Chronic Animal Inhalation Study of Short (<5 μm) Asbestos Fibers

Progress Report

by

Stanley F. Platek

David H. Groth

Division of Biomedical and Behavioral Science

National Institute for Occupational Safety and Health

Taft Laboratories

4676 Columbia Parkway

Cincinnati, Ohio 45226

Contract Number: 210-77-0151

International Research and Development Corporation

Mattawan, Michigan 49071

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An animal inhalation study was initiated in 1977 to study the chronic biological effects of inhalation of short chrysotile asbestos fibers. Rats and monkeys were exposed for 7 hours/day, 5 days/week for 18 months to specially prepared chrysotile. Based upon daily chamber measurements, the mean concentration of fibers in the chamber air was less than 1 mg/m^3. By phase contrast and electron microscopy, the ratio of the number of chrysotile fibers/cc <5 μm in length to the fibers >5 μm was established at approximately 265:1. Rats were autopsied for examination 1, 3, 6, 12, and 18 months after initiating exposures. Histopathological examinations of the lung tissue have so far revealed little or no pathological reaction to the inhaled asbestos. Although asbestos fibers could not be seen in lung tissues by light microscopy, they were seen in alveolar macrophages when examined by electron microscopy. Six months after the last exposure date, i.e., 24 months after initiating exposures, the remaining rats will be sacrificed for examination. The monkeys will be maintained and observed for signs of latent pulmonary disease for approximately an additional seven years.

INTRODUCTION

Asbestos has been implicated by numerous investigators as playing a major role in the debilitating human diseases of bronchogenic carcinoma, mesothelioma, and pulmonary fibrosis (1,2,3). Previous studies have focused on the inhalation of asbestos fibers greater than 5 μm in length and largely disregarded the effect of asbestos fibers less than 5 μm in length.

The purpose of this project is to study the relationship of exposure to chrysotile asbestos fibers less than 5 μm long and the development of chronic, asbestos-associated diseases. Chrysotile asbestos was employed in this project because more than 90% of the asbestos used industrially and commercially is chrysotile (4,5).

MATERIALS AND METHODS

Short Fiber Preparation

The chrysotile used in this study was type 7TF1 chrysotile obtained from the Johns-Manville Sales Corporation in Denver, Colorado. Short asbestos fibers were prepared by drying 500 grams of the fibers in an oven at 191°C. After cooling, the chrysotile was placed in a cylindrical ceramic (Burundum) ball mill (7" x 8½") with 120 cylindrical Burundum pellets, each pellet measuring 13/16" x 10/16". The mill was rotated at 73 rpm for 24 hours after which time the chrysotile was removed and again dried in an oven at 191°C for 24 hours. The asbestos was then cooled and stored in tightly secured, double plastic bags. By

this method of preparation, 99.98% of the resulting chrysotile fibers, as viewed and sized by electron microscopy, were less than 0.6 μm in diameter and about 20% were longer than 5 μm.

In addition to the large and short asbestos fibers, the ball-milling process produced amalgamated "balls" of chrysotile fibers which measured up to 10 μm in diameter. Figures 1, 2, and 3 are scanning electron micrographs (SEM) of the ball-milled asbestos showing the various sizes of fibers and "balls."

It has been reported that ball-milling is not a completely satisfactory method for preparing short asbestos fibers for biological studies (6,7), however ball-milling was the best method available for this project in which the production of more than 100 pounds of short asbestos fibers was required.

Animals

The experimental design incorporated 300 male Sprague-Dawley rats and 20 male cynomolgus monkeys. Each animal was individually identified and randomly assigned, half the rats and monkeys to an asbestos exposure chamber and the other half of the rats and monkeys to a control inhalation chamber. Each rat and monkey was individually housed and provided food (Purina Basal Rat Diet and Purina Monkey Chow, respectively) and water ad libitum, except during the hours of inhalation exposure.

All animals were exposed in 12 m^3 inhalation chambers for 7 hours/day, 5 days/week for 18 months. At the end of the 18 month exposure period, the surviving rats, both those exposed to chrysotile and the controls, were scheduled for a 6 month post-exposure observation period of which at the time of this report, 5 months have elapsed.

Table 1 shows the serial sacrifice schedule for the exposed and control rats at 1, 3, 6, 12, and 18 months after the initiation of the asbestos exposure. A terminal sacrifice of all remaining rats will be made at month 24.

TABLE 1

RAT SACRIFICE SCHEDULE

	Sacrifice Interval and No. of Rats Selected					
	1 mo.	3 mo.	6 mo.	12 mo.	18 mo.	24 mo.
Exposed rats	5	15	15	15	15	85
Control rats	5	15	15	15	15	85

The experimental design designates that all monkeys, 10 control and 10 asbestos exposed, will be maintained and observed for at least seven years after the last exposure day.

Animal Tissue Diagnostic Tests

Five rats from the exposed group and five rats from the control group were sacrificed at the end of one month's exposure. Lung tissue was taken for

scanning electron microscopy examination for asbestos fibers as well as liver, kidney, spleen, and tracheal and mesenteric lymph nodes for histopathological examination. At the 3, 6, 12, and 18 month exposure intervals, 15 rats/group were sacrificed. Of the 15 rats/group, 5 were used for evaluation by scanning electron microscopy and cytochemical determination. The relative amounts of cellular acid phosphatase, beta-glucuronidase, and lactic dehydrogenase were measured to determine the release of non-membrane bound enzymes and the extent of lysosomal exocytosis.

Samples of lung tissue, liver, kidney, spleen, and tracheal and mesenteric lymph nodes were preserved in 3.0% phosphate-buffered glutaraldehyde for scanning electron microscopy examination. Of the remaining 10 rats/group, blood and half of the left lung were analyzed for silicon (Si) by plasma emission spectroscopy. The other half of each left lung was evaluated for relative amounts of hydroxyproline. The right lung of each animal was fixed in 10% formalin and processed with other body tissues for gross and histopathological examination.

Rats to be sacrificed at 24 months will receive the same evaluations previously described for the interim sacrificed animals with 10 rats/group used for silicon analysis and the remainder for histopathological examination.

Exposed and control monkeys will have the complete cytochemical-silicon analysis-histopathological evaluations conducted at the time of their scheduled sacrifice.

Chamber Asbestos Analysis Methods and Results

Conditioned air (humidity and temperature modified) was used to disperse the asbestos into the dilution air of the 12 m^3 chamber and to prevent fibers from adhering to one another. The air flow through the chamber was regulated for six air changes per hour. The chrysotile asbestos in the exposure chamber was measured by three methods: gravimetric analysis, fiber length distribution analysis, and by scanning electron microscopy.

The gravimetric analysis consisted of drawing a sample of air from the exposure chamber at 5.3 liters/minute for 60 minutes through a 37 mm diameter fibrous glass filter. The pre-collection filter weight and post-collection filter weight were used to determine total asbestos collection for a determined volume of sampled air. Three samples were collected at evenly spaced intervals during each 7 hour daily exposure and the concentration expressed in mg/m^3. The mean concentration of asbestos in the chamber air for the entire study was $0.95\ mg/m^3 \pm 0.26$ (S.D.).

The NIOSH P and CAM 239 method was used in a modified form to determine the chrysotile fiber length distribution in the exposure chamber (8). The asbestos concentration was not determined by this method. A sample of chamber air was drawn at a flow rate of 5.3 lpm for 90 minutes through a 37 mm diameter, 0.8 µm pore size cellulose ester filter. The 5.3 lpm differed from the 1.7 lpm to 2.5 lpm prescribed in the NIOSH method. The procedures as described in the NIOSH method were only used to clear the cellulose ester filter and to count asbestos fibers longer than 5 µm in length.

A small wedge of the filter was placed on a microscope slide and the body of the filter was cleared with a reagent containing dimethyl phthalate and diethyl oxalate. The slide was then viewed at a magnification of 400X in a phase contrast microscope and the sizing of fibers accomplished with the use of a Porton graticule and hand counter. Three chamber atmosphere samples were taken at regular intervals during each 7 hour daily exposure. The mean number of chrysotile fibers/cc greater than 5 μm in length for the entire study was 0.79 fibers/cc $\pm$ 0.13 (S.D.).

Electron microscopy was used to monitor the number of fibers/cc less than 5 μm in length. On occasion, the >5 μm fibers were also measured by this method. Chamber air was collected in the same manner used for samples drawn for fiber length distribution except that a polycarbonate filter was substituted for the cellulose ester filter. The filter containing the collected sample was mounted on a carbon planchet and viewed through a scanning electron microscope. Photographs were taken at a magnification of 2,000X and the negatives used to print X5,000 enlargements of the field. The asbestos fibers were then counted and measured by hand. Figure 4 shows a typical photo taken of a sample prepared by this method. Short (<5 μm) and long (>5 μm) asbestos fibers can be seen as well as a few of the previously described asbestos "balls." Figure 5 shows a higher magnification (X20,000) of one of these generated asbestos "balls" collected from the asbestos exposure air. By electron microscopy, the number of fibers/cc <5 μm in length was estimated at 210 (a ratio of 265 fibers/cc <5 μm to each fiber >5 μm).

DISCUSSION

At the time of this report, there are less than two months of maintenance and observation to complete before the June, 1980, terminal sacrifice of the rats. The final steps are being taken to arrange the long-term holding of the control and exposed monkeys.

The monthly body weights of both exposed and control rats and monkeys have indicated normal weight gains. Forty-six rats (23 exposed and 23 control) have died or were sacrificed moribund. No pharmacotoxic signs were seen that could be associated with exposure to chrysotile. Lung tissue from serial sacrificed rats through the 18 month sacrifice has revealed little or no pathological reaction to the inhaled asbestos (Figure 6).

Some short asbestos fibers (0.5-1.0 μm) have been seen in the alveolar macrophages by scanning transmission electron microscopy. Due to the relatively small number of macrophages seen in the alveoli, pieces of rat lung 1.0 cc in size were ashed in a low-temperature plasma oven. The ash residue was then suspended in distilled water and filtered on a polycarbonate filter. Scanning electron microscopy examination of this filter revealed a number of short chrysotile fibers and "balls" as seen in Figure 7.

The positive identification of chrysotile asbestos in both alveolar macrophages and in the low-temperature, plasma-ashed rat lung tissue was accomplished by energy dispersive x-ray analysis.

The contractor is compiling the data on the interim rat sacrifice silicon assays and the other cytochemical tests for the final report due in September, 1980. No tests of tissues have been taken on the two groups of monkeys since none have been sacrificed or died thus far during the study.

It is hoped that the results of this study will be of significant value in the continuing assessment and updating of data on a highly hazardous industrial and commercial substance.

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PHOTOMICROGRAPH LEGENDS

Figure 1. Ball-milled chrysotile (original X400, SEM).

Figure 2. Ball-milled chrysotile showing numerous short asbestos fibers among a few large fibers and "balls" (original X5,000, SEM).

Figure 3. Chrysotile asbestos "balls" composed of numerous short asbestos fibers (original X20,000, SEM).

Figure 4. Inhalation chamber sample of chrysotile fibers (original X5,000, SEM).

Figure 5. Inhalation chamber sample of chrysotile "ball" (original X20,000, SEM).

Figure 6. Terminal bronchus of 12-month chrysotile exposed rat (original X200).

Figure 7. Chrysotile fibers and "balls" from low-temperature ashed asbestos-exposed rat lung (original X5,000, SEM).

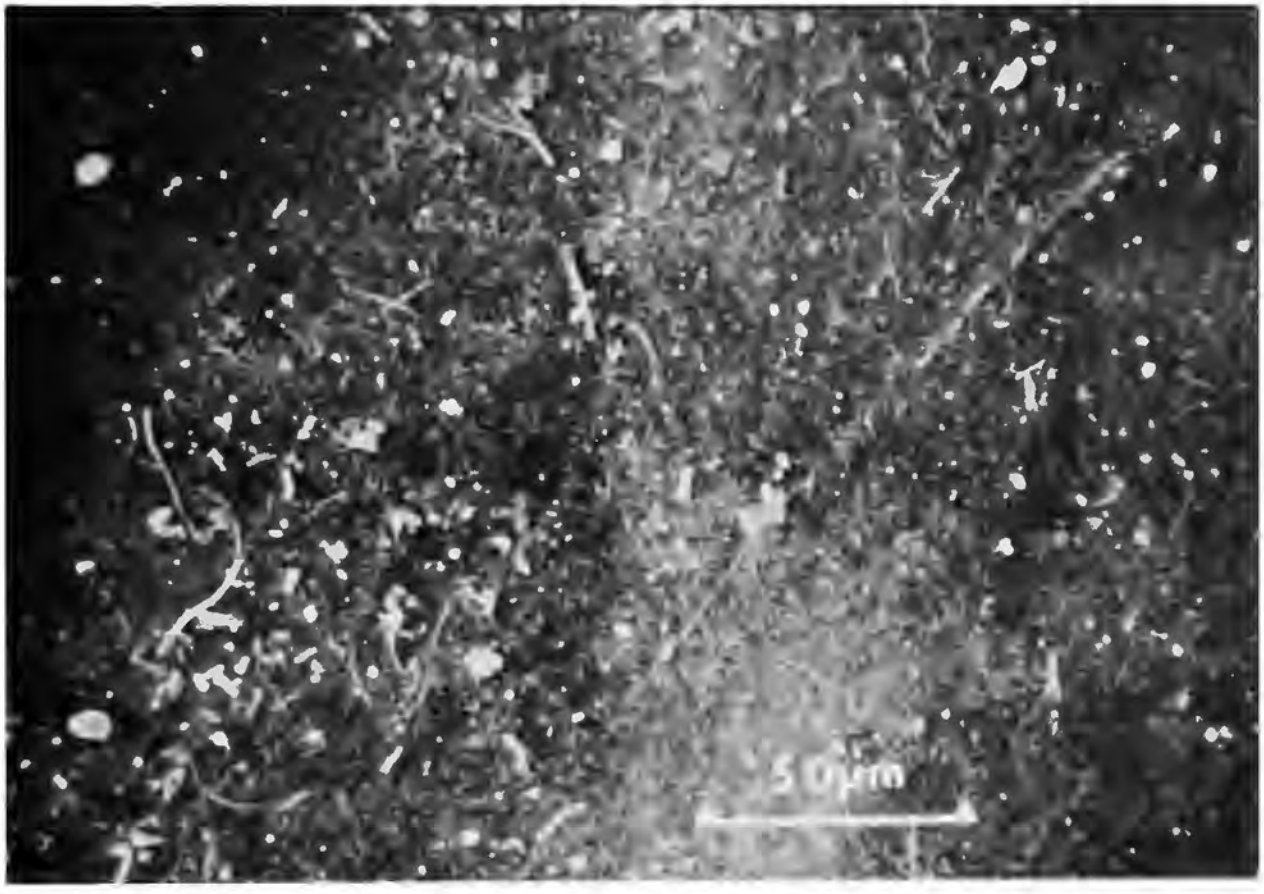
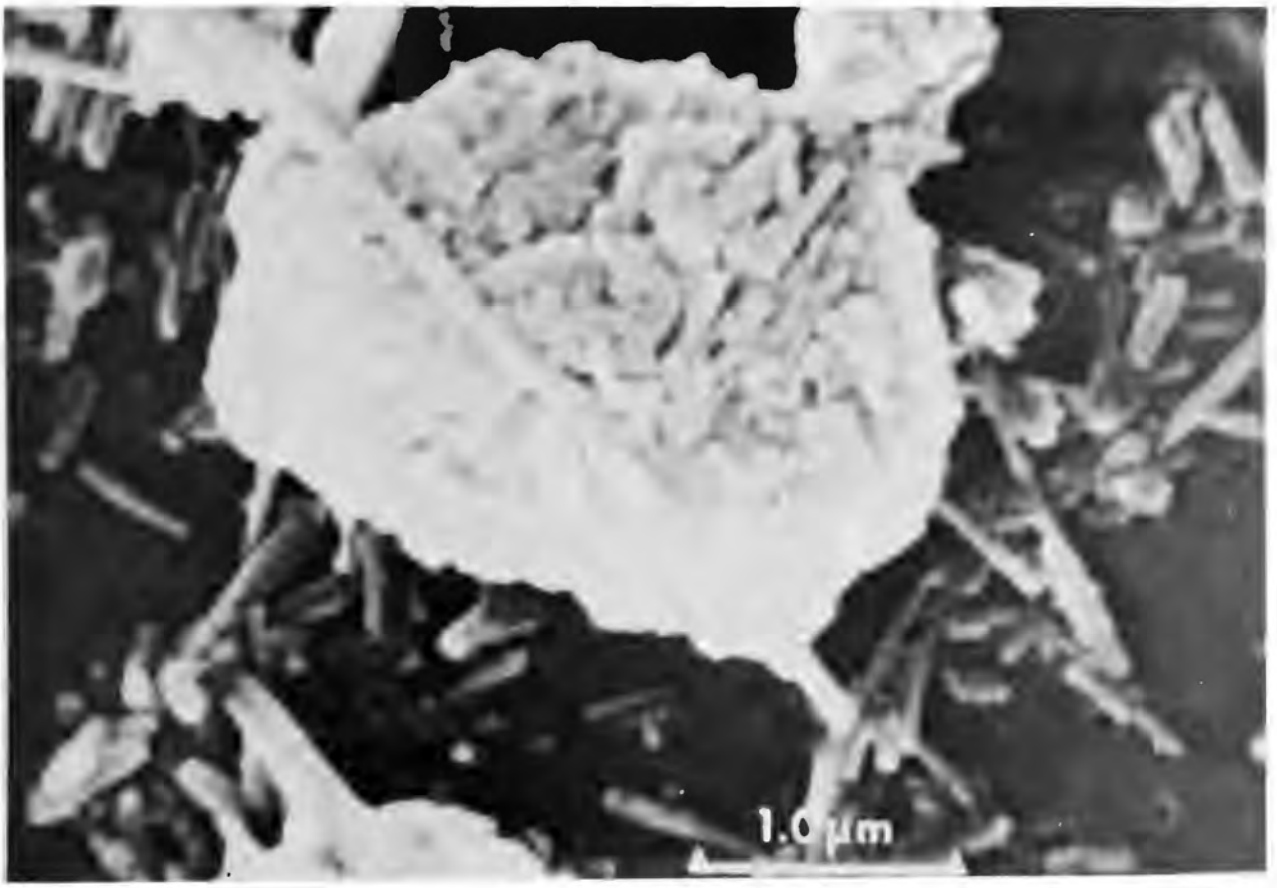


Figure 1

Figure 2



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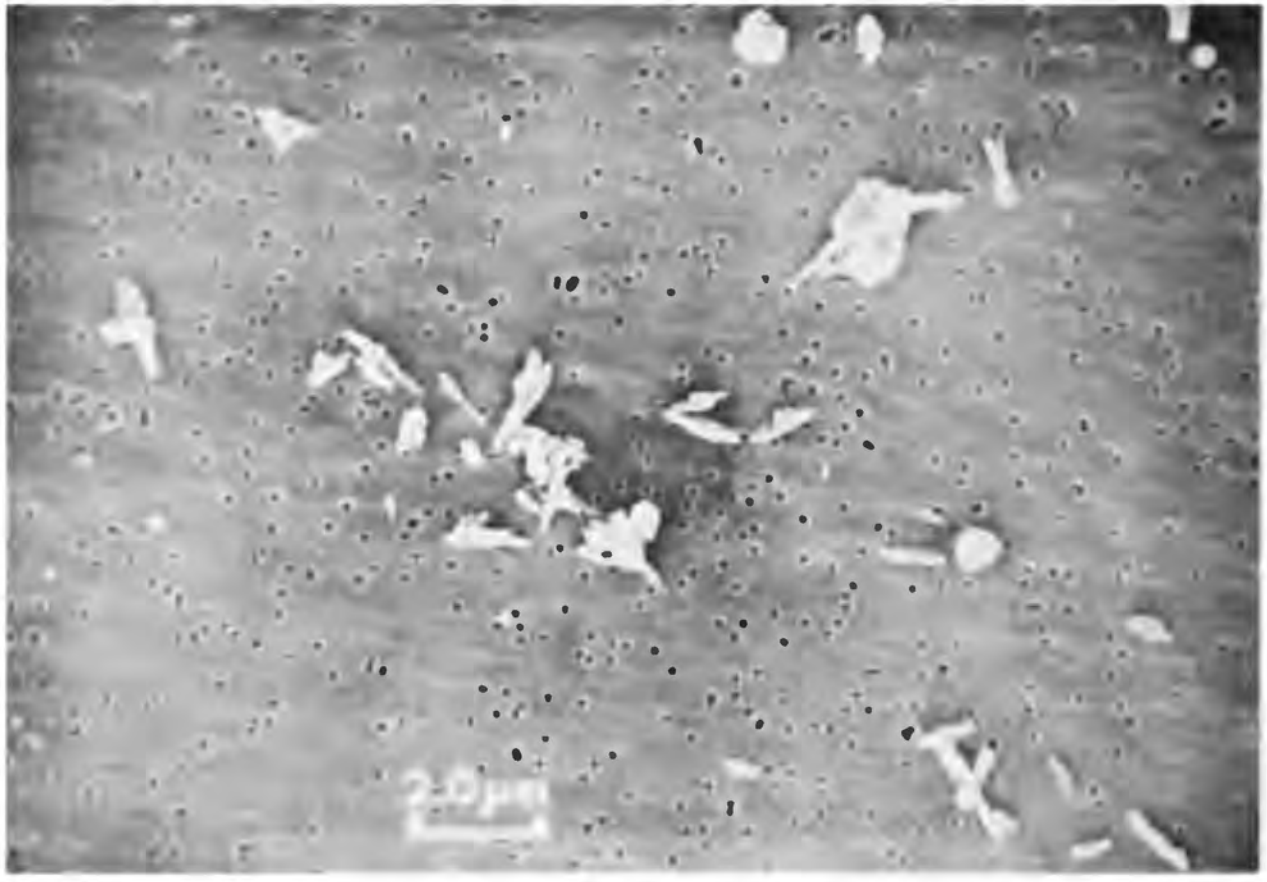

Figure 4

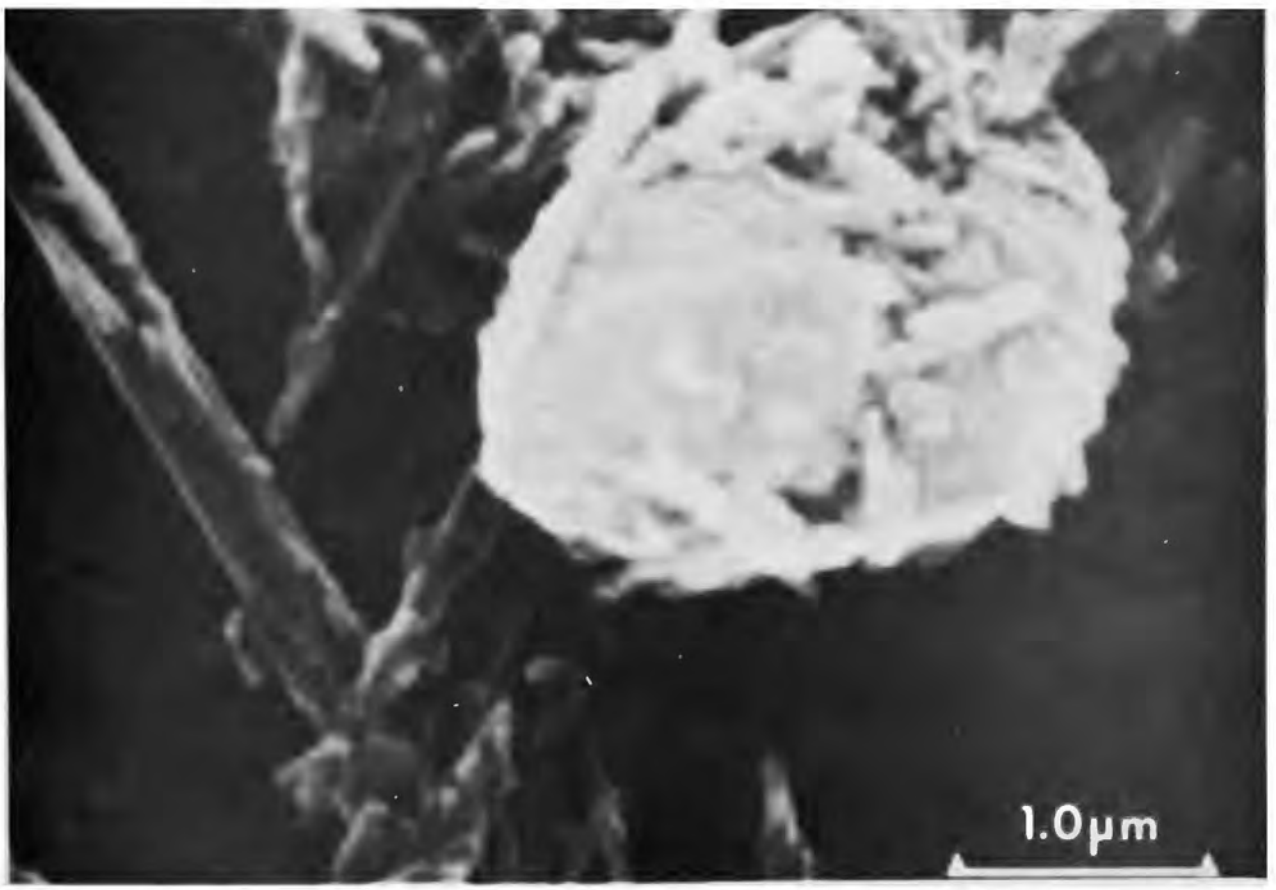

Figure 5

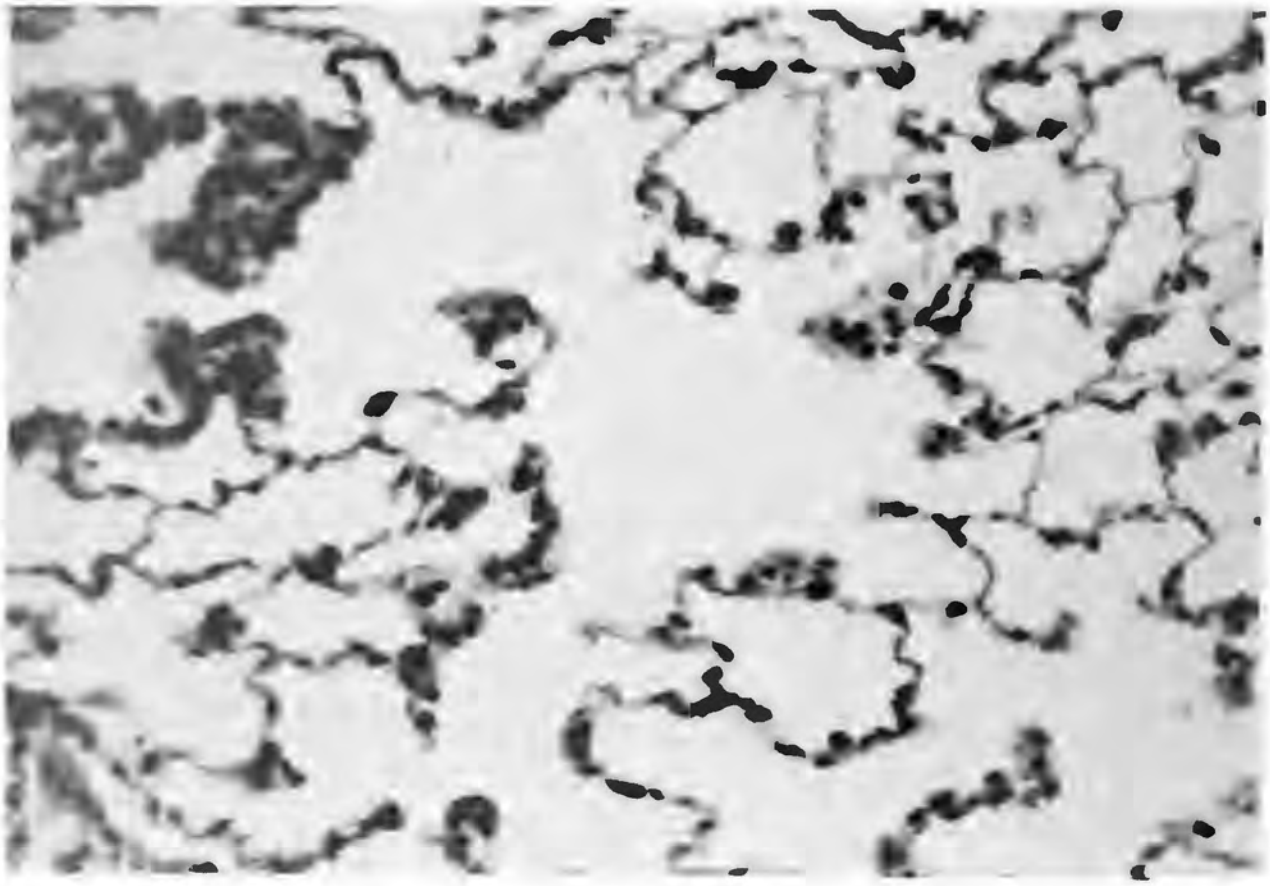

Figure 6

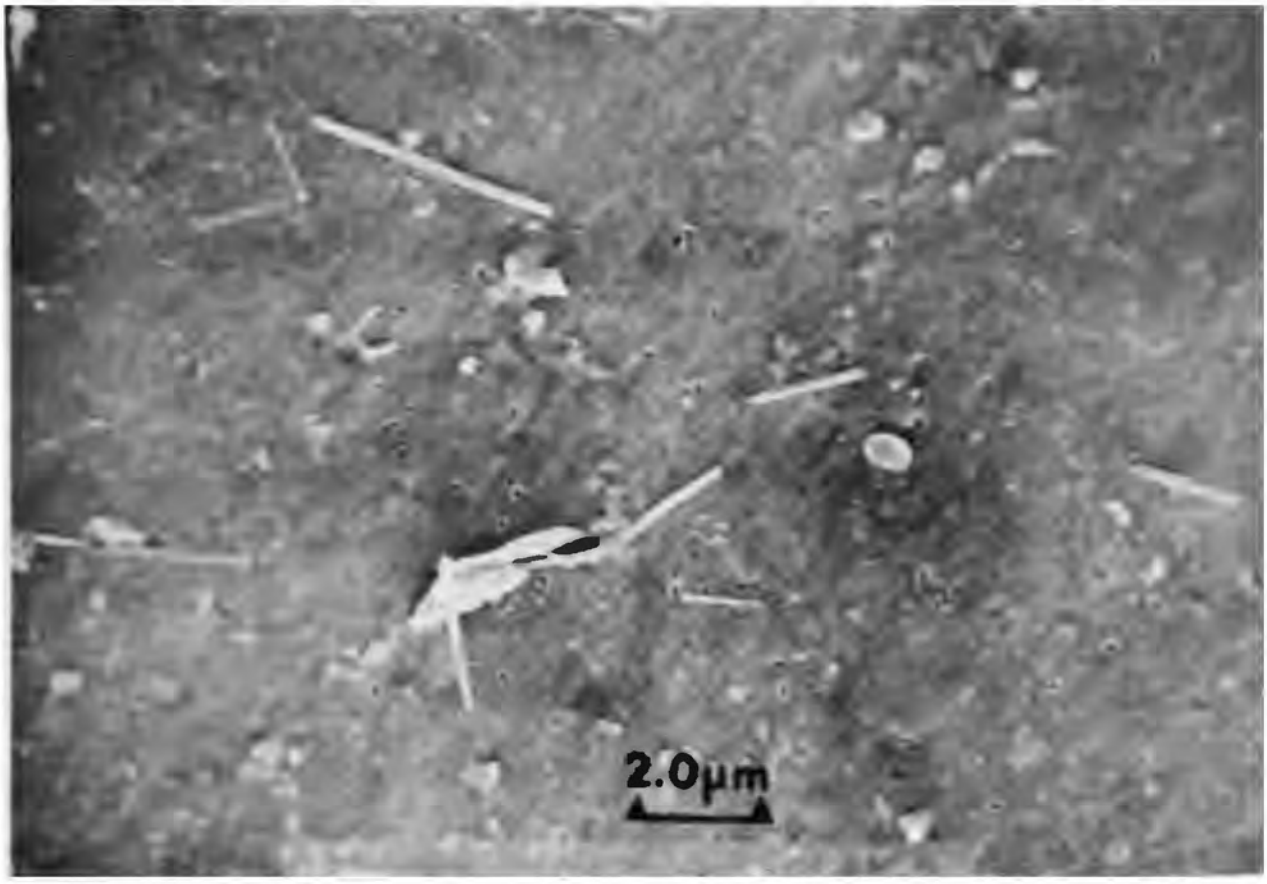

Figure 7

Dr. O'Conor, NCI: Yes, that is an elegant study. I have two questions. One, you say this is to test the chronic effects, I guess in these animals, but you are sacrificing them all six months after the first exposure, no, I mean after the termination of the exposure.

Mr. Platek, NIOSH: You must realize that the average lifespan of the rat is two years.

Dr. O'Conor, NCI: I was not thinking of the rats. I was thinking of monkeys.

Mr. Platek, NIOSH: Let me explain. I was appointed project director about one year ago. The design was set up by another gentleman at NIOSH. There is talk at this moment of possibly doing pulmonary function tests on these monkeys so the data will not be wasted. Granted the monkeys will live much longer than rats.

Dr. O'Conor, NCI: Have any of the monkeys been sacrificed to date?

Mr. Platek, NIOSH: No, none whatsoever. They will all be sacrificed or are scheduled for sacrifice the 25th of June.

Dr. O'Conor, NCI: Is there any possible consideration of just holding the monkeys rather than sacrificing?

Mr. Platek, NIOSH: I would have to speak with Dr. Groth on that question, but right now there is no consideration.

Dr. O'Conor, NCI: The other point is what do you think will be the implication, let us say that these experiments are negative; what do you think will be the implication in terms of the industry and regulatory action?

Mr. Platek, NIOSH: We were concerned in talking this over, that is, where would a worker be occupationally exposed to purely small asbestos fibers, and we don't know. Brake shoe repair operations might be one of the few places, but as I mentioned before, in any preparation or any exposure, long fibers will be present. However, this project was designed to test a standard of two fibers per cc, greater than 5 micrometers in length. In that standard you don't take into consideration any of these smaller asbestos fibers, and as you can see, our chamber samples here meet that standard and are below it. In fact, they are less than half of the federal standard. We can show that, but you can also see there are multitudes of these small fibers. This is why the project was conducted to determine when only these small fibers are present, do they have any adverse biological effect.

Dr. Burton, NCI: Was there any physical effect on the fibers of superheating them several times compared to fibers that might have been obtained in the original physical condition?

Mr. Platek, NIOSH: I know what you are saying. There have been numerous reports that heating, sonicating, even ball milling of the asbestos can create problems. We have done no tests to my knowledge to test whether the crystal structure of the asbestos was altered. This can be determined by x-ray and electron diffraction analysis. We have within the last year received the proper equipment on our transmission scope that we can do this type of analysis. That has not been done to date, but it will be performed and the results will accompany the final report.

Dr. Cameron, NCI: Just a further comment on the primate aspect. As a rhesus monkey they should have a lifespan--

Mr. Platek, NIOSH: Cynamolgus.

Dr. Cameron, NCI: Cynamolgus. They are still rhesus, the rhesus families have a lifespan in excess of 20 years. So that is minimal. I have a question though, are you aware of the NIEHS asbestos study at a local lab? I don't know the particulars. I wonder if you do?

Mr. Platek, NIOSH: I have been told, in fact, we just learned last week, there is evidently a lab that is doing an asbestos study; an intra-tracheal study of short asbestos fibers. I have no idea how they are doing it, but I have been told that it is a one-dose intra-tracheal injection study. I have no idea of the dose, how they prepared the asbestos, who their source was or the size range of the asbestos.

Dr. Lee, EPA: I would like to make a comment. If I am not mistaken, within the EPA at our TP laboratory we have also undertaken a chronic study of the asbestos in rats. I am not too familiar with this project. I wonder if Dr. Waters is at liberty to give us some information? Dr. Waters is not here. As I say, we understand that project is, also, near the end, and that is a two year study for the rats. You may want to get in touch with them. The project is headed by Dr. Coffin.

Dr. Brown, OSHA: Could you elaborate a little bit on the splitting of these particular asbestos fibers?

Mr. Platek, NIOSH: The splitting as far as the process we used?

Dr. Brown, OSHA: Right.

Mr. Platek, NIOSH: We did ball mill these asbestos fibers. I explained the method in which they were dried, ball milled for 24 hours and we dried them again, and they they were shipped to the contractor.

Dr. Brown, OSHA: Specifically what I mean was, was it vertically or horizontally splitting?

Mr. Platek, NIOSH: The ball milling split the asbestos bundles as well as breaking the fibers into shorter lengths. As I mentioned, asbestos "balls" were also created.

Dr. Brown, OSHA: The second question is where are the areas of the greatest deposition?

Mr. Platek, NIOSH: I honestly don't know yet. As I said, or pointed out in the slide that I showed a while back with the terminal bronchus, if you were seeing a lot of asbestos in that lung you would probably expect to see the asbestos fibers near the lymphatics and near the major blood vessels, but we have not seen them. Once again, we are talking about fibers that when viewed by the light microscope are going to be far beyond the range of light microscopy to resolve, and to develop a technique to do it by scanning electron microscopy is another interest of mine in this project.

Dr. Lee, EPA: One more question?

Dr. Hegyeli, NCI: There are studies indicating that the physical size, the diameter and the lengths of the fiber has much more importance in the physiological response than the nature, the chemical nature of the substance, including glass, metal and other fibers. So, my question is what does this study mean physiologically?

Mr. Platek, NIOSH: From what we have seen so far, as in the previous slide that you saw of the electron micrograph of the macrophage containing the asbestos fibers, it would appear the macrophage is doing its job, and it is engulfing the asbestos. There was no adverse effect in that macrophage that we could see, and it looks like they could be clearing themselves as they are supposed to do. That would be of significance in hopefully determining that the short fibers really don't produce the problems by remaining in the lung, and as you stated, it has been pointed out by numerous investigators that the macrophages have difficulty engulfing the long fibers, and therefore you have the influx of fibroblasts, the laying down of fibrin and then your fibrosis sets up. I hope that answers your question.

Dr. Hegyeli, NCI: My question is that in a practical sense you never encounter these type of fibers. You have mixed fibers, and most of them are in the range, and as you indicated with the macrophage, at least there are some scanning electron micrograph studies indicating that if it occurs up inside the cell, it might serve really as a factor.

Mr. Platek, NIOSH: I don't really know how to answer you any further than what I have on that one. I said that all environments with the possible exception of some of the brake shoe removal operations where the asbestos

is under extreme pressures and can be broken down into much smaller fibers, you are going to have large fiber lengths and the smaller ones in all exposures. This was, once again, mainly a project of testing a federal standard and not where will the worker be exposed to these short fibers because as of right now I do not know of a work environment that is strictly small fiber exposure.

NIOSH

PROCEEDINGS OF THE
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PROGRESS ON JOINT ENVIRONMENTAL AND
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MAY 6-8, 1980

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Appropriate portions of the discussions, working groups and plenary session were sent to the participants for editing. The style of editing varied, as could be expected. To the extent possible, we have attempted to arrive at a consistent format.

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