21

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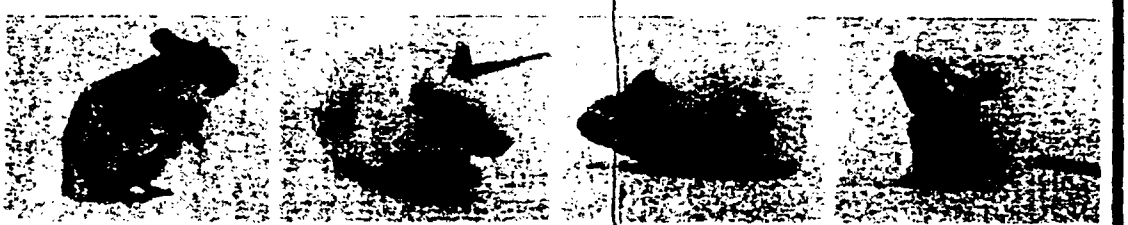
PROCEDURES FOR THE BIOLOGICAL EVALUATION OF ASBESTOS AND ASBESTOS-LIKE PARTICLES



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Research Triangle Park
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UNDER CONTRACT NO. 68-02-1567

Submitted By:

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PROCEDURES FOR THE BIOLOGICAL EVALUATION OF ASBESTOS AND ASBESTOS-LIKE PARTICLES

(REVISED)

July 1976

by

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FOREWORD

This report was prepared in response to a request from the Health Effects Research Laboratory of the Environmental Protection Agency (EPA). Included is a detailed plan of the research objectives and procedures to be followed in the accomplishment of Work Order 1.9 under EPA contract 68-02-1567.

This protocol was reviewed on July 8, 1976, at the New York Academy of Sciences, New York, by the EPA Ad Hoc Committee of the following participants:

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ABSTRACT

This report provides a detailed plan of the research objectives and operating procedures to be followed in future studies on the biological evaluation of asbestos and asbestos-like particles. Included in this report is background information on asbestos and asbestos toxicity, research objectives, capabilities of the research facility, and detailed laboratory operating procedures.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Section I

INTRODUCTION

The Scope of Work for Work Order 1.9 requires that Northrop Services, Inc., provide the services, facilities, and materials (except government furnished equipment) necessary to determine the health effects of air pollutants on laboratory animals from toxic compounds such as asbestos and asbestos-like particles.

Section II contains background information on the properties of asbestos and asbestos-like particles and the epidemiological and experimental history of asbestos toxicity in man and animals, as well as the study objective and research approach to be used in future studies. Section III includes the engineering design describing the capabilities of the facilities. Section IV describes the laboratory procedures in detail.

Section II

TECHNICAL PROTOCOL

This section consists of two basic parts. The first part contains background information on the properties of asbestos and asbestos-like particles and the epidemiological and experimental history of asbestos toxicity in man and animals. The second part contains the study objective and the research approach to be used in future studies.

2.1 ASBESTOS

More than nine-tenths of the earth's crust is comprised of silicates. Of the 30 mineral silicates which can crystalize in a fibrous form, some are called asbestos.[1] Mineralogically, asbestos is divided into two classes: serpentine, characterized as a sheet-shaped formation of silicate; and amphibole, built up of double chains of tetrahedral formations.

The amphiboles constitute an important group of rock-forming minerals which are estimated to comprise as much as 3 percent of the earth's crust and are associated with both igneous and metamorphic rocks. Amphiboles are divided into three groups: iron-magnesium (for example, anthophyllite and amosite), calcium (for example, tremolite and actinolite), and alkali (for example, crocidolite).[1]

The chemical compositions of serpentine and amphibole are:

Serpentine

Chrysotile $Mg_3[Si_2O_5](OH)_4$

Amphibole

Iron-magnesium

Anthophyllite $(Mg, Fe^{2+})_7[Si_8O_{22}](OH, F)_2$

Grunerite (Amosite)

$(Fe^{2+})_4(Fe^{2+}, Mg)_3[Si_8O_{22}](OH)_2$

Calcium

Tremolite $Ca_2Mg_5[Si_8O_{22}](OH, F)_2$

Actinolite $Ca_2(MgFe^{2+})_5Si_8O_{22}(OH)_2$

Alkali

Riebeckite (crocidolite)

$Na_2Fe_3^{2+}Fe_2^{3+}[Si_8O_{22}](OH, F)_2$

2.2 PATHOGENICITY IN MAN

Long-term epidemiological studies have now revealed beyond a resonable doubt that inhalation of asbestos dust can lead to pulmonary fibrosis and carcinoma of the lung and diffuse mesotheliomas of the pleura and peritoneum. Most data have been reported by groups associated with various asbestos-involved occupations. These professions include mining and milling operations, manufacture of asbestos-containing products (textiles, construction materials, and so forth), and jobs involved with application or removal of asbestos-containing insulating materials. Also, sufficient data on populations residing in areas adjacent to these industries are available.

Although asbestosis, a diffuse interstitial fibrosis of the lung, was first reported by Murray in 1907,[2] its relationship to human exposure to the mineral was not clearly recognized until 1920. The disease has since been investigated in epidemiological studies in several countries. Among the countries regarded suitable for such studies are the following: Australia (crocidolite), Canada (chrysotile), Cyprus (chrysotile), Finland (anthophyllite), Italy (chrysotile), South Africa (amosite, chrysotile, and crocidolite), United States (chrysotile and tremolite), and the Soviet Union (chrysotile). All forms of asbestos have been shown capable of producing asbestosis.[3] Studies evaluating comparisons of effects associated with various sizes and types of fibers have been carried out with workers in mining operations, as well as people residing in areas adjacent to such sites. By investigating the one type of fiber mined at a particular site, comparisons between mines have been made possible.

Pleural plaques are non-neoplastic lesions consisting of collagen arranged to give a characteristic "basket-weave" appearance which ceases abruptly at the

plaque margin. Pleural plaques are associated with occupational as well as nonoccupational exposure to asbestos. Epidemiological studies show that 40 percent of the population over 15 years of age in locations where anthophyllite was mined in Finland had pleural plaques.[4,5] Asbestos bodies were present in the lungs of 86 percent of the subjects with bilateral plaques. It was noticed, however, that if asbestos were present in abundance, plaques were regularly encountered.

Pleural plaques were also found in asbestos workers in Dresden[6,7], London[8], Bulgaria[9], and in Czechoslovakia[10]. Pleural calcification was observed in asbestos insulators in Copenhagen[11], New York City[12], and in Belfast[13], as well as in nonoccupationally exposed populations living in the neighborhood of anthophyllite mines in Finland[14], crocidolite and amosite mines in South Africa[15], and chrysotile mines in Italy[78].

The first suggestion that asbestos might be causally related to cancer of the lung was made by Gloyne in England in 1933[16] and Lynch and Smith in the United States in 1935.[17] However, it was not until 1947 that Merewether[18] proposed that asbestosis might be complicated by bronchial carcinoma. He reported 31 instances of cancer of the lung in 235 persons who died of asbestosis between 1924 and 1946. In 1951, Gloyne[19] further reported that out of 132 asbestos workers, 121 who had asbestosis also had cancer of the lung. The clear proof of the association between asbestosis and bronchogenic carcinoma came in a study by Doll.[20] He analyzed the causes of death among 105 men with histories of at least 20 years of work in asbestos textile plants. He found 18 cases of lung cancer, a figure which is 10 times higher than that for the general male population. In a recent analysis, Selikoff, Hammond, and Seidman[21] found that bronchogenic carcinoma accounted for approximately 45 percent of fatal neoplasms among asbestos insulation workers. It was suggested by the Senior Medical Inspectors Advisory Panel in 1967 that bronchial carcinoma is a complication of asbestosis rather than a result of exposure to asbestos.[22] British asbestos workers have exhibited no excess of bronchogenic cancer in the absence of asbestosis. In the United States, however, an excess mortality from bronchogenic carconima has existed in the comparative absence of asbestosis in

certain groups of workers exposed to asbestos.[23,24] In South Africa, bronchogenic carcinoma occurs in all South African asbestos areas.[25] These data have further been confirmed by several other investigators through epidemiological studies.[23,26-38]

It is now believed that there is a relationship between cigarette smoking and exposure to asbestos and incidences of bronchogenic cancer. Selikoff, Hammond, and Churg[32] demonstrated that asbestos insulation workers with a history of regular cigarette smoking suffered eight times the risk of lung cancer compared with cigarette smokers who were not exposed to asbestos and 90 times more than men who neither worked with asbestos nor smoked cigarettes. This effect was confirmed in a further study[39]. Exposure to asbestos did not seem to lead to an extremely high risk of bronchogenic cancer among nonsmokers. Also, Berry, Newhouse, and Turok[40] examined mortality from lung cancer in the asbestos textile industry over a 10-year period and compared them with the number of deaths due to lung cancer in the general population of the area. No significant excess was found in asbestos workers, whether smokers or nonsmokers, with low to moderate exposure; but among workers who smoked and who were severely exposed, the excess was highly significant.

The occurrence of cancers of the peritoneum and pleura (mesotheliomas) in association with asbestos was first reported in 1933 by Gloyne,[16] and then in 1960 by Keal[41] and Wagner[42]. In 1963, Wagner[43] collected 120 cases of mesotheliomas of the pleura which were confirmed by biopsy or autopsy. A large proportion of cases had been exposed to crocidolite in the Northwestern Cape. He also noticed that more than half of the group had never worked in the industry but had lived in the vicinity of mines and mills. Statistical evaluation by Oettlé[28] reveals that the people in the Northwestern Cape exposed to crocidolite have a distinct risk of developing mesotheliomas. The Northeastern Transvaal, where amosite and crocidolite are mined, were in the low risk area. To date, only a few cases of mesothelioma with possible past exposure to amosite in the region have been reported. Among those cases are the ones who were exposed solely to amosite in the United States and who had bronchogenic cancer and mesothelioma. No mesotheliomas have been reported due to anthophyllite from Finland[44,46]. Chrysotile seems to be the most actively involved etiological agent in the United States[30,31,34] and in Canada[38]. However, in

Swaziland and Rhodesia, where chrysotile is mined and milled, no cases of mesotheliomas have been reported[47]. Similar results have been reported in the U.S.S.R., Italy, South Africa, and Cyprus[38]. It is now accepted that mesothelioma can occur after a brief exposure to asbestos. In some cases, there was only a brief exposure in homes or simply as a result of living in the vicinity of asbestos factories or mills[48]. To date, there is no association between cigarette smoking and production of mesothelioma.

2.3 PATHOGENICITY IN ANIMALS

The current data linking asbestos exposure to cancer in man has precipitated considerable experimentation with animals. Lesions such as fibrosis of lung, squamous cell carcinoma of lung, and mesotheliomas have been produced in several species of animals by administration of asbestos particles. As in the case of human exposure, attention to fiber type and size must be taken into account. In addition, one must consider the variables augmented by several species and strains of animals.

A causal relationship between exposure to asbestos and pulmonary fibrosis in animals has been demonstrated by several investigators.[3,43,49-52] To date, the exact properties of the minerals that cause severe tissue damage and eventual fibrosis have not been adequately established. Miller and Harrington[53] have noted an increase in the amount of lysosomal enzymes after administration of experimental asbestos and suspect a causal relationship. The fibrogenic effect is not unique for asbestos. Davis[52] found that mineral fibers beside chrysotile, fibrous brucite, silica fiber, glass fiber, man-made insulation fibers, and other minerals (chlorite, chromite, forsterite, magnetite, olivine, pyronone, and taconite) all produced granulomas in the pleural cavities of mice. There was, however, a great variation in the magnitude of cell responses. For example, long-fiber samples produced large cellular granulomas that were eventually replaced by large amounts of fibrous tissue, and short-fiber samples or nonfibrous dust and finely ground dust revealed much less reaction.

Wagner[43] observed asbestosis in guinea pigs that had inhaled chrysotile dust over a period of 2 years. Holt, Mills, and Young[51] found that guinea pigs that had inhaled very fine particles of chrysotile asbestos showed a

well-developed bronchiolitis after a few days. Phagocytic multinuclear giant cells and monocytes were common features. After 30 weeks, there was cell degeneration and fibrosis. Collagen appeared first as a lining to respiratory broncioles and alveolar ducts. Numerous asbestos bodies appeared later. Similar lung damages were produced by crocidolite, amosite, and anthophyllite. Asbestosis is experimentally produced in rats[54-58], in hamsters[73], and in rabbits and monkeys[43].

The direct effect of exposure to asbestos and the synergistic effect of benzo(a)pyrene (BaP) (a known component of cigarette smoke) has been explored through animal experimentations by several investigators.[59-62] Miller, Smith, and Berliner[59] demonstrated that when amosite and chrysotile were injected intratracheally into hamsters, they produced squamous cell carcinomas of the lungs. When 5 milligrams of BaP was injected in conjunction with amosite, there was no increase in tumor production. When chrysotile was injected with the same dose of BaP, however, there was a considerable increase in tumor production. Vôsomaë[60] confirmed the promoting action of chrysotile established by Miller, Smith, and Berliner.[59] In Vôsomaë's studies, rats were administered intratracheal injections of BaP together with chrysotile (UICC standard reference sample). He demonstrated a considerably higher yield of malignant lung tumors in rats. Pylev[61] obtained a similar effect with chrysotile and BaP. Recently, chrysotile from Ural mines was tested by injecting particles intratracheally into lungs of rats.[62] Results revealed that when the rats were treated with chrysotile alone, an insignificant number of the animals developed precancerous lung lesions and no tumors were found. Analogous injections of chrysotiles together with adsorbed BaP resulted in a greater number of precancerous lung lesions, such as squamous metaplasia, reticulosarcoma and diffuse irregular hyperplasia of pleural mesothelium, and focal adenomatous growth of pleural mesothelium. The rats treated with chrysotile and BaP produced a larger number of precancerous lesions as well as lung papillomas, squamous cell carcinomas, and pleural mesotheliomas. The rats subjected to BaP alone produced precancerous lesions, but no tumors.

Diffuse mesotheliomas of the pleura have been produced by several investigators by means of intrapleural injections of asbestos. Wagner[63,43] demonstrated that amosite, chrysotile, and crocidolite can produce mesothelial tumors in hamsters when 50 milligrams of dust is injected into the pleural cavity. Peacock and Peacock[64] described a similar effect in fowl after injecting crocidolite, amosite, chrysotile, and tremolite. Later, Smith and Hubert[65] successfully produced mesotheliomas in hamsters with amosite and chrysotile.

Mesotheliomas have been produced by intraperitoneal injections. Scheuer, Hunth, and Pott[66] demonstrated in separate experiments that when UICC samples of amosite, anthophyllite, chrysotile A, and crocidolite were injected intraperitoneally, over 50 percent of the animals produced various diseases. These maladies include multicentric and malignant abdominal tumors such as fibrosarcomas, sarcomas, mesotheliomas, reticulum cell sarcomas, and adenocarcinomas. An effort was also made to investigate whether there is any combined effect of asbestos and BaP as in intratracheal innoculation. The data failed to show such a relationship via intraperitoneal injections. Malignant mesotheliomas were induced by single intraperitoneal injection of chrysotile or crocidolite asbestos fibers[67]. Davis[68] also produced mesotheliomas via intraperitoneal injections of crocidolite asbestos into rats and mice.

Comparative study of different UICC amphibole samples with different size was made by Wagner and Berry.[69] They used diameter distribution involving a decreasing order of fineness in crocidolite, amosite, and anthophyllite. The study demonstrated that anthophyllite produced the least number of mesotheliomas. Their interpretation of this study was that for a given weight of asbestos innoculated into the pleural cavity, anthophyllite provided fewer fibers than amosite, which in turn provided fewer fibers than crocidolite. Since chrysotile fibers are shaped like coils and amphiboles are shaped like needles, it is difficult to assess the state of chrysotile fibers in animals and to compare this with that of the amphiboles. Nevertheless, they pointed out that mesotheliomas are associated with the implantation of fine fibers in cells. The finer the fiber, the more tumors are produced.

Stanton and Wrench[70] and Stanton[71] produced mesotheliomas by implanting a fiberglass sheet impregnated by test particles into the thoracic cavities of rats. They introduced UICC standard reference samples of crocidolite and chrysotile A, two samples of fine fibrous glass with diameters of 3 millicrons or less, and aluminum oxide whiskers. They demonstrated that all produced mesotheliomas. The ability of glass fibers to produce mesotheliomas has also been shown by Pott and Friedrichs.[72] The authors suggest that the carcinogenicity is related to the ability of fibers to interact with the cell membrane without destroying the cell.

Respiratory tumors have also been produced in rats by chrysotile inhalation in work by Gross et al[73]. The animals surviving more than 10 months had adenocarcinomas, fibrosarcomas, squamous cell carcinomas, and mesotheliomas. The actual carcinogens responsible for these effects were ascribed by the authors[73] to increased amounts of trace metals introduced in or on the fiber during hammer-milling of the specimens.

Recently, Wagner et al[74] studied the effects of UICC standard reference samples, amosite, anthophyllite and crocidolite, Canadian samples of chrysotile, and Rhodesian samples of chrysotile on the lungs of rats. Significant differences were observed between various samples. Inhalation of anthophyllite and Canadian chrysotile resulted in the most asbestosis after exposure of 6 months or longer. Amosite resulted in the least asbestosis. Crocidolite and Rhodesian chrysotile produced intermediate results. An analysis was carried out to determine whether there was any relationship between the severity of asbestosis and the presence of lung tumors. It was found that the animals with lung tumors had significantly more asbestosis than those without. No tumors of the lung were observed within 300 days of the start of exposure. Adenocarcinoma and squamous cell carcinoma with some metastases and mesotheliomas were observed.

The amount of chrysotile retained in the lungs did not show any clear increase with increased dose in rats exposed for longer than 3 months. The weight of asbestos found in the lungs of rats exposed to amphibole was three

times greater than in those exposed to chrysotile. In spite of this fact, there was no evidence of either less carcinogenicity or less asbestosis in the groups exposed to chrysotile than those exposed to the amphiboles. UICC Canadian chrysotile produced as many mesotheliomas as the UICC crocidolite. Wagner et al[75], however, showed that after intrapleural innoculation, the risk of a mesothelioma occuring with UICC crocidolite is three times the risk incurred with chrysotile. No tumors were found in sites other than lungs.

2.4 STUDY OBJECTIVE

Massive amounts of noncommercial asbestos are found in the United States in mines used as sources of other minerals which have commercial value. The epidemiological and experimental data presented earlier give a clear indication of adverse health effects associated with exposure to asbestos. These data also raise an awareness that the health hazards may not be unique to commercial forms, but may also be associated with asbestos-like fibers found in other rocks and in man-made fibers. The most important question in the case of persons with nonoccupational exposures to asbestos and other amphiboles is whether there is an increased risk of malignancies. Since amphibole rock is estimated to comprise about 3 percent of the earth's crust, human beings may be at risk in many mining, quarrying, tunneling, and other hard-rock mining activities. Furthermore, nonoccupational exposure is possible by means of dust generated by rock crushing and milling as well as from tailing of mine wastes gaining access in the ambient air and water.

One of the mines containing amphibole matrices which is currently being exploited for taconite ore is the Reserve Mining Company's Peter Mitchell Pit in Minnesota. It was questioned by the Environmental Protection Agency (EPA) whether such a project is hazardous to the health of the exposed population in the surrounding community and litigation ensued. The defense in the EPA vs Reserve Mining case claimed that although the fibers were similar chemically to amosite asbestos, they were not amosite asbestos, stating that the fibers were too small to be regarded as asbestos fibers. Further, the mining company's representatives contended that there were insufficient medical data for the fibers to be regarded as a health threat.

It is pertinent that the potential toxicity of materials released in the mining and processing of taconite ore is yet to be determined. Such investigation should consist of studies of the physio-chemical properties of amphiboles and their biological effects with respect to geological classification. Moreover, this information should be of value not only to the specific problem associated with the particular taconite mine, but also should provide a means of judgment when similar problems arise from other mines and rock moving and processing generally.

2.5 RESEARCH APPROACH

The purpose of the proposed investigation is to evaluate whether amphiboles and other minerals processed during extraction and refining of taconite ore from Peter Mitchell Pit (PMP) are carcinogenic and/or cocarcinogenic. PMP particles and UICC ~~chrysotile~~ amosite particles will be administered to animals via intratracheal and intrapleural injections. The biological effects due to the PMP particles will be compared with those obtained by UICC ~~chrysotile~~ amosite samples in the presence and absence of a known carcinogen, benzo(a)pyrene. The following criteria will be used to evaluate such data:

- General health of the animals
- Lung pathology demonstrating asbestosis, fibrosis, bronchogenic carcinoma, and mesothelioma
- Metastasis to other organs
- Gastrointestinal tumors.

A backup contract has already been awarded to Illinois Institute of Technology Research Institute (IITRI) in Chicago to procure raw rock face and to identify them chemically and geologically. After such analysis, the contractor is to supply each geological type to Mt. Sinai Hospital, New York, for confirmation. A selection of samples will then be made according to the size, shape, and chemical properties of the particles. The samples will then be sent to this laboratory for the biological evaluation. All specimens will be held in Mt. Sinai laboratory as reference material.

2.5.1 Animal Selection

The type of animal to be used for asbestos lung studies was considered by the UICC Working Party in 1965. Various types of lesions, similar to those

occurring in human cases of asbestos mesothelioma and squamous cell carcinoma of the lung, can be induced in numerous species of animals. Therefore, a number of species were considered as research animals. The species determined most acceptable for such experimentation is Caesarean-derived (C/D) Wistar rat strains which are free of chronic murine pneumonia and which are kept under barrier controlled conditions.

2.5.2 Experimental Methods and Procedures

Six-weeks-old male rats of Caesarean-originated, barrier sustained Fisher strain will be supplied. Upon arrival, the bulk of an order of rats will be housed in a receiving area in an isolator. From each order, five randomly picked rats will be killed and autopsied to determine whether the animals are disease free. In addition, microbial and viral quality control will be established. Once the shipment is satisfactorily cleared, treatment will commence.

2.5.2.1 Preparation of Test Samples. Preweighed test samples will be received in sterile glass tubes. Since the supplier will be collecting the particles aseptically, the samples will not be sterilized upon arrival. However, the sterility of samples will be ascertained by conventional microbiological and viral assays. Immediately before use, sterile physiological saline will be added to the tubes to give concentrations of 2 milligrams per 0.2 milliliter. Care will be taken to avoid settling of the particles which might result in inaccurate dosage.

2.5.2.2 Preparation of Benzo(a)Pyrene Solution. Colloidal suspensions will be made of benzo(a)pyrene (BaP) in physiological saline at concentrations of 3 milligrams per 0.2 milliliter under sterile conditions. The suspension will be stirred continuously to keep BaP particles from settling.

2.5.3 Experimental Design

2.5.3.1 Procedure for Intratracheal Instillation. Before each treatment, the animals will be anesthetized with intraperitoneal injections of 0.4 milliliter of a 1-percent solution of sodium methohexital (Brevital). The abdominal wall will be disinfected with 70-percent ethanol. After the anesthetic is effected,

the rat will be transferred to a hood and placed on a specially designed platform. Here, the animal's mouth will be opened and the rat will be fixed to the platform by passing a rubber band through the lower incisors and fixing the band to the lower side of the board. The upper incisors will be fixed to the upper side of the board in the same manner. A selected volume of the test substance will then be drawn into a 1-cubic-centimeter tuberculin syringe with a blunt 19-gauge needle bent at a 135° angle. Special illumination in the hood will provide direct beam into the mouth, giving a clear view of the pharynx. The needle will be inserted into the tracheal lumen until a slight resistance is felt. The contents of the syringe will be discharged into the lungs and the animal will be removed from the platform and put into a cage.

2.5.3.2 Intratracheal Experiments. A series of intratracheal experiments is designed to provide information concerning the carcinogenic potential of PMP amphibole alone as well as to evaluate the differences between the synergistic effects of PMP amphibole, UICC ~~chrysotile~~ amosite, and iron oxide particle with BaP.

Series I -- Initial Range Finding Study. The dose regimen for Series II will be determined by this series of experiments. Ten animals will receive either 0.5, 1.0, or 2.0 milligrams of the test substance once a week for 12 weeks. The animals will be observed very carefully for any change in their general health and any weight loss for 3 months. After this period, the animals will be sacrificed and their pathology will be determined.

In case of a severe toxicity, the dose regimen will be reduced by individual dose and number of injections. The highest nontoxic dose will be determined.

Series II -- Chronic Intratracheal Testing of PMP Amphibole. The dose regimen will be determined from Series I.

I. Unknown Sample - PMP Amphibole - 600 Animals
II. Asbestos Control - UICC Amosite - 200 Animals
III. Negative Control - Saline and Gel - 200 Animals

Series III -- Chronic Interaction Studies by Intratracheal Instillations. The synergistic effects of chrysotile and BaP have been demonstrated.[59-62]

Shabad et al[77] demonstrated higher incidence of tumors in rats induced by mixing BaP and India ink powder than from BaP alone. Saffiotti et al[76] also induced lung tumors in hamsters by injecting iron oxide and BaP. Blair[45] has demonstrated similar results on rats. The results of these studies and others of a similar nature led to speculation that it was the presence of particles and not the nature of the particle which contributed to the cocarcinogenic effect. Miller et al[59], however, pointed out that while chrysotile demonstrated enhanced carcinogenic activity with BaP, amosite did not. These results have led to the suggestion that the synergistic effect between asbestos and a carcinogen may be property of specific types of asbestos.

The dose regimen for this series will be 3 milligrams of particle and 3 milligrams of BaP in 0.2-milliliter saline plus gel per week for 5 weeks. This dose is extrapolated from Blair's data to achieve 40-percent tumor incidence in the iron oxide plus BaP group.

I. PMP Amphibole plus BaP - 300 Animals
II. UICC Amphibole plus BaP - 300 Animals
III. Iron Oxide plus BaP - 300 Animals
IV. PMP Amphibole - 200 Animals
V. UICC Amphibole - 200 Animals
VI. Iron Oxide - 200 Animals
VII. BaP - 200 Animals

Statistical Consideration. A sample size of animals per group is adequate to detect differences between tumor incidence rates that equal or exceed 15 percent, provided that the lowest incidence rate among the three series is at least 10 percent. These calculations are based on controlling Type I and Type II errors at 0.05 and 0.20, respectively. A Type I error arises when the researcher declares that the difference is real when, in fact, this difference is zero. On the other hand, a Type II error consists of failing to declare the tumor rates significantly different when, in fact, they are different. A sample size of 600 in Series I is adequate to detect tumor incidence rate as low as 0.16 percent.

A general consideration in the statistical analysis of the results involves determining the number of animals considered to be at risk. Only those animals surviving for a period of time greater than, or equal to, the time of appearance of the first tumor will be included in the final analysis. The

animals that die early in a study from infectious diseases or other causes will be excluded.

2.5.3.3 Intrapleural Experiment. Tumors of mesothelial origin have been produced by many investigators by intrapleural injection of various preparations of particulate matter into experimental animals.[43,63-65] Although introduction of particles directly into the pleural space does not reproduce natural route of exposure to inhaled substances, this technique provides a convenient procedure for screening several particulate substances for their relative activity in the production of mesothelioma of the pleura.

2.5.3.4 Procedure for Intrapleural Injection. Rats will be anesthetized by intraperitoneal injection of 0.4 milliliter of a 1-percent solution of sodium methohexital (Brevital) as described earlier. The left thoracic wall will then be shaved properly and disinfected with 70-percent ethanol. A special 18-gauge needle with a blunt hollow trochar is required for this procedure. The needle will be introduced through the thoracic wall of the rat. Once the wall is punctured, the trochar will be pushed inside and the lung will be pushed aside. The needle will then be pushed farther into the thoracic cavity and the fluid inside the syringe containing the test particles will be discharged into the pleural cavity. After innoculation, the needle will be retracted from the thoracic cavity and the trochar will be removed.

Series IV -- Chronic Intrapleural Testing of PMP Particles. The dose regimen will be a single injection of 20 milligrams of the test substance in 0.5-milliliters of physiological saline.

I. Unknown Sample - PMP Amphibole - 150 Animals
II. Asbestos Control - UICC Amosite - 150 Animals
III. Negative Control - Saline - 150 Animals

Statistical Consideration. A sample size of 150 animals per group is sufficient to duplicate the findings of Wagner and Berry[69] with respect to intrapleural injection of various amphibole and chrysotile particles. Again, the Type I and Type II errors are controlled at 0.05 and 0.20, respectively.

Untreated Controls. Two Hundred animals from Group III of Series II and 150 animals from Group III of Series IV will be considered as controls for non-

pulmonary tumors. In addition, 250 animals will be maintained in the facility without any treatment.

2.6 LABORATORY OPERATIONS

All experiments will be conducted throughout the life span of the animals. The rats will be maintained in a specially designed microbiological barrier which is described in Section III. The three groups (namely, control, PMP particles, and asbestos particles) will be housed in separate rooms. Racks, tables, and various other items will be placed in the barrier and will be sterilized with formaldehyde gas before the animals arrive. All items entering the facility after the initial sterilization of the barrier will be sterilized by one of the several methods which are described in Section IV.

The animals will be fed autoclaved NIH Formula 31 and sterile water *ad libitum* and will be housed in a see-through suspended cage system with an automatic water supply. The cage system will consist of solid-bottom, polystyrene cages suspended in stainless steel racks. This system is equipped with a rear manifold with custom valves specially designed to avoid any water leakage within a cage. Water will be supplied by gravity from a 5-gallon stainless steel tank. This system will also have a special attachment to provide a water bottle if special treatment is required for the animals. The animals will be checked every day. During the treatment period, they will be weighed once a week and subsequently once a month.

When each animal dies, it will be autopsied, and its trachea and lungs will be removed and immersed in 10-percent buffered formalin. Other organs (such as liver, kidney, and gastrointestinal tract) will also be examined, and the samples will be preserved in 10-percent formalin. These sections will be sent out to a commercial histology laboratory for processing. Pathological evaluation will then be made on these slides.

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