular spaces were so thin that they consisted of two closely apposed layers of extended and flattened cells with no basement membrane between them. Where cells were supported by areas of fibrous tissue a basement membrane was

present. While no method has been developed for the direct quantification of this pleural metaplasia, its occurrence is closely related to the presence of advanced interstitial fibrosis or adenomatosis in the lung tissue and it is particularly common where patches of this type of parenchymal lesion have reached the surface. This means that in the present study it was much more frequently found in animals treated with long-fibre chrysotile than in those treated with the short-fibre preparation.

It is uncertain whether or not this vesicular pleural metaplasia is a potential precursor of pleural mesotheliomas or not. While almost all the chrysotile-treated animals surviving until the last 6 months of the present study showed some evidence of this type of change, only two definite pleural mesotheliomas were found. Both of these occurred in the long-fibre treatment group and neither showed histological patterns similar to the vesicular hyperplasia. One of these mesotheliomas had a papillary structure and the other consisted of spindle-shaped cells of fibrosarcomatos type.

No important differences were noted in the types of non-neoplastic disease arising in the extra-thoracic tissues of animals from the two chrysotile groups or from the controls. Since most of the animals were allowed to survive until of an advanced age, the majority of lesions present at autopsy were associated with senescence.

A large proportion of the rats in both chrysotile treatment groups and the control group developed tumours in non-pulmonary tissues as they reached advanced age. The numbers of tumours recorded are illustrated in Table 4 where individual tumours are recorded although a number of animals had more than one tumour present at autopsy. This applied to three animals in the long-fibre treatment group, six animals in the short-fibre group and six animals in the control group. There were no significant differences between the overall numbers of tumours found in the three groups of rats.

The lung content of chrysotile found in the long and short-fibre treatment groups at the end of the dusting period and 6 months later is illustrated in Table 5. Animals treated with short-fibre chrysotile retained nearly three times as much dust at the end of the exposure period as animals treated with long fibre. In the subsequent 6 months, however, 89% of the short-fibre chrysotile had been removed from the lung tissue compared to only 54% of

Table 4. Non-pulmonary tumours found in animals treated with long and short-fibre samples of chrysotile asbestos and in a control group of rats

| | Long chrysotile | | Short chrysotile | | Controls | |
|---|---|---|---|---|---|---|
| Number of rats examined | 41 | | 41 | | 47 | |
| Organ system | B | M | B | M | B | M |
| Digestive/peritoneal | 1 | 1 | 1 | 1 | 3 | 2 |
| Urinogenital | — | 1 | — | 3 | — | 1 |
| Endocrine | 6 | 6 | 4 | 8 | 13 | 4 |
| Musculo, skeletal and integumentary | 6 | 3 | 5 | 1 | 1 | 5 |
| Reticulo-endothelial/vascular | — | 6 | — | 6 | — | 3 |
| Central nervous system | — | — | — | — | — | 1 |
| Totals | 13 | 17 | 10 | 19 | 17 | 16 |

B, benign; M, malignant.

Table 5. Lung dust burdens from the chrysotile inhalation exposures

| Time post exposure | Mean lung burden of asbestos | | | |
|---|---|---|---|---|
| | Short-fibre chrysotile | | Long-fibre chrysotile | |
| | Left lung only | Both lungs** | Left lung only | Both lungs |
| 3 days | *392 (86) | 1019 (224) | 135 (75) | 351 (195) |
| 6 months | 42 (10) | 109 (26) | 62 (15) | 161 ( 39) |

* Units are micrograms of dust (s.d. in brackets) and are mean figures obtained from groups of four rats.

** Figures obtained by calculation from the left lung dust content.

the long-fibre dust. These differences in pulmonary deposition and clearance between the long and short-chrysotile preparations are both significant ($P<0.01$).

*Intraperitoneal injection studies*

*Sizing of dusts by scanning election microscopy.* The dust samples of long and short chrysotile used in these injection studies were collected from the inhalation chambers used in the inhalation studies by an elutriation process in order that they would be as comparable as possible with the dusts inhaled by the rats. It is recognized, however, that dust samples collected in this way will not be identical in fibre size distribution to airborne dusts and a separate SEM sizing exercise was undertaken with the dusts used for injection. The results obtained are illustrated in Table 6 expressed as fibre numbers per milligram of injected dust in each size range. Difficulties in determining from these figures an exact number of fibres in any size range needed to produce mesotheliomas are explained in the discussion section of this paper.

The numbers of peritoneal mesotheliomas found following injection of long and short-fibre chrysotile at three dose levels are listed in Table 7 together with the mean tumour

Table 6. Numbers of long and short-fibre chrysotile present in the dust samples used in the intraperitoneal injection study.

| Fibre length | Long-fibre chrysotile | Short-fibre chrysotile |
|---|---|---|
| All fibres | 92.7 | 343.0 |
| >1 | 57.1 | 162.0 |
| >3 | 21.2 | 24.4 |
| >5 | 15.2 | 7.4 |
| >8 | 10.2 | 2.3 |

These figures represent the number of fibres in each size range in each microgram of injected dust. They were obtained using scanning electron microscopy at a magnification of × 10 000.

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Table 7. Mesothelioma production following injection of long and short chrysotile dust

| Dose (mg) | Long-fibre chrysotile | | Short-fibre chrysotile | |
|---|---|---|---|---|
| | Animals with mesothelioma | Mean induction period (days) | Animals with mesothelioma | Mean induction period (days) |
| 25 | 23 | 361 | 22 | 504 |
| 2.5 | 22 | 511 | 8 | 675 |
| 0.25 | 16 | 736 | 0 | — |

Group size 24 rats in all cases.

induction periods for each group. At the highest dose level of 25mg, little difference was found in the numbers of tumours produced by the long and short-fibre chrysotile preparations. At dose levels of 2.5 and 0.25mg, however, the short-fibre chrysotile material was significantly less carcinogenic than the long ($P<0.001$). The 0.25mg dose of short-fibre chrysotile failed to produce any tumours in the group of 24 rats.

Within the experimental groups injected with either the long or short-fibre chrysotile preparation there was evidence of an increased tumour induction period with reducing dose of dust. This phenomenon has previously been reported for both chrysotile and amphibole asbestos (Bolton *et al.* 1984). Even at the 25mg dose level where tumour numbers were similar, the mean tumour induction period was different with the short-fibre preparation taking significantly longer to produce mesotheliomas ($P<0.001$). This increased tumour induction period with short-fibre chrysotile was also seen at the 2.5mg dose level where it was equally significant and would probably have been seen with the 0.25mg dose if the short-fibre chrysotile had produced any mesotheliomas at this level.

## Discussion

The present study has confirmed the importance of fibre length in the pathogenicity of chrysotile asbestos although the results are less clear cut than those recently reported for amosite where the inhalation of short fibres produced neither pulmonary fibrosis nor tumours (Davis *et al.* 1986*b*). The reason for this lies almost certainly in the quality of the short-fibre chrysotile preparation that was available for inhalation studies. In the original report of Jolicoeur *et al.* (1981) it was stated that the sedimentation techniques which had been developed allowed the production of chrysotile samples with all fibres below 8$\mu$m in length. It was found, however, that while this certainly applied to samples of a few milligrams, it was not practicable for the 1.5kg needed for long-term inhalation studies. In the airborne dust clouds at a respirable mass dose of 10mg/m$^3$, the short-fibre cloud had only five times fewer fibres $>5\mu$m in length as seen by PCOM than the long-fibre sample used for comparison. However, at greater fibre lengths the differences were more substantial with 20 times fewer fibres $>20\mu$m in length and 80 times fewer fibres $>30\mu$m in length.

This short-fibre chrysotile sample certainly represented the shortest chrysotile that has been available for long-term inhalation studies and has permitted a meaningful comparison to be made with long-fibre dust using both inhalation and injection techniques. Because of fibre levels of over 300

fibres/ml > 10μm in length, it is not surprising that the short-fibre dust cloud produced both pulmonary fibrosis and pulmonary tumours. However, the long-fibre dust with five times more fibres > 5μm in length and six times more fibres > 10μm in length produced approximately six times more advanced interstitial fibrosis in the oldest animals and three times more pulmonary tumours. These figures could indicate that whereas only the relatively long fibres stimulate fibrosis, the shorter fibres (< 5μm in length) which were present in much larger numbers in the short-fibre preparation, do have some carcinogenic potential. With an amosite preparation, however, where extremely few fibres were > 5μm in length, neither interstitial fibrosis nor pulmonary tumours developed (Davis *et al.* 1986*b*). It has been reported previously from this unit (Davis *et al.* 1986*c*) that asbestos-related pulmonary tumours in rats tend to develop from areas of interstitial fibrosis via a stage of epithelialization of airspaces which leads to adenomatosis. It is likely, therefore, that these stages of interstitial fibrosis and adenomatosis represent the direct response to the dust, with the progression of 'precancerous' lesions to definite tumours being a more haphazard process. If this is so then a fibrous dust that is unable to produce fibrosis, is unlikely to produce tumours.

An understanding of the precise number of fibres in any size range necessary to produce tumours has been an important research goal for many years. The problem is, however, beset with technical difficulties, particularly where inhalation studies are concerned. It is relatively easy to determine the numbers and sizes of fibres in a dust cloud but the proportions reaching the alveoli and retained in the lung for long periods will vary with fibre size and the chemical durability of the fibres. A number of workers including Wagner *et al.* (1974), Middleton *et al.* (1977) and Davis *et al.* (1978) have shown that following exposure to the same respirable dust mass, much more amphibole than chrysotile was present in rat lungs at the end of both long and short inhalation periods. It was suggested by Timbrell (1973) that this was due to the curly chrysotile fibres being more likely to impact on the upper airways and to be cleared on the mucociliary escalator within a few hours. Recent work with long and short amosite (Davis *et al.* 1986*b*) and the present study with long and short-fibre chrysotile have allowed a better understanding of the interrelationships between deposition, mechanical clearance from lung tissue, and fibre dissolution.

With both amosite and chrysotile, significantly more short-fibre material than long was present in the lungs at the end of a one-year dusting period, confirming that long fibres penetrate less easily into the lung parenchyma. The mean figures in question were 3570 and 5640μg/rat for amosite and 350 and 1020μg/rat for chrysotile. These figures demonstrate, however, that much more long-fibre amosite was present than the very much shorter chrysotile material, indicating that fibre penetration was not the only factor in determining the lung dust burden. During the 6-month period after the end of dusting the short-fibre amosite and chrysotile preparations cleared more rapidly than the long from the lung; at this time the long and short-fibre amosite burdens were 3080 and 4470μg respectively with the comparable figures for chrysotile being 161 and 109μg. This more rapid clearance of short fibres would be expected if mechanical removal of the short, easily phagocytosed fibres by pulmonary macrophages was the main process involved. However, the proportion of chrysotile removed was much higher than for the equivalent amosite preparations with clearance of long and short-fibre amosite at 14 and 20% respectively compared to 54 and 89% for the long and short chrysotile. It is possible that macrophages phagocytose and remove chrysotile fibres more readily than amosite but there is no evidence of this and it is more likely that these findings demonstrate that dissolution of chrysotile fibres in lung tissue is an important factor in the removal of this dust. These observations fit

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very well with findings from lung tissue where far more amphibole than chrysotile is found in the lungs of asbestos workers at autopsy, even when chrysotile has been known to constitute by far the bulk of dust inhaled (Pooley 1976, Gylseth *et al.* 1983).

The combination of variable fibre deposition and dissolution makes it impossible at present to calculate accurately the numbers of fibres in a dust cloud that will be available in the lung to cause disease many months later. Injection studies where there are no problems of deposition and clearance of dust should permit an easier examination of the number of fibres necessary to produce tumours. With chrysotile, however, the problem is still complicated by the separation of fibres into individual fibrils which may be a variable process when large masses of dust are injected. Electron microscope counting of fibres from a liquid suspension also presents technical difficulties. With all asbestos types it is difficult to be certain that the filtration of fibres onto nuclepore filters results in a completely even distribution of the material. With chrysotile this is particularly difficult and even in dilute suspensions the fibres tend to aggregate into clumps. One method of avoiding this is the use of a brief period of ultrasonication of the solution before filtration, a process adopted by most workers examining the fibre content of human lungs. There is a risk, however, that this treatment will cause some splitting of fibres, particularly chrysotile, and so increase the numbers counted.

In spite of these problems, it was still felt to be a worthwhile exercise to produce SEM counts from the long and short-fibre chrysotile preparations used in the injection studies (Table 6). For the reasons stated, they probably represent an overestimate of the number of fibres injected although with the separation of chrysotile into individual fibrils in the tissues, they will be an underestimate of the number of active fibre units available to stimulate tumour production. However, regardless of the level of accuracy of the chrysotile counts obtained by SEM, it is obvious that very large numbers of fibres must be injected into the peritoneum of rats to produce mesotheliomas. The dose of short-fibre chrysotile which produced no tumours in 24 rats was calculated to contain $8.6 \times 10^9$ fibres of all lengths and $57 \times 10^6$ fibres $> 8\mu m$ in length (Stanton *et al.* (1977) calculated that fibres $> 8\mu m$ in length were the most carcinogenic). While the number of animals injected was too small for it to be certain that this represents a definite zero response, the injected dose was still very substantial in terms of fibre number. Even allowing for the short life span of the rat, this seems to be strong evidence that substantial numbers of fibres must reach the pleura in order to make it likely that mesothelioma will develop. This may be of relevance to current fears of the possible carcinogenic effect of extremely low exposures of humans to asbestos, such as occur in the environments of public buildings and schools.

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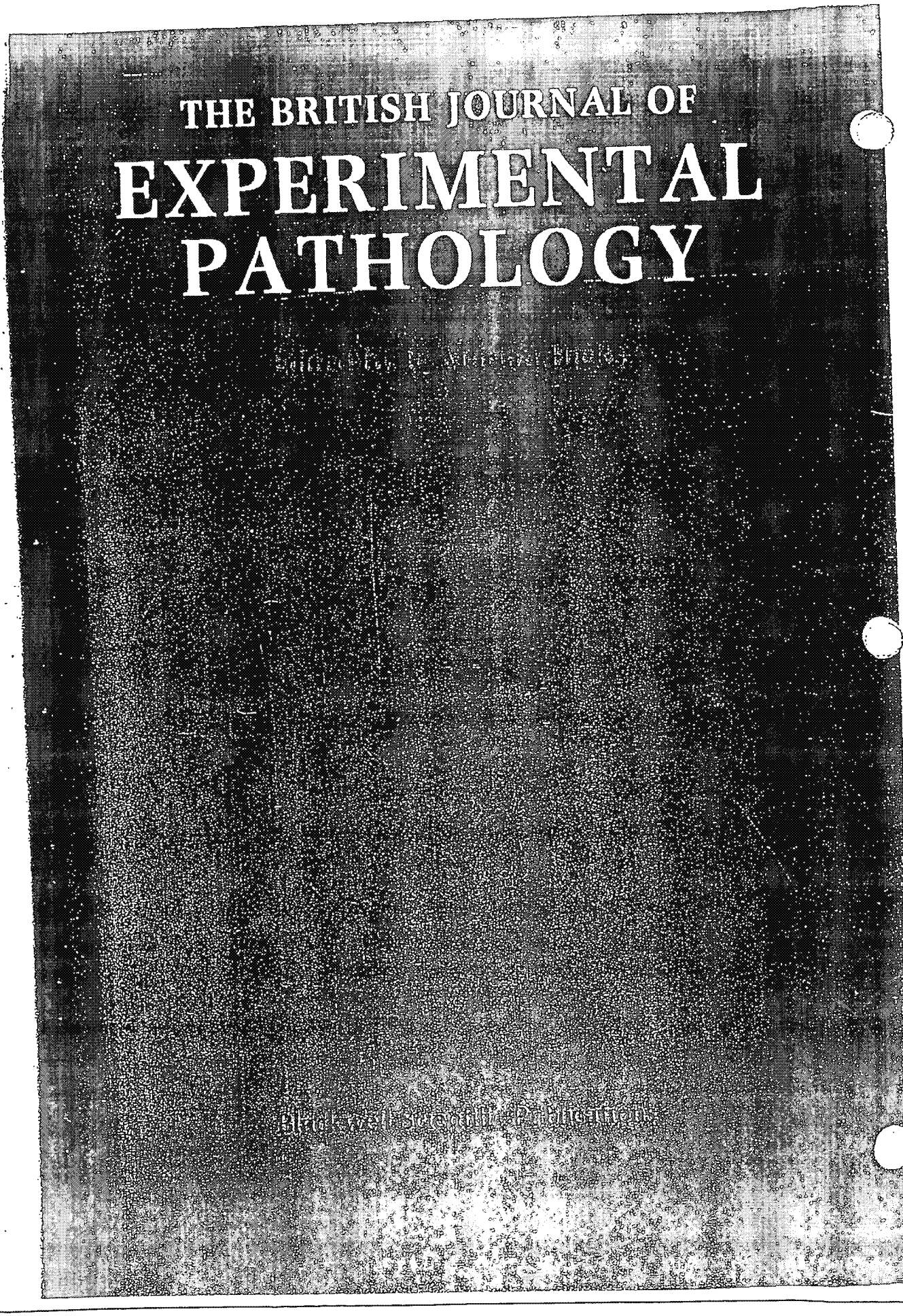


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