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EXHIBIT  
ASA-103

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



*Submitted To:*

**ENVIRONMENTAL PROTECTION AGENCY**

**Research Triangle Park  
North Carolina**



**UNDER CONTRACT NO. 68-02-1567**

*Submitted By:*

**NORTHROP SERVICES, INC.**

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

(REVISED)

July 1976

by

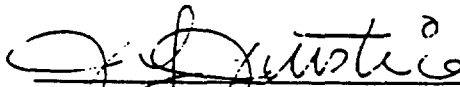
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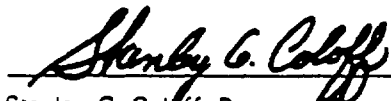
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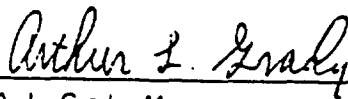
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## FOREWORD

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.

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 Cesarean-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 Cesarean-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. Prew weighed 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 <sup>amosite</sup> ~~chrysotile~~, 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. <sup>59-62</sup>

Shabad et al<sup>77</sup> demonstrated higher incidence of tumors in rats induced by mixing BaP and India ink powder than from BaP alone. Saffiotti et al<sup>76</sup> also induced lung tumors in hamsters by injecting iron oxide and BaP. Blair<sup>45</sup> 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<sup>59</sup>, 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.<sup>43,63-65</sup> 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 inoculation, 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<sup>69</sup> 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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