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Environmental Health Effects Research Series

STUDIES ON THE EFFECT OF AMMONIUM SULFATE ON CARCINOGENESIS

Health Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

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STUDIES ON THE EFFECT OF AMMONIUM SULFATE ON CARCINOGENESIS

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affadavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

Evidence exists that exposure to atmospheric "sulfate" may be associated with increased pulmonary disease in people. Biological studies indicate that the response to the various sulfates varies according to the specific compound, some compounds of sulfate having more effect than sulfur dioxide gas, others no effect whatever. Ammonium sulfate, which is numbered among the more reactive compounds in these studies, is of interest because it is generally believed to be one of the more common compounds of sulfur in polluted air. Sulfur dioxide has been shown to enhance the effect of a standard carcinogen (benzo(a)pyrene) when both compounds are given by inhalation.

The following EPA study evaluates the influence of ammonium sulfate as a cofactor in carcinogenesis studies employing benzopyrene as the prime agent. Additional information concerning influence on a metabolic enzyme aryl hydroxylase and the body's absorption and excretion of sulfate is also included. These factors are relevant to the overall EPA program since the agency is attempting to evaluate the importance of the sulfates in relation to other atmospheric factors and their possible health effects.

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ABSTRACT

This project was designed to evaluate health effects of ammonium sulfate* inhalation using experimental animals. The questions studied were:(1) Is inhaled ammonium sulfate co-carcinogenic. (2) What are the deposition and clearance patterns of inhaled ammonium sulfate? (3) What effect does ammonium sulfate have on pulmonary defensive mechanisms?

The carcinogenesis studies utilized hamsters given 5 mg intratracheal injections of Benzo(a)pyrene weekly for 15 weeks and 6 hour daily inhalation of ammonium sulfate at concentrations in the range of 200 $\mu g/m^3$ for 15 weeks. Incidence of respiratory cancer was 1.4% in unexposed controls, 2.9% in hamsters exposed to ammonium sulfate alone, 14.4% in those given only Benzo(a) pyrene injections and 11.8% in those given Benzo(a)pyrene injections and exposed to ammonium sulfate. The increased incidence of cancer with Benzo(a) pyrene was statistically significant ($p < 0.005$). Ammonium sulfate inhalation had no effect on the development of cancer and no effect on the development of other significant pulmonary diseases.

For deposition and clearance studies, S^{35}-labeled ammonium sulfate aerosols with high specific activity were used. A five minute exposure time and a short, reproducible time period in which tissues were obtained for the first analysis after exposure were necessary to determine deposition. Clearance was then assessed at 1, 3 and 6 hours after exposures. Hamsters, guinea pigs and rabbits were studied. Total respiratory tract deposition was greater with the larger particle size in all studies. Clearance patterns were similar for the three species regardless of particle size. The half time for clearance of ammonium sulfate from the lung was 18 to 20 minutes. Inhaled and injected sulfate was cleared via the urinary tract and by six hours after exposure 95% of the total collectable sulfate was present in the urine.

Pulmonary defensive parameters evaluated in this project were levels of aryl hydrocarbon hydroxylase activity in hamster lungs and pulmonary macrophage numbers. Ammonium sulfate exposure concentrations were in the range of 200 $\mu g/m^3$ and 1,000 $\mu g/m^3$. Enzyme activity was studied after 1, 3 and 10 weeks of exposure. Significant inducement of the enzyme was found with Benzo(a)pyrene at all analysis periods. Ammonium sulfate inhalation had no effect on this enzyme. Pulmonary macrophage number was not affected by ammonium sulfate inhalation.

Overall, ammonium sulfate was not found to be a deleterious air pollutant for the animal species assessed at the inhalation concentrations used.

* $(Nh4)2\ SO_4$

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SECTION 1

INTRODUCTION

An area of great concern in environmentally related research is the consideration of multifactorial determinants of human lung cancer. Animal models for induction of pulmonary neoplasia have employed both intratracheal instillations and inhalational exposure to a variety of organic or inorganic agents, alone or in combinations. Exposures to combinations of agents have been productive of large amounts of important data, but have in addition raised vital questions relevant to many environmental contaminants. Data already available from animal exposures have been useful in postulating pathogenic mechanisms as well as in elucidating specific human health hazards.

Presently, policy making and regulatory agencies are confronted with need for data relevant to the potential health effects of exposure to airborne particulate sulfates. This research project evaluates the role of these airborne particulates on the development of pulmonary neoplasia following exposure of Syrian Golden Hamsters to the ubiquitous hydrocarbon carcinogen, benzo(a)pyrene. This investigation combines exposure to aerosols of particulate sulfate with an intratracheally administered suspension of benzo(a)pyrene. The end points evaluated following the period of daily aerosol and weekly intratracheal exposures are the frequency of development and morphologic characterization of respiratory tract neoplasms in animals maintained for their natural life-time. The studies include use of radiolabeled airborne particles to evaluate the deposition and clearance of sulfate in the animal respiratory tract, and studies to evaluate pulmonary defense mechanisms operative in this model system.

SECTIONS 2 and 3

CONCLUSIONS AND RECOMMENDATIONS

The results of these studies on the health effects of exposure to airborne particulate ammonium sulfate indicate no deleterious effect from inhalation of this pollutant by Syrian Golden Hamsters at concentrations ranging from 200 $\mu g/m^3$ to 1,000 $\mu g/m^3$. There was no effect of ammonium sulfate inhalation on benzo(a)pyrene carcinogenesis. In addition, no other pathologic findings in the respiratory tract could be attributed to ammonium sulfate exposure. Pulmonary defensive parameters, including aryl hydrocarbon hydroxylase enzyme levels and pulmonary macrophage numbers, were not affected by inhalation of ammonium sulfate.

Deposition studies showed that airborne ammonium sulfate in particle size distributions of 0.3 μ and 0.6 μ MMAD reached the lung. However, a substantial portion of the total respiratory deposition was in the nose. The noses of the animals studied, therefore, acted as a protective mechanism against these submicronic particles. Clearance studies showed a rapid removal of sulfate from the lung with one half of the deposited amount cleared in 18 to 20 minutes. Clearance was via the blood to the urinary tract with 95% of the collectable sulfate in the urine within 6 hours of exposure. Therefore, when sulfate was inhaled in this highly soluble form, it was cleared very rapidly from the body.

This research project was part of a large program to evaluate the biologic effects of airborne sulfate. Data derived from this project does not support the contention that sulfate ion is a harmful pollutant. However, the data must be interpreted only in terms of the chemical form of sulfate studied, the concentrations studied and the animal species assessed. We recommend that evaluation of the findings of the entire sulfate research program should be done before proceeding to initiate additional research or develop policy.

SECTION 4

STUDIES ON THE EFFECT OF ATMOSPHERIC POLLUTANTS ON CARCINOGENESIS

I. GOALS AND OBJECTIVES OF THIS PROJECT

This project was initiated to determine the role of selected atmospheric pollutants as co-factors in carcinogenesis. During the project period, the goals and objectives of this program were reshaped from a broad based study on particulate pollutants as co-factors in carcinogenesis to a more definitive study designed to evaluate health effects of ammonium sulfate inhalation using experimental animal model systems. The questions studied were: (1) can inhaled ammonium sulfate act as a co-carcinogen? (2) What are the deposition and clearance patterns of inhaled ammonium sulfate? (3) What effect does ammonium sulfate have on pulmonary defensive mechanisms?

The rationale for testing ammonium sulfate as a co-carcinogen was based on evidence from three studies. Findings from the EPA-CHESS program (1, 2) indicated that levels of suspended atmospheric sulfates, much lower than sulfur dioxide levels, were associated with increased prevalence of pulmonary diseases. Amdur and her associates (3) had used the parameter of increased pulmonary air flow resistance of guinea pigs as an indicator of irritation. They studied a number of compounds and found only those containing sulfate ions produced an irritant response. No epidemiologic studies had been done correlating lung cancer incidence and levels of suspended sulfate. However, Laskin (4) had used sulfur dioxide as an irritant in carcinogenesis studies and found it to be a promoting agent of carcinogenesis. It was, therefore, quite possible that sulfate ion, a more potent irritant in the epidemiologic and guinea pig studies, could be a promoting agent of carcinogenesis. The choice of ammonium sulfate was based on Amdur's demonstration of the irritative effects of this compound and evidence that suggested this was a pollutant likely to increase in ambient air in the future (2, 5).

Deposition and clearance studies were needed to determine if the size of ammonium sulfate particles planned for use in the carcinogenesis studies would reach the lungs of our animals. No data was available on deposition and clearance of ammonium sulfate or other water soluble hygroscopic particles in hamsters. These studies required development of methodology. Once this was achieved, it became apparent that questions of deposition and clearance had implications beyond our carcinogenesis studies. Since we had the methodology and facilities, we extended these studies to guinea pigs and rabbits, animals frequently used in assessment of pollutant effects on health.

Pulmonary defensive studies were a major portion of the original proposal. We were attempting to elucidate the mechanism of co-carcinogenic activity of pollutants by assessment of their effect on aryl hydrocarbon hydroxylase enzyme and pulmonary macrophage activity. These studies were done

using ammonium sulfate at several different concentrations.

II. CARCINOGENESIS STUDIES

A. General Plan

The carcinogenesis studies utilized Benzo(a)pyrene (BaP), a known carcinogen (6, 7), given intratracheally. This compound instilled in this manner had been shown to result in a low incidence of pulmonary cancer (8, 9). Ammonium sulfate was given by inhalation to determine if this irritant could increase the incidence of cancer in this experimental model system. There were four study groups: (1) BaP alone, (2) sulfate alone, (3) BaP-sulfate and (4), unexposed control. In these studies four groups of 80 hamsters, all from the same litter date, from Lakeview Hamster Colony, were used.

Hamsters in the group receiving BaP alone were given 5 mg doses of the carcinogen by intratracheal injection on Wednesday of each week for 15 weeks. Monday through Friday of each week these animals were placed in a chamber without food or water at a negative pressure of 0.15 inches of water for 6 hours. Filtered room air passed through the chamber at a rate of one complete air change per minute. In this way, these animals were held under the same conditions as those in the sulfate exposure groups. The 5 mg dose was selected on the basis of Saffiotti's findings (6).

The sulfate alone group was exposed for 6 hours each day, Monday through Friday without food or water, in individual holding cages at a negative pressure of 0.15 inches of water. Exposures lasted 15 weeks. Three sulfate samples were taken each day, and samples for particle sizing were done intermittently. Sulfate concentrations in the chamber were in the range of 200$\mu g/m^3$. This level was selected as a conservative extrapolation of known sulfate levels of major metropolitan areas (10).

The BaP-sulfate group was exposed to sulfate daily and BaP weekly. The sulfate exposure followed the same protocol used for the animals exposed to sulfate alone. The BaP injections followed the same procedure used for animals receiving BaP alone.

Unexposed control animals were maintained in the same animal facility with the exposed groups.

All animals were earmarked for identification, facilitating rotation of animals through different areas within the chamber each day, and for easy identification when weighing the animals. Animals were weighed every month during their lifetime. They were observed daily and moribund animals isolated to prevent canabalism on death. Dead animals were necropsied. At necropsy, gross observations were made on all internal organs, including the brain, nasal structures and larynx. Histologic sections were routinely done on the noses, larynx, trachea, bronchi and all lobes of the lungs. All tissues from animals were saved until the end of the study. Animals not dying spontaneously were sacrificed 2 years after completion of exposure. Necropsy evaluation was the same as for animals dying spontaneously. All necropsies were done under the direction of a pathologist. The tissues were prepared

for histologic study by routine methods. Hematoxylin and eosin-stained slides were evaluated independently by two pathologists. Observations on all tissues studied were recorded and then coded for computered evaluation of the findings.

We proposed two carcinogenesis studies to evaluate the effect of ammonium sulfate as a co-carcinogen. The first was the study outlined above; the second study was to use a concentration of sulfate determined by the preliminary findings of the first study.

In studies that attempt to relate experimental environmental exposure to adverse health effects, a number of factors are important. Temperature and relative humidity must be within normal ambient ranges. If particulates are studied, these must be respirable by the test species. However, the most important parameter is the concentration level of the pollutant in the exposure system. Studies using concentrations that could not be reasonably extrapolated to the natural situation are only of value in showing that an effect could take place. They are not useful in developing air quality standards based on adverse health effects. Carcinogenesis and co-carcinogenesis studies can be sensitive indicators of a most significant health effect. However, the concentrations used in the experiments determine their relevance to air quality standards. These studies are expensive and time consuming, so that careful selection of concentration level is critical. In our first carcinogenesis experiment, the exposure concentration was based upon a conservative extrapolation of the ambient sulfate level in the Philadelphia area. The exposure range of 200 $\mu g/m^3$ for 6 hours per day was used. In carcinogenesis experiments, the number of animals is also important and must be selected so that expected results will yield statistically significant data. The estimated malignancy rate with BaP alone was in the range of 10%. Therefore, using 80 animals per group, a doubling of this rate in the BaP-sulfate groups would be significant at the 5% level. Since both the rate of malignancy and the exposure level were chosen empirically in the first study, a second carinogenesis study was planned. The exposure parameters of this experiment were to be based on the 6 month findings of the first experiment. At that point, malignancies had been seen in the BaP group at a rate of 10%. No malignancies were seen in the BaP-sulfate group. Obviously, there had been no enhancement by sulfate. In fact, there was a statistically significant reduction of the malignancy rate in this group. Since our original hypothesis predicted enhancement, it would have been unreasonable to go to a lower sulfate dose which had been planned if enhancement had been seen. Significantly increasing the concentration level would not have been in keeping with the objective of this experiment, since we would not then be working with environmentally relatable levels. We, therefore, decided to repeat the experiment at the same concentration so that data from these experiments could be evaluated both independently and in combination. The combined data would have twice the number of animals in each group so that the significance of small differences among treatment groups could be determined with greater statistical certainty. Therefore, the protocol of the second carcinogenesis study was the same as the first.

B. Technical Details

1. Inhalation Facility--

The Medical College of Pennsylvania Inhalation Laboratory consists of two 322 liter stainless steel and glass exposure chambers (11), a 42" x 30" x 30" stainless steel and glass glove box and a 6' x 3' x 3' plexiglass glove box. All of these units are connected to exhaust ducts used exclusively for this system. Each unit has two absolute filters (99% efficiency on all particles greater than 0.1μ MMD) connected in series to trap all particles emanating from the system. The exhaust fan has an emergency back up fan connected in parallel that can be activated by loss of the required pressure at strategic points in the system. Each unit has a valve to control air flow into the exhaust system. These units are connected to the sewer system for easy cleaning, but this drainage system can be sealed off from the units by valves so that air cannot be drawn from the sewer system.

The stainless steel glove box was used for manipulations involving BaP. The milling apparatus for grinding this carcinogen to fine particulate size was housed here. The glass front of this glove box was covered with aluminum foil to protect the BaP from photodecomposition. Operating pressure in this unit is minus 0.50 inches of water with air flow through the unit set at 10 cubic feet per minute.

The flow diagram in Figure 1 outlines the parts and connections of the aerosol generating and exposure system.

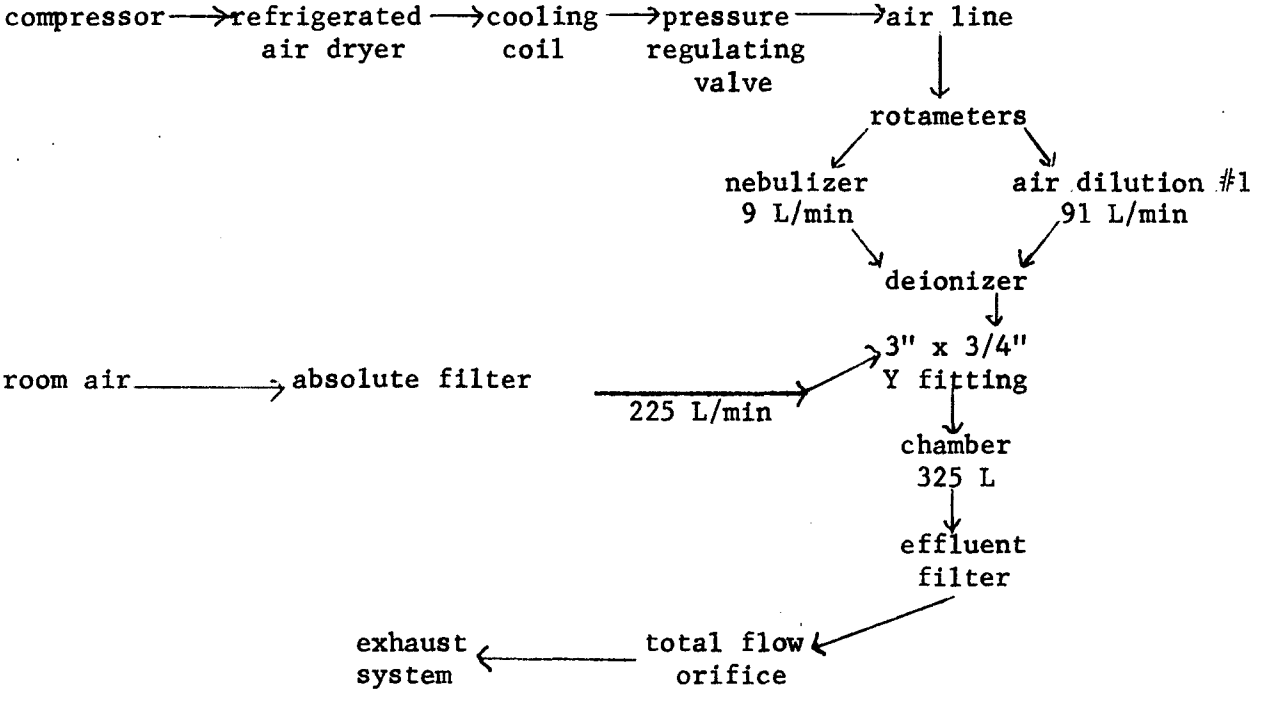

Figure 1. Flow diagram of ammonium sulfate exposure system.

The exposure chambers received air from two sources, one source providing approximately 1/3 of the total air flow was a Gast model 7HDD oilless compressor that ran continuously producing 200 liters of air at 35 PSIG. This source

was used for aerosol production and one air dilution of the aerosol. Air from this compressor passed through a Deltech refrigerated air dryer which removed as much as 100 ml of water from 12 m^3 of air. At each chamber, an auxiliary cooling coil (50' of ¼" copper tubing coiled to a diameter of 3", 12" in length) was present on this air line. This coil further lowered the temperature of the air when it was immersed in ice. Beyond the coils, each air line had a pressure regulating valve to maintain constant pressure to the aerosol nebulizer and a Watts coalescing air line filter that removed all solid particles 0.01μ and larger. The second source of air to the chamber was ambient air from the room. Two thirds of the total chamber air flow was drawn through an absolute filter into the chamber and used as a second air dilution of the aerosol. An air flow of one air change per minute was found necessary to assure endogenously produced ammonia levels below 100 $\mu g/m^3$. Chamber pressure in this system was negative and ranged between 0.14 and 0.16 inches of water.

The aerosol generator used was a Collison nebulizer obtained from Environmental Research Corporation, St. Paul, MN. At an incoming air pressure of 35 PSIG, the output was 9 liters/min from this nebulizer. The deionizer had a sealed Krypton 85 source of energy to neutralize static electrical charge of the aerosol particles.

Preliminary studies over several weeks of operation showed that temperature and relative humidity were dependent on ambient conditions. Temperature ranged from 22° - 27° C and relative humidity ranged from 28% to 62%. This represented less control of these parameters than had been planned. However, the higher air flow using room air was necessary to assure low ammonia levels in the chamber.

2. Aerosol Sampling--

Sampling to determine sulfate concentration in the aerosol as well as the size of the particles was done with a stainless steel ½" tube positioned isokinetically within the chamber. For concentration sampling, the tube was connected to a millipore filter unit for air sampling, containing a 47 mm diameter 0.8μ pore size millipore filter. A vacuum pump drew 14 liters of air per minute through the filter. Air was sampled for 15 minutes. The filter was then placed in a 60 mm diameter disposable Petri dish containing 2 ml of deionized water and allowed to soak for 1 hour. The solution was then collected into sealed tubes and stored until analyzed. Particle sizing was done by connecting an Andersen Sampler to the tube and drawing air through the sampler for 15 minutes. This sampled 424.5 liters of air in this period. The plates of the sampler and the final filter were then placed in 100 mm disposable Petri dishes with 10 ml of deionized water and soaked for 1 hour. The solution was then collected and stored in sealed tubes until analyzed.

Sulfate samples were analyzed by the method of Melnicoff et al (12). This is an autoanalyzer method using sodium rhodizonate to colorimetrically determine sulfate concentration. This technique was developed for analysis of samples from this study. However, it also has broader applications in chemical analysis for sulfate.

3. Benzo(a)pyrene Preparation--

Before we began the carcinogenesis studies, the BaP to be used had to be characterized and prepared in an injectable form. BaP was obtained from Aldrich Chemical Corp., Milwaukee, Wis. Purity was determined by spectral analysis in benzene. The spectrum obtained matched that published by Sawicki et al (13) and using the extinction coefficients determined by these authors, concentration could be determined. Purity exceeded 99% by these methods.

BaP was given by intratracheal injection in a sterile saline gelatin suspension with the BaP particles less than 1μ MMD. This suspension was prepared in the following manner. A solution was prepared containing 0.5% gelatin (Difco Laboratories, Detroit, Mich.) and 0.9% sodium chloride. This solution, with 5 mm glass beads, was autoclaved in a Wheaton tissue culture roller bottle. BaP was then added to make a 2.5% suspension. The bottle was then placed on a Norton jar mill and turned continuously at 12 revolutions per minute for two weeks in a darkened unit. After milling, the suspension was separated from the glass beads and transferred sterilely in 10 ml aliquots into injection vials.

Intratracheal injections of 0.2 ml of suspension were given through blunt tipped needles at a 45° angle. The hamsters were suspended on a slant board by the upper incisors, the tongue held outward with forceps, the larynx visualized and injection made into the trachea.

4. Evaluation--

The following gross observations were made at necropsy of each animal:

a. Presence or absence of lung cancer.
b. Pathologic changes in all internal organs as well as the brain, nasal structures and larynx.
c. Overall assessment of animal condition prior to death.

The following histologic observations were made on tissues of each animal:

a. Larynx, trachea and bronchi.
    (1) The presence or absence of cancer: if present, histopathologic types.
    (2) Condition of the epithelium: normal, atrophic, hyperplastic, anaplastic, carcinomatous, ulcerated.
    (3) Condition of mucous glands: normal, atrophic, hyperplastic.
    (4) Condition of goblet cells: normal, increased, decreased.
    (5) Presence or absence of inflammation - type.

b. Lungs:
    (1) Presence or absence of cancer.
    (2) Pattern of particulate accumulation, nodes, interstitial, obliterative.
    (3) Condition of bronchial, bronchiole and alveolar epithelium: normal, ulcerated, atrophic, hyperplastic, anaplastic, carcinomatous.

(4) Presence of inflammation: peribronchiolar, intraalveolar, interstitial - type.
(5) Maintenance of pulmonary architecture - presence of emphysema or fibrosis.

c. Other structures:
(1) Presence or absence of cancer. Metastasis or primary?
(2) Other pathologic changes.

Both these descriptive observations and the slides from which they were made were then reviewed and the findings used to make specific diagnoses. These included: the histologic types and locations of cancers, the presence and location of benign cellular proliferations, the presence of bronchitis, pneumonia, emphysema, fibrosis or other pulmonary diseases, and the presence of other significant non-pulmonary diseases.

C. Results

Chi square analysis of mortality rates, body weights, development of cancer and histologic types of cancer in the two carcinogenesis studies showed no differences between these two studies. Review of protocols and exposure parameters (see Table I) did not reveal differences between the two studies. Therefore, all data from the two studies have been combined for presentation in this report.

Parameter	Carcinogenesis I		Carcinogenesis II	
Exposure	Sulfate alone	BaP ± sulfate	Sulfate alone	BaP ± sulfate
Sulfate concentration	189 ± 39 $\mu g/m^3$	204 ± 37$\mu g/m^3$	181 ± $\mu g/m^3$	190 ± 30 $\mu g/m^3$
Temperature	24.5 ± 1.9° C	25.6 ± 1.5°C	24.0 ± 1.6°C	26.0 ± 1.6°C
Relative humidity	39.2 ± 12.2%	37.2 ± 9.0%	39.0 ± 11.2%	43.8 ± 15.1%
Particle size MMAD	0.30μ	0.30μ	0.29μ	0.31μ

Table I. Exposure Parameters of Carcinogenesis - Studies I and II.

The mean weights of the hamsters in each treatment group throughout the study period are illustrated in Figure 2. It can be seen that the animals given BaP weighed less throughout the study than those animals that did not receive this carcinogen. However, ammonium sulfate exposure did not have any effect on body weight when comparisons were made between the unexposed control group and sulfate alone or BaP alone and BaP-sulfate groups.

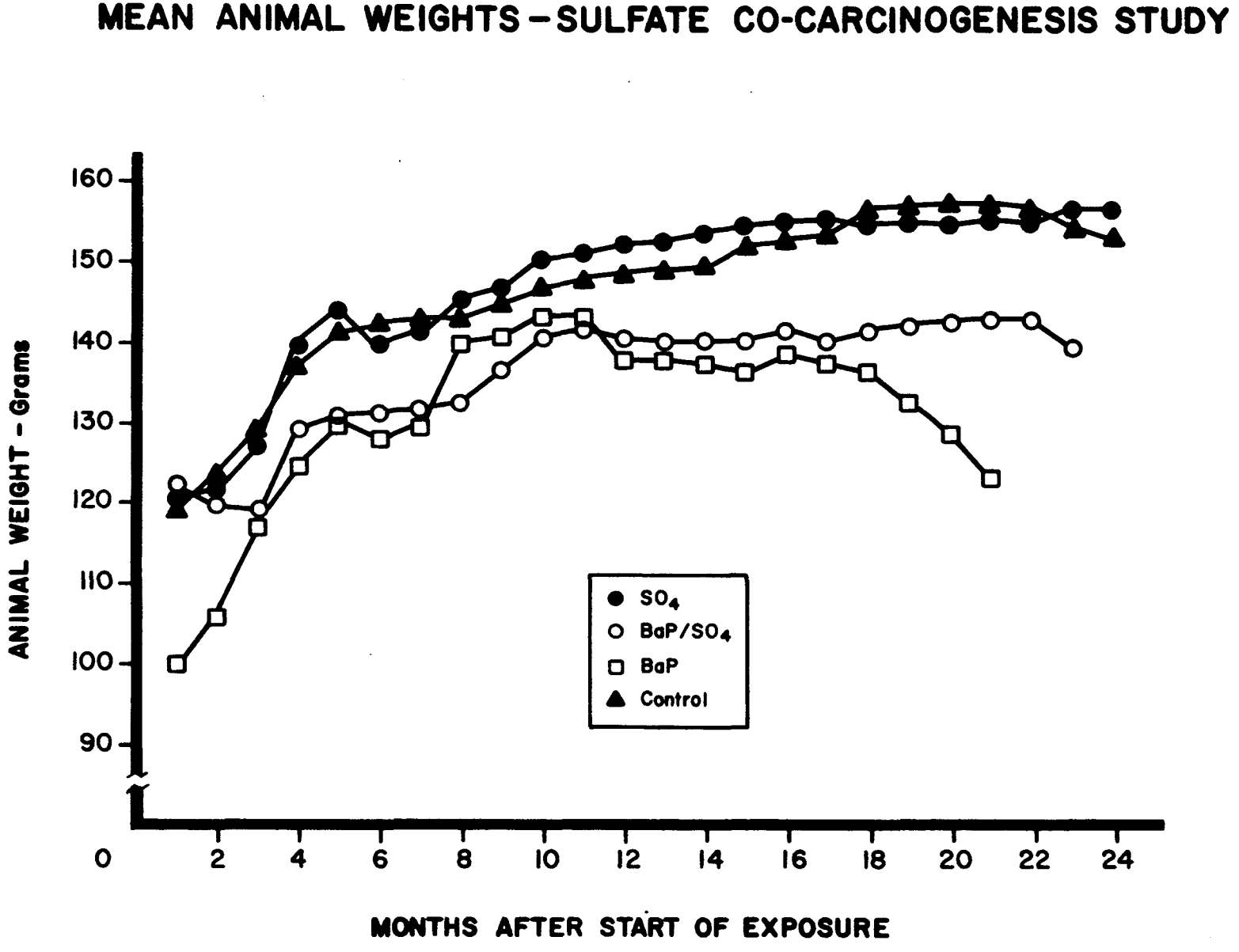
MEAN ANIMAL WEIGHTS – SULFATE CO-CARCINOGENESIS STUDY
ANIMAL WEIGHT - Grams
160
150
140
130
120
110
100
90
0
2
4
6
8
10
12
14
16
18
20
22
24
MONTHS AFTER START OF EXPOSURE
SO4
BaP/SO4
BaP
Control

Figure 2.

Cumulative mortality throughout the study period is illustrated in Figure 3. The BaP groups had higher mortality throughout the study. This can be traced to higher mortality rates in the BaP alone group between the first and fifth month after the start of exposure and in the BaP-sulfate groups in the fifth through the eighth month. Many of the deaths were due to malignancy in these time periods. We have no explanation for the earlier development of cancer in the BaP alone group.

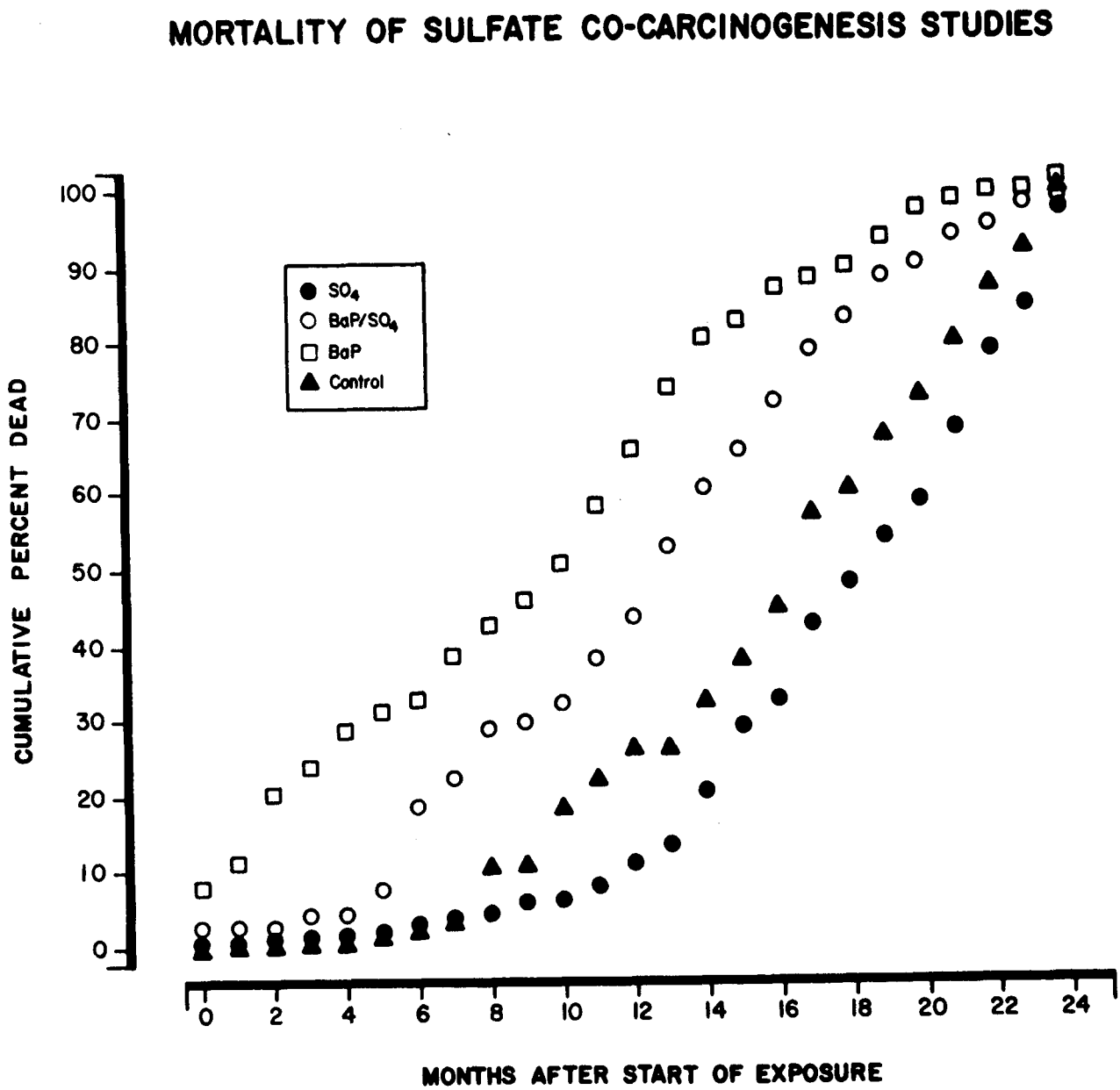

Figure 3.

Table 2 illustrates the percentage of hamsters with cancer in each exposure group, as well as the percentage of hamsters with cancer of the respiratory tract.

Table 3 lists the Chi square and p for comparisons between treatment groups in the development of cancer.

TABLE 2. INCIDENCE OF ALL CANCER AND RESPIRATORY CANCER

Treatment	Cancer	Respiratory
Control	5.9%	1.4%
Ammonium sulfate	4.0%	2.9%
BaP	34.6%	14.4%
BaP-sulfate	27.9%	11.8%

TABLE 3. CHI SQUARE AND p FOR COMPARED TREATMENT GROUPS

Total Cancer			Respiratory Cancer		
Comparison	Chi Square	p	Comparison	Chi Square	p
Control-sulfate	0.04	N.S.	Control-sulfate	0.03	N.S.
Control-BaP	17.09	< .001	Control-BaP	8.03	< .005
Control-BaP-sulfate	10.95	< .001	Control-BaP-sulfate	5.98	< .02
Sulfate-BaP	28.07	< .001	Sulfate-BaP	10.16	< .005
Sulfate-BaP-sulfate	19.03	< .001	Sulfate-BaP-sulfate	7.30	< .01
BaP-BaP-sulfate	0.72	N.S.	BaP-BaP-sulfate	0.29	N.S.

From Table 3 it can be seen that a statistically significant incidence of cancer was found with the instillation of BaP. Ammonium sulfate inhalation had no effect on the development of cancer.

Table 4 lists the percentage of cancers by location in the respiratory tract in each exposure group. Table 5 lists the histologic types of cancer found in each exposure group.

TABLE 4. LOCATION OF RESPIRATORY TRACT CANCERS.

Treatment	Nasal	Trachea	Lung
Control	0	0	100*
Sulfate	0	50.0	50.0
BaP	0	40.0	60.0
BaP-sulfate	9	45.5	45.5

* Numbers indicate percentage of total respiratory cancers in each location.

TABLE 5. HISTOLOGIC TYPES OF CANCER IN EACH TREATMENT GROUP

Treatment	Squamous	Adenocarcinoma	Undifferentiated	Lymphoma	Other
Control	0	0	25.0*	75.0	0
Sulfate	50.0	0	25.0	25.0	0
BaP	38.9	13.9	13.9	16.6	16.6
BaP-sulfate	42.3	11.5	15.4	23.1	7.7

*Numbers indicate percentage of total cancers with each treatment.

The finding of nasal cancer in the BaP-sulfate groups is interesting. These lesions included both squamous carcinomas and small cell undifferentiated lesions. Cancer developed in the trachea of animals in the treatment groups, but differences were not significant between those exposed and not exposed to sulfate. The cancers of the respiratory tract were predominantly squamous. Adenocarcinomas were in the gastrointestinal tract. Undifferentiated tumors were often the large cell type of the lung. A larger than expected proportion of lymphomas was noted in all treatment groups. These usually involved many nodes, the liver, spleen, and in some cases, the lung and skin. Histologic types of lymphoma were predominantly the poorly differentiated diffuse lymphocytic variety, but histiocytic types were also noted. Other tumors included rhabdomyosarcomas, hepatomas and fibrosarcomas.

Table 6 compares benign cellular proliferation in the exposure groups. In the respiratory tract, these benign lesions ranged from Type II hyperplasia associated with inflammatory lesions to squamous metaplasia and hyperplasia in the bronchial tree. These lesions were seen in approximately one third of the hamsters in all groups. In the "total" group, many of these benign lesions were hemangiomas of the liver. Adrenal hyperplasia was also noted in several animals.

TABLE 6. PERCENTAGE OF ANIMALS WITH BENIGN PROLIFERATION AND PERCENTAGE OF ANIMALS WITH RESPIRATORY BENIGN PROLIFERATION

Treatment	Total Percentage Present	Total Percentage Respiratory
Control	25.4	19.4
Sulfate	32.3	22.2
BaP	33.6	30.8
BaP-sulfate	33.3	31.2

Table 7 compares the non-neoplastic lung diseases found in the animals of each exposure group.

Table 8. lists the percentages of other significant but non-neoplastic and non-pulmonary diseases found in each exposure group.

TABLE 7. PERCENTAGE OF ANIMALS WITH OTHER SIGNIFICANT PULMONARY DISEASES

Treatment	Pneumonia	Emphysema	Fibrosis	Other
Control	25.3	9.0	1.5	1.5
Sulfate	25.2	15.2	3.0	4.0
BaP	30.8	24.0	1.0	1.0
BaP-sulfate	25.8	20.4	0	0

TABLE 8. PERCENTAGE OF ANIMALS WITH NON-PULMONARY SIGNIFICANT DISEASES

Treatment	Percentage
Control	38.8
Sulfate	36.4
BaP	30.8
BaP-sulfate	30.1

Chi square analysis of these findings indicated that the development of emphysema was statistically significant in the groups given intratracheal in-

stillation of BaP. Ammonium sulfate exposure was not relatable to the development of non-neoplastic pulmonary diseases. Other significant pulmonary diseases included cases of bronchitis and vasculitis of the pulmonary arteries and arterioles. Bronchitis was found rarely in this study despite expectations of this being one of the major disease entities to be encountered. Vasculitis was seen in control and sulfate alone groups. It occurred in a group of animals which died about the same time so it may have been infectious. However, no other evidence for infection was noted in these animals. Other significant non-pulmonary diseases included infectious gastrointestinal and renal disease, heart failure and trauma.

The following figures illustrate some of the typical pathologic findings of this study.

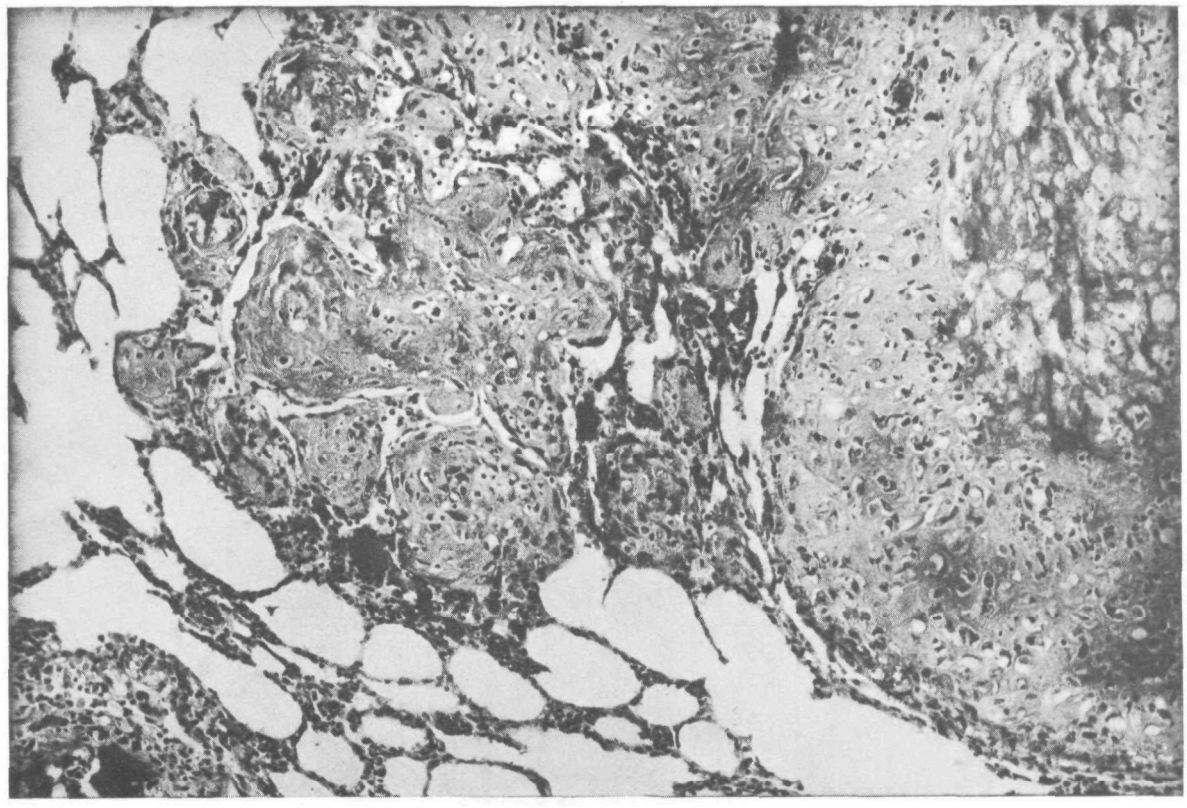

Fig. 4. Squamous carcinoma arising in the lung of hamster exposed to BaP-sulfate (160X).

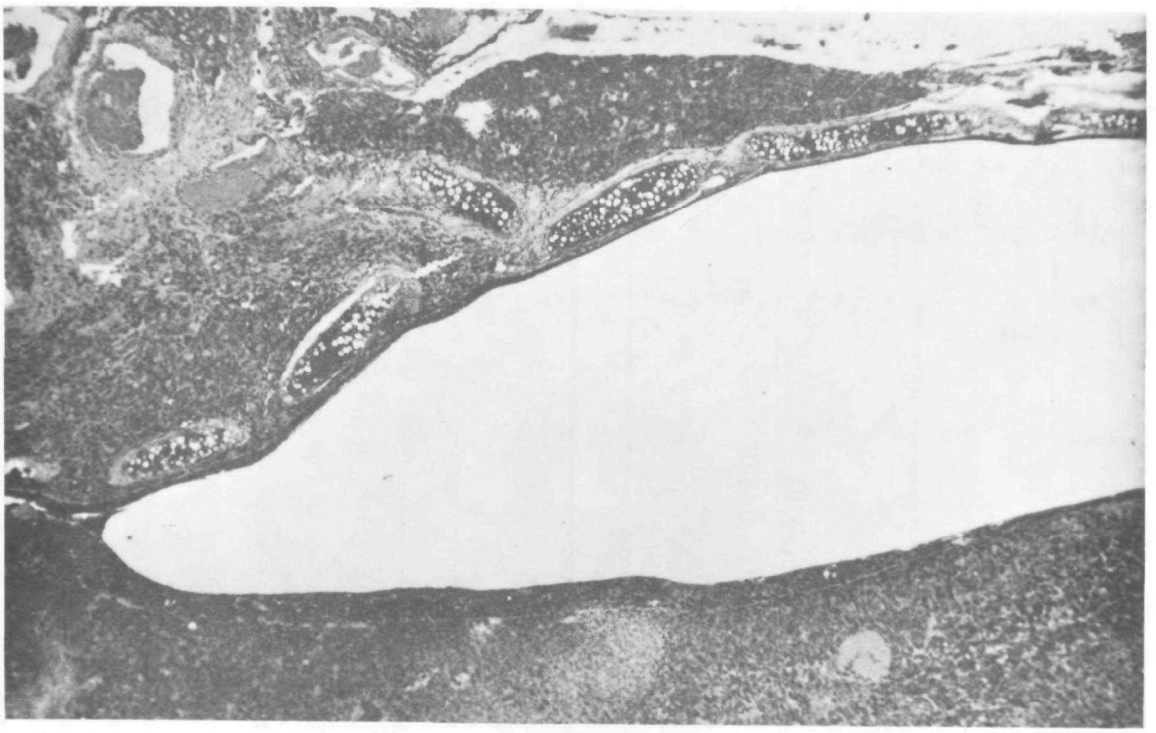

Fig. 5. Poorly differentiated squamous carcinoma of the bronchus in hamster exposed to BaP alone (40X).

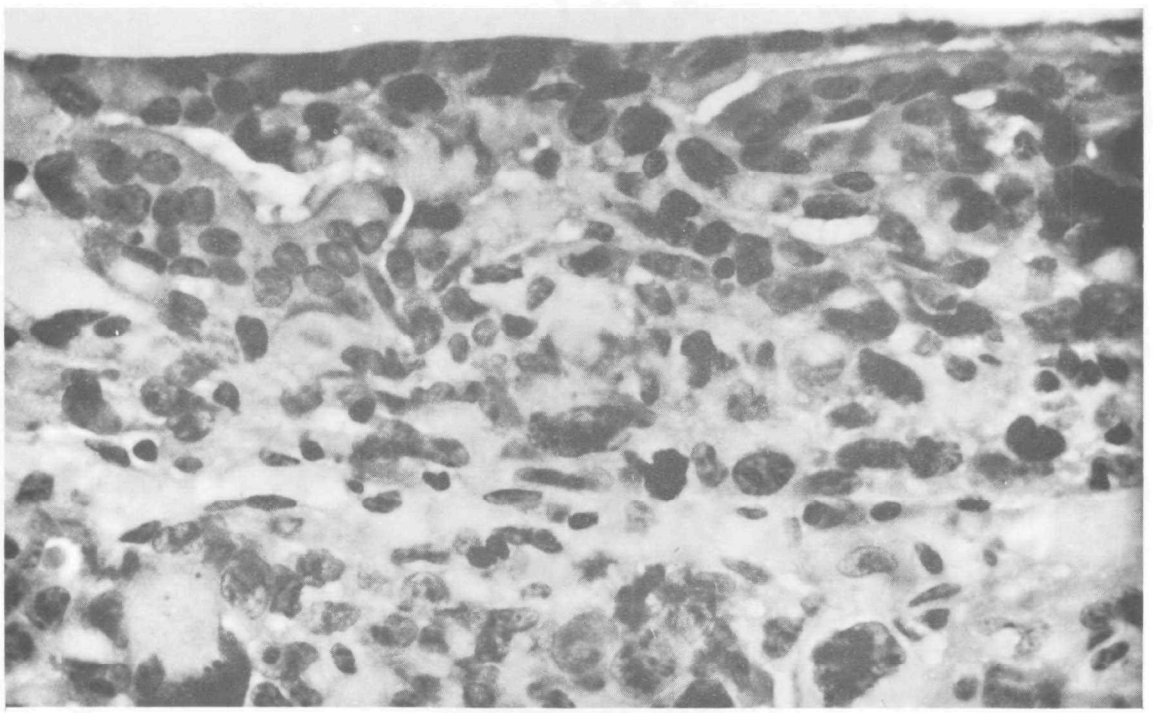

Fig. 6. Higher magnification of cancer seen in Fig. 5 (640X).

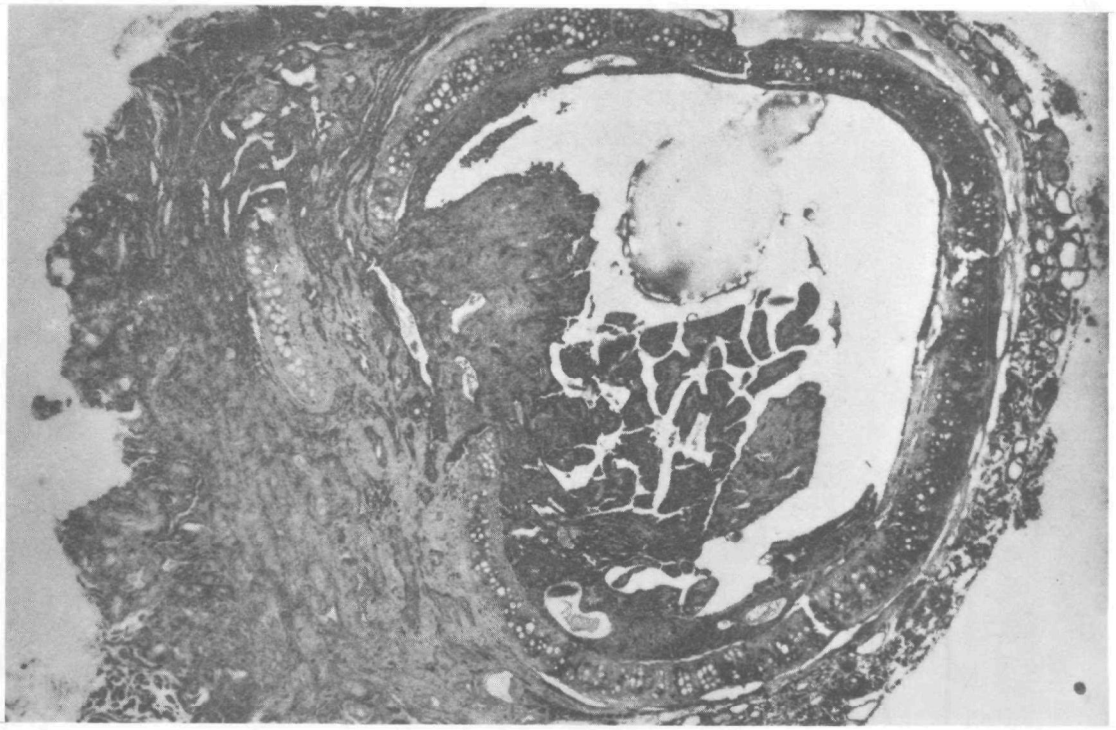

Fig. 7. Papillary and invasive squamous carcinoma of the upper third of the trachea in hamster exposed to BaP alone (40X).

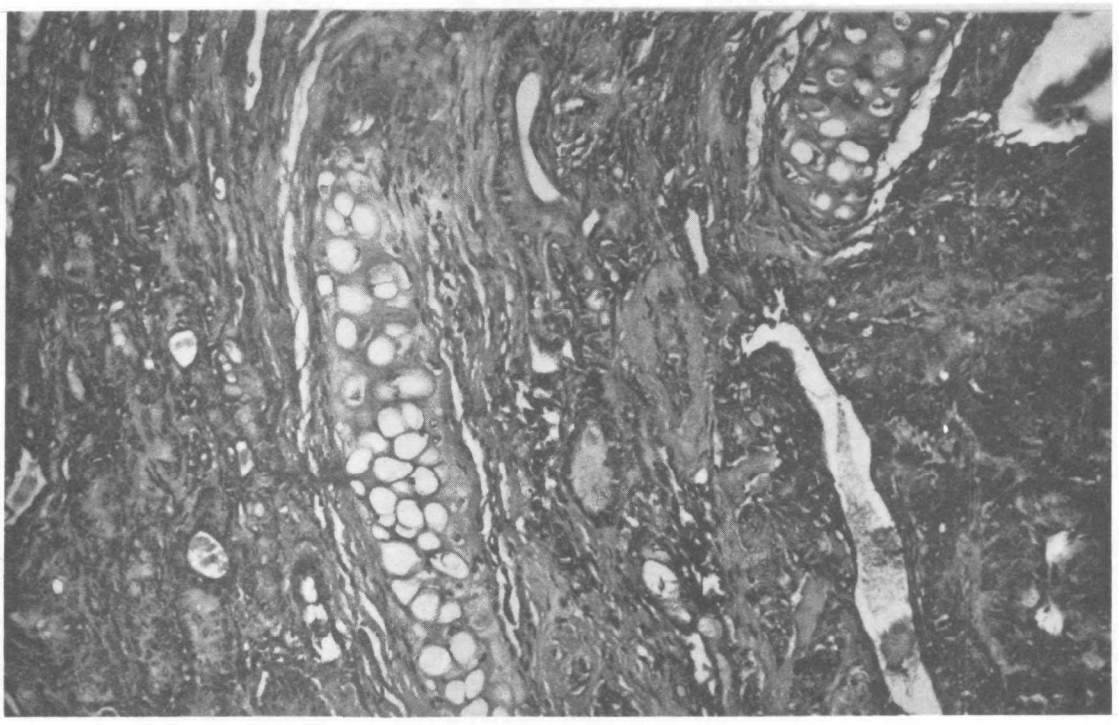

Fig. 8. Higher magnification of cancer seen in Fig. 7 (160X).

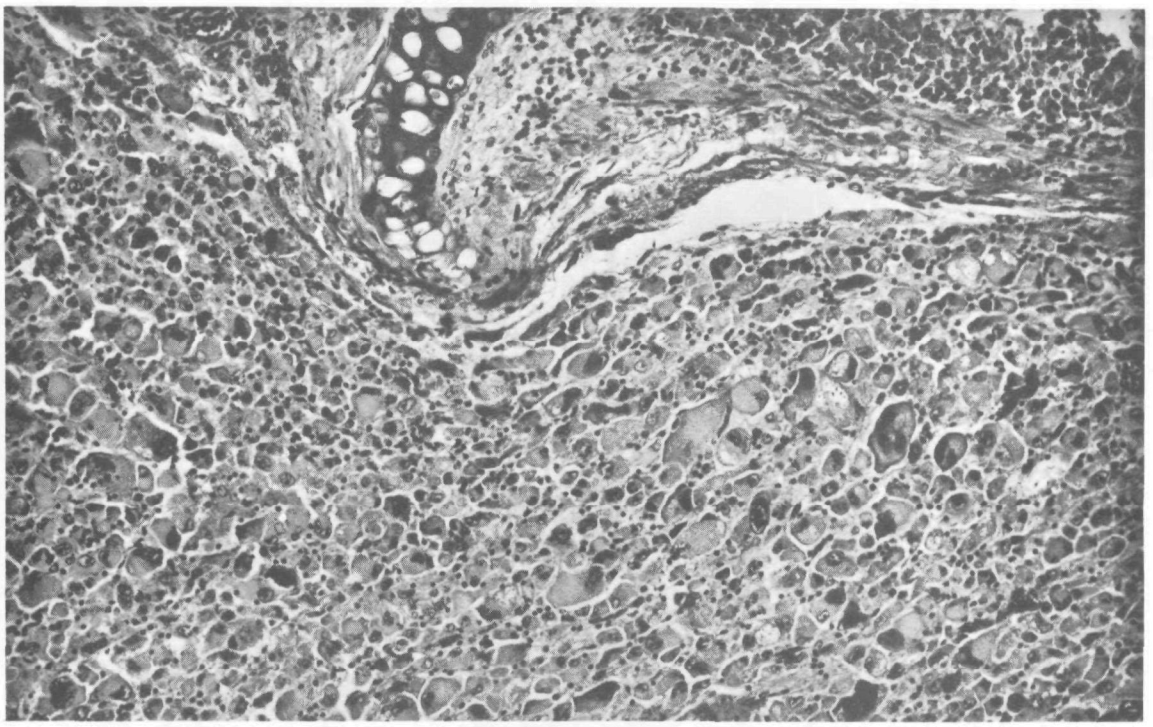

Fig. 9. Undifferentiated giant cell malignancy in the lung of a hamster exposed to BaP-sulfate (160X).

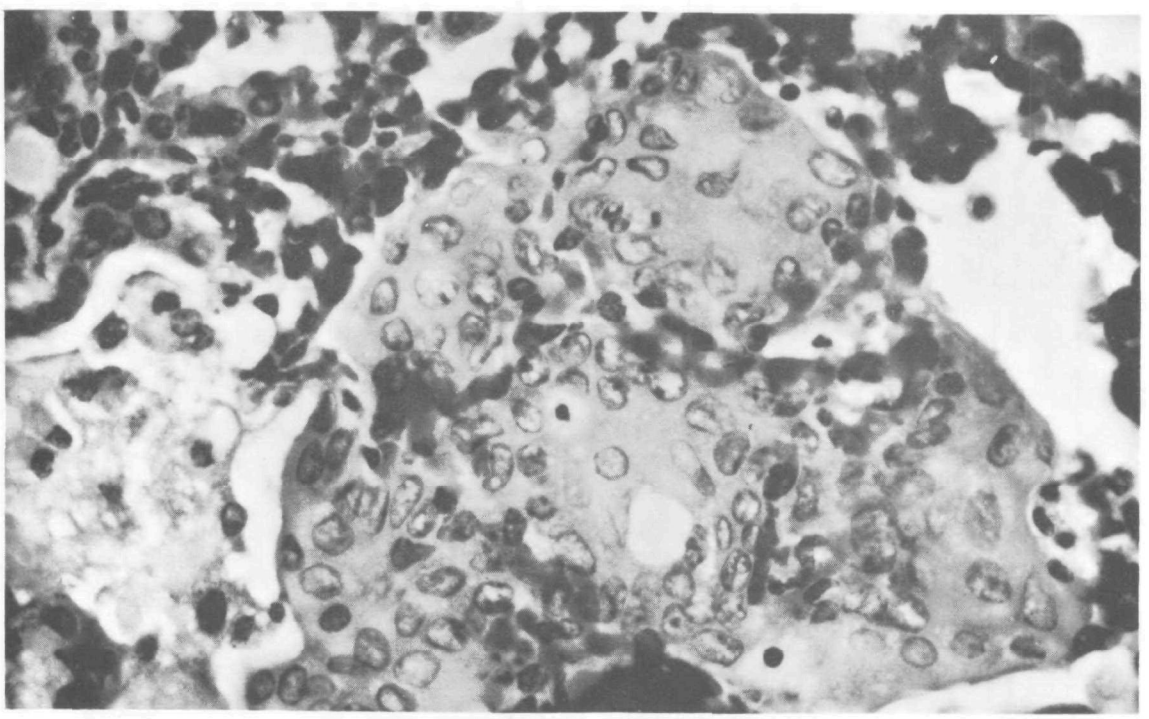

Fig. 10. Benign cellular proliferation with squamous metaplasia seen in a hamster exposed to BaP alone (640X).

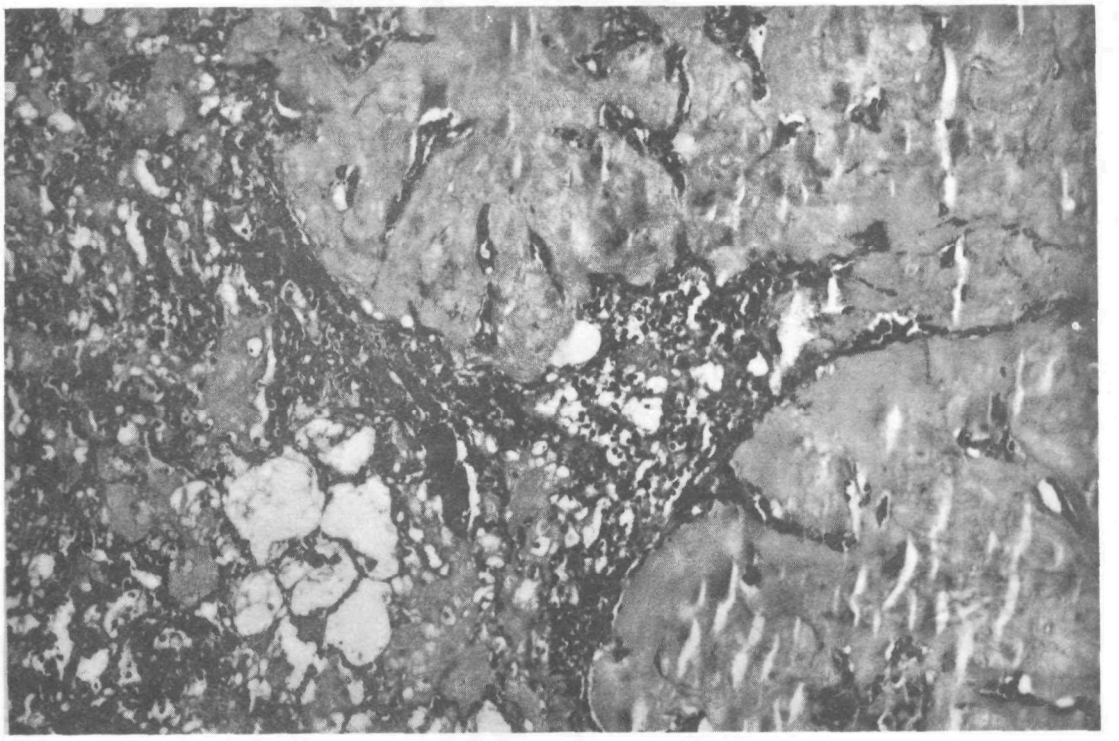

Fig. 11. Area of benign cellular proliferation and inspissation of hyaline-like material in the lung of a hamster exposed to ammonium sulfate alone (160X).

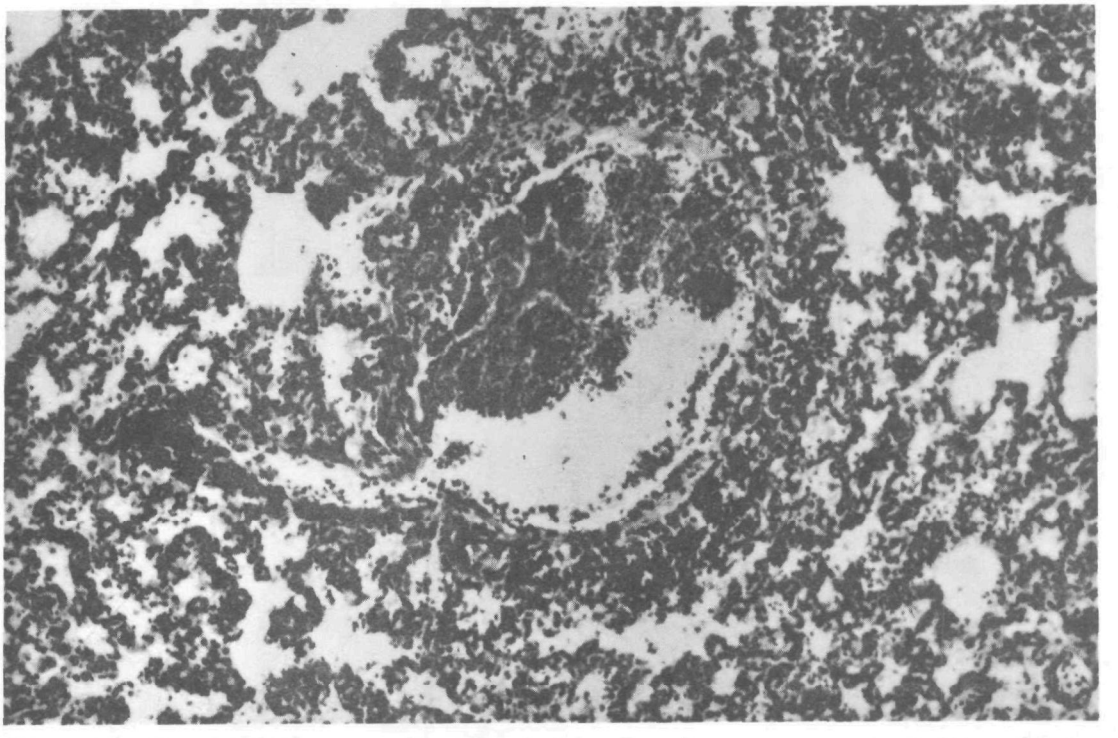

Fig. 12. Vasculitis seen in the lung of a hamster exposed to sulfate alone (160X).

D. Discussion

These studies were designed to determine whether inhalation of ammonium sulfate enhances the development of cancer. The studies utilized intratracheal injections of BaP from which a low incidence of cancer was expected and inhalation of ammonium sulfate for 6 hrs/day, 5 days/week, for 15 weeks, at a concentration of 200 $\mu g/m^3$. BaP instillation resulted in both respiratory and non-respiratory malignancy. Kobayashi (9) had reported an incidence of respiratory tract cancer with BaP alone to be 36% using slightly lower total doses than used in our studies. Others have reported incidences of 0% (14), 21% (15) and 53% (16). Our overall incidence of 13% falls within this wide range. Both the location and types of respiratory cancer observed in our studies were similar to previously reported studies (6, 8, 9, 15). Our findings of a substantial incidence of non-respiratory cancers have not been reported by others. However, published pulmonary carcinogenesis studies have not usually included complete autopsy findings.

Ammonium sulfate inhalation did not result in enhanced carcinogenesis, nor did it result in other significant pathologic changes. The concentration of 200 ug/m^3 used in these experiments can be viewed in two ways. First, in terms of acute concentration, it is 20 times greater than usual ambient levels. However, since the exposures were for 6 hours a day, 5 days per week, one can compare this experimental exposure to ambient levels in terms of a time-weighted average over one week. In this case, the experimental concentration was only 3 times greater than ambient exposure. These levels viewed in either way are easily relatable to air quality standards. Should higher concentrations be used in subsequent studies? With higher experimental concentration levels, a positive effect would be useful only from a qualitative viewpoint. Perhaps, this should be the first question raised in these kinds of studies. Laskin's study (4), using sulfur dioxide inhalation as a co-carcinogen, was done at concentrations acutely 100 times higher than those in our study. Levels this high are not quantitatively relatable to air quality standards. Therefore, in reporting our findings, we conclude that ammonium sulfate inhalation had no effect at concentrations that were acutely 20 times greater than ambient levels, and an average of 3 times greater than ambient levels.

III. DEPOSITION AND CLEARANCE STUDIES

A. Hamster Deposition Studies

Studies of deposition and clearance were proposed as preliminary experiments to assure that the ammonium sulfate particle size we planned to use in our carcinogenesis studies would reach the deep lung. These studies using hamsters were necessary since very little information was available on deposition of water soluble hygroscopic aerosols in man or animal models. We expected these deposition studies to be difficult since clearance occurs almost simultaneously with deposition. Our approach to the problem utilized an S^{35}-labeled ammonium sulfate aerosol with high specific activity, a five minute exposure time, and a short, reproducible time period after exposure in which the animals were killed to obtain tissues for analysis.

1. Methods--

The diagram in Fig. 13 outlines the exposure system used for hamster studies.

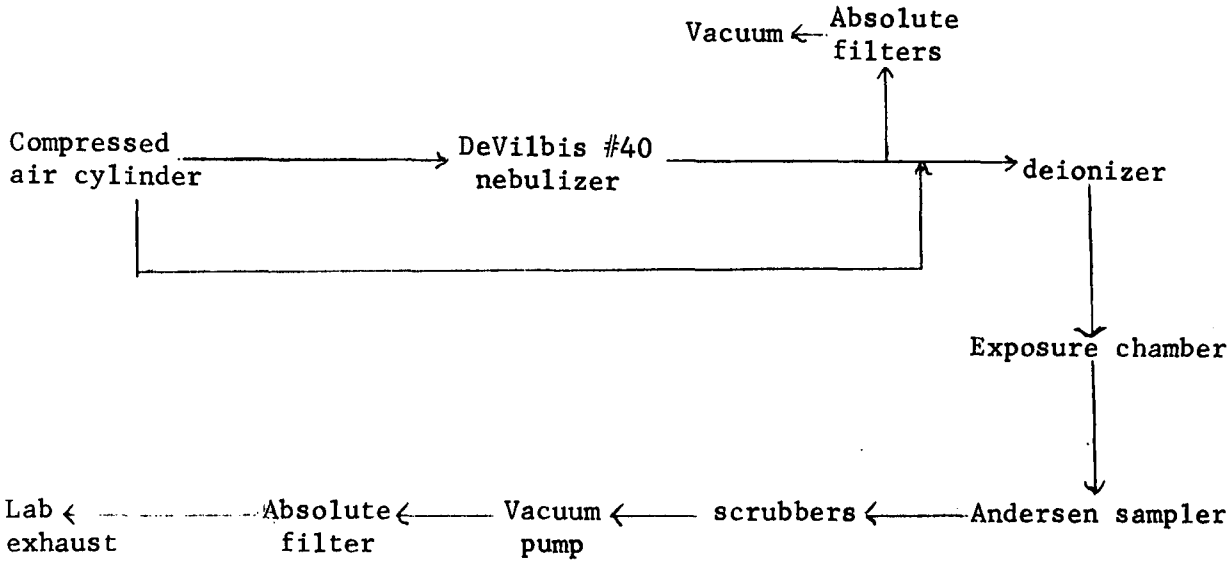

Fig. 13. Flow diagram of exposure system used with radioactive aerosol.

This system operated at 0.15 inch of water negative pressure. Nebulizer flow was 8.5 liters/min. Ammonium sulfate concentration in the nebulizer was 0.05% for smaller particle size distributions and 0.5% for larger size distributions. When the higher concentration was used, a portion of the aerosol was drawn off so that the same aerosol concentration was achieved with each particle size distribution. The exposure chamber for hamsters consisted of a plexiglass tube 30" long, 4" in diameter, with eight animal-holding tubes mounted perpendicularly along the side. The openings from the holding tubes into the central tube were ½" in diameter. The holding tubes were 5" long and closed by a rubber stopper. Hamsters appeared most comfortable when their noses were positioned in the ½" opening into the central tube through which the aerosol passed. Temperature and relative humidity were monitored in the chamber. After passing through the chamber, the aerosol went directly into the Andersen sampler which was calibrated at 28.3 liters/min. This rate equaled the total air flow in the system.

Sulfate concentration and particle size were determined by an eight stage Andersen sampler with a backup filter. The entire aerosol to which the animals were exposed was sampled. The sampler plates and filter were soaked in water and the radioactivity on each plate was quantitated by counting an aliquot of the wash water in a liquid scintillation counter. The concentration of sulfate in the aerosol and the amount of sulfate on each plate could then be calculated from the specific activity of the ammonium sulfate solution used

to make the aerosol. The aerosols were polydisperse and all had distributions with greater than 99% of the particles less than 2.1μ. Aerosols with an MMD of 0.3μ had 98% of the particles less than 1.1μ. Significant radioactivity was never found in the scrubbers or cold trap (see Fig.13), but these were in line as precautionary measures.

Immediately following a 5 minute exposure, the hamsters were killed with an overdose of sodium pentobarbital injected intraperitoneally. The fur of the animals was washed with a decontaminating detergent (Contrad-70 (R)) to remove any radioactive sulfate, and final drippings were collected and analyzed to be certain there was no external contamination of internal organs. Blood was obtained from the heart. The lungs were removed and oven dried for 48 hours. The head was removed; the lower jaw, muscles and all fur were dissected away. The nose was lavaged with water and then the nose and cranium were placed in water to soak for 24 hours. Urine was collected and added directly to scintillation cocktail for analysis. The esophagus, stomach, and small intestine were coarsely minced and placed in water to soak for 24 hours.

Sulfate was quantitated in the lung using a Thomas-Ogg combustor and the method of Charles et al (17). Quantitation of sulfate in other areas was done by adding trichloracetic acid to blood samples, nasal fluid and gastrointestinal tract fluid, making a final TCA concentration of 5% which precipitated protein. The precipitates were then washed three times and all supernates were collected. A vacuum flask evaporator was then used to concentrate all supernates to 5-10 ml. Two ml of this was then used to quantitate radioactivity.

All radioactivity determinations were done in an Intertechnique scintillation counter using Instabray (Yorktown Research) cocktail, into which up to 2 ml of sulfate solution could be placed for analysis. In analysis of biologic materials, quench corrections were necessary and these were done using an internal standard. Preliminary studies using known amounts of sulfate added to blood or injected directly into excised lungs showed a recovery of 97.9 ± 3.66% with the technique outlined here. To be certain that the nasal lavage was adequate for recovery of sulfate in the nose, an additional wash was collected separately and quantitated for S^{35}. In no case was any S^{35} found in these samples.

To study deposition, hamsters were exposed for five minutes to two particle size distributions. S^{35} was quantitated in blood, lung, nose, urine and GI tract immediately after exposure. Eight animals were exposed at one time. Three exposures were used for each particle size distribution. Table 9 lists the exposure parameters used.

Both particle size distributions were in the respirable range. The concentrations, temperatures and relative humidities were not significantly different.

TABLE 9. EXPOSURE PARAMETERS IN HAMSTER DEPOSITION AND CLEARANCE STUDIES

Parameter	Group I	Group II
Particle size, MMAD	0.65 u	0.36 u
Concentration (mean ± SD)	1.69 ± 0.40 ug/l	1.32 ± 0.11 ug/l
Temperature	22° C	22° C
Relative humidity (mean ± SD)	58 ± 2.2%	63.7 ± 4.6%

Results and interpretation--

Table 10 outlines the deposition found in lung, nose, urine, blood and GI tract for the two particle sizes.

TABLE 10. DEPOSITION OF S^{35}-LABELED AMMONIUM SULFATE IN HAMSTERS

	Group I	Group II
Particle size	0.65 u	0.36 u
Deposition in: lung	35.1 ng	24.0 ng
nose	21.8 ng	5.7 ng
GI tract	140.0 ng	35.3 ng
blood	76.5 ng	24.0 ng
urine	1.43 ng	0.7 ng
Total sulfate deposited	274.8 ng	89.7 ng
Expected sulfate deposited	386.0 ng	356.0 ng
% expected deposited	71.1%	25.1%
% deposited in lung	12.8%	26.7%
nose	7.9%	6.4%
GI tract	50.9%	39.4%
blood	27.8%	26.8%
urine	0.52%	0.8%

Mean total deposition in the nose and lung was greater with particles 0.65 μ than with particles 0.36 μ MMD. An unusually large amount of sulfate was found in the GI tract of hamsters exposed to both particle sizes. During

exposure, the hamsters were actively chewing and licking the apparatus. This was most likely the source of the sulfate found in the GI tract. The total blood levels were extrapolated from blood concentrations using published data (15) for total hamster blood volume. With our methods, animals were killed immediately after exposure and all samples of tissue were collected within 5 minutes after the end of exposure. In less than 10 minutes from the first encounter with radiolabeled sulfate, the amount of sulfate found in the blood was the second highest in the body. This indicated extremely rapid uptake into this compartment regardless of whether the source was from the respiratory or gastrointestinal system. Also, the small amounts of sulfate in the urine indicated clearance from the blood had begun within this short period. The ratio of lung to nose deposition in the larger particles was 1.6 to 1.0. With the smaller particles it was 4.2 to 1.0. This suggested that an ammonium sulfate aerosol with an MMD of 0.65 μ can be trapped in the nose to a greater extent than one with an MMD of 0.36 μ.

We decided to use the smaller particle distribution for our carcinogenesis studies based on the above findings. This also seemed to correlate with the data of Amdur and Corn (19), who showed zinc ammonium sulfate particles at 0.3 μ MMD to be 100 times more potent in increasing airway resistance in guinea pigs than these particles in the same concentration at 0.7 μ MMD.

B. Guinea Pig Deposition Studies:

Since we had developed the methodology, we subsequently proposed to study deposition and clearance of S^{35}-labeled sulfate in guinea pigs so that our data could be directly correlated with findings using the airway resistance model.

1. Methods--

The study of guinea pigs necessitated slight modifications of our methods. These animals were exposed to S^{35}-labeled ammonium sulfate in our 322 liter exposure chamber described earlier in this report. The animals were exposed for five minutes in groups of 12 at a time. The animals were placed inside individual wire cages that allowed them to breathe without restraint and their bodies were covered to the neck with cotton orthopedic stockings to minimize fur contamination by S^{35}.

Aerosols were generated from DeVilbis #40 nebulizers. A single nebulizer containing 0.5% $(NH_4)_2\ SO_4$ was used to produce the large particle aerosol and five nebulizers, each containing 0.05% $(NH4)_2SO_4$, were used to produce an aerosol of the same concentration but with a smaller particle size. The aerosols were monitored by Andersen sampling throughout the entire 5 minute exposure. The sulfate concentration and particle size were measured as previously described for the hamster studies.

Animals were killed immediately following exposure by an overdose of sodium pentobarbital. The fur of the face and chest was then washed to reduce any possible chance of contamination and the final washings quantitated for radioactivity. Twice background was considered an acceptable level. A sample of blood was taken from the heart. The lungs were removed, lavaged with saline 5 times followed by 3 water lavages. The whole lungs were then homogen-

ized. The head was removed, the lower jaw separated and the nasal cavity lavaged from both the pharynx and the external nares, first with saline, then with water. Nasal lavage fluid usually totaled 500-700 ml. Urine was collected from the bladder, gastric contents from the stomach.

Trichloracetic acid was added to the pulmonary lavage fluid, lung homogenates, blood samples, nasal lavage fluid and gastic material, making a final TCA concentration of 5% which precipitated protein. These samples were then handled in the same manner as described for the hamsters.

2. Results and interpretation--

Table 11 lists the parameters used in the guinea pig exposures.

TABLE 11. EXPOSURE PARAMETERS IN GUINEA PIG DEPOSITION AND CLEARANCE STUDIES

Parameter	Group I	Group II
Particle size, MMAD	0.65 μ	0.31 μ
Concentration	1.42 μg/l	2.01 μg/l
Temperature	22.2° C	22.2° C
Relative humidity	53%	40%

Total respiratory tract deposition at each particle size in guinea pigs was not significantly different, although there was slightly more sulfate found in animals exposed to the larger particles. The large particle to small particle deposition ratio was 1.2 to 1.0. In comparing lung to nose ratios, the larger particles were again trapped to a greater extent in the nose (L/N ratio 1.5), whereas the smaller particles had less nasal entrapment (L/N ratio 2.0). Significance of the difference in nasal deposition at the two particle sizes is $p < 0.05$. In correlating these data with studies of Amdur and Corn (19), we find a number of factors to be considered. First, a larger proportion of smaller particles are deposited in the lung than in the nose. However, more sulfate was recoverable from the lungs of animals exposed to the larger particles (123 ng larger - 107 ng smaller). When the simultaneous clearance factor is considered, it can be seen that more sulfate passed into the blood in animals exposed to the smaller particles (53.7 ng larger - 74.9 ng smaller). However, if the amount in the lung and the amount in the blood are added for each particle size, the totals are remarkably similar (176.7 ng-larger - 181.9 ng-smaller). This suggests that the amount of sulfate reaching the deep lung at these two particle sizes was not strikingly different. Perhaps the differences in effect noted by Amdur and Corn (19) are better explained by the particle number which should be greater in the small particle aerosol. Another possible explanation would suggest a difference in the interaction of the smaller particles with the tissues.

Table 12 outlines the deposition of sulfate in the lung, nose, GI tract, blood and urine.

TABLE 12. DEPOSITION OF S^{35}-LABELED AMMONIUM SULFATE IN GUINEA PIGS

		Group I	Group II
Particle size		0.65 μ	0.31 μ
Deposition in:	lung	123 ng	107 ng
	nose	79 ng	53 ng
	GI tract	62.3 ng	32.3 ng
	blood	53.7 ng	74.9 ng
	urine	0.63 ng	0.35 ng
Total sulfate deposition		318.6 ng	276.6 ng
Expected sulfate deposition		1136	1616
% expected deposition		28.0%	16.6%
% deposition in	lung	38.6%	40.0%
	nose	24.8%	19.8%
	GI tract	19.6%	12.19%
	blood	16.9%	28.0%
	urine	0.19%	0.1%

C. Rabbit Deposition Studies

We also proposed to study deposition in rabbits so that our findings could be correlated with studies of pulmonary responses in rabbits underway in other laboratories.

1. Methods--

Deposition studies in rabbits were carried out using both nose only and whole body exposure. The exposure chamber for nose only exposure consisted of a central plexiglass tube (4" in diameter) with two rabbit-holding tubes mounted on each side perpendicular to the central tube. Two inch in diameter openings into the central tube provided the rabbits with nose only exposures, while loose fitting collars held the rabbit's head and nose in place. Rabbits were selected to fit in the apparatus so that their breathing was not restricted.

Whole body exposures were done in the same way as described for guinea pigs. Analysis of tissues for sulfate was also the same as described for guinea pigs.

Table 13 lists the exposure parameters used for the nose only exposures.

TABLE 13. EXPOSURE PARAMETERS IN RABBIT "NOSE ONLY" DEPOSITION STUDIES

Parameter	Group I	Group II
Particle size	0.5 μ MMD	0.3 μ MMD
Concentration (mean ± SE)	1.88 ± 0.40 ug/1	2.27 ± 0.15 ug/1
Temperature (mean ± SD)	23.0 ± 1.38° C	23.5 ± 1.73° C
Relative humidity (mean ± SD)	52.5 ± 4.39° C	51.5 ± 2.12° C
Estimated amount of sulfate entering respiratory system	9.4 ± 1.99 ug	11.38 ± 0.78 ug
Amount of sulfate recovered from nose, lung, blood, urine and stomach	2.03 ± 1.03 ug	2.12 ± 1.48 ug
Percent of expected recovered (mean ± SE)	23.75 ± 13.4%	20.25 ± 14.9%

Since rabbits were exposed individually, there was variability in concentrations but concentrations at the two particle sizes were not significantly different. Temperature and relative humidity were held constant within a narrow range. The total amount of sulfate recovered from animals in each group was not significantly different. The percent of expected recovered varied widely as can be seen by the mean and standard error shown. This was probably

related to variation in breathing patterns of the animals.

2. Results--

Table 14 outlines the findings of pulmonary versus nasal deposition at the two particle sizes.

TABLE 14. PULMONARY VERSUS NASAL DEPOSITION IN RABBITS

	Group I (0.5 μ MMD)	Group II (0.3 u MMD)
ug/nose	0.72 ± 0.16	0.44 ± 0.17
ug/lungs	0.25 ± 0.05	0.61 ± 0.35
% nose	73 ± 4%	43 ± 2.5%
% lungs	26 ± 4%	57 ± 2.5%
Significance:	$p < 0.001$ Nose Group I vs Nose Group II	
	$p < 0.001$ Lung Group I vs Lune Group II	

Total mean respiratory deposition with 0.5 μ MMD particles was 0.97 ± 0.19 and with 0.3 μ MMD particles was 1.05 ± 0.58. These differences were not significant. On the other hand, the differences in amounts in the nose and lungs of these animals, as shown in Table 14, are highly significant ($p < 0.001$).

Blood concentrations of sulfate resulting from the exposure were 1.00 ± 0.10 ng/ml in animals exposed to 0.3 μ MMD particles and 0.60 ± 0.17 ng/ml in those exposed to 0.5 μ MMD particles. These concentrations were not significantly different ($p = < 0.1$). However, when total rabbit blood volume was calculated (18) and the total amount of sulfate in blood due to exposure was determined, the percentage of this compartment in relationship to the total amount recovered in the animal was significantly different for each group. These values were 5.9 ± 1.0% for animals exposed to 0.5 μ MMD particles and 16.9 ± 1.0% for animals exposed to 0.3 μ MMD particles. These percentages were significantly different ($p < 0.001$). The finding of radiolabeled sulfate in the blood of either group indicated the rapidity with which sulfate was cleared from the respiratory tract. This blood level was probably not due to gastrointestinal absorption since the amount quantitated in this area never exceeded 10% of the total respiratory deposition. In many animals, no radiolabeled sulfate was quantitated in the gastrointestinal tract, yet these animals had blood levels equal to or greater than those with the highest GI levels. Animals who had higher deposition levels of sulfate tended to have sulfate from the exposure present in the urine even after so short an exposure. However, again many animals did not yet have any sulfate detectable in the urine at this early time.

Table 15 lists the exposure parameters for the whole body exposures.

TABLE 15. RABBIT EXPOSURE PARAMETERS FOR WHOLE BODY EXPOSURES

Parameters	Group I	Group II
Parameter size, MMAD	0.60 μ	0.32 μ
Concentration (mean ± SD)	1.12 ± 0.08 μg/1	1.28 ± 0.09 μg/1
Temperature (mean ± SD)	22.2 ± 1.9° C	21.6 ± 2.7° C
Relative humidity (mean ± SD)	56 ± 0.71%	55 ± 8.5%

Table 16 outlines the deposition of sulfate in the lung, nose, GI tract, blood and urine.

TABLE 16. DEPOSITION OF S^{35}-LABELED AMMONIUM SULFATE IN RABBITS USING WHOLE BODY EXPOSURE

	Group I	Group II
Particle size	0.60 μ	0.32 μ
Deposition in lung	456 ng	127 ng
nose	107 ng	90 ng
GI tract	1.05 ng	41 ng
blood	83.3 ng	60.3 ng
urine	46.4 ng	1.8 ng
Total sulfate deposited	703 ng	320 ng
Expected sulfate deposited	5992 ng	6848 ng
% expected deposited	11.7%	4.7%
% deposition in lung	66.1%	39.7%
nose	15.2%	28.1%
GI tract	0.15%	12.8%
blood	11.8%	18.8%
urine	6.6%	0.56%

The findings in rabbits by these two modes of exposure were so different that we felt it was necessary to do another group of rabbit exposures. We therefore repeated the nose only exposures. Table 17 lists the parameters of

these exposures.

TABLE 17. SECOND RABBIT NOSE ONLY EXPOSURE

Parameter	Group I	Group II
Particle size MMAD	0.63 μ	0.34 μ
Concentration	2.88 μg/l	3.30 μg/l
Temperature	23.1° C	23.3° C
Relative humidity	50.3%	49.1%

Table 18 outlines pulmonary and nasal deposition in these experiments.

TABLE 18. PULMONARY VERSUS NASAL DEPOSITION IN SECOND NOSE ONLY EXPOSURE

Parameter	Group I	Group II
Particle size	0.63 μ	0.34 μ
Deposition in lung	371 ng	255 ng
Deposition in nose	389 ng	220 ng

This repeat study showed that the larger particle size had a larger total respiratory tract deposition than the smaller particle size and nasal and pulmonary deposition fractions were almost equal. These findings were different from the two previous studies. We then examined all our deposition studies from the viewpoint of animal to animal variation. Table 19 lists mean ± SD of pulmonary and nasal deposition of the studies.

TABLE 19. PULMONARY AND NASAL DEPOSITION OF ALL DEPOSITION STUDIES (ng SULFATE ± SD)

Study	Area	Large Particles	Small Particles
Second rabbit study nose only	Lung	371 ± 231	255 ± 79
	Nose	389 ± 222	220 ± 100
Rabbit chamber study	Lung	465 ± 352	127 ± 66.0
	Nose	107 ± 62.3	90.2 ± 37.0
First rabbit study	Lung	250 ± 50	615 ± 352
	Nose	723 ± 165	441 ± 171
Guinea pig study	Lung	122.8 ± 36.5	107 ± 14.0
	Nose	79.0 ± 12.8	53.1 ± 8.6
Hamster study	Lung	35.1 ± 10.5	24.0 ± 6.4
	Nose	21.8 ± 3.9	5.7 ± 1.9

By looking at standard deviation it can be seen that much greater differences were present from animal to animal in the rabbit studies than in those of hamster and guinea pig. The first rabbit study seemed to have the least variation, while subsequent studies had much more. These findings were difficult to explain. It is possible that since the rabbits were larger animals, they were not as amenable to our methods of analysis as smaller animals and the variation seen was a methodologic problem. In any case, we did not further pursue the rabbit studies.

D. Clearance Studies in Hamsters, Guinea Pigs and Rabbits

To study clearance, animals were exposed for five minutes to two particle size distributions. S^{35} was quantitated in blood, lung, nose, urine and the GI tract immediately, one hour, three hours and six hours after exposure. Quantitation methods were the same as used for the animals in deposition studies. In fact, clearance and deposition were usually studied in the same group of animals so that exposure parameters for these studies were those listed earlier for each animal group.

The patterns of sulfate clearance in the three species studies are described by Figures 14 through 19. In contrast to deposition, the clearance patterns of sulfate were similar in all three species tested. All animals showed rapid sulfate clearance from the lung. The rate of clearance, as expressed by the slope of the lung graph from 0 to 1 hour, was the same for the three species. $T_{\frac{1}{2}}$ was 18-20 minutes. The rate of clearance from the lung was the same for large and small particles. Within species, the clearance pattern for each tissue studied was almost identical for large and small particles. In all animals studied, the sulfate cleared from the lung, nose and GI tract, and then ultimately appeared in the urine. In preliminary studies, using 24 hour urine collection, it was found that greater than 95% of sulfate that appeared in urine was there by six hours. There was a difference in the clearance from the GI tract of the hamster and the two larger species studied. The hamster had a large initial GI deposition, as explained earlier, which was followed by rapid clearance, almost parallel to that of the lung. The rabbit and guinea pig showed a maximum in GI at one hour after exposure. This pattern was possibly due to clearance into the GI from the nose and lungs, followed by the clearance of sulfate from the GI to the urine. This clearance pattern is consistent with the clearance model used by the ICRP for soluble inhaled substances (Task Group in Lung Dynamics) (20). This model predicts that 50% of inspired soluble particles rapidly pass into the gastrointestinal tract and 25% of these particles promptly enter the circulation.

The blood concentration of sulfate varied somewhat throughout all the deposition and clearance studies, but without any definitive pattern. The variation in blood was much less than for any other tissue studied. This mild flux was most likely due to blood being the route of sulfate transport from the lung, nose and GI tract to the urine.

DEPOSITION AND CLEARANCE OF AMMONIUM SULFATE IN HAMSTERS

(Concentration-1.69 μg per liter; Particle size-0.65μ MMAD)

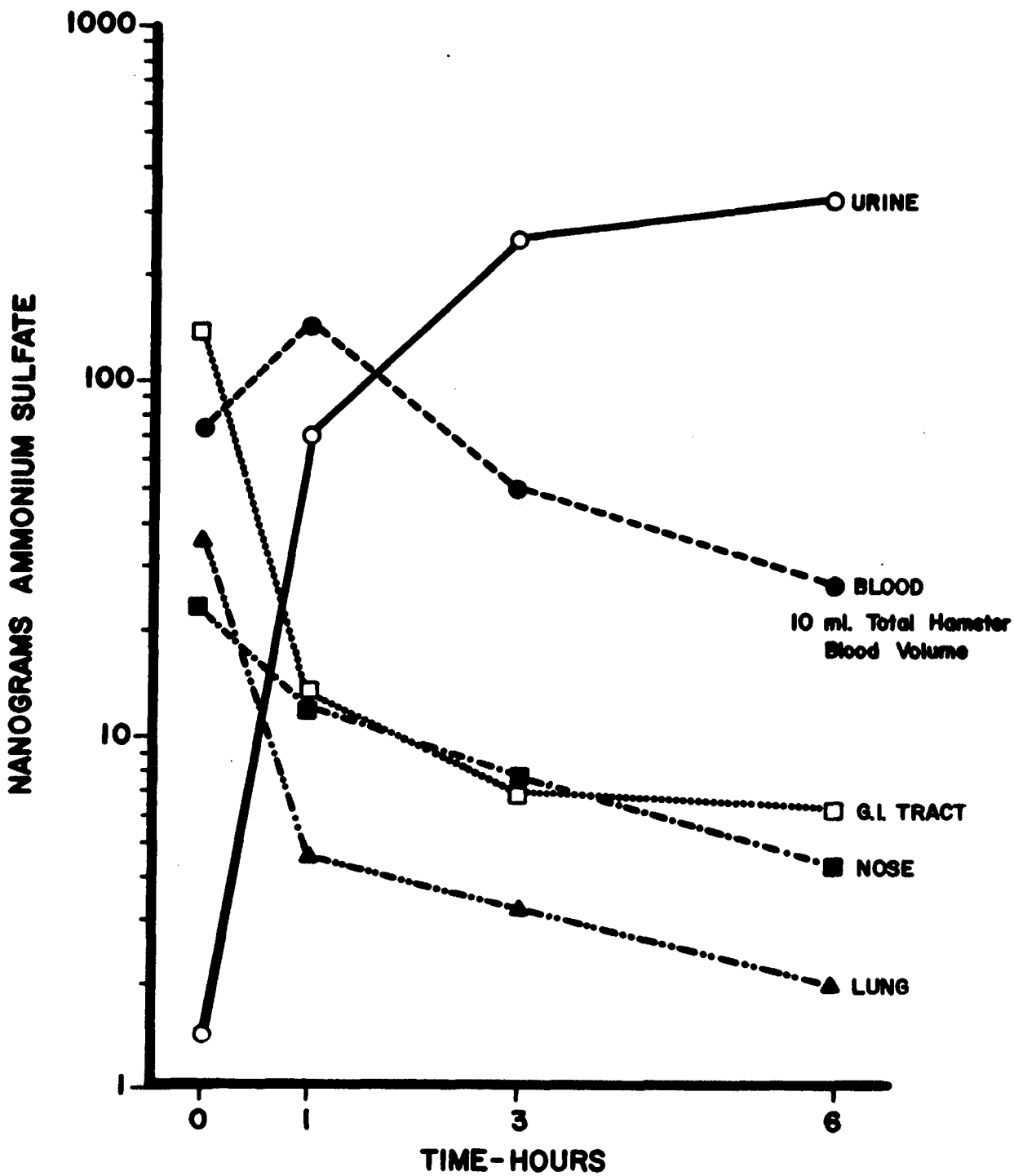

Fig. 14. Large particle deposition and clearance in hamsters.

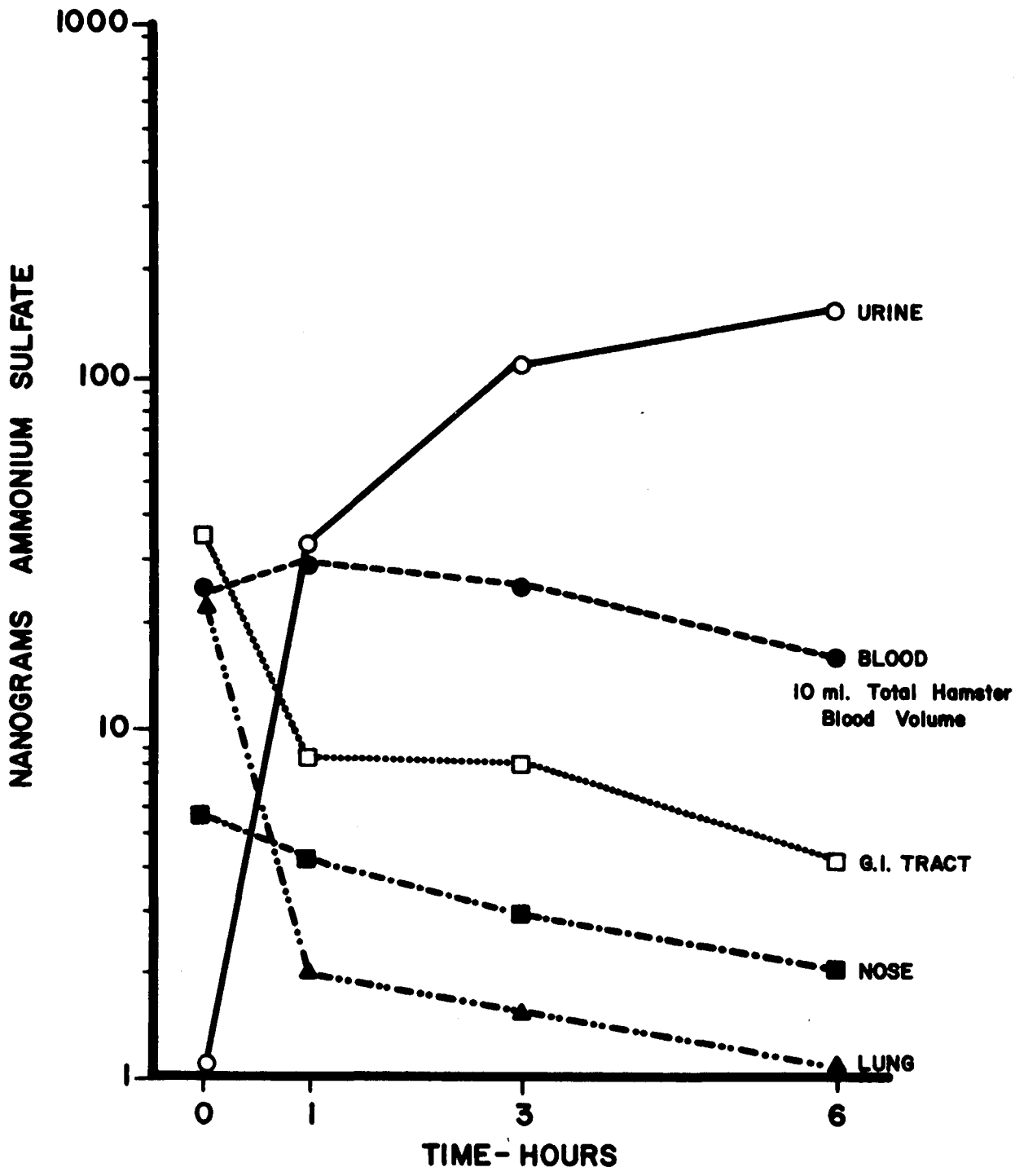

Fig. 15. Small particle deposition and clearance in hamsters.

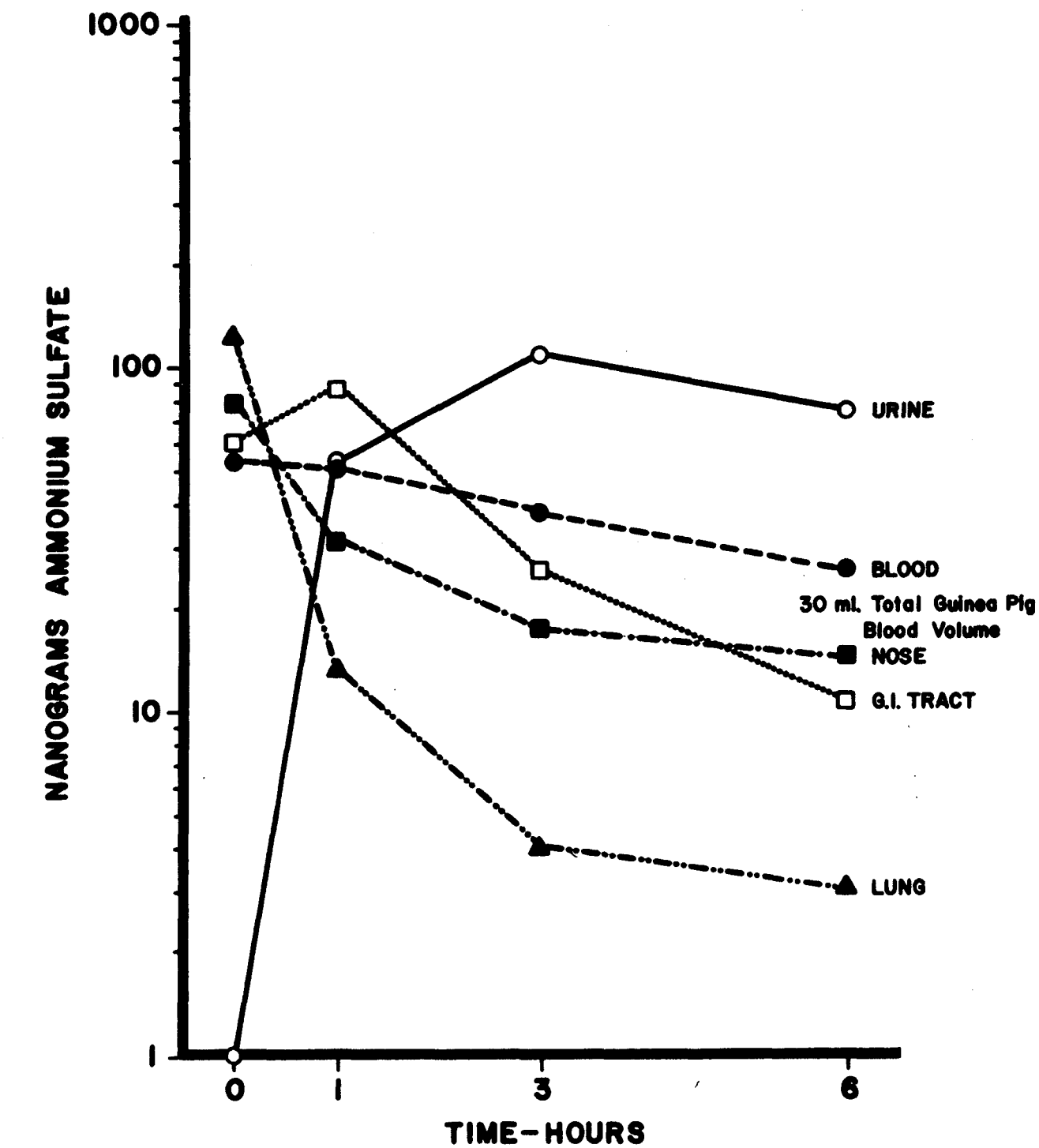

Fig. 16. Large particle deposition and clearance in guinea pigs.

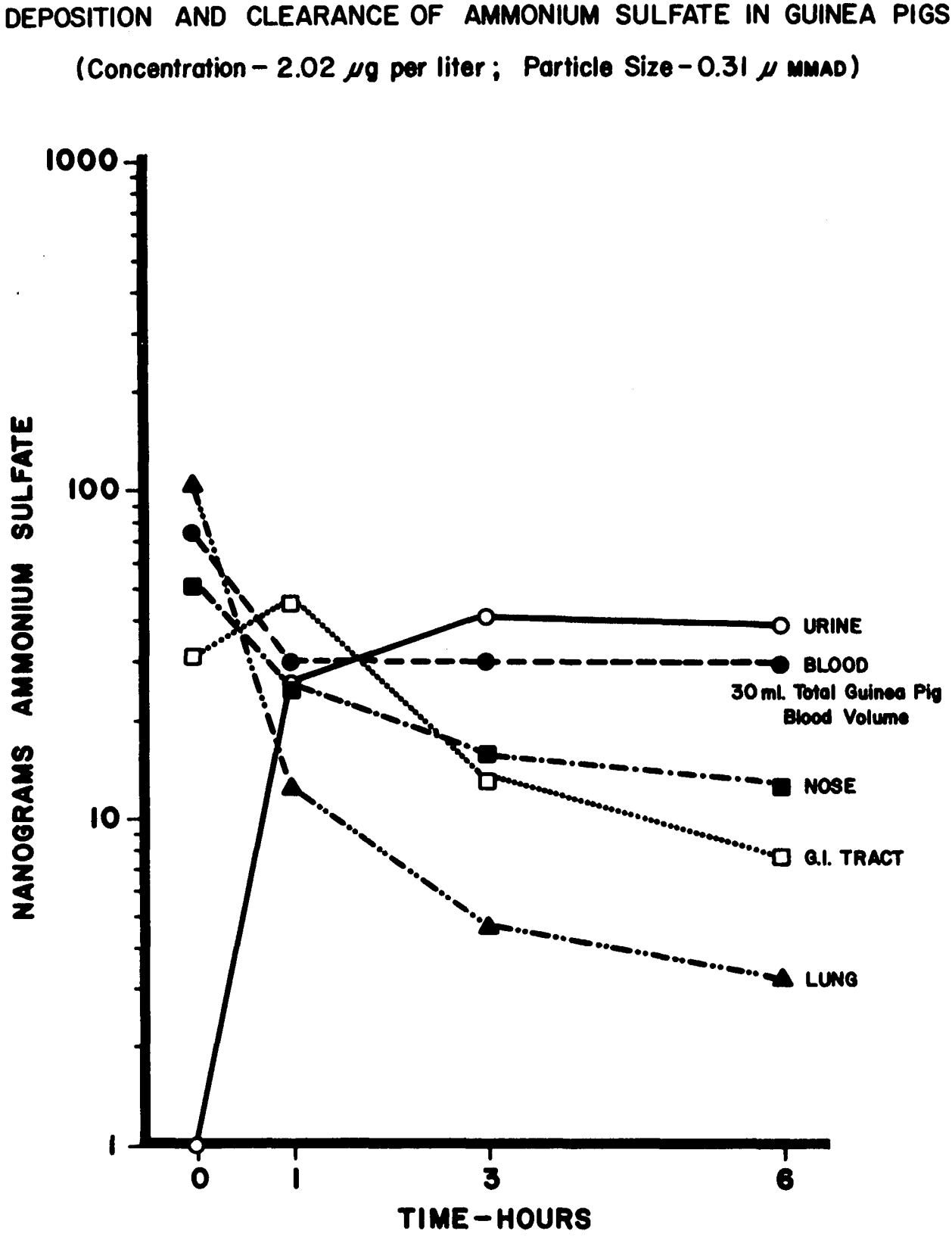

Fig. 17. Small particle deposition and clearance in guinea pigs.

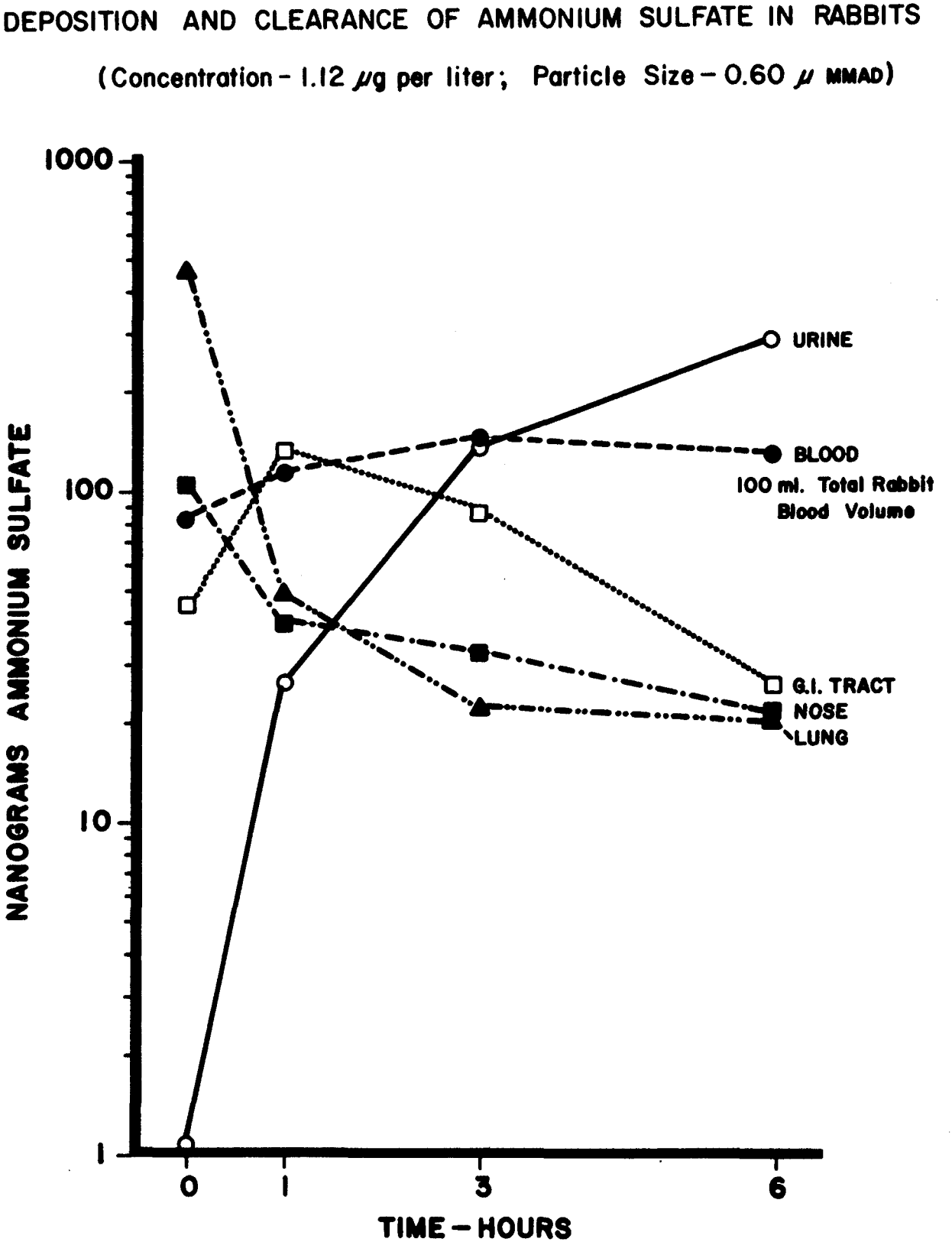

Fig. 18. Large particle deposition and clearance in rabbits from whole body exposure.

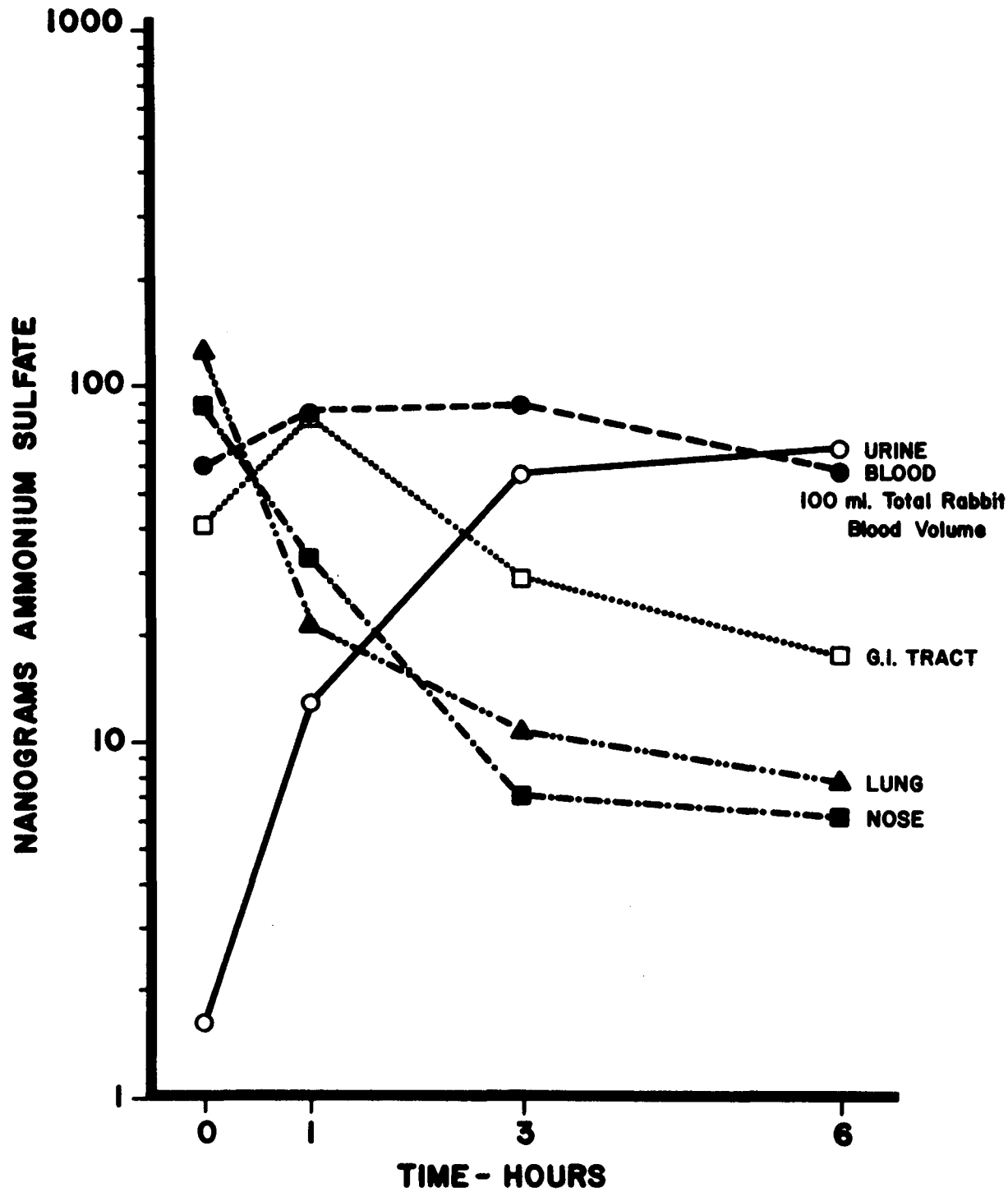

Fig. 19. Small particle deposition and clearance in rabbits from whole body exposure.

IV. PULMONARY DEFENSE STUDIES

A. Aryl Hydrocarbon Hydroxylase Studies

Aryl hydrocarbon hydroxylase is an enzyme that acts in the metabolism of BaP and other carcinogens. Its role is to render these organic substances more hydrophilic and thereby to aid in excretion. It is an inducible enzyme, and has been shown to be inhibited in the lung by oxidant air pollutants (21). Our purpose was to determine whether ammonium sulfate had an inhibiting influence on this enzyme, which could be important in the clearance of carcinogen and consequently in carcinogenesis.

Animals were exposed using basically the same protocol as in the carcinogenesis experiments. Four groups were studied: control, sulfate, BaP and BaP-sulfate. Animals were exposed to ammonium sulfate at a concentration of 189 $\pm$ 30 μg of sulfate per cubic meter for 6 hours per day, five days per week. Animals received BaP intratracheal injections on Wednesday of each week, using a 5 mg dose in saline-gelatin suspension. Exposure temperature and relative humidity for animals exposed to sulfate were 24.3 $\pm$ 1.1^{o} C and 48.1 $\pm$ 6.6%. These parameters for BaP only and control animals were 23.3 $\pm$ 0.75^{o} C and 39.0 $\pm$ 9.8%. Animals were killed for enzyme assay at the end of the full week's exposure. Six animals per group per time period were studied. Sodium pentobarbital overdose was used to kill the animals. This had no effect on enzyme levels in preliminary studies. The lungs were removed en bloc and quick frozen with dry ice for subsequent analysis.

Aryl hydrocarbon hydroxylase assays were performed by the method of Okamoto et al (22). Six determinations were done on each animal. Three were 0 time assay controls without reduced triphosphopyridine nucleotide (TPNH), and three were determinations at 20 minutes with TPNH. Preliminary studies with this enzyme assay showed the amount of product produced with time was linear from 10 to 25 minutes.

Table 20 outlines the results of these studies.

TABLE 20. EFFECT OF AMMONIUM SULFATE AEROSOL ON ARYL HYDROCARBON HYDROXYLASE ACTIVITY IN HAMSTER LUNGS

Treatment	AHH Activity after 1 week	AHH Activity after 3 weeks
Control	1.33 $\pm$ 0.737 units[1]	1.60 $\pm$ 0.801 units
Sulfate	1.38 $\pm$ 0.980 units	1.59 $\pm$ 0.879 units
BaP	11.0 $\pm$ 1.34 units	13.2 $\pm$ 4.04 units
BaP-sulfate	11.8 $\pm$ 3.39 units	10.9 $\pm$ 3.85 units

1. One unit is equivalent to the fluorescence of 50 ug of quinine sulfate/ml 0.1 N H_2SO_4 when converted to 3-OH BaP by 10 mg lung tissue/20 min. (Okamoto, T., P. Ahan and B. So, Life Sciences 11, II, 733-741, 1972).

As expected, benzo(a)pyrene injection induced significant increases in aryl hydrocarbon hydroxylase activity. However, exposure to ammonium sulfate at the levels used did not affect this enzyme in stimulated or unstimulated animals.

Studies were then done exposing hamsters to concentrations of sulfate five times greater and continuing the exposures for 10 weeks. Actual exposure parameters were: sulfate concentration - 1.35 ± 0.15 mg/m^3; temperature - 23.9 ± 2.4° C; relative humidity - 34.7 ± 4.7% and particle size - 0.04 μ - MMAD. Assays were done in the same way as the previous study.

TABLE 21. EFFECT OF AMMONIUM SULFATE AEROSOL ON ARYL HYDROCARBON HYDROXYLASE ACTIVITY IN HAMSTER LUNGS

Treatment	AHH Activity after 1 week	AHH Activity after 10 weeks
Control	0.471 ± 0.264*	0.246 ± 0.0530
Sulfate	0.583 ± 0.309	0.319 ± 0.413
BaP	7.53 ± 2.52	8.50 ± 1.02
BaP-sulfate	4.62 ± 0.904	8.20 ± 1.40
	n=6 for each group	

*Units are the same as in Table 20.

Exposures to ammonium sulfate at a higher concentration and for a longer time also had no effect on aryl hydrocarbon hydroxylase activity.

B. Pulmonary Macrophage Studies

The pulmonary macrophage is an important defensive cell in the lung. Its role in bacterial clearance is well known, but its role in chemical carcinogenesis is debatable. It undoubtedly plays a major role in clearing deposited carcinogen from the lung. However, there has been speculation that perhaps pulmonary macrophages, in clearing carcinogens, concentrate these chemicals and thereby produce an undesirable situation in which a more concentrated carcinogen is redeposited on pulmonary epithelium. The effect of several known co-carcinogens on pulmonary macrophage number has been studied. Using standardized methods of quantitation, Brain et al (23) have shown that ferric oxide decreases macrophage numbers in the lung in some species. Our studies were designed to determine whether ammonium sulfate inhalation had any effect on macrophage number using a change in number as an indication of pulmonary irritation or toxicity.

Hamsters were exposed to 860 μg/m^3 sulfate as ammonium sulfate for 12 hours. Mean relative humidity was 43% and temperature was 22.7° C. The particle size was 0.3 μ MMAD as determined by Andersen sampling. To achieve this particle size at this concentration, the aerosol was generated by four Collison atomizers arranged in parallel. Control hamsters were held in the same environmental conditions except that their air contained no detectable sulfate.

Six sulfate-exposed animals were killed by sodium pentobarbital overdose immediately after exposure, and six, 24 hours later. Control animals were studied in the same manner. Pulmonary macrophages were collected and counted, and are reported (see Figs. 20 and 21) by the standardized methods of Brain and Frank (24).

No difference could be seen between the shape of the washout curve of controls and those of the post-exposure groups. Similarly, no significant differences were seen in the macrophage number of control and post-exposure hamsters. Data points in Figs. 20 and 21 represent the mean of six animals $\pm$ standard error. Although there was statistically no difference between the three groups, the standard error was greater in the exposed groups than in controls. The significance of this change is speculative. The study was repeated with similar parameters and the same results were obtained. Therefore, based on these data, it can be concluded that ammonium sulfate inhalation at this level had no significant effect on pulmonary macrophage numbers.

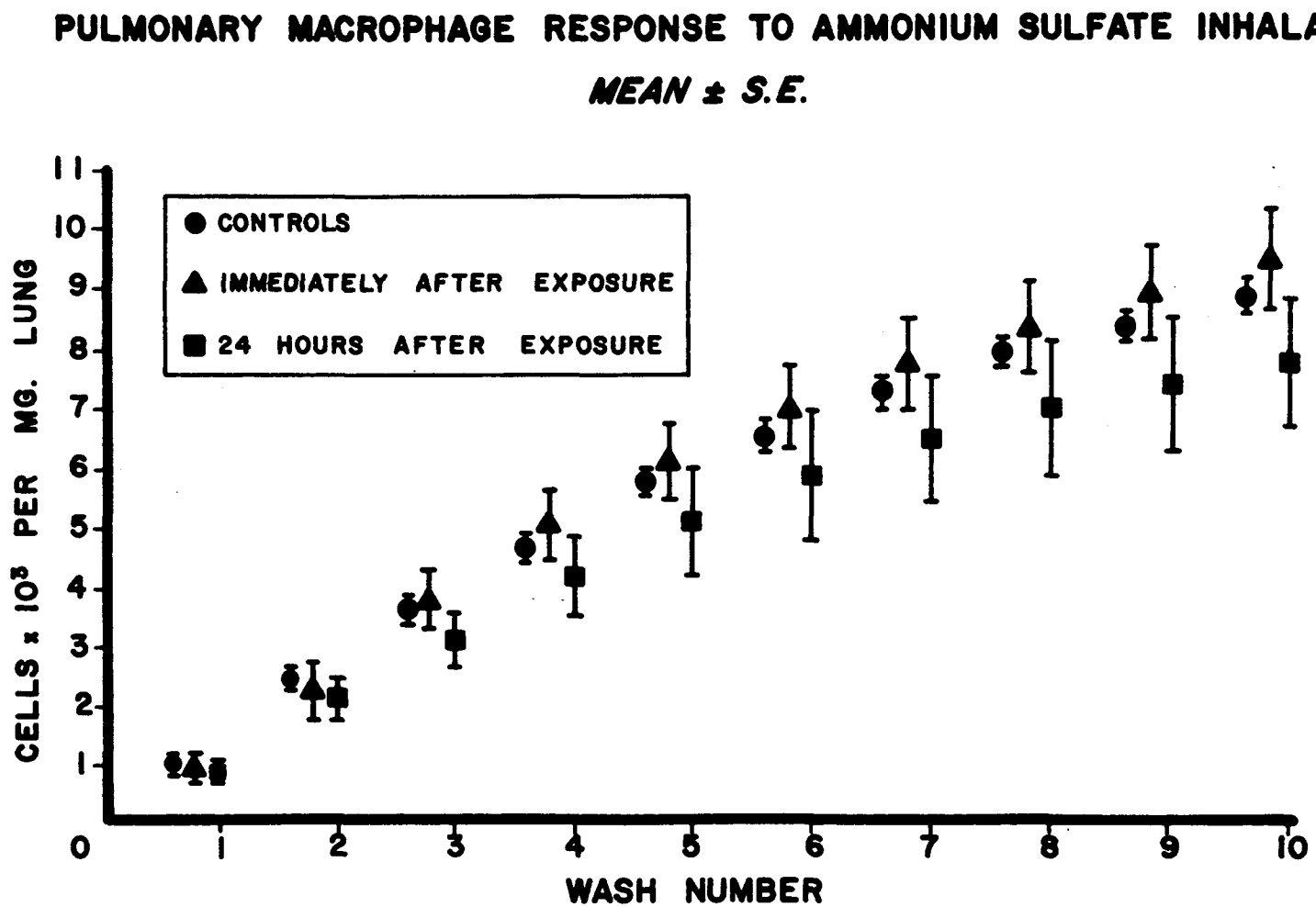

Fig. 20. Macrophages obtained by lavage in control and ammonium sulfate exposed hamsters. Mean ± S.E.

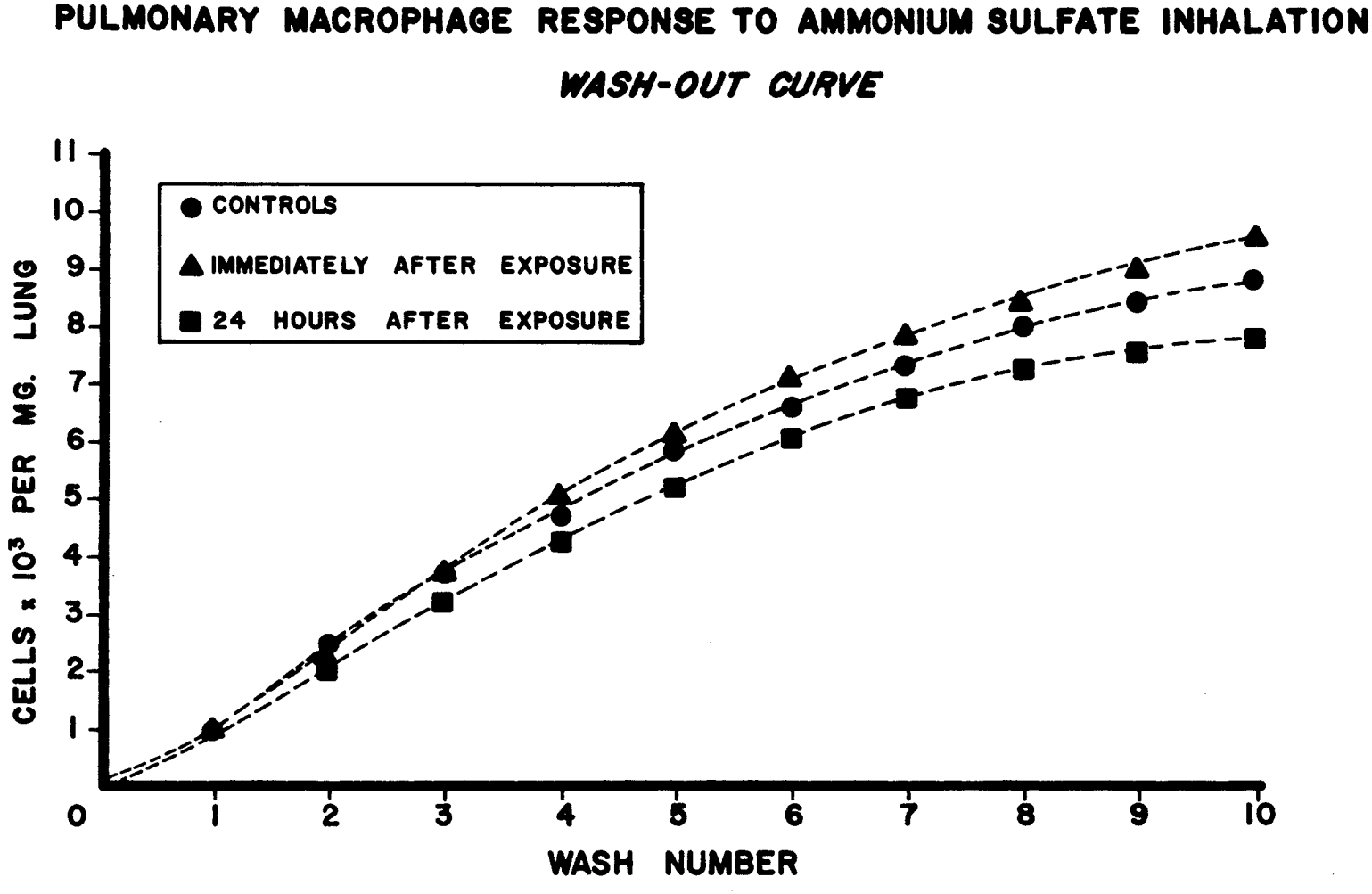

Fig. 21. Macrophages obtained by lavage in control and ammonium sulfate exposed hamsters: Comparison of washout curves.

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

This project was designed to evaluate the health effects of ammonium sulfate $(NH_4)_2SO_4$ inhalation using experimental animals. The questions studied were: (1) Is inhaled ammonium sulfate co-carcinogenic. (2) What are the deposition and clearance patterns of inhaled ammonium sulfate? (3) What effect does ammonium sulfate have on pulmonary defensive mechanisms?

The study showed that ammonium sulfate inhalation had no effect on the development of cancer and no effect on the development of other significant pulmonary diseases in hamsters.

Hamsters, guinea pigs and rabbits were studied for deposition and clearance of inhaled ammonium sulfate. Total respiratory tract deposition was greater with the larger particle size in all studies. Clearance patterns were similar for the three species regardless of particle size. The half time for clearance of ammonium sulfate from the lung was 18 to 20 minutes. Inhaled and injected sulfate was cleared via the urinary tract and by six hours after esposure 95% of the total collectable sulfate was present in the urine. Pulmonary macrophage number was not affected by ammonium sulfate inhalation.

17. KEY WORDS AND DOCUMENT ANALYSIS

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