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A Review of Carcinogenicity Studies of Asbestos and Non-Asbestos Tremolite and Other Amphiboles.

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ABSTRACT

Experimental animal studies comparing asbestos and non-asbestos varieties of tremolite indicate tremolite asbestos is markedly more carcinogenic. By direct analogy, the differences in carcinogenicity between tremolite asbestos and non-asbestos prismatic tremolite should be the same for the other types of amphibole that also crystallize in the asbestos and non-asbestos habits. The earliest of the experiment animal studies, done more than 25 years ago, have design limitations by modern standards including the use of injection or surgical implantation as the route of administration rather than the more relevant route of inhalation. However the differences in the carcinogenicity of amphibole asbestos and non-amphibole asbestos are sufficiently large to be clearly discernable even with the study limitations. Together with later studies on these and related minerals, there is strong evidence of a much lower hazard associated with the shorter, thicker fibers of the non-asbestos amphiboles, than is found for the asbestos analogues of the same mineral. It is possible that the non-asbestos amphiboles are no more hazardous than other silicate minerals widely considered nuisance dusts.

INTRODUCTION

We will define some basic asbestos terminology to clarify the terms used. The glossary in 'The Health Effects of Mineral Dusts' produced by The Mineralogical Society of America (Guthrie & Mossman 1993) has the following definition: "Asbestos is a term applied to asbestiform varieties of serpentine and amphibole, particularly chrysotile, 'crocidolite', 'amosite', asbestiform tremolite, asbestiform actinolite, and asbestiform anthophyllite. The asbestos minerals possess asbestiform characteristics". The Mineral Society's glossary goes on to define asbestiform as: 'an adjective describing inorganic materials that possess the form and appearance of asbestos. When applied to a mineral, the term 'fibrous' is applied when it 'gives the appearance of being

composed of fibers, whether the mineral actually contains separable fibers or not' (Veblen and Wylie 1993). Asbestiform is a subset of fibrous, where asbestiform implies relatively small fiber thickness and large fiber length, flexibility, easy separability, and a parallel arrangement of the fibers in native (unprocessed) samples. Often, asbestos fibers occur in bundles, i.e., they are often polyfilamentous. From the definition it is clear that not all fibers or fibrous minerals are asbestiform and not all fibrous minerals called asbestiform are asbestos.

A convention has developed that a fiber is any particle with an aspect ratio equal to or greater than 3:1. This stems from the fiber definition in the early UK and US fiber counting methods (Asbestosis Research Council 1969, Asbestos Textile Institute 1971, Langer et al. 1991); it could just as easily have been 5:1 or 10:1. In using these methods, the microscopist had to make a decision to count or not count a particle depending on whether the shape and size met certain size criteria. The decision was more easily and consistently made for particles with aspect ratios just higher or lower than 3:1, and much more difficult with the higher aspect ratio thresholds. Similarly, a minimum fiber length of 5 μm was arbitrarily introduced for a fiber to be counted by these methods.

The inclusion of the abundant short fibers (less than 5μm length) in the count would have made it much less consistent or reliable. Since the aim of the fiber counting rules was to differentiate between asbestos and total particles aspect ratio and length cut-off chosen was one that produced consistency and not the ratio or length that might have had greater toxicological significance. By convention then, for a fiber to be counted it has to have an aspect ratio equal to or greater than 3:1 and a length equal to or greater than 5μm (and in some rules a diameter less than 3 μm, e.g. WHO 1985).This counting strategy has nothing to do with a definition of asbestos per se; it is simply helpful to microscopists doing fiber counting method. Since fiber counting analysis is performed using a phase-contrast light microscope at a magnification of 400-450x, the minimum width that can be counted is 0.2-0.25 μm.

Many non-asbestos particles, including non-asbestos amphiboles and other minerals can have aspect ratio greater than 3:1, but that does not make them ‘asbestos’ even though they are technically fibers. However, it does mean that they would be counted *as if they were an asbestos fiber* when seen in the course of a count of fibers in a membrane filter sample of airborne dust. In addition, asbestos will produce asbestos dust particles that mostly have aspect ratios equal to or greater than 3:1, but it will also produce particles that have lower aspect ratio. That does not mean that these low aspect ratio particles are not asbestos, but simply that they would not be counted as asbestos in the membrane filter method. The same is true for asbestos fibers with lengths shorter than the 5 μm minimum specified in the fiber counting method.

The adoption by some scientists and regulatory agencies of the fiber counting protocol using a 3:1 aspect ratio and a length of 5 μm or greater as being in some way a definition of asbestos has no scientific basis. It has been useful an improved metric when compared to just counting particles for assessing workplace exposure to airborne fiber dust leading to better epidemiological correlations between asbestos exposures with disease.

MINERALOGY

Tremolite is one member of the calcic amphibole group of minerals that all possess similar crystal structures, basic chemical formula, although the various crystal forms have profoundly different physical properties. The group is characterized by a crystal structure described as a double chain of silicon oxide tetrahedra that is common to all members of the group. Within this chain structure are between 7 and 8 metal cations allowing wide range in elemental composition that still maintains the basic crystalline form (Deer et al. 1997). This has produced the large number of named variants or species within the amphibole group (Leake et al. 1997, 2004) Actinolite and ferro-actinolite are part of a solid solution series with tremolite and differ only in the amount of substitution of magnesium by iron.

All of the amphibole minerals, and particularly tremolite, are very resistant to chemical attack by strong acids and bases (Addison & Davies 1990) so that their biopersistence when inhaled would be expected to be very high. In addition to the chemical variability there is further variability in what is known as the crystal habit of the minerals that may arise independent of chemistry (Dorling & Zussman 1987). The habit of a mineral is a description of the way that the crystals are commonly formed, and might otherwise be described as morphology.

The most common crystal habit for any amphibole is that called prismatic; elongate prisms with a lozenge shaped cross section that grade one way into short stocky prisms and in the other way into fine needle-like crystals or ultimately fine hair-like crystals (sometimes known as byssolite). The prismatic habit is the normal form for amphiboles in igneous and metamorphic rocks and is very widespread throughout the continental crust of the planet. Some amphiboles are also found in the habit that is termed asbestiform; this means that they have crystallized as bundles or matted masses of extremely fine fibers. The appearance of these forms usually implies some sort of secondary modification such as shearing and faulting or hydrothermal alteration. These may be found in three types of geological situations; 1) cross-fiber veins where the fibers have filled planar fissures, such as in the riebeckite (crocidolite) asbestos and grunerite (amosite) asbestos mines of South Africa; 2) in shear planes where slip fiber has formed in the plane of movement of a fault or shear plane; or 3) as disseminated fiber formed by hydrothermal alteration, such as in Libby, Montana (Meeker et al. 2003).

The differences in the manner of the formation of asbestos amphiboles, compared to the prismatic and other forms, have led to subtle differences in the details of the crystal structure that, while not sufficient to warrant a different mineral name, nevertheless lead to profound differences in physical properties (Langer et al. 1991). The commercial exploitation of the asbestos amphiboles depended upon these properties, including their capacity to be readily split into long, thin fibers with high tensile strength. These physical differences also lead to

differences in the size distributions of dusts formed when the minerals are crushed, and arguably properties which impact the pathogenic potential of the material, especially their carcinogenic properties when these dusts are inhaled. Cleavage planes are planes of relative weakness along which certain minerals tend to fracture and are determined by the crystal lattice geometry. Mica, for example, is described as having a single perfect cleavage because it splits easily along the silicate sheet structure. Calcite has three perfect cleavages that form perfect rhombohedra when the mineral is crushed. Amphiboles have two sets of cleavage planes at 126º to each other and parallel to the long axis of the crystals (and parallel to the dominant prismatic crystal faces). In addition they also have a cleavage plane on (100).

These are not perfect cleavages; they are not persistent across or along the crystals and tend to be more widely spaced than the separations between the fibers of the asbestos amphiboles. The prismatic amphiboles, including byssolites, have relatively low tensile strength and the thin needle-like crystals fracture easily across the length. They also fracture along cleavage planes that are parallel to the length of the crystals. When prismatic amphiboles are crushed a relatively small proportion of the fragments formed are elongate with faces determined by the cleavages along which the crystal fractures. These elongate particles will often meet the regulatory size criteria for an asbestos fiber within the asbestos permissible exposure limits, but differ from the asbestos fibers in critical ways. The cleavage fragment fibers often show the typical lozenge shape cross section as determined by the cleavage faces, at 126º degrees to each other. The cleavage fragment fibers tend to be thicker than asbestos fibers because of the spacing of the cleavage planes, and for any given length the cleavage fragment fibers are roughly twice as thick as asbestos fibers. Very few, if any, of the cleavage fragment fibers longer than 10μm will have diameters less than 1μm. With cleavage fragment fibers the width distribution is much broader and width increases with length so aspect ratios tend to be lower and of narrower distribution. In overall size distributions the asbestos fibers have a very narrow width distribution and the width

of fibers is largely independent of length. As a result, the aspect ratio of fibers increases with length.

Since the cleavage fragments and asbestiform fibers tend to be morphologically defined by somewhat different crystal surfaces it is tempting to speculate that this may go some way to explaining the apparent differences in toxicological properties as described below.

EXPERIMENTAL ANIMAL STUDIES

Five *in vivo* experimental animal studies provide information on the variation in carcinogenicity of dusts derived from prismatic or non-asbestos tremolite and tremolite asbestos. Davis et al. (1985) remains the only inhalation experiment to be carried out using tremolite asbestos. Previously, Smith et al. (1979) used a variety of tremolite types for intrapleural injection in hamsters; Stanton et al. (1981) used two different tremolites for intrapleural implantations in rats, while Wagner et al. (1982) report on three different tremolites for intrapleural injection in rats. Later, Davis et al. (1991) used six tremolites of different morphology for intraperitoneal injections in rats. If the actinolite and ferro-actinolite amphiboles are included the number of studies increases slightly but is still small. Coffin et al. (1978, 1982, 1983) and Cook et al. (1982), used a fibrous ferro-actinolite in intrapleural injection and intratracheal instillation into rats. Pott et al. (1974, 1989, 1991) reported results from intraperitoneal injection of a granular actinolite and (later) an asbestiform actinolite. A lifetime (including exposure to the dams and gavage during the neonatal period) oral ingestion study (1% in the diet) in rats of ‘blocky’ tremolite did not to show evidence of carcinogenic activity (NTP 1990, McConnell et al. 1983).

Other studies might also be considered as contributing to the debate about the relative carcinogenicity of amphiboles and their asbestiform varieties. Berman et al. (1995) reviewed the size distributions of all of the asbestos dust exposures used in the Institute of Occupational Medicine inhalation studies over many years, including the Korean asbestos tremolite, and

concluded that, while no univariate measure of exposure could be found to predict lung tumor incidences, the concentration of total structures longer than 20 μm provided the best fit. Furthermore the best estimate for the carcinogenic potency of fibers greater than 0.5 μm in width was zero. The inhalation and intraperitoneal injection experiments of Davies et al. (1986) with long and short fiber amosite, the inhalation studies of various sized chrysotile (Ilgren and Chatfield 1998, McConnell et al. 1984, Wagner et al. 1984), and the cell studies of Donaldson et al. (1989, 1991), Donaldson and Golyasnya (1995), Brown et al. (1986) were aimed at understanding the relative importance of fiber length in carcinogenicity and fibrogenicity. Other mechanistic studies such as those by Kane (1991), and reviews such as those by Oberdorster & Lehnert (1991) and Jaurand (1991), among others also have a bearing on the understanding of the different reactions observed between asbestos particles and other particles with the same mineral chemistry but different morphology.

Inhalation Experiments

Davies et al. (1985) exposed rats (SPF male Wistar, whole body exposure) to a commercially mined tremolite asbestos form South Korea at concentrations of 10 mg/m^3, around 1600 f/mL, (>5 μm) for 12 months. Having produced very high levels of pulmonary fibrosis as well as 16 carcinomas and two mesotheliomas (rarely found in rat inhalation experiments) among the 39 treated animals the tremolite asbestos was described by them as the most dangerous mineral ever studied at the Institute of Occupational Medicine, UK. The Korean tremolite asbestos is the same one used later in the intraperitoneal injection experiments (Davis et al. 1991) for which full size distributions of the respirable dust were given, as shown in Figure 2.

The important feature of the size distribution of the Korean tremolite asbestos is that the vast majority of fibers are less than 0.5 μm in diameter and shorter than 5 μm in length, which is typical of asbestos amphiboles. The geometric mean diameter for Korean tremolite asbestos

fibers longer than 0.4 μm was 0.24 μm (SD 1.6) and the mean length was 1.97 μm (SD 2.11) which are somewhat longer and thicker than airborne fibers in crocidolite mining (GM diameter 0.076 μm, GM length 0.98 μm, Hwang & Gibbs 1981).

The high carcinogenicity of the Korean tremolite asbestos was attributed to the much higher airborne fiber concentration for fibers longer than 5 μm (1600 f/mL) which was almost twice that of the UICC amphiboles at the same 10 mg/m^3 dust mass concentration used grunerite (amosite) asbestos 550f/mL and riebeckite (crocidolite) asbestos 860 f/mL, Davis et al. 1978). This also is a reflection of the finer diameter of the Korean tremolite asbestos.

Injection and Implantation Experiments

Smith et al. (1979) injected a range of tremolites and tremolitic talcs intrapleurally into hamsters (of unspecified type) at doses of 10 mg and 25 mg. The samples were identified as follows**:**

The animals were allowed to survive up to 600 days after which the final survivors were sacrificed for necropsy. No tumors were found in the final survivors. The samples used by Smith et al. 1979 and described as asbestos or asbestiform produced higher levels of fibrosis and numbers of mesotheliomas in the hamsters than those described as tremolite or tremolitic talc. Most of the tumors were diagnosed as mesotheliomas.

Campbell et al. (1979) examined some of the tremolites used by Smith et al. 1979 and described two of the tremolites (275 and FD72) in more detail. The images of the fibers clearly show FD72 (tumor rate 5/23 and 3/13) to be asbestos and 275 (tumor rate 0/31 and 0/34) to be a prismatic amphibole. This is reflected in the numbers of fibers of length > 10 μm and diameters less than 1μm in the tremolite asbestos, and their absence in the non-asbestos minerals. Similarly, in tremolite FD72 many more of the fibers longer than 5μm had aspect ratios greater than 10:1 than in tremolite 275 (23 – 0, and 19 – 1 using the petrographic microscope and the Scanning Electron Microscope respectively).

Non-asbestos tremolite 14 (FD 14, tumor rate 0/35) was later evaluated by Wylie et al. (1993) and confirmed to be a tremolitic talc with very few tremolite fibers in the size ranges longer than 5 μm and less than 1μm diameter.

This study was criticized for being deficient in a number of ways (Federal Register, 1992). In particular, the fiber size measurements and fiber characterizations were found to be inadequate for the purposes of identification of the materials as tremolite asbestos or prismatic tremolite. The later characterizations by Campbell et al. (1979) and by Wylie et al. (1993) improved on the original ones and the classification of the mineral types appears established. The higher carcinogenicity of those materials described as asbestiform compared to those of tremolitic talc or non-asbestos tremolite is without doubt.

Wagner et al. (1982) used a tremolite from the California talc deposits (A), a prismatic tremolite from Greenland (B) and a tremolite asbestos from Korea (C, probably from the same source as the one in Davies et al. 1985) for a series of intrapleural injection experiments with SPF Sprague-Dawley and Wistar rats and a range of *in vitro* tests. The rats were 8 –10 weeks old when injected and were allowed to live out their lives. Median survival times after injections were 644, 549, and 557 days respectively for samples A, B and C.

The value of the Wagner et al. (1982) injection experiments was impaired by the poor survival rates as a result of infection of the positive control animals injected with riebeckite (crocidolite) asbestos. Nevertheless, the tremolite (C) asbestos was the only one the three tremolites that showed carcinogenic activity producing mesotheliomas in 14 of 47 rats (30%). Neither of the other non-asbestos tremolites produced any tumors in the 31 and 48 rats used. The fiber size data as presented are not amenable to numerical evaluation, but measurements taken from the published diagrams show that in the tremolite (C) asbestos about 25% were longer than 10 μm and less than 0.6 μm in width. The non-asbestos forms had no fibers at all in that size range

(Sample A California, or Sample B, Greenland). Table 2 shows Wagner's figures for the numbers of particles, fibers longer than 1 μm, and fibers longer than 8 μm with widths less than 1.5 μm; the differences are obvious with Tremolite C containing many more long fibers.

The *in vitro* tests used by Wagner et al. (1982), including mouse peritoneal macrophage lactic dehydrogenase (LDH) and B-glucuronidase (BGL) release, cytotoxicity to V79-4 cells and giant cell stimulation with A549 cells confirmed the relative toxicity of the different tremolite morphologies *in vivo*. So, while the study remains limited by the poor survival of the positive controls, it is nevertheless useful in that it reproduces the general findings of Smith et al. (1979).

Stanton et al. (1981) described a series of 70 experiments where a wide range of different fibers were implanted at doses of 40 mg in hardened gelatin on to the left pleural surface of Osborne-Mendel rats by thoracotomy. It should be noted that in contrast to intrapleural or intraperitoneal injection, the use of the "hardened gelatin" exposure technique literally holds the fibers in contact with the target tissue (pleura) and does not allow for potential macrophage phagocytosis and clearance of the particles. Therefore this technique may create the highest effective dose of all of the exposure methods used for assessing the potential carcinogenicity of fibers. Stanton et al. 1981 reported on two tremolite asbestos samples from the same lot, described as "in the optimal range of size for carcinogenesis" and "distinctly smaller in diameter than the tremolite fibers used by Smith et al. (1979)". As they anticipated the two tremolites produced mesotheliomas in 21 and 22 animals out of the 28 used, with a 100% tumor probability. The tremolites contained very high numbers of fibers in the Stanton size range (>8 μm in length and <0.25 μm diameter) with 1.63×10^{8} and 2.76×10^{7} respectively *in each dose* for tremolites 1 and 2. In addition, the talc (No 6), which produced no tumors in the Stanton study, was actually New York State tremolitic talc (Wylie et al. 1993) with 40-50% non-asbestos tremolite and talc fibers, in fact the same material as used by Smith et al. (1979) and identified as FD 14. The general relationship between the probability of developing a tumor in these experiments and the

common logarithm of the number of fibers > 8μm in length and less than 0.25 μm in diameter per microgram of implanted dust was highly significant (Figure 3).

There were however a number of problematical experiments in the Stanton series where tumors developed for test materials with no fibers in the critical size range, and one where no tumors had developed even with large numbers of critical fibers present. Some of these results were attributed to large numbers of fibers with sizes close to the critical range, and others to problems of clumping and fragmentation in the fiber preparations for transmission electron microscopy analysis

Figures 3 and 4 show the general relationships developed, and described by Stanton as highly significant, between the numbers of fibers per microgram in the dose and the probability of tumor development. The statistical relationships between the fiber numbers in the different sets and probabilities of tumor development have not been evaluated but the diagrams show that the correlation for the shorter classes of fiber is much weaker than that for the longer fibers. It is reasonable to suggest that there must be more short fibers per microgram in the short fiber dusts than in the longer fiber dusts so the poorer correlation for short fibers is, if anything, even more indicative of their lack of importance in tumor development.

The size distributions given in Stanton et al. (1981) do not make it easy for full comparison with other size distributions of known asbestos minerals because the size classification was relatively crude and the method of exposure (hardened gelatin) was unique. The two tremolite samples however have sufficient numbers of long fibers with diameters less than 0.5 μm to indicate that their identification as asbestos is reasonable. The size distributions are somewhat unusual for pure asbestos as is seen in Figure 5 which shows Tremolite 2 to have a bimodal distribution which suggests that it is actually a mixture of tremolite asbestos and prismatic

tremolite. Such an occurrence in poor commercial quality tremolite asbestos formations is common.

Wylie et al. (1993) re-examined Tremolite 1 and 2 as well as Talc 6 that were used in the Stanton studies. They state that Tremolites 1 and 2 are the same material, tremolite asbestos from California, with all the characteristics of commercial amphibole asbestos. The two size distributions given by Stanton differ somewhat but they are similar and have the appearance of a mixed asbestos – prismatic fiber assemblage.

In contrast, the size distribution of Stanton's Talc 6 shows the much thinner, shorter distribution (Figure 6) not typical of a prismatic tremolite fiber population even though it consists of 40-50% tremolite. Talc 6 produced no tumors despite containing more fibers in the "Stanton fiber" range than Tremolite 2, and almost as many as Tremolite 1, both of which had a 100% probability of producing tumors.

This talc (6), or tremolitic talc, was reported by Wylie et al. (1993) as being identified in Stanton's laboratory notes as Nytal 300. Pure talc is a specific mineral with a closely defined chemical composition and crystal structure. Commercial producers however often named their products as 'talc' even though they contained less than 50% of the mineral talc.

Davies et al. (1991) used six tremolites of differing morphologies in a series of intraperitoneal fiber in saline injection experiments with male SPF Wistar rats. These were identified as follows:

1. Tremolite asbestos from Jamestown, California, United States;
2. Tremolite asbestos from Korea;
3. Tremolite asbestos from National Coal Board Laboratory, Swansea, Wales, Great Britain;

4. Tremolite, long needle-like crystals from Ala di Stura, N. Italy;
5. Tremolite, short needle-like crystals from Dornie, NW Scotland, Great Britain;
6. Tremolite, prismatic crystals from Shinness, N. Scotland, Great Britain.

The tremolite from Korea was the same material as was used in the earlier tremolite inhalation and injection experiments by Davis et al. (1991). The fiber size distributions were assessed by counting and measuring 300 fibers of all sizes in a known weight of sample deposited on to a polycarbonate filter using Scanning Electron Microscopy. At 10,000 times magnification the effective minimum diameter that is visible is 0.1 μm, so the effective minimum length of a counted fiber was 0.4 μm. This was followed by the counting and measurement of a further 100 fibers longer than 5 μm. The data were combined to calculate the numbers of fibers in a series of length and diameter classes in the 10 mg dose administered to the rats. In addition, the numbers of particles (Aspect ratio less than 3:1) were also counted and estimated for each dose.

The rats were allowed to live out their full life span or until they showed signs of debility or tumor formation. Statistical analysis of the times at which death from mesothelioma occurred was used to calculate survival curves and these were correlated with the fiber doses received by each animal.

Table 3 shows the relative hazard ranking, the numbers of mesotheliomas and the fiber numbers in the doses. The relative hazard was derived from Cox's proportional hazards model (Cox & Oakes 1984) and is a function of the numbers of animals developing mesothelioma and their median survival times. The values given in the table differ from those shown in Davis et al. (1991) only in that the hazard is expressed arithmetically as a multiple of the lowest hazard, and the fiber numbers are expressed as those in the dose.

The main conclusions of the study were: 1) that all of the materials appeared to have some potential to cause mesothelioma by intraperitoneal injection in rats; 2) that fiber numbers alone were not sufficient to explain the differences in response, nor were the fiber numbers in the 'Stanton' fiber class able to fully explain the response; and 3) that the Dornie and Shinness material would be unlikely to pose a risk of mesothelioma to humans from inhalation of the dust. The spontaneous occurrence of peritoneal mesothelioma in male rats of this strain may account for the small numbers of tumors found in the animals injected with the latter two dusts (Pott et al. 1991**)**.

Coffin et al. (1978, 1982, 1983) and Cook et al. (1982) confirmed that ferro-actinolite asbestos has a high potency for generating mesothelioma in rats. In each case the ferro-actinolite asbestos had large numbers of fibers in the 'Stanton' range. The papers by Coffin and his colleagues were based on experiments using intratracheal instillation and intrapleural injection of an actinolite asbestos from the Mesabi Range (USA) iron ores in comparison to UICC amosite. The result**s** were problematical in that the response from the amosite was lower than expected from previous experiments (Stanton et al. 1981). In Coffin et al. (1983) 33.6% of F344 rats injected intrapleurally with 20 mg of UICC amosite developed mesothelioma. The response to the actinolite asbestos was lower than that from the UICC amosite or amosite in general in terms of the mass dose used, but the response relative to the numbers of Stanton fibers was higher. Cook et al. (1982) explained the relatively high response from the ferro-actinolite as resulting from shortening and splitting of the fibers in the lungs and on the pleural surface of the rats.

Pott et al. (1988) reported more than 80% of rats with tumors two years after intraperitoneal injection of 0.3 mg of a German actinolite although the given size distribution of the actinolite is not provided. Pott et al. (1989) then reported 56% of rats with tumors after an injection with 0.25 mg of (presumably) the same German actinolite. The size distribution is not detailed but shows 90% of the fibers as less than 0.2 µm in length and 10% longer than 4.2 µm. In contrast,

when a dose of 4 x 25 mg of 'granular' actinolite was used in similar experiments (Pott et al. 1974) no tumors were found.

Grunerite (Amosite) Asbestos Studies

The inhalation and intraperitoneal injection experiments of Davies et al. (1986) used long and short fiber amosite asbestos. These were produced from the same bulk batch of amosite, the short form by ceramic ball milling and the long by elutriation. Importantly, TEM examination showed no loss of crystallinity in the milled short fiber sample. In the inhalation studies rats were exposed for one year (224 days in 12 months) to 11.9 and 11.6 mg/m^3 of respirable dust for the long and short fiber types respectively**.** The aerosol contained 2,060 and 70 f/mL for fibers longer than 5 μm, and 1,110 and 12 f/mL for fibers longer than 10 μm. In the injection studies two batches of rats received doses of 10 mg and 25 mg of the respirable dust collected from the inhalation experiment chambers using a vertical elutriator.

The results showed that rats exposed to the long fiber grunerite (amosite) asbestos developed significantly higher levels of pulmonary fibrosis and more lung tumors than rats exposed to the short fiber grunerite (amosite) asbestos**.** In fact the animals exposed to the short fiber developed no more fibrosis than did the control animals, no pulmonary tumors and only one peritoneal mesothelioma that was considered to be unrelated to the dust exposure as the type had previously been reported in untreated rats. The animals exposed to the short fiber had significantly higher burdens of asbestos in their lungs immediately after the inhalation period, and they remained higher throughout the following six months of clearance. The injection experiments produced mesothelioma in 88% and 95% of rats treated with 10 and 25 mg respectively of the long grunerite (amosite) asbestos, while the short fiber grunerite (amosite) asbestos produced 0% and 4% (1 animal) tumors with the same respective doses (mass) (Table 4). The short fiber grunerite

(amosite) asbestos contained about 0.1% of fibers longer than 10 µm and about 2% longer than 5 µm while the long fiber grunerite (amosite) asbestos contained more than 11% longer than 10 µm and 3% longer than 25 µm. The diameter distributions were very similar with about 50% less than 0.5 µm in width.

These results were taken as an indication that the short fiber grunerite (amosite) asbestos showed a much lower relative pathogenicity than the long fiber grunerite (amosite) asbestos.

In Vitro CELL STUDIES

The cell culture studies of Donaldson et al. (1989, 1991, and 1992) Brown et al. (1986) and Hill et al. (1995) have generally confirmed the impression that fibers shorter than 5 µm, and indeed possibly less than 10 µm, have little pathologic effect other than what might be expected from a general respirable silicate mineral dust. Tumor necrosis factor released from macrophages was shown to be dependent on fiber length as demonstrated by the long and short fiber grunerite (amosite) asbestos (Donaldson et al. 1992). The same minerals showed that release of superoxide anions by macrophages differed significantly (Hill et al. 1995). Since such factors are associated with the development of inflammation, pulmonary fibrosis, and tumor formation, this supports the view that fiber length is an important element in determining the pathogenicity of fibers.

OTHER RELEVANT STUDIES

The studies at IOM (Miller et al. 1999a, b and Searl et al.1999) confirm that biopersistence was a significant factor controlling the pathogenicity in animals of a wide range of different synthetic mineral fibers, but for durable fibers the most important factor was fiber length. The fibers used were: glass microfiber, JM 100/475; MMVF 10, 21, 22 and Refractory ceramic Fibers 1, 2, and 3, from the Thermal Insulation Manufacturers Association repository of size selected fibers; a

silicon carbide whisker fiber and the long fiber grunerite (amosite) asbestos as used by Davis et al. (1986). In the intraperitoneal injection studies the best correlation with capacity to produce mesothelioma was with the *in vivo* biopersistence factor (derived from measurement of fibers before and after intratracheal instillation) and the number of fibers longer than 20μm with diameters less than 0.95 μm. In the inhalation studies with the same suite of fibers the pulmonary tumor production (lung cancer) was best predicted by a function of the dissolution rate (measured in continuous flow through with simulated physiological saline solution) and the numbers of fibers in the length range greater than 20 μm with diameters less than 0.95 μm.

DISCUSSION

The main question that has been asked of these studies is to what extent they support the hypothesis that the carcinogenicity of fibers depends upon morphology. A second question that is being debated to what extent the short mineral fibers contribute to the carcinogenicity in humans. There are limitations to the injection or implantation assessments of carcinogenicity that reduce their ability to predict the outcome of inhalation of the same materials by humans (US EPA 1986). These include the avoidance of normal defence mechanisms of the inhalation process, the unnatural introduction of large doses to sensitive tissue sites, possible clumping of dusts introducing even higher doses at some sites, and the reduction of normal lung clearance mechanisms. However, the net result of these limitations is to over-estimate carcinogenicity by these methods, so that a negative finding is a strong indication that a given mineral dust is unlikely to be carcinogenic when inhaled by humans.

The early studies of Wagner et al. (1982) and Smith et al. (1979) are limited by poor survival and uninformative size distribution measurements. However, both experiments showed no potential for prismatic amphibole fibers to cause tumors by inhalation or by injection. So while limitation do exist, they ought not to be seen as grounds for disregarding the results and general concepts

derived from the experimental animal studies indicating that amphibole asbestos minerals are carcinogenic while the prismatic amphiboles or cleavage fragments are markedly less active.

Some questions have been raised about the interpretation of the Davis et al. (1991) study which we will answer. For example, the authors stated that the response from the Shinness fiber was no more than would be expected from control animals, and that the non-asbestos tremolites were unlikely to pose a specific mesothelioma risk to humans by inhalation. It was suggested that these two tumors, with the non-asbestos Shinness dust, were significant since there were no tumors among animals in many other experiments from the same laboratory (IOM) (Federal Register 1992). The experiments referred to in the Federal Registry were inhalation experiments with other asbestos fibers, and that, other than with the Korean tremolite, these have rarely produced mesotheliomas in rats. The background mesothelioma incidence is higher when the route of administration is by injection. Furthermore, Stanton et al. (1981) implantation studies have shown a percentage of animals with tumors in the range of 0 to 10% may well be within the expected range for a 40 mg dose of mineral particles of any type not introduced by inhalation.

The size distributions of the fiber types show that the tremolite asbestos from different geological locales as exemplified by the Californian (Jamestown) sample, are dominated by very much thinner fibers than the prismatic tremolites, as exemplified by the Shinness sample, which contain almost no fibers longer than 8 μm and less than 1 μm in diameter. While it is true that the response could be explained simply as a dose response to the numbers of Stanton fibers, yet this fails to explain all of the variance in the results between the various growth habits in which tremolite naturally occurs. It is a distinct possibility that, as with Stanton's experiments, the low responses from the non-asbestos Shinness fibers and the Dornie fibers are inert dust responses.

A second criticism in the interpretation of these results stems from the high tumorigenicity of the Italian (Ala di Stura) tremolite (Davis et al. 1992) This was described in the paper as a spicular

(the same as acicular, a sub-type of prismatic) non-asbestos variety of tremolite which would not be expected to produce tumors; so the high tumor rate has been used to suggest that acicular and byssolite amphiboles do indeed have a similar carcinogenicity to the asbestos amphiboles. It has been shown that the Ala di Stura tremolite sample contains a sub-set of asbestiform tremolite fibers that appear as extremely long and fine fibers but which, because of the limitations of fiber sizing methodology, are not fully expressed in the fiber numbers as reported in the study (Figure 1b).

The tumor response from the Ala di Stura tremolite was unusually high compared to the number of Stanton fibers in the sample, but an important factor in the response was the timing of the mesotheliomas in the life spans of the animals. Two thirds of the rats exposed to the Ala di Stura tremolite developed mesothelioma, but very late in life (median survival time was 755 days). In contrast the three asbestos samples had much shorter median survival times ranging from 301 days to 428 days. (The Korean tremolite asbestos had a median survival time of 428 days compared to 325 days in the earlier study with a 25 mg dose). The median survival time for those animals that develop mesothelioma appears to be inversely related to dose, as seen in Davis et al. (1991), so the response from this dust could be simply that which might be expected from a trace asbestos component in a dust injected into animal at high concentrations.

It was also pointed out in the original report that the tremolite asbestos from Swansea had produced a response that was much higher than expected given the number of Stanton fibers in the dose. Both the Swansea tremolite asbestos and the Korean tremolite asbestos produced the maximum response in mortality but the high Hazard Index of the Swansea asbestos, calculated in the statistical analysis, was the result of the much faster tumor induction. It was suggested that this may have been the result of a masking of the response to simple fiber numbers by the overdose of asbestos forms, and that a multi-dose-response experiment might produce a clearer picture of the relative potencies of these types.

The Stanton studies confirmed the high tumorigenicity of tremolite asbestos and identified the Stanton Fiber range, fibers > 8 μm with diameters < 0.25 μm, for which the correlation between fiber numbers and mesothelioma generation was highly significant. Had the size classes and instillation method been different**,** the 'Stanton Fiber' critical size may well have been different. The authors stated that shorter and thicker size classes also correlated with mesothelioma potency, and that it should not be assumed that they had no potency. However, as can be seen in Figure 7, the numbers of fibers in the different classes are strongly correlated.

So it is to be expected that if the tumorigenicity is correlated strongly with numbers in the long, thin class of fibers it will also correlate with the fiber numbers in the shorter classes. That does not necessarily imply a causal relationship, and these short fibers may indeed have insignificant tumorigenicity. Even particulates that are considered relatively innocuous, e.g. FeO, magnetite can produce tumors by injection techniques if the dose is high enough (Pott et al. 1991).

As can be seen in Figure 8 many of the mineral and glass fibers in the experiments had less than 10 % probability of generating mesothelioma despite having huge numbers of fibers in the administered dose in the size range of 4 –8 μm length with no fibers in the longer classes. It is noteworthy; the fibrous talc minerals (5 and 7) produced no tumors despite having large numbers of short, thin fibers. The halloysites produced only 5 and 4 tumors despite having among the highest numbers of short fibers. Halloysite has the same tubular morphology as chrysotile asbestos despite having a little thicker fundamental diameter (0.07 μm). The attapulgites (palygorskite) produced few (2/29) tumors with similarly high numbers of fibers shorter than 8 μm. However, one long fiber attapulgite has been found by Wagner et al. (1987) to be capable of producing large numbers of mesotheliomas in rats by intraperitoneal injection. Both halloysite and attapulgite have been described as asbestiform but neither fiber-type is asbestos.

The size distributions of the various fibers used by Stanton et al. (1981) are in many cases highly unusual but a detailed discussion of all their full fiber size distributions is beyond the scope of this paper; some contained no long fibers, some contained no short fibers, some contained no fibers thinner than 0.5 μm, and others contained no fibers thicker than 0.5 μm. The tremolites however were unusual in having bimodal distributions consistent with a mixture of tremolite asbestos and prismatic non-asbestos tremolite (Figure 5).

One important factor in the Stanton studies that has implications for many other injection and implantation experiments is the range and distribution of the results found. There are a large number of dusts producing between 0 and 10% mesothelioma in experimental animals even though many of these samples contained more than 100,000 fibers per microgram of implanted dust. In a 40 mg dose implanted there are 40,000 times more fibers present than in a microgram. It is reasonable to conclude that this range of tumor production may be the "normal" background for his mineral dust implantation technique. In addition, Stanton's implantation controls had a 2.8% incidence of pleural sarcomas and all controls had an age-adjusted rate of 7.7$\pm$4.2%. Also, Pott et al. (1991) using intraperitoneal injection stated that tumor rates of below 10% in small groups should be regarded as spontaneously occurring or induced non-specifically. The background rate of his non-injected controls is 0%, but up to 10% for saline injection controls, which is highly significant when compared to non-injected animals.

One implication of this observation would be that the testing of materials by the implantation or injection of unrealistically high doses might be useful a screening test for mesothelioma potency in humans by inhalation. In addition, both routes of exposure do not allow for normal physiological removal as would be expected after inhalation (McConnell, 1995). The Stanton method is particularly problematic in this regard because the fibers are 'held in place', i.e. in contact with the mesothelium in the gelatin vehicle. For these reasons the methods is very useful when a negative result is obtained for the assessment of fundamental differences between fiber-

types and concepts of carcinogenic activity. But positive results are of limited use as predictors of the risk to humans from inhalation of more general dusts. Furthermore, the doses to which the animals are exposed are probably many orders of magnitude higher than would be expected from exposure of humans to airborne dust.

CONCLUSIONS

The conclusion that should be drawn from the evaluations of this set of studies is that there is very little evidence of carcinogenicity from exposure of animals to mineral fragments or short fibers formed from normal prismatic amphibole minerals. No positive carcinogenicity has been found with any experiment using non-asbestos amphibole dust (Ilgren 2004). Furthermore, when genuinely short fiber amphibole asbestos has been used in inhalation or injection experiments they have also been shown to have no carcinogenic properties. Evidence from experiments with other mineral fibers suggests those fibers in excess of 20 μm and with diameters less than 1 μm are necessary to cause cancer. This is probably because such long fibers cannot be phagocytized by resident macrophages and therefore, cannot be removed from the lung (Lippmann et al. 2000). This explains the lack of carcinogenicity of cleavage fragment fibers of amphiboles since these rarely if ever contain fibers of these critical dimensions.

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Figure 1. Typical prismatic form of amphibole showing main cleavages and prism faces.

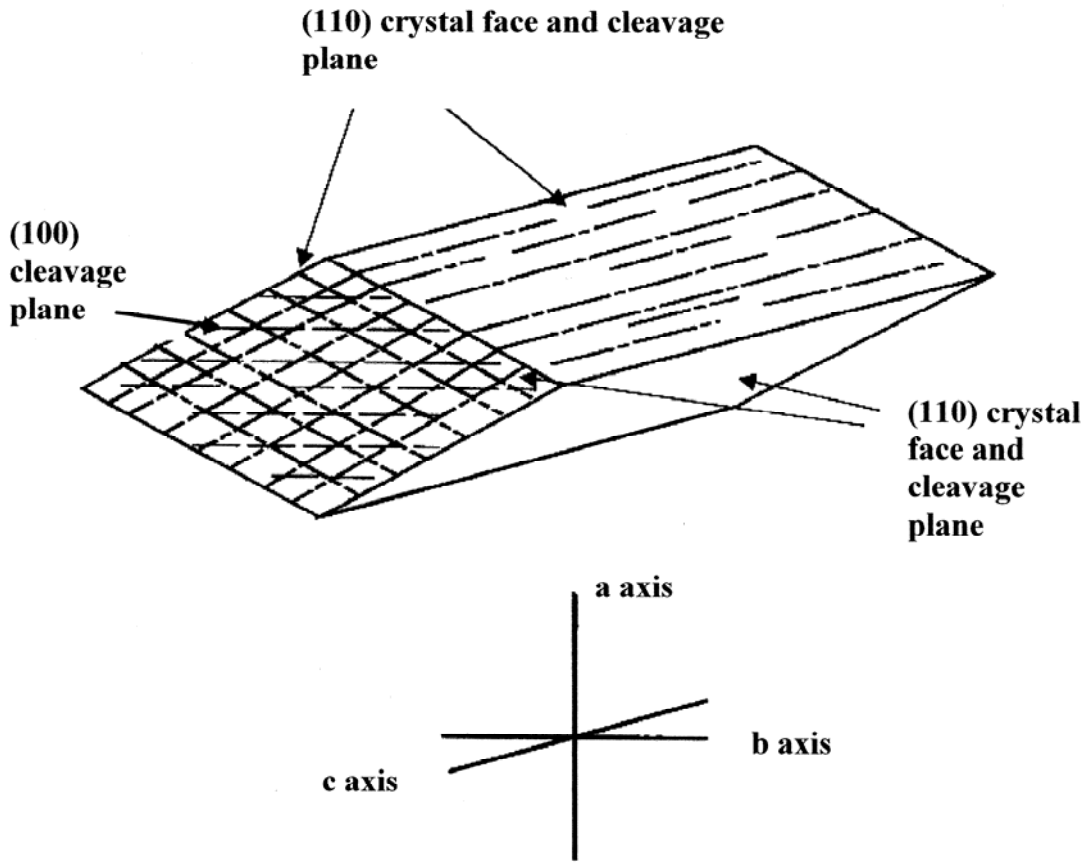


Figure 1b. Scanning Electron Microscope microphotograph of Ala di Stura tremolite showing a large prismatic crystal with cross section with a cross section shape determined by (110) crystal faces; also evident are the traces of the (100) cleavage planes. Thin asbestiform tremolite fibers with diameters finer than 1 micron are also visible on the right hand side of the image.

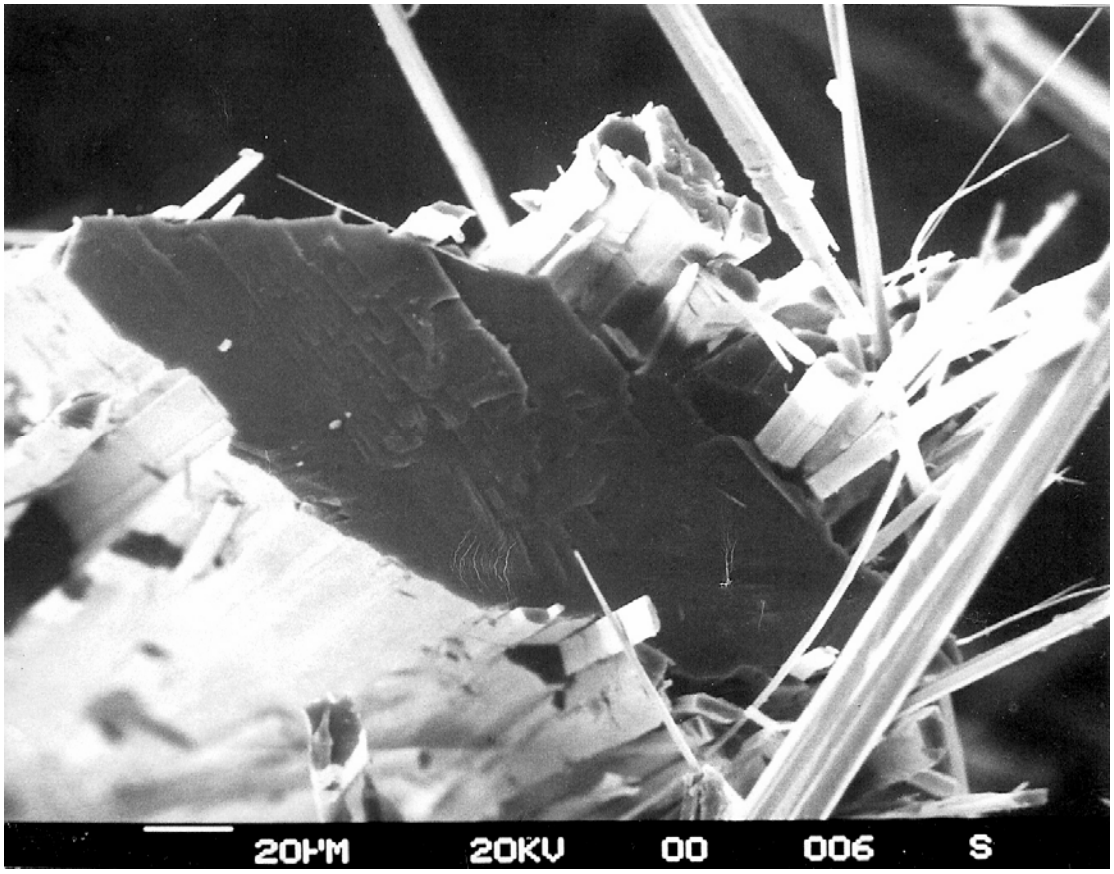


Figure 2. Length and width distribution of fibers (microns) in elutriated respirable dust of the Korean Tremolite Asbestos. This is a typical asbestos fiber size distribution with most fibers less than 5 microns in length and less than 0.5 microns in diameter. There are however some thin fibers with length greater than 10 microns, and some cleavage fragment fibers with diameters greater than 1 micron.

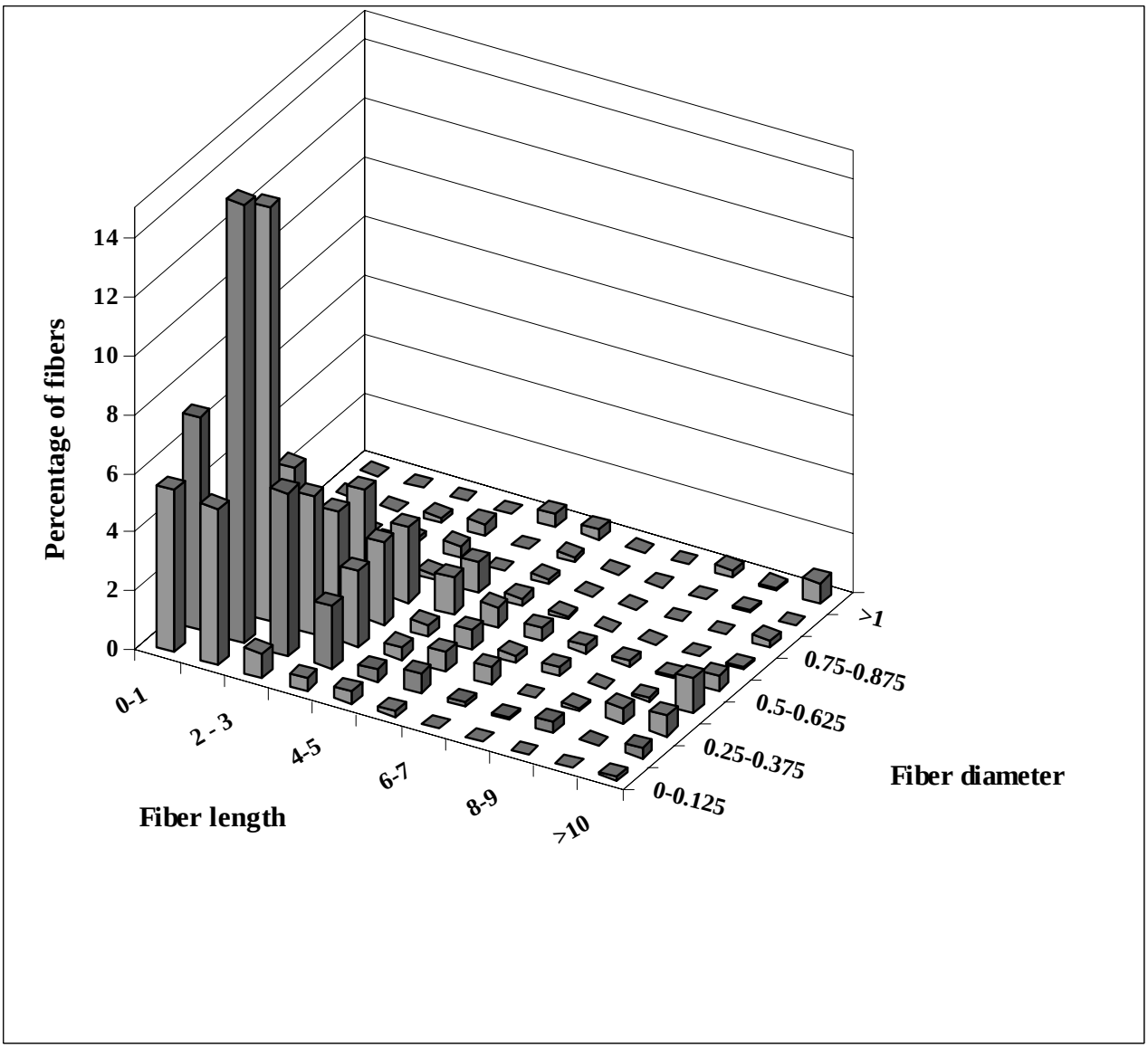
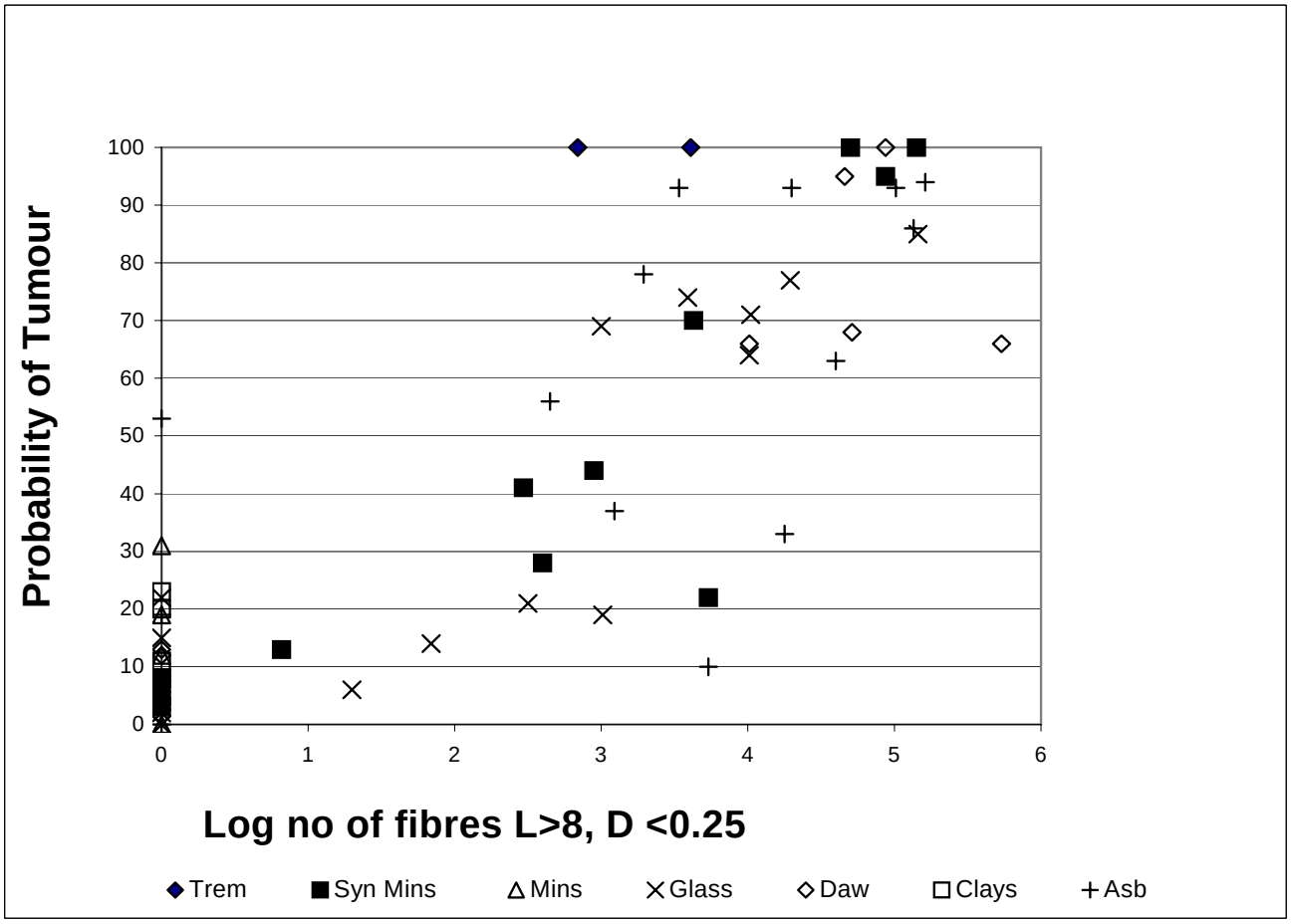


Figure 3. The probability of fibre generating a tumour compared to the log of numbers of fibre per microgram longer than 8 microns with diameter greater than or equal to 0.25 microns. This is the same data as in Stanton et al (1981).



Tremolite: Tremolite
Syn Mins: Synthetic minerals, Silicon carbide, Aluminum oxide, Potassium octatitanate
Mins: Minerals, Wollastonite, Talc
Glass: Borosilicate glass fibers
Daw: Dawsonite (synthetic)
Clays: Attapulgite, Halloysite
Asb: Asbestos (mostly crocidolite)

Figure 4. Probability of fibres generating mesothelioma compared to the numbers of fibres per microgram in dose within the size range of 4 – 8microns long with diameters in the range 0.01 – 1.5 microns. Data from Stanton et al (1981)

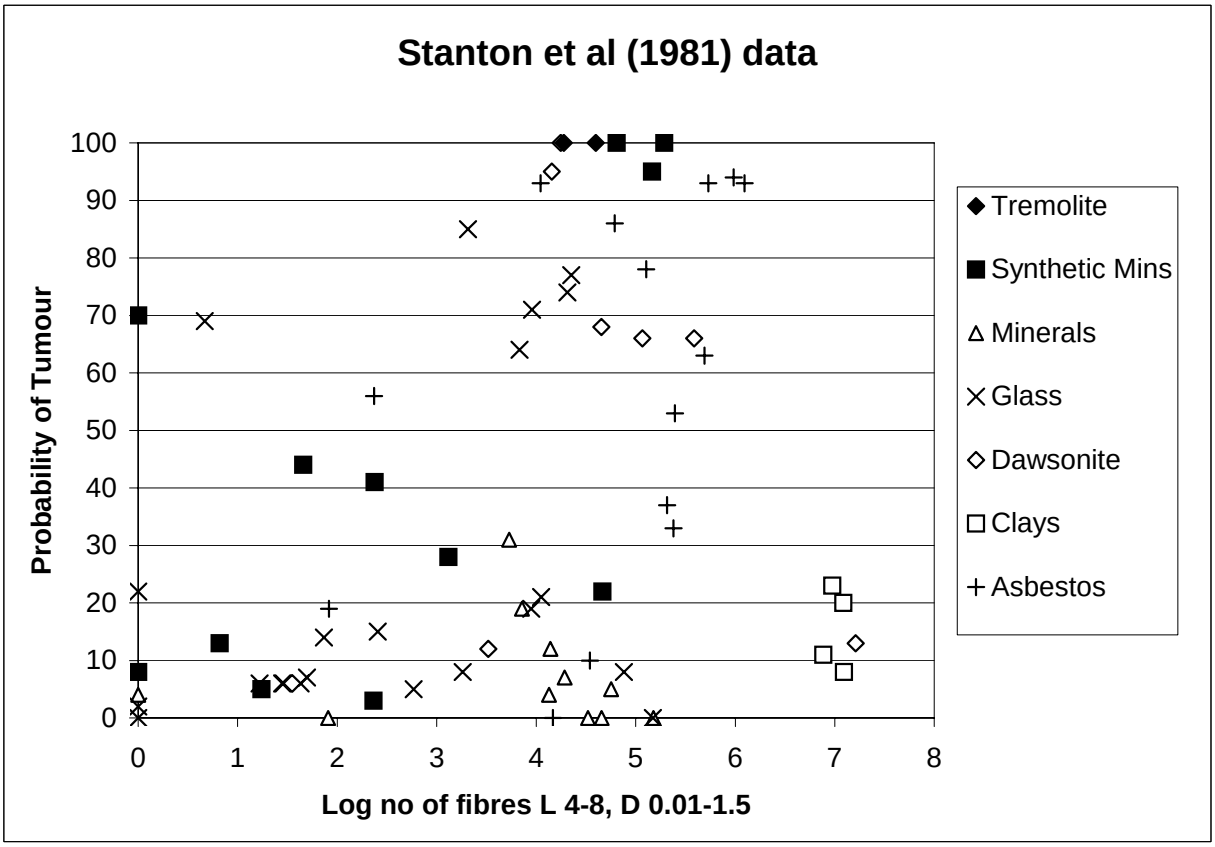


Figure 5. Length and diameter distribution of Tremolite 2 from the experiments of Stanton et al (1981) showing the bimodal distribution of the fibres. Based upon the Stanton data.

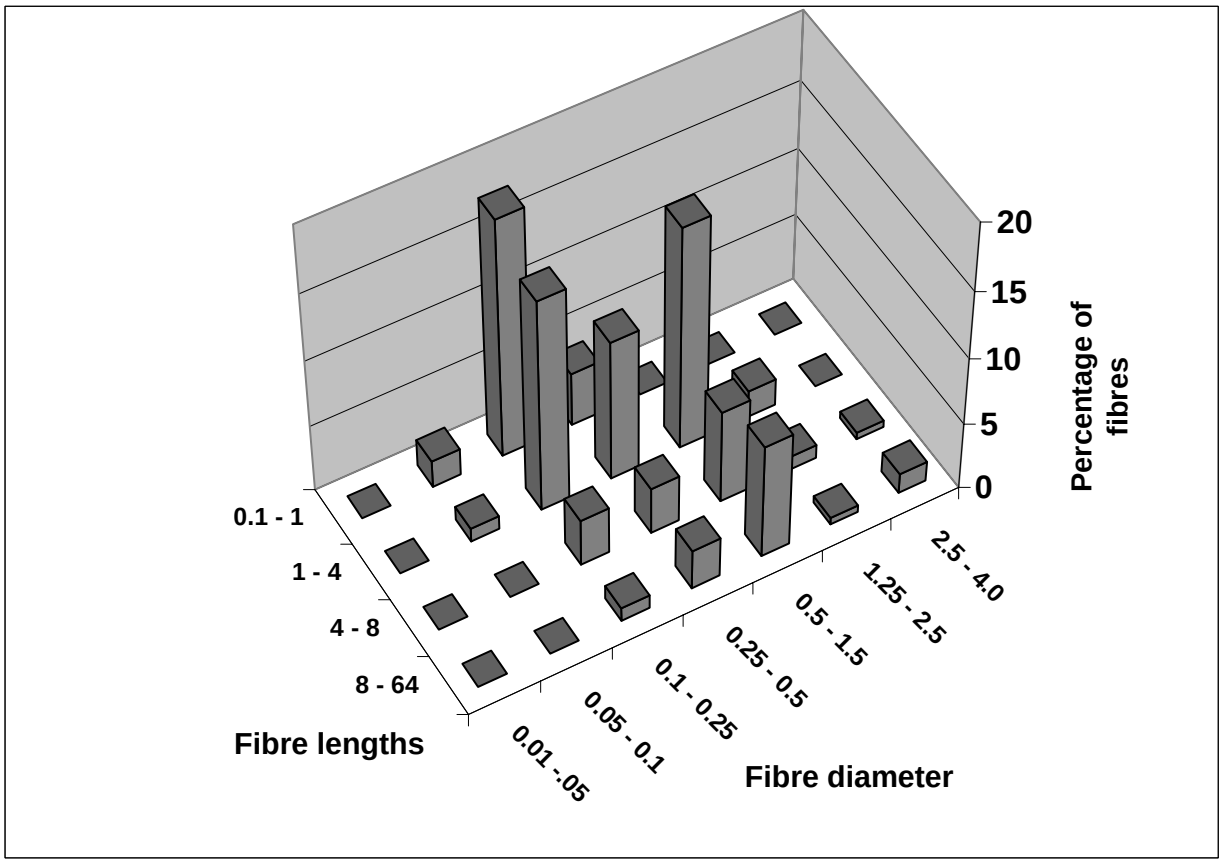


Figure 6. Size distribution of Talc 6 using the size data from Stanton et al (1981)

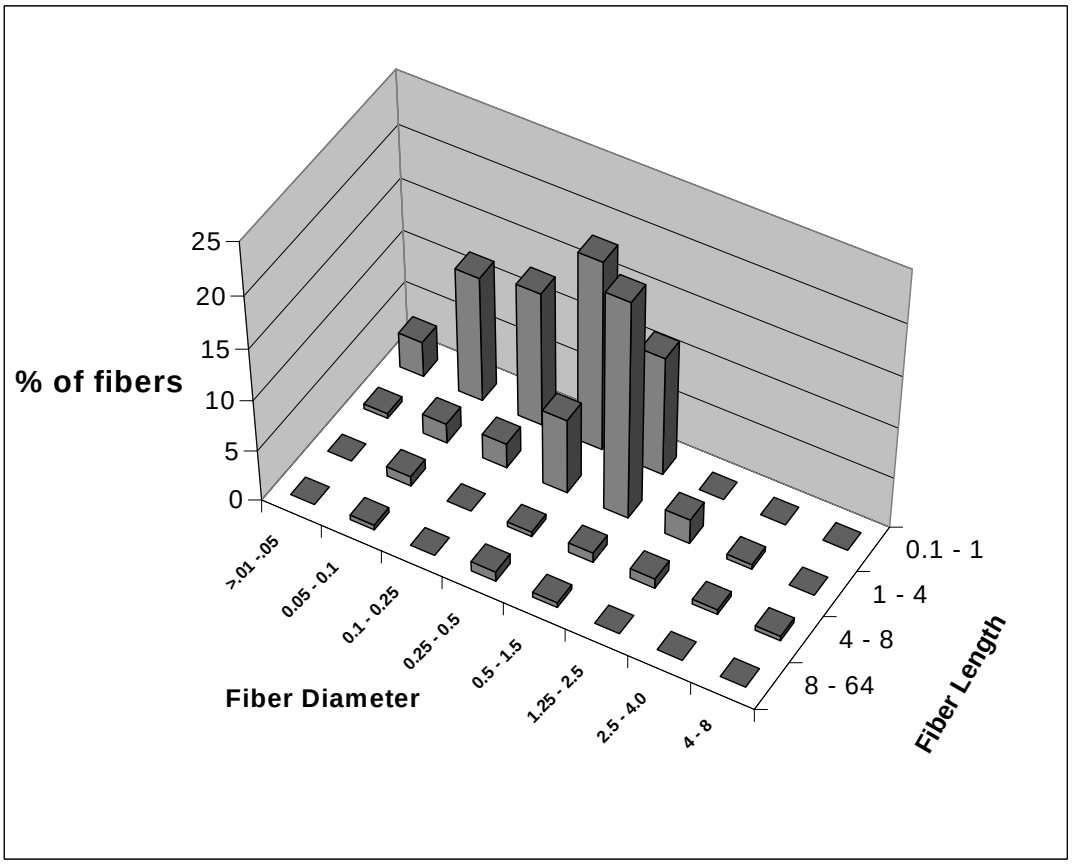


Figure 7. Numbers of 'Stanton Fibers' per microgram compared to the numbers of fibres in the size range 4 –8 microns long and 0.01 – 1.5 microns diameter showing an obvious relationship (Correlation coefficient 0.74). Fibers with no fibers in either class have been omitted. Data from Stanton et al (1981)

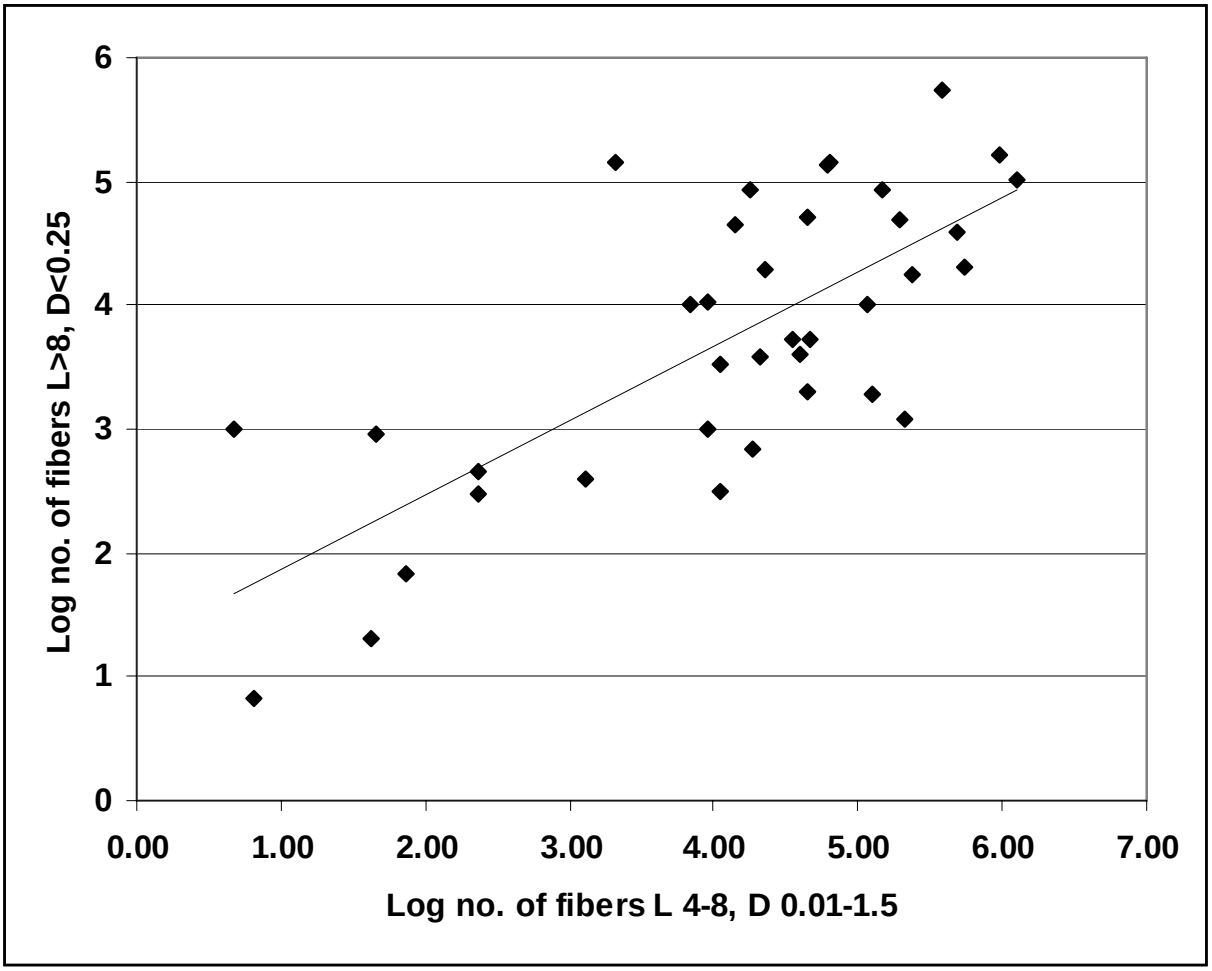


Figure 8. Probability of producing tumor vs number of fibers per microgram in the dose that were longer than 4.0 microns with diameter between 0.1 and 1.5 microns (and no fibers longer than 8 microns). Data from Stanton et al (1981). Very large numbers fibers of clay minerals and dawsonite produced relatively low response.

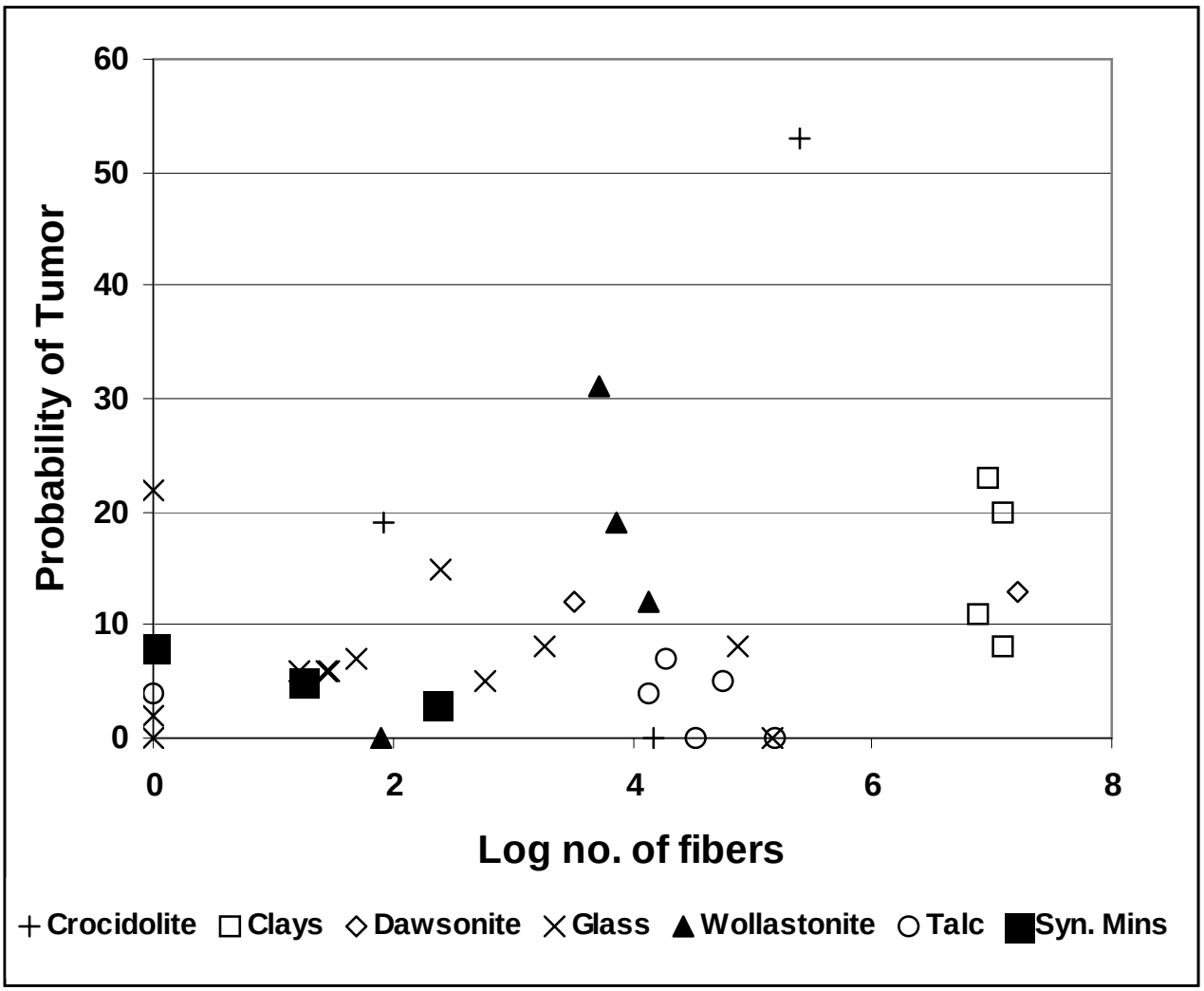


Table 1. Summary of the samples and results of the toxicological testing of Smith et al. (1979)

Sample Number	Descriptor	Tumor Incidence		Composition
		10 mg dose	25 mg dose	
14 (or FD-14)	Tremolitic talc, New York State,	-	0/35	50% non-asbestos tremolite
275	Tremolite selected from NY Tremolitic talc	0/34	0/31	95% non-asbestos tremolite
31	Tremolitic talc, unspecified location in W. USA	1/42	6/30	90% tremolite, possibly asbestiform
72 (or FD72)	Asbestiform tremolite, unspecified location	3/13	5/23	95% tremolite asbestos
72N	Asbestiform Tremolite	6/25	11/26	95% Tremolite Asbestos

Table 2.

Tremolite particles per microgram of injected dose in Wagner et al. (1982).

Sample	Non-fibrous Particles x 10^4	All fibers x 10^4	Fibers > 8 μm long and <1.5 μm wide x 10^3
A	6.9	5.1	1.7
B	20.7	4.8	0
C	3.3	15.5	56.1

Table 3.

Results of the Davis et al. (1991) intraperitoneal injection experiments with tremolites of differing morphologies.

Tremolite Source	N° of Animals	N° of Mesothelioma	Median Survival Time (days)	Relative Hazard	Millions of Fibers in Dose Injected	Millions of fibers, (length >8 µm and diameter <0.25 µm)
California Asbestos	36	36	301	346,939	1,3430	121
Swansea Asbestos	36	35	365	183,673	2,104	8
Korea Asbestos	33	32	428	51,020	7,791	48
Italy Non-Asbestos	36	24	755	1,020	1,293	1
Dornie Non-Asbestos	33	4	§	6.4	899	0
Shinness Non-Asbestos	36	2	§	1	383	0

§ Insufficient animal death for calculation

Table 4.
Results from Davis et al. (1986) inhalation and intraperitoneal injection experiments with long and short fiber grunerite (amosite) asbestos.

Injection Experiments	**Long Grunerite (Amosite) Asbestos**		**Short Grunerite Amosite (Amosite)**	
Dose	10 mg	25 mg	10 mg	25 mg
Number (%) of Animals with Mesothelioma	21 (88%)	20 (95%)	0	1(4%)
Mean Tumor Induction Period	535	520	N/A	837
Millions of Fibers in Dose Length ≥5 µm	1731	4327	60.3	150.75
Millions of Fibers in Dose Length ≥10 µm	932	2330	10.34	25.85
Inhalation Experiment	**Long Grunerite (Amosite) Asbestos**		**Short Grunerite Amosite (Amosite)**	
Number (%) of Animals with Mesothelioma	14(35%)		1(2.4%)	
Lung contents (mg) Immediately after dusting (Std Dev)	3.57 mg (1.59)		5.64 mg (0.37)	
6 months clearance (Std Dev)	3.08 mg (0.37)		4.47 mg (0.58)	