THE TOXICOLOGY OF SULFUR OXIDES AND THE *IN VITRO* RESPONSES OF LUNG MACROPHAGES

by

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ABSTRACT

A general overview of sulfate air pollution is presented with the conclusion that further basic research is needed to determine the exact relation of sulfate pollution to pulmonary disease. The importance of fossil-fueled electric power generating plants as a source of sulfur oxides and sulfate particulates is discussed. Details of aerosol generation for *in vivo* animal exposure are given. However, emphasis is placed on the importance of lung macrophages in the pulmonary response to inhaled sulfate aerosols. Experimental work with the lung macrophage in an *in vitro* culture system comparing the response to metal sulfates versus metal oxides is presented. Improvements and modifications of the experimental system, especially enzyme determinations, are suggested.

Experimental investigations reported herein are of a preliminary nature to establish possible future areas of investigation.

CHAPTER 1

INTRODUCTION

The poet-philosopher W. H. Auden once remarked that today in the technologically advanced countries there is little to fear from nature; the only serious problem is man, "his mastery over nature has, as it were, brought man back to the same point at which he stood when he was utterly helpless before her." It is no longer the creatures and forces of the natural world, or even such historic diseases as the pox and plague, that threaten us most. Instead it is the chemical pollutants of our own making. They are all around us--in our air, our water, our food and on the things we touch.

Some of the more important points about the effects of pollutants on human health and society's attitude toward them are:

1) Risk potential rose markedly with the advent of the industrial revolution while the level of awareness of health problems remained at a relatively primitive level. Society seemed to accept the tenet that a certain risk was necessarily linked to the process of making a living. Environmental contamination was tacitly considered an integral part of

progress and prosperity. In the expanding industrial and technological age a keynote about pulmonary toxicology was a profound apathy.

2) Over the past few decades, especially since the end of World War II, we have released into the environment a vast volume of new chemical compounds with little or no knowledge of their health effects, and with virtually no effort to determine those effects or to regulate the release of many chemicals that might be hazardous. Not until disastrous pollutant episodes such as Donora, London or the Meuse Valley were any investigations into the effects of the sulfates and sulfur dioxide introduced.

3) The World Health Organization estimates that 60-90% (Trakowski, 1976) of all cancers are a result of environmental factors. Yet the odds are that only a relatively small portion of the chemicals in our environment pose any serious health threats.

4) We are now concerned with the varying quality of health, much as we were concerned with distinctions between health and clinical disease in the past. We are interested in an optimally healthy environment for all levels of human behavior and response. Standards are rising everywhere with a corresponding increasing demand that something be done now about the vague, chronic ill-health that has been regarded as the inevitable lot of those past middle age. The belief

is developing that causes can be discovered and controlled and that the multiple, minor, and repeated environmental insults we have unwittingly brought upon ourselves--"bronchitis," "heart trouble," "rheumatism," "fatigue," or "allergies"--may be contributors to such ill-health. We are concerned also about possible delayed or long-range effects from exposure to environmental pollutants which do not produce acute or prompt responses.

5) A large and growing share of diseases that cripple and kill us--emphysema, anthracosis, and lung cancer--are caused by environmental factors, in the broadest sense of the word. These diseases are going to take an increasingly heavy toll upon our lives and well-being unless and until we stop trying to deal with them by treating them after they occur and start taking serious steps to prevent them from occurring in the first place.

We remain almost entirely unable to discover how harmful a chemical can be until years after it has become a significant factor in our economy. Although it is clear that there is a wide range of "safe" concentrations and that substances range from single pure gases through vapors and fumes to solid dusts, and there may be differences of toxicity between chemically identical materials.

There are threats of global climate changes caused by atmospheric pollutants. Of equal importance are the

direct inhalational consequences of atmospheric pollutants, such as acid mists, because the respiratory tract is a major route of entry of gaseous vapors into the body.

We must consider "benefit versus risk factor," concerning ourselves more with latent risks associated with certain obvious benefits. At times we may have used our limited technology and tradition of short-term thinking as blinders to potential hazards. While the benefits and rewards of a rapidly advancing society are large, tangible, and immediate, the penalties to be paid for such progress may be often clearly unavoidable, though delayed in time and less visible. There is little complacency in the notion that what doesn't show doesn't matter.

We have been in the fortunate position to afford the absence of effective control authority on an international scale. The question is, can we afford this luxury any longer?

Underlying the concern for the implications of the global effects of pollution is the impact of environmental requirements on the world economy. Can the ever-increasing needs of a world population that may double by the year 2,000 afford pollution control?

One author, Trakowski (1976) believes that environmental requirements in the United States are not damaging

our economy, but instead are a growing source of strength within it. In hearings before the U.S. Congress (1975), Ashford expressed the opinion that pollution control requirements are a stimulant to industrial development and investment, and is creating a whole new field of industry and concomitantly more jobs and new products--clean air, clean water and clean land--which . . . is of immeasureable value to the American public. On the other hand, a recent poll by Pollution Engineering (1978) of pollution control manufacturers reveals that in many cases the Environmental Pollution Agency (EPA) is setting such stringent specifications for pollution control equipment that little new equipment is being developed. Also, EPA interference in the selling of pollution control equipment and the lack of tax breaks to those meeting or exceeding EPA requirements have led to the use of inferior pollution control equipment and a heavy economic burden upon the regulated industries.

Given the potential seriousness of pollution health hazards, it is a matter of urgency that we move rapidly to expand and coordinate the control efforts of industry, nations and specialized international agencies. This includes greater efforts in disseminating information and performing research.

The sources of noxious materials subject to inhalation and the circumstances under which exposure may occur

are highly variable. For example, an industrial worker may be subject to a baseline low-level exposure during work periods with a potential for episodic higher exposures, while the effluent from the plant of the same process presents a much-lower-level continuous exposure to those living in the region of the industrial plant. Wide dissemination of the toxicant represents a still lower exposure, but to greater numbers of people. All of the variables that may influence the relation of dose to response (sex, age, etc.) are applicable to inhalation toxicology. In inhalation studies, however, the true dose to lung or other tissues often is difficult to ascertain, and from a practical viewpoint the respiratory tract is often subject to mixtures of chemicals and multiple sets of exposure characteristics. Therefore, a study of a basic system, e.g., lung macrophages, known to be one of the first biological areas of exposure and response to irritant pollutants is essential to understanding lung pathology resulting from chronic or acute exposure.

CHAPTER 2

ENVIRONMENTAL CONCERNS RELATING TO SULFATES

The irritant potency of sulfates is of considerable interest in air pollution toxicology because suspended sulfate particulates are prevalent in the atmosphere in many areas of the United States.

Epidemiology

Sulfate-containing aerosols are broadly distributed throughout large regions of the eastern and midwestern United States. Sulfates contribute a larger portion of the sulfur compounds measured in the western urban sites than in eastern urban sites (Altschuller, 1973). Fly ash produced by coal-fired power generating plants is an ubiquitous source of aerosols containing trace elements (Charles et al., 1977). The potential for interaction with sulfate particulates or aerosols is high because acidic aerosols of sulfuric acid are produced by several environmentally important processes including stationary power generating plants, smelters, petroleum refineries, and catalytic converters on motor vehicles. The cationic sulfates and sulfuric acid from these activities account for 5-20% of the total suspended particulate in air.

Sulfur dioxide, the more predominant of the sulfur oxides, exists in community atmospheres as a result of burning of fossil fuels (coal and oil) containing sulfur. Accordingly, meteorologic conditions being equal, the SO_2 levels in the air will vary with the amount and type of fuel burned. In the past, higher SO_2 levels were experienced in coal-burning communities (London, Birmingham, England); lower levels, in oil-burning communities, typical of cities in the United States (Stokinger, 1969).

Until very recently, SO_2 was commonly released into the air. In addition, special devices such as tall stacks or stack gas heaters, were used to deliberately spread these obnoxious wastes over large areas (Meyer, 1977). Thus, an acute local problem was transformed into a chronic regional nuisance. As a result, large segments of regional populations have been exposed to the effect of toxic or objectionable gases. During unfavorable weather conditions, the emission can be trapped and accumulates in inversion layers reaching critical concentrations which adversely affect health (Shrenk et al., 1949). During the last ten years, no acute episodes have occurred, but earlier, such episodes used to be quite common. The incidents of the Meuse Valley of Belgium in 1930 (Firket, 1936), in Donora in 1948 (Shrenk et al., 1949), and in Tokyo, London (Holland, 1974), and New York during the 1950's and early 1960's are still

well-remembered. In several of these places, mortality above the statistically predicted rate could be demonstrated and autopsy data and cardio-respiratory distress in clinical patients was well-established (Shrenk et al., 1949) and eventually led to large scale legal action and to the study of the health effects of pollution.

Sources of Sulfate Pollution

Numerous sources of sulfate pollution exist. Among the most prominent are smelters, catalytic converters, and fossil fuel-fired electric power plants.

Smelters

Sulfur participates in many important reactions. Smelting of copper ore yields (Meyer, 1977):

$$CuS + Air + NH_3 \longrightarrow Cu + (NH_4)_2SO_4$$

In this reaction, sulfate is a by-product. Depending on the chemical path chosen, sulfate acts as an air pollutant, as solid waste, or as sellable fertilizer.

One report (Hansen et al., 1974) indicates that the airborne particulate collected near smelters contains 4 times the ratio of sulfite to sulfate. In that case the activity of sulfite with metal catalysts may be much more important than synergism of sulfates with metal ions. Brosset (1973), in his review of the atmospheric chemistry of sulfur dioxide, indicates that sulfite is merely an

intermediary in the formation of sulfuric acid and sulfates. Meyer (1977) claims that smelters emit such highly concentrated sulfur dioxide, up to 5%, that unabated emission causes acute local damage (e.g., vegetation and health).

Catalytic Converters

Although catalytic converters do a good job of reducing the emissions of carbon monoxide and hydrocarbons, they also catalyze the oxidation of sulfur, a normal constituent of gasoline, to sulfite which leads to the formation of sulfates and sulfuric acid aerosol (Amdur, 1971). It has recently been suggested by a number of researchers, (Charles and Menzel, 1975; Hansen et al., 1974), that catalytic devices installed on 1975 model and later cars may prove to be a significant source for ammonium sulfate. Amdur and Corn (1963) observed that ammonium sulfate (1.0 mg/m^3 for one hour) was highly irritating to guinea pigs causing a bronchoconstricting reaction similar to that seen in humans.

Fossil-fueled Power Plants

Unprecedented, often unrestricted, growth in industrial activity throughout the world results in the consumption of high quantities of fossil fuels. Fossil-fueled power plants are considered to be those utilizing gases, liquids, or solids as primary fuels derived, respectively, from natural gas, oil, or coal. Gaseous emissions from

fossil-fueled power plants generally contribute more material to the atmosphere than do particulate emissions, except in the now rare case of uncontrolled coal combustion. One of the major emissions, in terms of mass, involves the sulfur oxides (National Air Pollution Control Administration Publication No. AP-50, 1969; Natusch, 1978). The impact is rarely acute, but the delayed release of sulfur dioxide, and its oxidation products, contributes to annoying health and environmental problems (e.g., asthma and air opacity).

The world's coal reserves are well established and immense. Coal contains an average of 1.5% sulfur, most of which is released during combustion as sulfur oxides (Pattle and Cullumbine, 1956). Presently, sulfur released from coal combustion about equals all other forms of sulfur production. If coal combustion becomes more important, sulfur released from coal will make a major impact, regardless of whether it is recovered or released to the atmosphere. Air pollution control policy as embodied in the Clean Air Act Amendments of 1970 and implemented by the Environmental Protection Agency is progressively reducing our usable coal base by tightening the limits on sulfur content (Mullan, 1974). The immediate answer was the use of low-sulfur fuels until the necessary emission control technology reached the commercially ready stage. However, as Amdur (1971) states, the difficulty lies in the fact that the fuel in shortest supply

is that naturally low in sulfur. Large reserves of low-sulfur fuel exist, but they are primarily in the West, undeveloped and generally beyond the economical transport reach of the heavily industrialized East. Governmental response to economic pressure has been to relax limits on sulfur content of coal. Therefore, further use of coal in an economically feasible manner could result in continued sulfate insult to our environment.

Sulfur released from coal is an inadvertent by-product of industries which traditionally consider all their combustion products as wastes. As shown in Figure 1, almost all power plant by-product sulfur enters the atmosphere as sulfur dioxide. Sulfuric acid content of the effluent from the plant is lower, but remains in the air longer because it is present as an aerosol or mist which is much less mobile than a gas (Amdur, 1971). A little sulfur dioxide remains in the ashes as sulfate (Meyer, 1977). Since the sulfur dioxide gas is heavier than air, it does not mix well, and about 90% returns to the soil within a day and usually within a radius of less than 50 miles. In rare cases, it contributes to the fertility of the soil in those cases where there is a nutrient sulfur deficiency; more commonly, it damages forests and lawns, and is considered a nuisance or even a menace. Present solutions include taller stacks or catching the SO_2 in lime and dumping it.

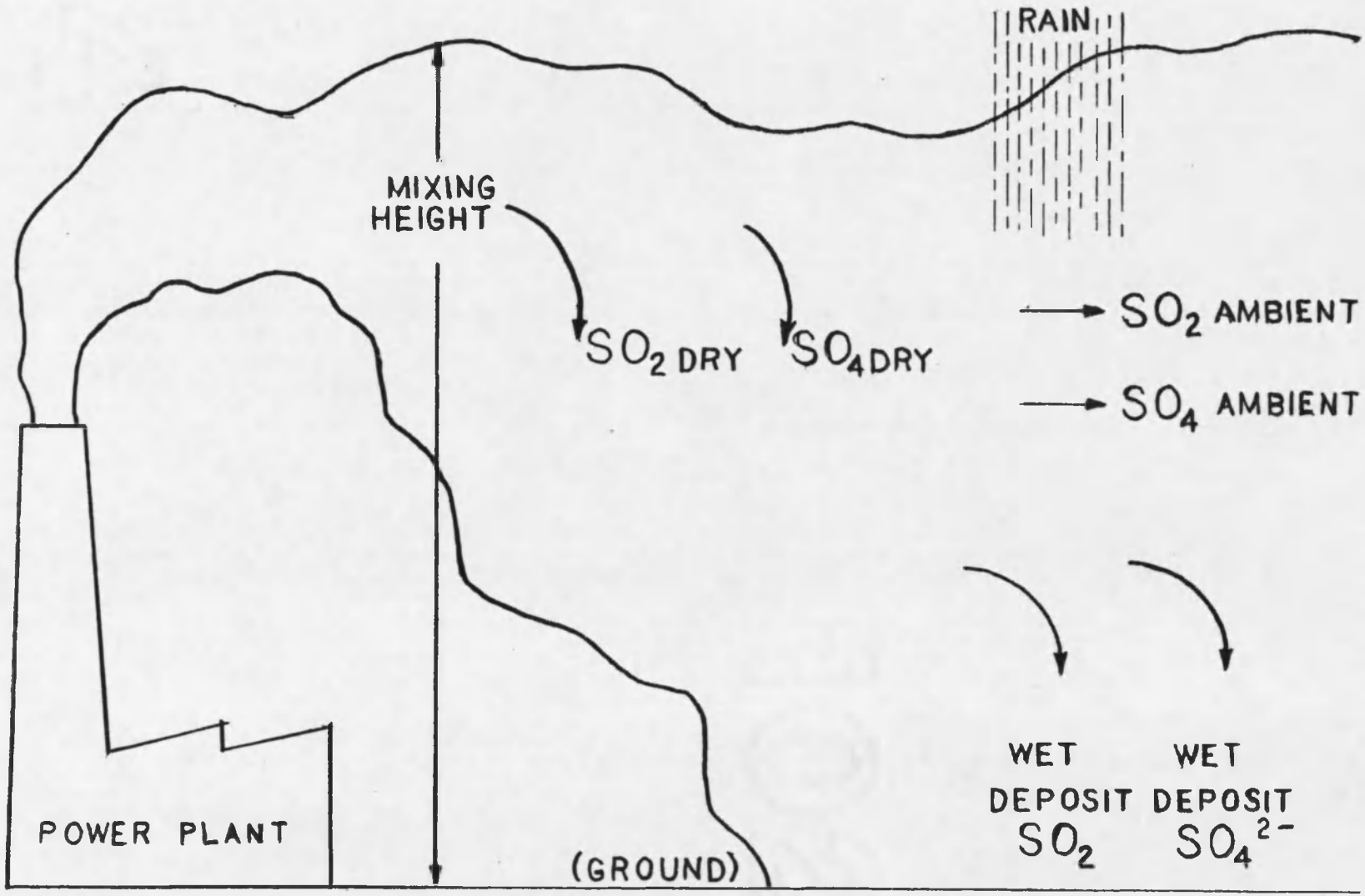

Figure 1. Fate of sulfur dioxide from power plants (Meyer, 1977).

Neither solution is permanent. Dumping may be worse because the waste persists longer. Taller stacks result in release of the SO_2 to a regional zone, with complex, uncontrollable, and often unforeseeable interactions, instead of confining the SO_2 to a local microclimate with reasonably predictable interaction. Thus, if the local damage is reduced by dilution, the exposed area is increased, and the damage is merely spread because even low concentrations can cause substantial damage. A viable solution depends on integrating sulfur dioxide emission in a useful cycle in which the sulfur has a positive purpose and use. Such a cycle can only be found if combustion products are considered as raw materials, rather than as waste. This sounds fine; however, mining industry attempts to market sulfuric acid produced from sulfur dioxide emissions have been notably unsuccessful.

Natusch (1978) reports that, in the absence of control, the amounts of sulfur oxides emitted from a fossil-fueled power plant are directly related to the sulfur content of the fuel burned. In this case, typical SO_x emissions lie in the range 500-3000 ppm with 1000-2000 ppm being most commonly encountered (Natusch, 1976). Nowadays, however, most major installations utilize control equipment which typically achieves 80-90% removal of SO_x. Generally, about 2-3% of the emitted sulfur oxides are in the form of SO_3 which reacts

rapidly with water vapor to produce sulfuric acid mist, part of which may be deposited on the stack walls and the rest of which is emitted to the ambient air (Brosset, 1973). A small amount of the SO_2 is also chemisorbed by fly ash particles to form metallic sulfates. In addition, stack gases and urban air can yield smog, primarily H_2SO_4 (Meyer, 1977). The rate and extent of sulfur dioxide conversion to sulfuric acid mist and solid particulate sulfate in a power plant plume are unknown; however, current thinking is that these processes occur fairly extensively, so that a significant proportion of the gaseous sulfur oxides produced actually occur in urban atmospheres as sulfuric acid mists or as particulate sulfate (American Chemical Society, 1969). This is an important consideration, since it means that the health hazard presented by gaseous sulfur oxides may be partly manifest through inhalation of sulfuric acid aerosol and sulfate particles.

Sulfate Atmospheric Chemistry

The key to the role of sulfur dioxide in air pollution toxicology lies not in the gas itself but in its atmospheric chemistry. Sulfur dioxide is chemically unstable, and thus possesses the ability to react catalytically or photochemically with materials in the atmosphere (National Air Pollution Control Administration Publication No. AP-50, 1969). The most widely studied reactions are oxidation of

sulfur dioxide to sulfuric acid and sulfate particulates (Charles and Menzel, 1975), reaction of sulfur dioxide with ammonia, and the reaction of sulfur dioxide with air pollutants, i.e., trace elements. The presence or absence of humidity plays an important role in these reactions (Novakov, Chang, and Harker, 1974).

In all systems, gaseous sulfur dioxide converts to non-volatile sulfate which forms aerosols. The aerosols can affect the opacity of the air, they can participate in reactions with other pollutants or catalyze reactions in the polluted atmosphere, and they are known to aggravate pre-existing health problems (Environmental Protection Agency, 1974). There is substantial controversy about the nature of the conversion of sulfur dioxide to sulfate; the reaction mechanisms in the atmosphere are still not well understood. The following discussion attempts to summarize what is known or surmised about atmospheric reactions of sulfur oxides.

Oxidation of Sulfur Dioxide (SO_2)

During combustion of sulfur-containing fuels, the sulfur is primarily converted to SO_2. The behavior of this product, as illustrated in Figure 2, follows two different reaction patterns, (a) and (b).

Approximately 2-3% of the SO_2 is oxidized in the stack and converted to concentrated H_2SO_4, part of which may be deposited on the stack walls and the rest emitted to the

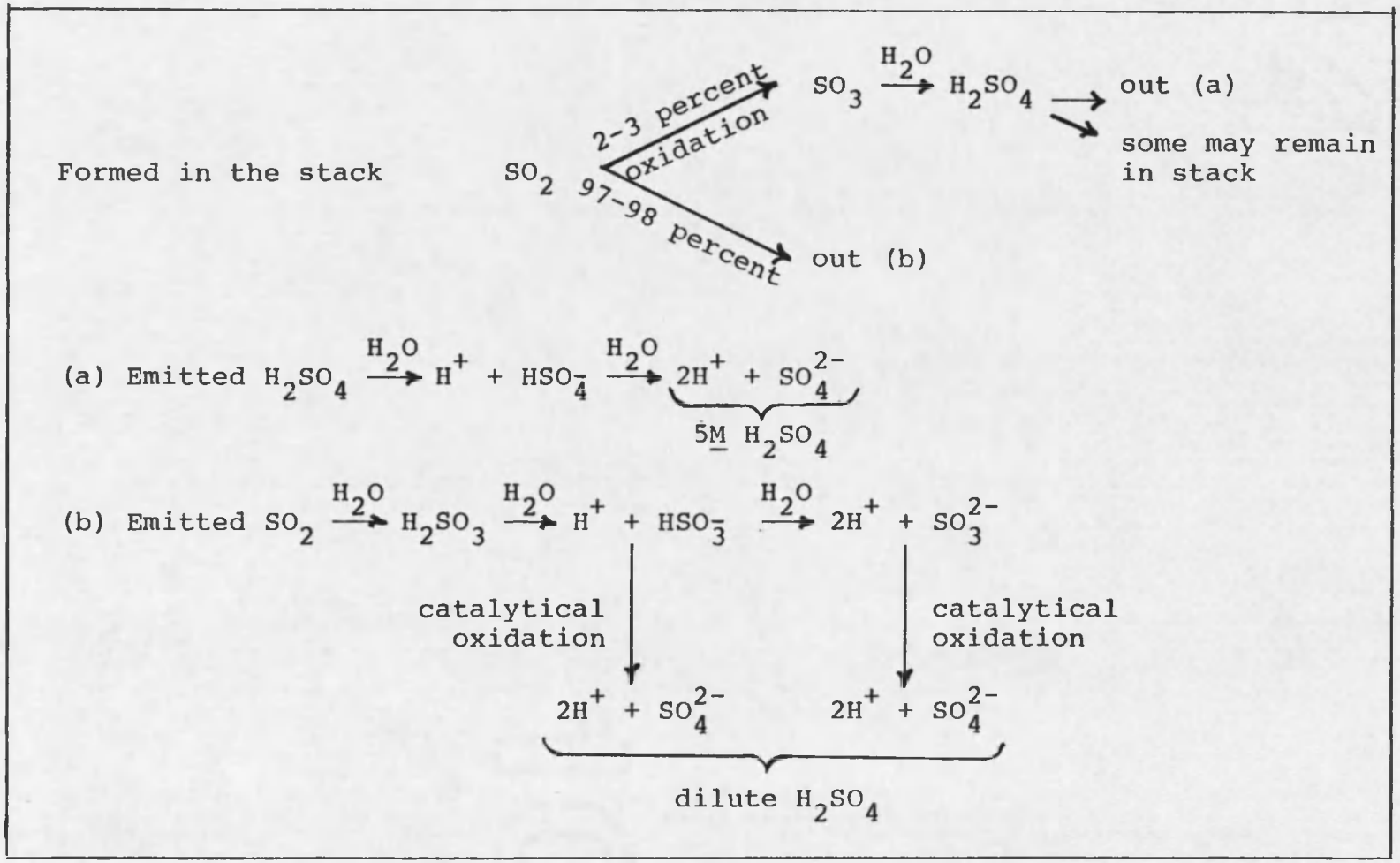

Figure 2. Catalytic oxidation of sulfur dioxide (Brosset, 1973).

Oxidation occurs slowly at 400°C without a catalyst (National Air Pollution Control Administration Publication No. AP-50, 1969).

ambient air. Deposition in the stack is usually not desirable, due to the corrosive effects, and is avoided by releasing the flue gases at a sufficiently high temperature. As discussed previously, heating the stack causes increased dispersal of the flue gases and is, therefore, undesirable. Electrostatic precipitators are currently in use in smelter stacks to reduce sulfur oxide emissions.

When emitted to the ambient air, drops of concentrated H_2SO_4 react according to (a) they pick up water and eventually, due to the water vapor pressure in the atmosphere, form drops of approximately 5 M H_2SO_4; the SO_2 that is not oxidized in the stack reacts according to (b) in the atmosphere SO_2 comes in contact with very small particles covered by an aqueous film or with water drops. The film on the drops may contain dissolved or suspended substances, e.g., iron compounds. SO_2 is dissolved while forming H_2SO_3, which is a medium-strong acid in the first step, and is dissociated to H^+, HSO_3^-, and SO_3^{2-} ions.

Under the influence of iron oxides or chromic oxide acting as catalysts (National Air Pollution Control Administration Publication No. AP-50, 1969), HSO_3^- and SO_3^{2-} are rapidly oxidized to H_2SO_4 (Johnstone and Moll, 1960). This means that H_2SO_3 is replaced in the drop by H_2SO_4, which is a strong acid. Because of the increase in acidity of the solution, the dissociation of H_2SO_3, formed through

additional absorption of SO_2, is restricted and the oxidation slows down. According to Junge and Ryan (1958), the process ceases entirely when the solution reaches pH = 2.2.

Junge and Ryan (1958) also showed that for catalytic oxidation (a) the maximum possible sulfate formation is a linear function of the partial pressure of SO_2 in the atmosphere; (b) sulfate formation can account for observed sulfate concentrations in rain, fog, and smog only if some neutralizing cations in the aerosols or traces of NH_3 in the air are present; (c) no appreciable oxidation takes place in the absence of a catalyst; and (d) initial oxidation is rapid and controlled by SO_2 concentration.

Drops of sulfuric acid formed through reaction (a) or (b) can come in contact with, for instance, iron oxide particles. Reaction (b) may also start on such iron oxide particles (National Air Pollution Control Administration Publication No. AP-50, 1969). Novakov, Chang, and Harker (1974) confirmed this by demonstrating that moist surfaces of particulates are such effective catalysts for the oxidation of SO_2 to SO_4^{2-} that ambient concentrations are sufficient to account for most of the air pollution effects which formerly have been explained by gas phase mechanisms. This is not astonishing, as the particulates accompanying sulfur dioxide from power plant stacks consist of fly ash (Meyer,

1977) which contains traces of transition metals as shown in Table 1.

Some metal oxides will oxidize SO_2 directly to sulfate. These include Fe_2O_3, ZnO, Mn_2O_3, and CuO. A sulfide is also formed as a product if the metal ion is not reduced to a lower valence state (National Air Pollution Control Administration Publication No. AP-50, 1969).

Table 1. Trace metals concentrated in fly-ash particulate emitted from a fossil-fueled power plant.[a]

Trace Metal	Concentration µg/g of Particles	Comment
Cadmium	35	Concentrated
Nickel	1,600	Concentrated
Zinc	13,000	Concentrated
Iron	15 wt. %	Only limited trend towards concentration in respirable particles
Manganese	470	
Cobalt[b]	---	Not particularly enriched in respirable particles, but values of 130 µg/g have been reported

[a]Source (Charles et al., 1977).

[b]Cobalt is the most potent cation for increasing absorption of sulfate ions as demonstrated by intratracheal instillation.

Photochemical oxidation of atmospheric sulfur dioxide to sulfur trioxide proceeds in the presence of oxygen

moist air and sunlight (Amdur, 1971). Sulfur trioxide, at all vapor pressures in air except the very low, immediately combines with water to form sulfuric acid; therefore sulfuric acid, rather than sulfur trioxide, is normally found in the atmosphere. Sulfur trioxide is a strong acid and readily reacts with metallic oxides to form sulfates. Metal oxides, such as MnO_2 and Fe_2O_3, catalyze the oxidation, therefore increasing the rate of reaction (Amdur, 1971; Barrie and Georgii, 1976). Thus photochemical oxidation in smog is faster than oxidation in "pure" fog.

Ammonia and Sulfur Dioxide

Ammonia and sulfur dioxide are both present in the atmosphere; their reaction has been invoked to explain the atmospheric behavior of sulfur dioxide, and is used in air pollution abatement systems (Meyer, 1977). It should be pointed out that the sulfuric acid formed in water droplets as a product of SO_2 oxidation can be partially neutralized if the particle contains acid soluble oxides and if NH_4^+ is taken up from the atmosphere (Arrowsmith, Hedley and Beer, 1973; Treon et al., 1950). This reaction results in drops containing concentrated solutions of $(NH_4)_2SO_4$ together with a number of hydrated metal ions (Brosset, 1973).

Gas phase reactions are different in that if SO_2 is in great excess, a 1:1 compound of NH_3:SO_2 forms, probably amidosulfurous acid (NH_2SO_2H), but the large amounts of NH_3

normally present in the atmosphere lead to the formation of several 2:1, NH_3:SO_2, compounds all of which oxidize to $(NH_4)_2SO_4$ (Scott, Lamb and Duffy, 1969). Therefore, ammonium sulfate is still considered to be a principal component of sulfate residues in the atmosphere (Atmospheric Analysis Laboratory, 1975; Environmental Protection Agency, 1974). Based on the acid-base nature of sulfur dioxide and ammonia, such a relationship is to be expected.

The present output of sulfuric acid by auto exhaust catalytic converters is probably converted to a mixture of ammonium sulfate and sulfuric acid by environmental ammonia. In addition, second generation auto exhaust devices include the addition of a reducing catalyst to convert NO_x to nitrogen. Carbon monoxide serves as the source of electrons and during the incomplete reduction of NO_x ammonia is the favored product, resulting in greater amounts of ammonium sulfate output to the atmosphere (Charles and Menzel, 1975). At a relative humidity of approximately 80% $(NH_4)_2SO_4$ undergoes a transition from the dry crystal to a solution droplet (Orr, Hurd, and Corbett, 1958).

As stated earlier, the physical and chemical interactions among the particles, gases and vapors in the atmosphere are complex, constantly changing and poorly understood. The sciences of aerobiology, chemistry, and physics have only recently come of age in this respect, so

that knowledge of any given exposure to air pollutant mixtures is largely inadequate. The next section will attempt to explain aerobiologic and physical factors relating to sulfate particulates.

Irritant Properties of Sulfate Aerosols

Amdur (1971) has produced convincing evidence that not only is the response to a given aerosol concentration greater as the particle size decreases, but also that the dose-response curves are steeper as the particle size decreases. This means that a small increase in mass concentration produces a larger increment in biological response as the particle size is decreased. This emphasizes the very important point that if a compound is dispersed as a particulate material, a measure of mass concentration alone does not permit assessment of irritant potency. If the particle size is essentially equivalent, then sulfuric acid is a more potent irritant than sulfate aerosols (Amdur, 1971). On the other hand, if zinc ammonium sulfate is present, dispersed as a fine aerosol, then it is the more irritant (Amdur, 1971; 1974). There is a need for more data relating particle size to irritant potency.

Particle Size and Pulmonary Deposition

The aerodynamic size of a particle is a major function in determining the efficiency with which it can be

collected by control equipment, its atmospheric transport characteristics and lifetime, and its deposition and clearance behavior when inhaled (Natusch and Wallace, 1974). In addition, the size of a particle determines the specific surface area which can come into intimate contact with body fluids and tissues (Natusch, 1978). The size distributions of particles produced by different power plants exhibit considerable variation; however, a typical size distribution of fly ash emitted from a coal-fired power plant equipped with an electrostatic precipitator falls in the respirable size range (Jones, 1972). In fact, a large portion of the total pollution aerosol, and the dominant portion of the aerosol of submicron size, is formed within the atmosphere from gaseous precursors associated with combustion sources (Lippmann and Altshuler, 1975).

Relatively few measurements have been made of particle size distributions in power plant plumes. Natusch (1978) made a rough determination that particulate material collected at a distance of 5 miles downwind from a coal-fired power plant plume under stable plume conditions has an aerodynamic mass median diameter in the range 0.08 - 0.025 micrometer. Such samples usually exhibit a bimodal distribution, with the two modes being centered around 0.04 micrometer and 0.3 micrometer. The smaller modal particles are thought to represent a secondary aerosol consisting

primarily of sulfate particles. Similar general behavior could be expected for oil or natural gas-fired power plants.

Inhaled particles deposit in the component regions of the respiratory tract (Casarett, 1972; Green, 1974; Lippmann and Altshuler, 1975) according to:

1) their size, density and aerodynamic drag factor,
2) the average rate and time pattern of flow,
3) the geometry of passageways and spaces.

The single most important feature of atmospheric particles is size. The smaller the particles, the greater the irritant action at any given mass concentration (Amdur and Corn, 1963). When the number of particles per unit volume is equal, the larger sized aerosols are more irritant (Amdur and Corn, 1963).

Although there are many sampling devices and numerous means of expressing the size characteristics of particle populations, one convention is used most often and most effectively. This is either the count median diameter (CMD) or the mass median diameter (MMD) (Casarett, 1972). This usage derives from the observation that most natural (and many generated) aerosols exhibit patterns approaching log-normal distributions.

Several other important characteristics of particles are shape, hygroscopicity, and density. Customarily shape of particles is assumed to be spherical. That this is not

true is obvious. The manner in which the shape of a particle may alter its behavior in the respiratory air stream is complex and not subject to clear means of prediction.

Hygroscopicity of particles is of importance largely because of its influence on size. As usually measured, particles are not saturated with water. On entry into the respiratory tract with its saturated atmosphere, particle size may change markedly. This is true of sulfuric acid, sodium chloride and glycerol (Lippmann and Altshuler, 1975). Thus the true size as "seen" by the respiratory tract may be considerably different from the measured size outside the tract. All other factors being equal, hygroscopicity is of less significance with particles of high density.

The physical diameter of a particle is not usually a suitable measure of its behavior in the respiratory airstream. The aerodynamic diameter of a particle is a function of both the physical diameter and the density of the substance. For example, given two particles of the same physical size, that which has the higher density is, aerodynamically, a larger particle. The aerodynamic diameter as defined and discussed provides the best available measure against which to examine regional deposition in the respiratory tract (Casarett, 1972).

The major subdivisons within the respiratory tract differ markedly in structure, size, function, and response

to deposited particles. They also have different mechanisms and rates of particle elimination as will be discussed in Chapter 3. Thus, complete determination of an effective tissue dose from an inhaled aerosol depends on:

1) regional deposition,
2) retention times at the deposition sites and along the elimination pathways,
3) the physical and chemical properties of the particles and their biological effects (Lippmann and Altshuler, 1975).

The remainder of this discussion centers on the deposition pattern of inhaled particles and selected factors which determine it.

A simple stylized summary of the deposition pattern and processes involved is presented. In Figure 3, a geometric presentation of a respiratory tract is presented with a few salient features. The particle sizes, in microns, do not represent the physical sizes of the particles but rather their aerodynamic size. Further, the sizes are intended to be modal, i.e., they represent size ranges within which the probability of deposition in the stated compartment are highest, except as noted below. The sizes of the respiratory compartments given in Figure 3 have no quantitative significance, but qualitatively represent the increasing volume and surface area of the respiratory tract from the

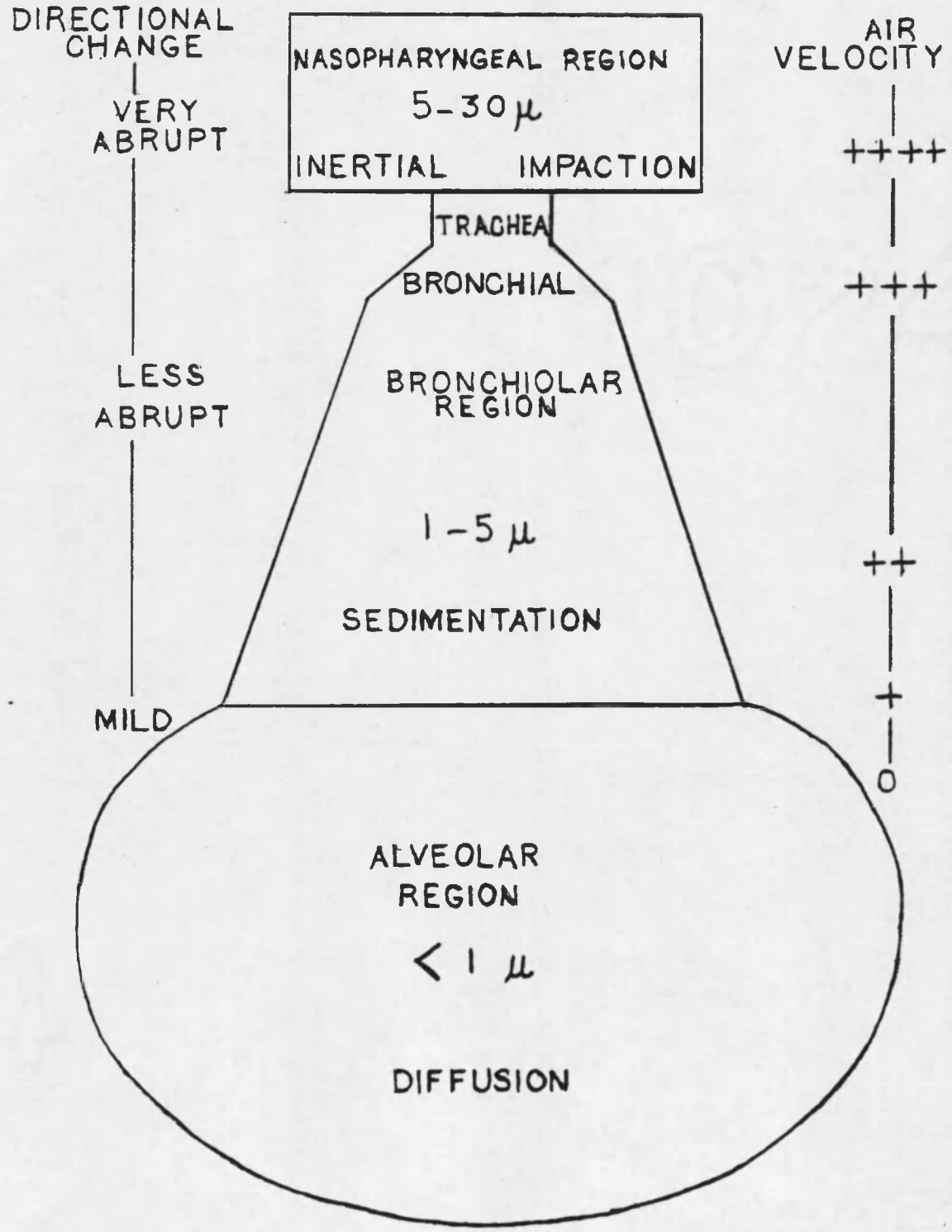

Figure 3. Approximation of selected parameters influencing deposition of particles in the respiratory tract (Casarett, 1972).

nares to the alveoli. Finally, the divisions of sizes and processes are neither abrupt nor mutually exclusive, depicting predominant features in the gamut of sizes deposited throughout the tract and the principal influences in the deposition process.

Particles deposit in the various regions of the respiratory tract by a variety of physical mechanisms. According to Lippmann and Altshuler (1975), deposition efficiency in each region depends on the aerodynamic properties of the particles, the anatomy of the airways, and the geometric and temporal patterns of flow through them. Impaction is important in particle deposition where air flow rapidly changes direction while following the tortuous path through the nose and pharyngeal region because the momentum of the particles tends to keep them in their prior directions, which can carry them onto airway surfaces. The particle size with detectable deposition in this region extends to about 1 micron and deposition is virtually complete at sizes greater than 10 microns. With decreasing air velocity and less abrupt changes in the direction of airflow, there is greater residence time of particles which have penetrated the nasopharyngeal region and gravitational sedimentation becomes an important mechanism for deposition in the smaller bronchi, the bronchioles and the alveolar spaces where the airways are small and the air velocity low. The particle

sizes in this region extend over a range of 10 microns. In the alveoli, despite the fact that air velocity approaches zero and directional changes are not significant, gravity is less important because only very small particles reach the alveolar region. These submicron particles then undergo a random motion caused by the discrete impacts of gas molecules, which results in diffusion onto the alveolar surface. This Brownian motion increases with decreasing particle size and becomes more important.

Because of the dependence of deposition on effective falling speed, the penetration of a particle into the lung is influenced by air velocities and breathing patterns. Increasing velocity augments impaction deposition, but decreases sedimentation and diffusional deposition by decreasing residence time. Central deposition of particles is favored by deep inspiration, slow respiratory rate, breath-holding, and airways obstruction. It thus becomes apparent that the risk of exposure to an airborne pollutant will depend, in part, on the respiratory pattern of the exposed individuals, e.g., mouth-breathing in sleep, and on the level of physical activity, a factor that has great influence on respiratory breathing patterns (Wolff et al., 1975). In view of the fact that only micronic particulates penetrate to the depths of the lung, only small total volumes of liquid aerosols can

be expected to reach the distal portions of the tracheobronchial tree (Casarett, 1972).

Production of a Respirable Zinc Ammonium Sulfate Aerosol

As just discussed, the toxicity of an airborne material is related to particle size, in addition to its atmospheric concentration. Determination of the size of sulfate particulates has shown that the major portion are smaller than 0.5 micron in diameter (Brosset, 1973). However, the respirable range is up to 5 microns and zinc ammonium sulfate has been estimated to have occurred in the Donora fog episode of 1948 (Hemeon, 1955) in the respirable range as one of the primary irritant aerosols. In addition, zinc and sulfur dioxide emitted simultaneously from fossil-fueled power generating plants (Charles et al., 1977) will combine to form irritant particulates (Amdur and Corn, 1963; Amdur, 1974).

Experimental Objective. The purpose of this study was to determine the concentration of zinc ammonium sulfate needed to produce a respirable aerosol and to obtain a 10-20-fold range of concentration within a 44 liter animal inhalation exposure chamber.

Methods and Materials. A schematic of the dynamic exposure system used is presented in Figure 4. A stream of generating air was passed through a silica gel drying

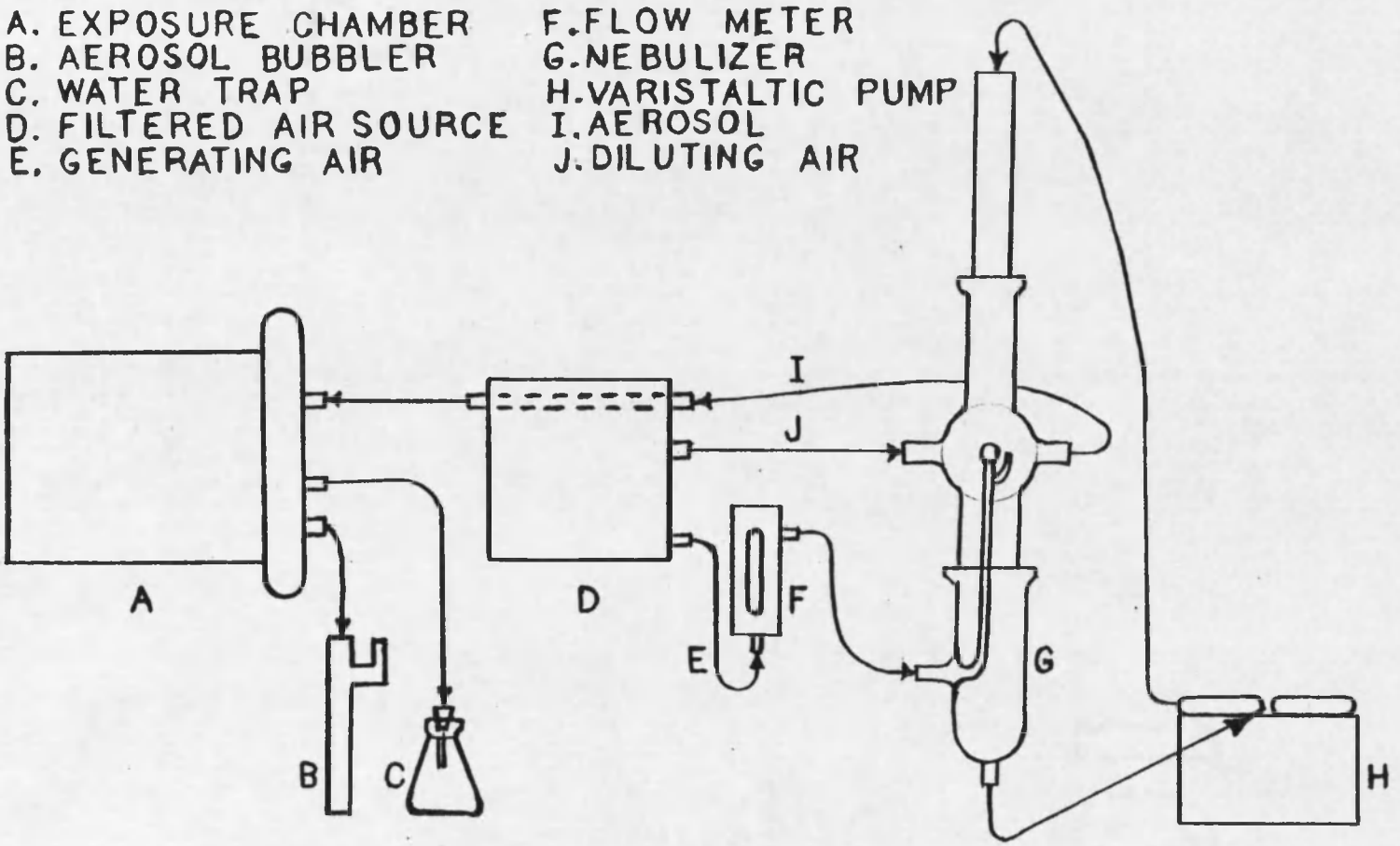

Figure 4. Schematic representation of the zinc ammonium sulfate aerosol exposure apparatus.

chamber and into the base of the nebulizer to exit from the hollow glass ball within the nebulizer. Fifty milliters of the zinc ammonium sulfate solution dripped at a constant rate, determined by the varistaltic recirculating pump, onto the outer surface of the hollow glass ball. The zinc ammonium sulfate aerosol formed as the solution passed over the orifice through which the generating air exited the hollow glass ball. The resultant aerosol impinged on a second smaller solid glass ball at a distance of 3/16 inch from the orifice in the first ball to produce a respirable particle size. The zinc ammonium sulfate aerosol was driven through a glass column by a stream of diluting air at a constant rate into a 44 liter Pyrex bell jar. The aerosol was exhausted through an outlet at the bottom of the chamber door into a water trap.

An airtight, easy access into the chamber was provided by fastening a 1/2 inch thick lucite plate to the chamber with 16 1-3/4 inch x 1/4 inch hex bolts. The bolts were fixed in 3/4 inch x 1-1/2 inch lucite expoxied to the outer circumference of the chamber. Two rubber tubing 0-rings insured an adequate seal.

The zinc ammonium sulfate aerosols were generated by nebulizing a 5.00 g/100 ml zinc ammonium sulfate solution. An aerosol sample drawn at (rabbit) nose level was bubbled, at a rate of 960 ml/minute, through a 100 ml 7.5×10^{-7}

hydrochloric acid (HCl) solution for 5 minutes. A dilute acid solution was necessary to prevent absorption of the zinc onto glass surfaces of the aerosol bubbler. The zinc ammonium sulfate concentration was determined from a 20 ml sample of the bubbled dilute HCl, containing trapped zinc ammonium sulfate, by atomic absorption spectrophotometry (University of Arizona Analytical Center). The concentration of zinc in the 20 ml sample was read from a standard curve, made with each atomic absorption run, of concentration of standard versus peak height from the atomic absorption readout. The amount of zinc ammonium sulfate ($ZnSO_4(NH_4)_2SO_4 \cdot 6H_2O$) in the sample was determined from:

$$\mu g \text{ zinc ammonium sulfate} = \mu g \text{ Zn/ml} \times \frac{\text{FW zinc ammonium sulfate}(401.66)}{\text{FW Zn } (65.37)}$$

$$= \mu g \text{ Zn/ml} \times 6.144$$

Aerosol concentration was adjusted by manipulating the air flow rates into the nebulizer and thence into the chamber. An increased generating air flow and/or decreased diluting air streams produced the higher concentrations. Conversely, a decrease in generating air flow rates and/or increase in the diluting air flow rates produced a lower concentration. Increasing zinc ammonium sulfate solution concentrations also increased aerosol concentration.

The aerosol particle size (Mass Median Diameter) was determined using an eight-stage cascade impactor. The zinc

ammonium sulfate aerosol was drawn through the cascade impactor at a rate of 735 ml/minute for 15.0 minutes. The effective cut-off diameter for each stage was determined by Gomez (1978):

$$\text{Effective cut-off diameter} = 1265 \times \frac{W^3}{F}$$

where: W = hole diameter in centimeters

F = flow rate in cubic cm/minute

Zinc ammonium sulfate accumulation on each stage was determined by atomic absorption. The percent cumulative distribution was plotted against the effective cut-off diameter. The 50% intercept is equivalent to the aerodynamic mass median diameter (AMMD). The AMMD was converted to the Mass Median Diameter (MMD) by:

$$MMD = \sqrt{\frac{AMMD}{\rho} + 0.01} - 0.1$$

where ρ = solution density 1.018.

Results. A 5.00 g/100 ml zinc ammonium sulfate solution at three different combined generating and diluting air flow rates gave average aerosol concentrations of 8.65, 94.17, and 159.06 mg/cu m zinc ammonium sulfate. At 8.65 mg/cu m, a particle size of 1.41 micron was obtained; while at 94.17 mg/cu m, a particle size of 1.19 micron was obtained. No data was obtained for the highest concentration aerosol. The complete results are given in Appendix A.

Comment. A usable range of zinc ammonium sulfate aerosol concentrations with respirable size particles can be

produced using a nebulizer. Since particle size drops with increasing air flow rates, the particle size range at the highest concentration obtained with the 5.00 g/100 ml zinc ammonium sulfate solution will contain a higher proportion of submicron particles than the lower concentration aerosols, considerably changing deposition characteristics in the respiratory tract of the exposed animal. To generate particles of a size comparable to that obtained for the two lower concentrations, while maintaining a higher total aerosol concentration, it might be necessary to increase concentration of the zinc ammonium sulfate solution and use the same diluting and generating air flow rates previously used to produce the mid-range, 94.17 mg/cu m aerosol.

Nebulizers characteristically produce heterogenous droplets which evaporate and leave heterogenous particle residues. Ideally for a toxicologic study, it is desirable to have an homogeneous solid aerosol. However, by inserting an impactor device downstream (Amdur and Corn, 1963), it is possible to remove the largest droplets from the droplet spectrum produced and thus narrow the resulting particle size distribution. This would be an advisable modification of the apparatus in Figure 4 since the concentrations of zinc ammonium sulfate on the various stages of the impactor showed a wide spread of particle sizes.

Concentrations obtained in the chamber are reported as averages because a consistent observation was a markedly lower concentration during the first 5 minutes of aerosol generation, rising to a constant level by 15 minutes. This is due to the necessity for, first, mixing of the aerosol with the ambient air of the chamber and, second, due to surface effects (Silver, 1946) of the chamber on the aerosol. Silver (1946) therefore recommended that animals to be exposed should be introduced into the chamber through a door after the desired aerosol concentration has been established. Opening the door results in a decrease in chamber concentration, but this can be minimized by higher rates of air flow, use of the smallest possible door, and expeditious placement of the experimental animal in the exposure chamber. It is also important that animal volume not be greater than 5% of chamber volume.

Modification of the aerosol apparatus in Figure 4 with an impactor downstream from the nebulizer, inclusion of a quick opening door (other than the present chamber seal) and observation of the precautions noted by Silver should make this exposure system operable with comparative efficiency.

Irritant Sulfates and Synergistic Effects

All estimated injury by urban sulfur oxides is due to chronic exposure in conjunction with moisture,

particulate, and other gases. These synergistic mixtures are found in ambient air, especially in the vicinity of fossil fuel flues. At concentrations above present threshold limit values they can form acute irritants. Table 2 lists the present air quality standards for these pollutants singly.

Some studies of mixtures of gases have been carried out, for example, ozone with nitrogen dioxide (Murphey et al., 1964). Most often the effects of mixtures appear to be simply additive or substitutive.

A strong trend toward realistic pollutant exposure is readily detectable in the literature. A review by Heiman (1967) cites a number of animal studies of naturally occurring atmospheres through 1966. The sometimes equivocal results of epidemiologic studies on smogs since 1966 (Environmental Protection Agency, 1974; French et al., 1973) makes it obvious that some caution is ncessary before attempting to perform "smog" exposures because it is rarely possible to select the degree to which each component of the atmosphere contributes to observed effects. Atmospheres certainly vary from one location to another; in chronic studies the constituents may vary considerably from time to time in the same location. Therefore, Casarett (1971) suggests that a degree of realism may fruitfully be abandoned for controlled studies of mixtures of compounds approximating real

Table 2. U. S. national ambient air quality standards.[a]

Pollutant	Primary Standards	Secondary Standards
Sulfur Oxides (Sulfur Dioxide)	(a) 80 micro g/m^3 (0.03 ppm) annual arithmetic mean	(a) 60 micro g/m^3 (0.02 ppm) annual arithmetic mean
	(b) 365 micro g/m^3 (0.14 ppm) maximum 24-hour concentration not to be exceeded more than once a year	(b) 260 micro g/m^3 (0.1 ppm) maximum 24-hour concentration not to be exceeded more than once a year
Particulate Matter	(a) 75 micro g/m^3 annual geometric mean	(a) 60 micro g/m^3 (0.02 ppm) annual geometric mean
	(b) 260 micro g/m^3 maximum concentration not to be exceeded more than once a year	(b) 150 micro g/m^3 maximum concentration not to be exceeded more than once a year

[a]Source (Clean Air Act, 1971).

Note: Congress in process of preparing revision.

atmospheres and a comparison with effects noted from exposure to the individual components.

Gases and vapors are distributed along the respiratory tract in proportion to their solubility in the respiratory tract fluids lining the airspaces (Green, 1974). Soluble gases, such as SO_2, tend to be totally absorbed in nasal passages, while insoluble gases, such as ozone and nitrogen dioxide, penetrate along with the respiratory gases to the respiratory bronchioles and alveoli. This simplistic concept is complicated by the interaction of soluble gases with accompanying particulates, present as both solid particles and liquid droplets, and again by chemical interaction of component gaseous materials. The pioneer work of Amdur and associates with aerosols and SO_2 is of paramount importance because particulate material is always present in the atmosphere and its role in the effects of air pollution is therefore accountable in inhalation toxicology studies if the studies are to be interpreted in relation to the real environmental circumstances (Amdur, 1957; Amdur and Clayton, 1969; Amdur and Corn, 1963; Amdur and Underhill, 1968).

As discussed previously, matters are complicated even further by air pollution components, such as SO_2, which undergo transformation in the environment, including oxidation to sulfates, liquefaction to micronic particulates, solubilization in water and other vapors, and adsorption on

solid particles. According to Green (1974) such transformation processes greatly alter the characteristics of the airborne exposure and the ability of a soluble gas, such as sulfur dioxide, to penetrate to the depths of the lung as a sulfate particulate or as a solubilized gas or as a vapor absorbed on a particle.

The fate and effects of sulfur dioxide have been studied to a greater extent than those of any of the other pollutant gases. SO_2 reaching the lungs diffuses rapidly to the bronchial walls and its concentration rapidly diminishes with depth (Lippmann and Altshuler, 1975). SO_2 can be delivered to the bronchioles and alveoli when adsorbed on inert particles which are small enough to reach and deposit in these airways (Amdur and Underhill, 1968). "Among the particles to be found in the atmosphere, carbon is most common, and plentiful, but metallic substances presenting in smaller concentrations are of overriding significance" (Casarett, 1972, p. 13). Aerosols of soluble metallic salts known to catalize oxidation of sulfur dioxide, such as iron, manganese, vanadium, and presumably other transition metals, to sulfuric acid, which would become liquid droplets when entering the moist respiratory tract, have produced major potentiation of the response to sulfur dioxide, whereas insoluble oxides of these same metals were inactive in potentiating the irritant effect (Amdur and Underhill, 1968).

Many of these metals, such as lead, titanium, cadmium, aluminum, selenium, arsenic, zinc, nickel and chromium, are found concentrated 100- to 1000-fold in submicron-sized particles of fly ash (Davison, Natusch and Wallace, 1974). These inert particles may serve as nuclei for sulfate particulates or may be inhaled simultaneously with sulfate aerosols (Charles et al., 1977).

Toxicologic studies, single, short-term and repeated, long-term inhalation exposure of animals to single and binary combinations of sulfur dioxide with particulates and several sulfate compounds demonstrated that sulfur dioxide alone and at ambient levels, was only a mild respiratory irritant (Alarie et al., 1970; Alarie et al., 1973; Amdur and Clayton, 1969; Amdur and Underhill, 1968; Speizer, 1969), whereas sulfate and sulfuric acid aerosols are more potent irritants (Amdur, 1971; Amdur and Clayton, 1969).

The combination of sulfur dioxide with other aerosols has been proved injurious to the lung, causing changes in upper respiratory flow resistance, decline in ventilatory capacity, increased respiratory rate and lowered arterial oxygen tension (Alarie et al., 1970). The concentrations of sulfur dioxide alone required to affect flow resistance in human subjects are at least several times higher than those found in the atmosphere (Amdur and Underhill, 1968; Andersen et al., 1977; Frank et al., 1962; Speizer and Frank, 1966).

Increased pulmonary flow resistance reflects the broncho-constrictive effects of the pollutant.

The irritant properties of sulfuric acid and the sulfate particulates relate to particle size, duration of exposure, total dose, relative humidity, and temperature (Amdur and Clayton, 1969; Amdur and Corn, 1963; McJilton and Frank, 1973; Pattle, Burgess and Cullumbine, 1956). Furthermore, it has been shown that sulfuric acid aerosol produces significantly greater lung injury and decrements in respiratory function than any combination of sulfur dioxide and other contaminants, e.g., fly ash emitted simultaneously from power plant flues (Alarie et al, 1973; Amdur, Schultz and Drinker, 1952; Pattle, Burgess and Collumbine, 1956).

Although sulfite ion and sulfurous acid have previously been discussed and dismissed as no more than intermediates in the oxidation of sulfur dioxide to sulfate or sulfuric acid, Hansen et al. (1974) believe that Fe(III)-$SO_3^=$ and Cu(II)-$SO_3^=$ complexes may play an important role in respiratory health problems. Hansen et al., postulate that formation of these complexes by Fe(III), Cu(II), and presumably other transition metals, may account for synergistic effects of sulfur dioxide and aerosols.

Amdur, in a careful review of all literature available up to 1969, states: "If sulfur dioxide present as an air pollutant remained unaltered until removed by dilution,

there would be no evidence in the toxicological literature suggesting that it would be likely to have any effects on man at prevailing levels (Amdur and Clayton, 1969, p. 638)." Unfortunately, sulfur dioxide emerges from sources jointly with particulates, other noxious gases, and moisture. Thus, in establishing air quality standards, it is insufficient and improper to regard the reported effects of sulfur dioxide gas alone as the sole criterion upon which to base air quality standards. For protection of the public health, the standards must be set with due consideration to the potential of sulfur dioxide under certain atmospheric conditions to form these more irritant particulate materials (sulfuric acid and particulate sulfates). The importance of this cannot be emphasized too much, as the following discussion of respiratory disease related to sulfate pollution illustrates.

Sulfate Pollution in Relation to Pulmonary Disease

Airborne pollutants are potentially responsible for more of our ills than are water- and food-borne together. As a rule, pollutants express their effects seldom singly but through interaction with other pollutants or with some preconditioning factor(s) within the host (Stokinger, 1969). Table 3 is a partial list of preconditions which, according to a U. S. National Academy of Sciences study (Carnow and

Table 3. Populations at high risk due to SO_2 and particulate because of pulmonary deficiencies.[a]

Genetically Deficient
Asthma
Cystic fibrosis
Cystic disease of lung
Chronic Disease
Chronic bronchitis
Emphysema
Kyphoscoliosis
Advanced tuberculosis
Bronchiectasis
Other Environmental Factors
Cigarette smoking
Workers exposed to dusts, fumes, etc.
Others
Prematures
Newborns
The obese

[a]Source (Carnow and Meier, 1973).

Meier, 1973), are to be considered especially vulnerable to the effects of sulfur dioxide.

A major contribution of the effects of sulfates and ozone is seen as a break in the body defenses, producing acute disease in concert with infectious agents, viruses and bacteria (Anderson et al., 1977; Coffin and Gardner, 1972). Also, chronic effects on the airways, producing perhaps defensive responses to the sulfate compounds, results in irritation of the mucous membranes leading to narrowing of the lumen of the bronchi due to edema, mucous secretions and impairment of the function of the ciliary apparatus (Alarie et al., 1973; Hirsch, Swenson and Wanner, 1975; Wolff et al., 1975).

Whether the reactions are classified as acute or chronic, there is general agreement that there is a relation between air pollutants and chronic bronchitis, atmospheric asthma, lung cancer, pulmonary edema, and increases in respiratory infections (Kilburn, 1975; Meyer, 1977). These adverse health effects should be attributed to suspended sulfate levels rather than observed concentrations of sulfur dioxide and suspended particulates because of the consistency of the relationship between symptoms aggravation and sulfate levels, and the lack of consistency of this relation with other pollutants (Environmental Protection Agency, 1974).

The findings in French and associates' studies (1973) clearly demonstrate that ambient air pollution contributes to increased frequency and severity of acute respiratory disease in human populations. Sulfur dioxide and suspended sulfate air pollution was linked to excess morbidity for the acute respiratory disease group as a whole and for both upper and lower respiratory tract infections. The observation that excessive acute respiratory disease was largely restricted to those individuals who were exposed to heavy pollution for a minimum of three years may indicate that an acute effect may be a manifestation of chronically impaired resistance to disease. Other researchers confirm that increased atmospheric concentrations of sulfur dioxide and suspended sulfates are clearly associated with an excess frequency of respiratory illnesses in children (Chapman et al., 1974; Hammersmith et al., 1974; Shy, 1974). Although other factors such as socioeconomic status and cigarette smoking contribute to excessive, acute respiratory disease, the effect of pollution was found to be independent of these variables (French et al., 1973).

Thus, the weight of evidence is in favor of a correlation between sulfate and sulfur dioxide pollution and several forms of respiratory disease: chronic respiratory disease, aggravated lung disease, asthma attacks, and children's lower respiratory disease.

Asthma

Asthma, defined as intermittent, reversible airways obstruction, is a syndrome that represents, at a clinical level, a response of the airways to noninfectious immunogenic particulates, infectious agents with immunogenic potential and organic and inorganic irritant gases and particles (Green, 1974). A number of experimental studies with animals and humans have indicated that inhalation of sulfur dioxide, sulfates, sulfuric acid, particulates, and nitrates is capable of initiating the bronchoconstriction characteristic of asthma (Amdur and Clayton, 1969; Cohen et al., 1972; Frank, 1964; Jeffrey, Widdicombe and Reid, 1975; Pattle, Burgess and Cullumbine, 1956; Yoshida et al., 1974).

And, while many people can adjust and can continuously work with 5-10 ppm sulfur dioxide in the air, most people react to that concentration and severe bronchospasm may be the result in sensitive individuals, especially those with latent disposition to asthma and similar bronchial complaints (Amdur and Clayton, 1969; Meyer, 1977); however, no information on the duration of exposure was given in either report. Thus, sulfur dioxide must be classified as a primary irritant. Customarily, pulmonary irritants are separated into primary and secondary irritants. Primary irritants are those which are sufficiently reactive with tissue to produce pulmonary effects so marked that the

systemic actions are obscured or much less important. Secondary irritants are those whose action on tissues of the respiratory tract are less pronounced than the systemic effects resulting from the same toxicant (e.g., volatile chlorinated hydrocarbons affect the liver and central nervous system).

In contrast to the accepted relation between sulfate pollutants and asthma, Ramsey (1976) concluded that a reduction in ambient temperature and barometric pressure appears to be more instrumental in promoting tendencies to asthmatic dyspnea than do ambient exposures to air pollutants. However, sulfates interact to contribute to the detrimental effects of low temperature on asthmatics. In his analysis, Ramsey made no attempt to determine if exposure, especially to the lower respiratory tract, was increasing with decreasing temperature. In order to reduce the morbidity of asthma therefore, significant changes in air pollution will be required.

Bronchitis

Bronchitis is a localized condition with a high relative incidence due to acid gases, particulates, respiratory infection, and inclement climate (French et al., 1973; Stokinger, 1969). Sulfur dioxide not only contributes to bronchitis directly by increasing the numbers of mucous secreting cells after extended exposures (Knauss et al.,

1976), but also indirectly through contributing to childhood respiratory illnesses which predispose children to develop chronic bronchitis in later life (French et al., 1973). A generally deleterious effect of sulfur dioxide upon elimination of dust from the lungs may also contribute to the genesis of bronchitis (Einbrodt, 1975).

Emphysema

Emphysema is a general condition with a medium relative incidence due to airborne respiratory irritants and familial tendency. Always elusive and consequently overlooked because of its complexity, heredity, in altering susceptibility, looms as a governing factor in diseases influenced by environmental pollutants (Stokinger, 1969). This is so because environmental pollutants exist in such extremely low concentrations, the hereditarily hypersusceptible are the most prone to react. At least as far as the familial form of pulmonary emphysema is concerned, the tendency to acquire it has been traced to a genetic defect expressing itself as a serum antitrypsin deficiency. This deficiency is expressible at an early age (27 to 35 years) in the homozygous without stimuli from environmental pollutants. However, it becomes manifest in the heterozygous upon exposure to respiratory irritants (Stokinger, 1969). In addition to this, Stokinger (1969) reports that special units for the management of emphysema were doubled in 1967 in the

52 Veterans Administration hospitals in the United States as a reflection of the rate at which the disease is increasing. Of course, not all of this increase is due to pollution, much of it is caused by smoking. Emphysema will be discussed further in Chapter 3 under macrophages in relation to pulmonary disease.

Lung Cancer

When considering pollution-related development of the more serious chronic diseases, cancer appears to be the most important. Epidemiological evidence, although well documented with regard to cigarette smoking, is largely lacking in relation to air pollution, this despite an increasing mortality due to lung cancer (Laskin et al., 1975).

The induction of lung cancer in animals has been demonstrated using sulfur dioxide in combination with benzo-[a]pyrene, a known inhalation carcinogen (Laskin et al., 1975). However, sulfur oxides are not, in themselves, thought to be carcinogenic (Gardner, 1966; Natusch, 1978) and there is some dispute over the carcinogenicity of benzo-[a]pyrene in the urban atmosphere (Shy, 1974).

Sulfur oxides are, however, quite reactive and are known to react with, for example, polycyclic aromatic species and to promote lung damage when associated with airborne particles derived from high temperature combustion or smelting operations, e.g., automobile exhaust and blast

furnace dusts (Natusch, 1978). Probably most important is the fact that sulfur oxides are emitted in high concentrations with, and react with several known and suspected carcinogenic transition metals that are highly concentrated in respirable fly ash particulate from fossil-fueled power plants (Natusch, 1978). Table 4 compares concentrations of these metals and sulfur oxides in urban and rural atmospheres.

Conclusion

Many of the conditions attributed to exposure to environmental pollutants are either accelerations of the aging process or are associated with aging. This is particularly true of the air pollutants; ozone and photochemical oxidants merely add to and hasten the oxidative destruction of the lung (Stokinger, 1969); respiratory irritants hasten the onset of emphysema, bronchitis, and appear to promote cancer of the respiratory tract, all diseases of aging man (Meyer, 1977). That these problems are common to man, especially aging man, is undoubted; however, the question arises as to whether these respiratory ailments would exist at the same high levels free of the complicating factors introduced by respiratory irritants.

At present, without an improved perspective, only the crudest estimate of the total over-all effect is possible. Morbidity data are lacking, and mortality and

Table 4. Concentrations of known and suspected inorganic cases and particulate carcinogens in urban and rural atmospheres.[a]

Substance	Status	Urban Air Range ng/m^3	Urban Air Average ng/m^3	Rural Air Range[b]
Cd	Recognized	4-250	10	--
Co	Suspected	0.5-15	2	< 0.5-2
Cr	Recognized	5-120	15	< 1-10
Cu	Suspected	10-4000	60	1-280
Fe	Suspected	1000-2000	1400	10-1000
Ni	Recognized	10-1000	100	< 10
Pb[c]	Suspected	500-3000	1500	10-100
SO_x[d]	Reactant[e]	20-1200	70	0.1-5
SO_4^{2-}	Suspected	1000-100,000	5000	--
V[f]	Suspected	50-2000/1-100	500/10	< 1-50

[a]Source (Natusch, 1978).

[b]Most values represent 24-hr. averages established over periods ranging from several days to one or more years.

[c]Values listed for lead refer to concentrations in countries utilizing lead alkyl gasoline additives. Significantly lower values are encountered in countries which do not utilize leaded gasoline.

[d]The only inorganic case (gas) listed, measured in $\mu g/m^3$.

[e]Natusch (1978) defines reactant as those substances considered likely to be involved in chemical reactions which may result in the production or removal of carcinogenic species or which may interact synergistically with known carcinogens.

[f]The two sets of values refer, respectively, to urban areas where considerable use is made of fuel oil for power generation and domestic heating, and to urban areas where oil burning is minimal.

longevity data relate to all causes, not to just pollutant effects. We have thus, only the few following signposts (Stokinger, 1969). There is no evidence of any marked increase in recent years in longevity in the United States, particularly for males (who have the greatest exposure to pollutants) although more people are living to riper years. Those people with the greatest longevity are not urban dwellers, who constitute more than 60% of the American population (Stokinger, 1969). How much pollution contributes to this ceiling on longevity for city dwellers and how much nervous tension, relative lack of exercise and city habits of excessive smoking, drinking and eating contribute cannot obviously be disassociated at this time.

Because long term animal and human exposures by pollutants are generally necessary to produce clinical lung disease, the need for <u>early</u> detection of lung response is enhanced. If the lung macrophage system responds early in the exposure, as postulated by Clayton, Fernando, and Behnke (1976), then the biochemical activity related to macrophage function should be indicative of potential lung disease. This possibility will be explored further in Chapter 3.

CHAPTER 3

LUNG MACROPHAGES

"With an alveolar surface of 1 m^3/kg body weight, the lung has the largest surface of the body exposed to the environment (Witschi, 1976, p.267)." And, due to the airborne nature of sulfate compounds, the primary route of exposure of such compounds is by this large respiratory surface area.

Lung macrophages occupy a pivotal position in maintenance of alveolar function. The macrophages are free cells located on the surface of the pulmonary alveoli. The response of alveolar cells to inhaled aerosols is central to an appraisal of at least two major processes (Casarett, 1971:

1) Alveolar changes in cellularity and function are intimately associated with the processes and mechanisms of clearance of particles from the lungs.

2) Early changes in alveolar cells may be considered as initial events related to the etiology of ultimate pulmonary effects (e.g., overt pathologic states).

Lung macrophages are involved in both of the above processes to a great extent; just how great an extent has not yet been fully explored. Their principle activity is

alveolar clearance to maintain a sterile surface for gas exchange. If the deposition of particles in the alveolar zone overwhelms the macrophages, toxic materials may persist for long periods due to various mechanisms, e.g., physical, or chemical binding (Kilburn, 1975).

Many aspects of particle-cell interaction, especially in relation to alveolar injury, are most profitably and conveniently approached through use of an *in vitro* system as will be discussed later in this chapter. Lung macrophages are recommended for use in such systems owing to the ease with which they may be obtained and cultured, and because they are more germane to research on pulmonary injury than the frequently used peritoneal macrophages (Dannenberg and Burstone et al., 1963).

Origin and Morphology

The origin of lung macrophages is still being debated. Some authors (Casarett, 1972; Green, 1974; Moore and Schoenberg, 1964) still favor a mixed origin; from both the bone marrow promonocyte via circulating monocytes and locally derived from lung epithelium. Evidence for the latter source is mainly theoretical. Whereas the generally accepted bone marrow hypothesis is proven by several studies using chromosome markers (Brunstetter et al., 1971; Virolainen, 1968) and differential irradiation of bone marrow and lungs and tritiated thymidine labelling (Velo

and Spector, 1973) demonstrate that lung macrophages are all derived from hematopoietic tissues.

Bone marrow promonocytes rapidly replicate and give rise to monocytes, which are released a short time later into the peripheral blood stream to travel to the pulmonary interstitial tissue where they mature in 2 or 3 periods of mitosis (approximately 3 days) into a much larger cell, the lung macrophage, with a relatively long lifespan of more than 60 days (Allison, 1975; Bowden, 1973; Cohn, 1972; Velo and Spector, 1973). Figure 5 outlines the lung macrophage compartments. Whether the macrophage divides in the lung once it has differentiated is as yet undetermined. It is agreed that the lung macrophage is the final cell stage, "the ultimate scavenger of the respiratory system," and is destroyed and cleared from the body at the end of its useful lifespan.

During the maturation process the lung macrophage develops a more complicated ultrastructure. On stained smears mature lung macrophages are large, irregularly-shaped cells, about 15-30 μm in size. The larger cells have an indented nucleus and the smaller have a round nucleus with a few indistinct nucleoli (Bowden, 1973; Virolainen, 1968). Initially the cells are rounded or have few pseudopods, but, due to their long lifespans, the majority of macrophages present are mature with pronounced membrane ruffling along

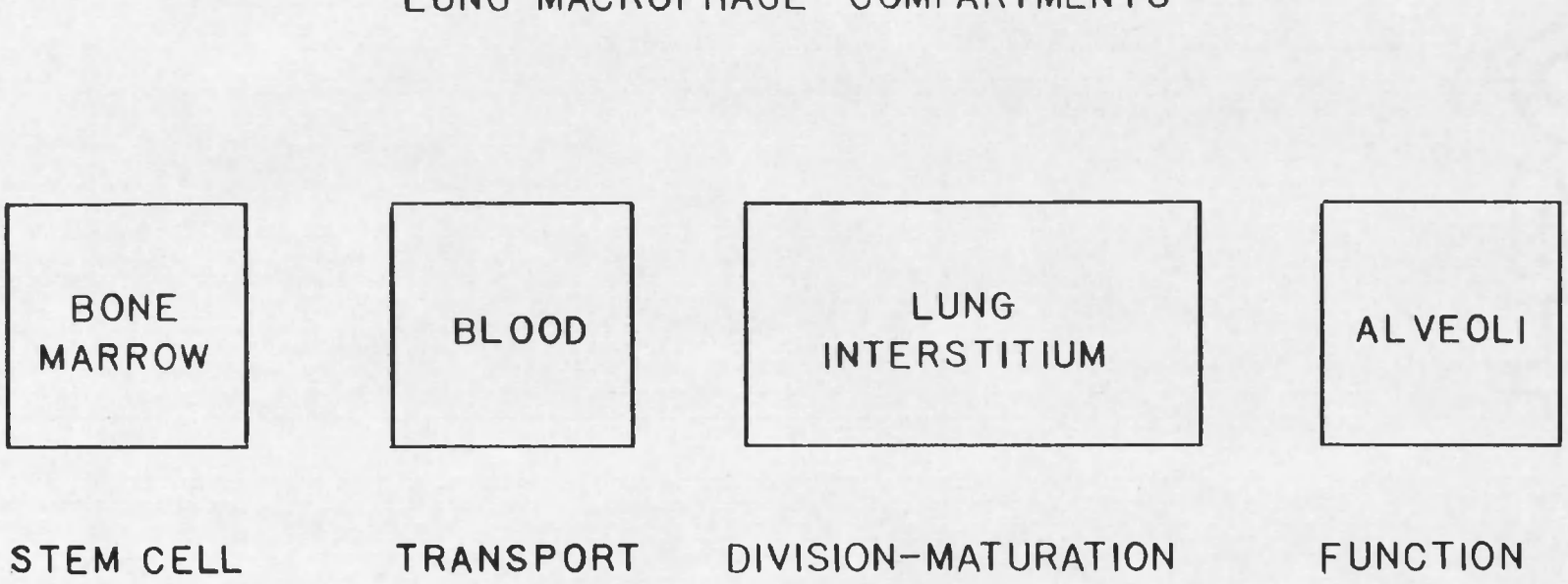

Figure 5. The pulmonary interstitial cells provide an essential link between the circulating monocytes and the alveolar macrophages.[a]

[a]Source (Bowden et al., 1969)

the cell margin and/or slender cytoplasmic projections which aid in phagocytosis (Fedorko and Hirsch, 1970; Virolainen, 1968). In fact, some investigators have observed the presence of pulmonary surfactant in lung macrophages. It is thought to be there because of an excess or due to loss of surfactant activity (Zeligs et al., 1977). Lung macrophages are facultative aerobes with a constant oxygen uptake under aerobic conditions and increased levels of glycolysis under anaerobic conditions (Harris and Barclay, 1955).

Lung macrophage function, in general, is considered to be the intracellular breakdown and disposal of particulate elements. In this regard, they have been shown to contain a wide variety of hydrolytic enzymes which are normally contained within numerous cytoplasmic granules or lysosomes. The major acid hydrolases, which have been isolated from rabbit lung macrophages, are lysozyme, acid phosphatase and, to a lesser degree, β-glucuronidase. Cathepsin, lipases and esterase are also present, but no importance has as yet been attached to them (Cohn and Wiener, 1963a and 1963b; Gordon and Cohn, 1973; Heise, Myrvik and Leake, 1965; Myrvik, Leake and Fariss, 1961b). A dose-related increase for both lysosomal and non-lysosomal enzymes, with secretion, has been shown by several investigators upon in vivo or in vitro stimulation (Davies, Page and Allison, 1974; Gordon and Cohn, 1973; Heise, Myrvik and Leake, 1965). One of the most

important non-lysosomal enzymes is elastase because of its role in the etiology of emphysema (Ackerman and Beebe, 1974).

Lung macrophages are continually mobilized into the alveoli in response to inhalation of dust, bacteria, particulates, irritants, and possibly to extravasation of the occasional erythrocyte. These cells move slowly across the alveolar surface due to a large pseudopodial surface area closely applied to the epithelial surface of the alveolus. Therefore, they are not quite "free" cells, although they are easily removed from the lung by lavage (Brain and Frank, 1968; Myrvik, Leake and Fariss, 1961a) and possess a large migration capacity. In addition, lung macrophages form monolayers on glass due to their strong binding capability for glass (Bowden, 1973; Cohn and Benson, 1965a). For young macrophages, which are hard to separate morphologically from lymphocytes when lavaged, monolayer formation is one of the best means of identification.

The lung macrophage possesses a different set of attributes compared to macrophages from other sources. For example, the ultrastructure of the mature lung macrophage reveals more lysosomes and less endoplasmic reticulum and golgi apparatus than peritoneal macrophages. This reflects the different bias in activity of these two related cells.

As shown in the rabbit, lung macrophages exhibit a moderate degree of functional heterogeneity due to:

1) Phase of cellular activity (Bowden, 1973).

2) State of maturation. Both young and old cells will be present, but response will be determined by older cells because of the long lifetime of the lung macrophage.

3) State of health, including past disease states, nutrition and current health.

4) Previous history, including pollutant exposure, both gaseous and particulate (Knauss et al., 1976; Murphey et al., 1975).

In its activity as a part of the reticuloendothelial system, the lung macrophage performs many functions:

1) Phagocytosis of exogenous particulate.

2) Phagocytosis of degenerative cell products.

3) Synthesis and secretion of enzymes.

4) Immune response.

5) Pinocytosis.

Some of these activities will be examined in more detail in the following discussion.

Endocytosis

Lung macrophages represent the most critical cells governing the pulmonary response to large numbers of noxious environmental inhalants. The response of these cells depends on their primary function as phagocytes; which has long been recognized as depending on macrophage motility and ability to ingest particles and fluid. However, only during the

past few years has information been obtained about the underlying mechanisms.

Generally the endocytic response involves both the number and the distribution of macrophages, as well as the interaction between particles and cells and concomitant biochemical changes. These changes include increased oxygen consumption, glucose conversion to carbon dioxide, increased lactic acid production, increased phosphatase activity and hydrogen peroxide formation (Dannenberg, Walter and Kapral, 1963; Gabor, Anca and Zugravu, 1975; Rabinovitch, 1968). Phagocytosis and pinocytosis are variant forms of endocytosis apparently identical in all processes subsequent to the state of membrane interiorization. Endocytic activities of the lung macrophage, described below, are presented in Figure 6.

Pinocytosis

There are two types of pinocytic activity that are seen in macrophages. One that is most evident, and is called macropinocytosis, is a type of pinocytic activity that is seen most easily in the ruffled pseudopods of the cell; it occurs when ruffled membrane comes in apposition and fuses to form a vacuole. Another type is micropinocytosis, or pit formation. The determinants of micropinocytosis are unclear. The "macro" form is the most prominent in macrophages and results in vesicles which may reach 0.8

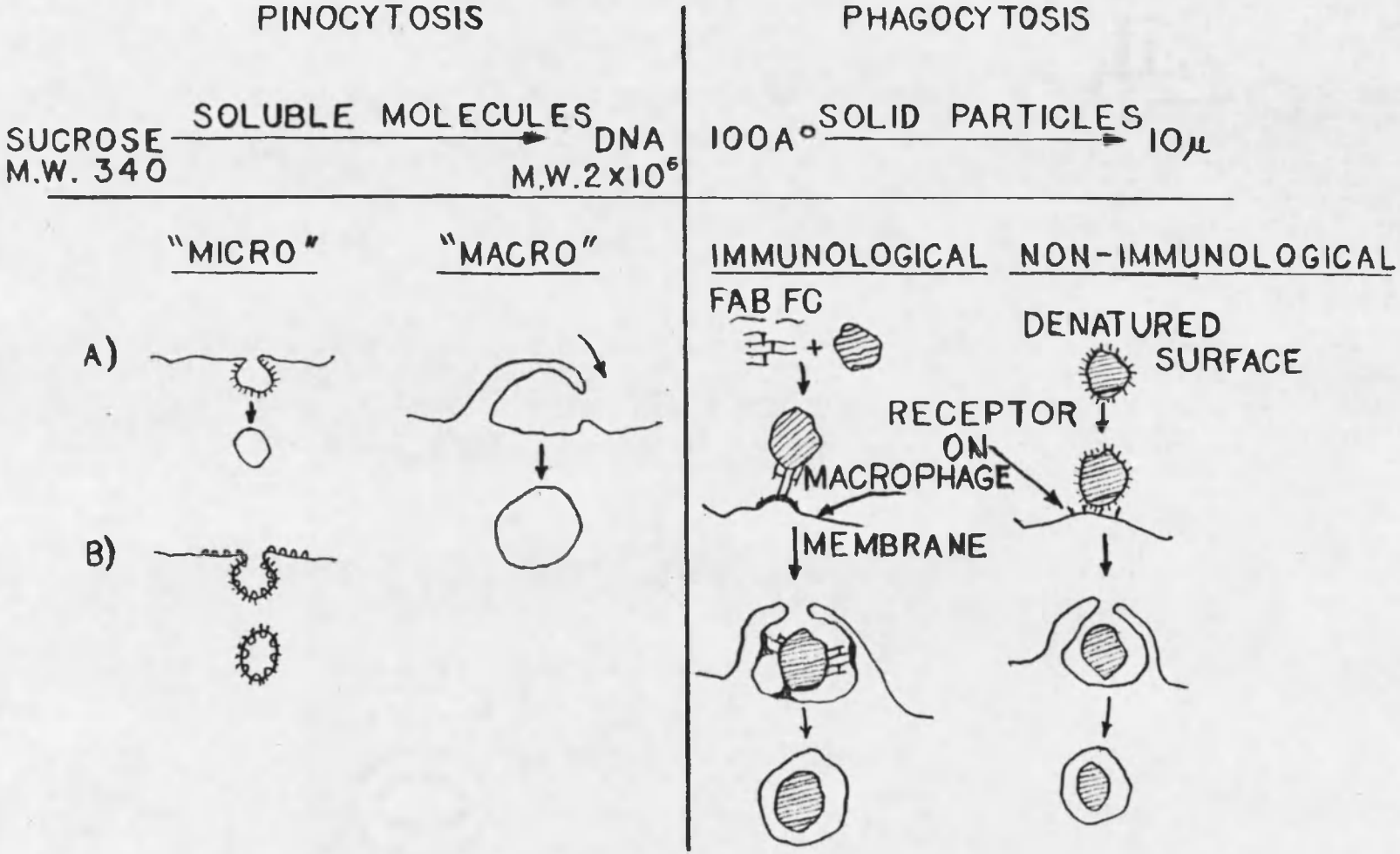

Figure 6. Endocytic activities of lung macrophages. A schematic representation of the uptake of both soluble and particulate substances by macrophages.[a]

[a]Source (Cohn, 1972).
Note: FAB and FC represent the antigen binding and FC receptor-binding, respectively, ends of an IgG antibody.

microns in diameter (Cohn, 1972). The uptake of soluble molecules depends upon a number of factors. Solutes which bind to the plasma membrane are taken up at rates which may be 1000 times greater than unbound molecules. Inadquately bound material is taken up at a rate proportional to its extra-cellular concentration. The presence of a specific Fc (fragment-crystallizable) receptor which binds IgG antibody at its Fc end aids pinocytosis. The other end of the IgG antibody is the Fab (antigen-binding) which binds to biologic materials and solid particles coated with denatured protein. Non-specific, functional receptors also are important in binding materials for uptake. The nature and topography of phagocytic receptors is poorly understood, and the term receptor is used in an operational sense.

Pinocytic activity occurs very actively in areas of inflammation, but nothing else is known about what stimulates pinocytic activity *in vivo* (Cohn, 1972). *In vitro* it is known that there is a high energy requirement due to the constantly undulating membrane, thus anaerobiosis inhibits the necessary glycolytic and respiratory pathways, resulting in reduced pinocytosis. Low temperature also prevents engulfment (Cohn, 1966) and serum proteins are required. Determination of the number of vesicles present in the cytoplasm is most conveniently made by phase contrast microscopy of glutaraldehyde-fixed cells (Cohn, 1966). This permits only

larger vacuoles to be observed. Electron microscopy is required to observe the entire size spectrum of vacuoles formed in micropincytosis.

Studies by Axline and Cohn (1970) have shown that pinocytic vesicles are converted into secondary lysosomes in the centrosphere region of the cytoplasm by fusion with golgi vesicles and/or pre-existing secondary lysosomes. In addition, Axline and Cohn demonstrated that the fusion of the secondary lysosome and endocytic vacuole is not the stimulus for lysosomal enzyme induction, but did not rule out the possibility that fusion of the primary lysosome and endocytic vacuole may act as the control site. Thus, environmental materials such as metal sulfates (soluble particulates), sulfur dioxide (soluble gases), and sulfuric acid mist (fluid aerosols) may increase the rate of pinocytic activity resulting in both increased numbers of lysosomes and increased levels of lysosomal enzyme. The accumulation of acid hydrolases in dense granules or digestive bodies during the course of endocytosis is a reversible process. The mechanism for the reduction of intracellular enzymes is not thought to depend upon the extrusion of enzyme into its environment, because Cohn (1972) could not find enough enzyme in the control medium to account for these changes. The most reasonable explanation to date is that lysosomal enzymes are themselves degraded within the confines of the digestive

vacuole. However, this may just be one phase of activity in a very complex system, because Davies, Page and Allison (1974) recorded definite extracellular increases in acid hydrolases within 12 hours of in vitro exposure to streptococcal group A cell wall substance (PPG).

Phagocytosis

Phagocytosis begins with chance contact between the inhaled particle (biologic or non-biologic) and the cell surface. Adherence of the particle to the cell membrane is the first step in the process, and this is enhanced by specific membrane binding sites and/or antibodies or opsonins coating the particle (Al-Ibrahim et al., 1976; Cohn, 1972; Green, 1974). After adherence, the particle is enveloped by cell membrane, in part by the contractile action of cytoplasmic myofibrils (Axline and Reaven, 1974). The phagocytic process at the cell surface is accompanied by bulk interiorization of large segments of the plasma membrane, a process that is dependent upon continuing membrane synthesis (Cohn, 1975). This continuing replacement of the plasma membrane is closely associated with production of digestive hydrolases by the cell (Cohn and Wiener, 1963b).

Once particles are ingested, the external membrane becomes sealed off within the cell as a phagosome. One or more lysosomes rapidly move into contact with the phagocytic vacuole and empty their acid hydrolase enzymes into the

phago-lysome. This allows for delivery of highly active enzymes to the ingested substance without subjecting the cell's cytoplasm to potentially injurious effects (Goldstein et al., 1976). The lung macrophage is particularly rich in the quantity and variety of proteolytic enzymes, as described previously, that are sequestered in the lysosomal membrane bound packets. In addition to the digestive capabilities of the lysosomal enzymes, potent bactericidal activity may be exerted through the presence of abundant hydrogen peroxidase (Green, 1974). Though active in polymorphonuclear leukocytes, the role of the hydrogen peroxide system in the lung macrophage is uncertain (Gee, 1970).

The difference in response of lung macrophages to particulate materials may be due to a number of factors:

1) Difference in sign or magnitude of charge on the surface of particles. If a similarly charged particulate is presented to the macrophage, binding may be inhibited by charge repulsion.

2) Size and shape of the particulate is a mechanical determinant of the ease of engulfment. The size of the macrophage itself dictates an upper limit to the size of the particulate that it can engulf without new membrane synthesis. In the case where engulfment can start but not be completed, premature fusion with lysosomes can occur, resulting in the extracellular release of enzymes to be discussed later.

3) Particle:cell ratio. Hahn et al. (1974) demonstrated a linear increase in measured phagocytosis over a particle:cell ratio up to about 10 followed by a diminution of effect of particle concentration. This suggests that, within the period of functional integrity of the lung macrophages, there is a limit of simultaneous adsorption of particles or a maximal rate of particle entry into the cell or both.

4) It should be emphasized that particles can interact with and be coated by small or large components of the medium, contributing greatly to the complexity of the already complex phagocytic response.

Processes which compete, particularly at high particulate loading, for the phagocytic capacity of the lung macrophage include:

1) Phagocytosis of worn out or malignant body cells.

2) Clearance of virus and bacteria organisms from infective sites.

3) Processing of antigenic materials as a prelude to immune responses.

4) Phagocytosis of competing non-antigenic foreign materials.

5) Transport of particles from the alveolus.

Phagocytosis is effectively blocked by inhibitors which inhibit glycolysis. Phagocytosis is not affected by

those agents known to decrease respiratory energy or oxidative phosphorylation. Anaerobic environment, cyanide, and Antimycin A have very little effect in reducing the phagocytosis of particles in the mouse macrophage, whereas they are effective inhibitors of pinocytic uptake in the same cell (Cohn, 1966). The mechanism of this difference in susceptibility to inhibitors is not clear. However, intense membrane activity is occurring at the site of pinocytosis. Phagocytosis is not dependent upon the same type of movement and occurs quite readily after the attachment of the particle to the membrane, which triggers the interiorization process. Inhibitors of respiration and oxidative phosphorylation are very effective in stopping membrane movement.

Clearance

It is generally agreed that lung clearance of particles is a very complex process, involving different mechamisms whereby the alveolar walls are kept clear of different inhaled or endogenous substances which otherwise could interfere with gas exchange. With rare exception, the inhalation of insoluble particulate material is followed by a measurable clearance which follows one or more exponential patterns. A generalized "classic" clearance curve is represented in Figure 7, in which four phases are depicted.

Disregarding material deposited in the nose and pharynx, phase I is generally assumed to be clearance of

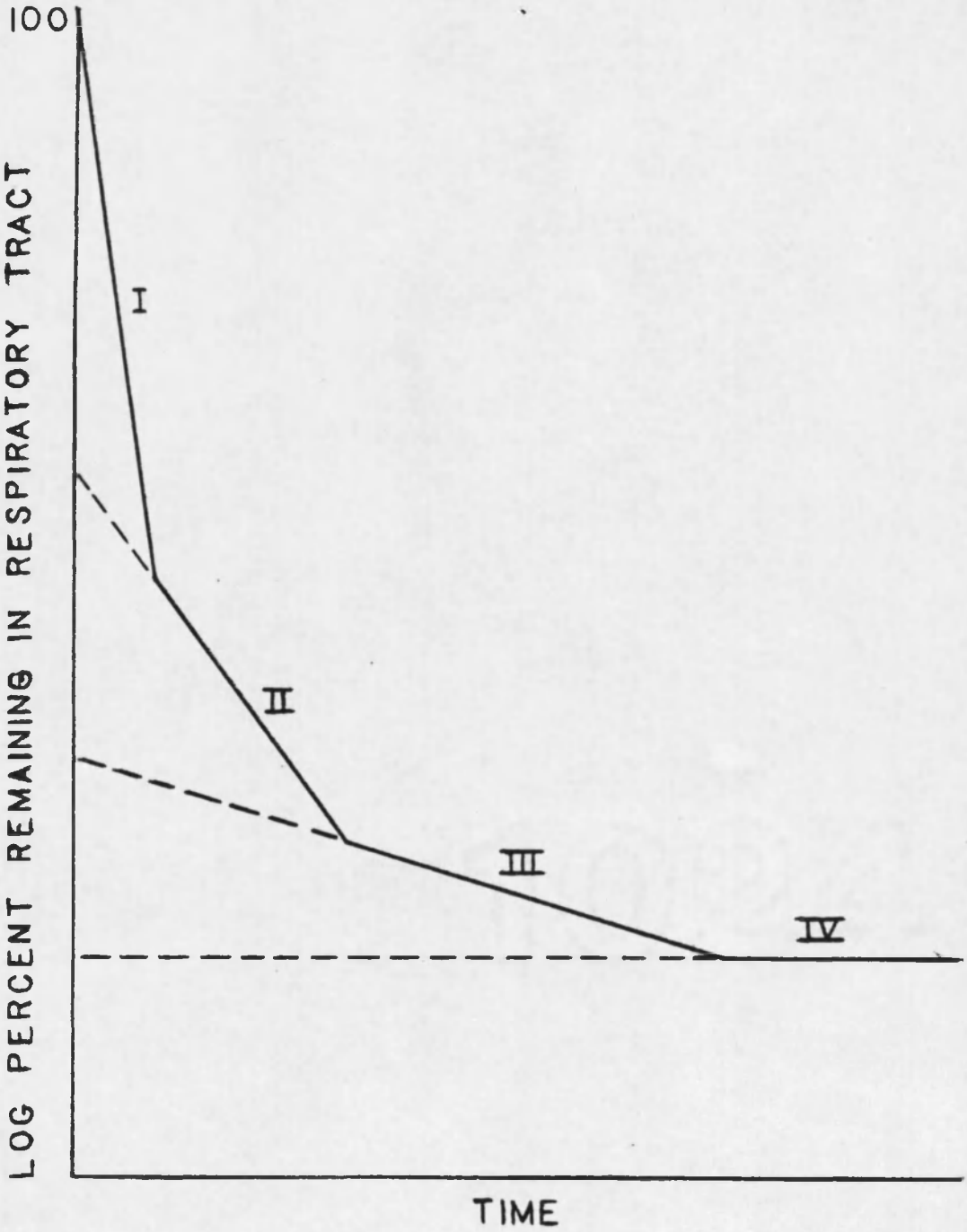

Figure 7. Stylized model of rates of clearance of inhaled particulate material from the respiratory tract.[a]

[a]Source (Casarett, 1972).

material from the upper part of the tracheobronchial tree (Ferin, 1975). The rate of clearance of this phase, expressed as a "half-time," is remarkably constant at about 12-24 hours.

With a high frequency of sampling during the initial period after inhalation, it is highly likely that three separate rates might be distinguishable (Casarett, 1972). One of these is related to a very rapid clearance of the nasopharyngeal region (minutes) and another to the clearance of material deposited in the trachea and bronchi and several generations of bronchioles (minutes to hours). A third subphase, mechanistically, is relatable to the clearance of material from the deepest bronchiolar structures (hours) with an additional contribution, perhaps, from the action of readily mobilized active macrophages (hours to days). The possible involvement of lung macrophages as part of phase I is consonant with the concept of simultaneous operation of the components of the clearance process.

The second phase is of particular interest because it is predominantly regulated and controlled by the phagocytic response and mobilization of phagocytic elements. When clearly defined from subsequent clearance, this phase can be represented by half-times most often lying roughly between two and six weeks.

Material which continues to reside in the lung may, at this stage, be considered as "sequestered." If a third phase is found, the half-time is usually of the order of months and the movement of material is largely a slower continuation of processes operative in phase II, namely, movement of particle-laden macrophages to the ciliary epithelium and a continued lymphatic uptake. During this period one may also expect some solubilization of the sequestered material subjected to body fluids (Morrow, 1973). Although other processes may continue, solubilization is probably a reasonable basis for a fourth phase if it is found and the rate of clearance for this phase is expressed in half-time values of months to years.

Morrow (1973) critically assessed various clearance concepts and reviewed the salient physiological and anatomical considerations that underly these concepts. He categorized the clearance mechanisms into those affecting the removal of the readily transportable materials, e.g., dissolved or monomeric substances, by passive and active absorption processes; and those removing the more persistent, less easily transportable materials, e.g., "insoluble" dusts, by endocytosis and dissolution. In addition to this general categorization, a number of other variables are superimposed on the curve presented in Figure 7. Some of these may relate to parameters already mentioned, such as particle size, locus

of deposition, the distribution of the mass of the aerosol in the respiratory tract, and the solubility of the compound. Other sources of variation are species, the condition of the clearance mechanisms, effects of additional materials in the aerosol (e.g., irritant gases), the total quantity of material deposited, and the reactivity or cytotoxicity of the compound.

The latter factors are of special concern. Numerous studies, previously discussed, indicate that SO_2, a highly soluble irritant gas, and sulfuric acid, an irritant aerosol, affect the mucociliary transport system adversely. Charles et al. (1977) demonstrated augmented sulfate ion absorption in the rat lung on simultaneous inhalation of heavy metals. Absorbed sulfate may result in release of histamine leading to increased bronchoconstricting activity (Charles and Menzel, 1975). In addition, if a material is highly reactive or is given in a dose which produces changes in the lung which are manifest as either hyperemia, inflammation, or transudation of fluids and proteins, this is a reasonable basis for concluding that the permeability of the alveolar structure has been altered. This is tantamount to a conclusion that clearance of particles under these conditions will be different from that which might be obtained with a non-reactive dose of the same or another kind of particle. A cytotoxic compound may influence clearance in several ways; two

examples will serve. First, a particle that, when phagocytized, has the capability to damage or destroy the cell, will decrease the functional capability of the macrophages to participate fully in the clearance process. Second, a cytotoxic compound may create a reaction in the lung (e.g., silicon dioxide) that may segregate a collection of particles in the reaction area, making them less subject to removal. Also, the appearance of dust particles in pulmonary interstitial, lymphatic, and lymphoid tissue may be evidence, a priori, for a failure of the lung macrophage to function optimally due to a cytotoxic action of the dust. Changes in dust removal are generally attributed to some action involving lung macrophages.

Particles deposited in the alveoli, which contain no cilia, must invoke other processes for removal. Several possibilities arise. For one thing, it is conceivable that particles may be moved from the alveolus to the area of the terminal bronchioles by alveolar movements during respiration, coupled with a shearing effect of the layers of secretory fluids on the surface, or an influence of the fluid movement by cilia at some distance. Having arrived at the terminal bronchiole, particles would then be subjected to movement by cilia. Thus, macrophage activity is not regarded as the singular clearance mechanism for the alveolar region. However, a dominant feature of the clearance of the

alveoli is the phagocytosis of particles by lung macrophages (Casarett, 1972; Morrow, 1973).

Following phagocytosis, the fate of the inhaled material is dependent on the disposition of the cells in which it is contained. Two prominent pathways are (a) migration (or movement) to the ciliated epithelium where they are expelled or swallowed, and (b) entry into the lymphatic system via interstitial penetration in the alveolus (Allison, 1975; Casarett, 1972). The process by which macrophages move from the alveoli to the ciliated airways is not clearly understood. Mobile phagocytic cells would be expected to effect this area transfer, at least, on the basis of random travel, and, at best, by virtue of facilitated tropism. The latter is most likely due to the presence of an alveolar fluid layer. In the latter case, then, both directional and mechanical assistance could be derived from the fluid flow. Whatever the process of lung clearance, clearly the macrophage plays an important role in particle clearance to maintain a near-sterile, functional respiratory surface. Thus, the basic interactions of particle and cell can be viewed as an important fundamental area of investigation.

In Vitro Exposure of Lung Macrophages

The interactions of macrophages with other cell types and their products in an organ such as the lung are so complex that analysis *in vivo* of macrophage activity is

difficult. The technique of pulmonary lavage makes available large numbers of lung macrophages without artificial stimulus. Normally more than 95% of the cells obtained by lavage are lung macrophages (Coffin and Gardner, 1972). It is therefore convenient to study the interactions of particles and other materials with macrophages in cultures.

The discussion in Chapter 2 emphasized the prevalence of sulfate pollution and its importance in pulmonary distress. In addition, transition series metals were discussed in relation to their synergism with sulfates. Several researchers have demonstrated the toxicity of the metal-sulfate interactions and metallic oxides (Bowden, 1973; Charles et al., 1977; Mustafa et al., 1971; Waters et al., 1975). The purpose of the study presented below was to examine the feasibility of a simple in vitro lung macrophage exposure system and to compare the toxicity of selected transition series metal sulfates and oxides as a function of time.

Methods and Materials

The stock metal solutions were MnO_2 (0.5514 g/l), and $MnSO_4 \cdot H_2O$ (0.955 g/l), Co_2O_3 (0.7944 g/l) and $CoSO_4 \cdot 7H_2O$ (1.008 g/l), $NiO \cdot Ni_2O_3$ (1.6414 g/l) and $NiSO_4 \cdot 6H_2O$ (1.01 g/l), Fe_2O_3 (0.5909 g/l) and $Fe(NH_4)_6SO_4 \cdot 12H_2O$ (0.5258 g/l), CdO (0.9124 g/l) and $3CdSO_4 \cdot 8H_2O$ (1.025 g/l), Cr_2O_3 (0.8368 g/l) and $Cr_2(SO_4)_3 \cdot xH_2O$ (0.5975 g/l),

CuO (1.8160 g/l) and $CuSO_4 \cdot 5H_2O$ (0.4726 g/l), Zno (1.0664 g/l) and $ZnSO_4 \cdot 7H_2O$ (1.01 g/l).

Lung macrophages were harvested according to the method of Myrvik, Leake and Fariss (1961a), as modified by Weissbecker et al. (1969). A schematic of the modification of the lavage apparatus to utilize gravity flow control, used in this experiment, is presented in Figure 8. Rabbits were chosen as experimental animals because large numbers of lung macrophages may be obtained from rabbits and extensive in vitro studies of lung macrophages have been performed.

The rabbit was sacrificed by 30 ml air bolus in an ear vein and the lungs immediately excised in toto with the trachea and heart. Both the lungs and liver were examined for signs of lesions that might indicate increased phagocytic activity (Rabinovitch, 1968). If lesions were observed, the lungs were discarded. Normal lungs were lavaged 4-5X with approximately, 30 ml. 37°C, Hanks Balanced Salt Solution (HBSS). The lavage solutions were combined, diluted to 150 ml with HBSS, and then added to 1 liter of Eagles' Minimum Essential Media (MEM--available from Flow Laboratories) with additives (100 units Penicillin-G/ml, 100 ug Streptomycin/ml, 0.1% L-glutamine). The cell-MEM suspension was apportioned in 20 ml aliquots to 56 previously silyated (by treatment with diphenyl dichlorosilane or dimethyl dichlorosilane) screwtop test tubes with a concentration of

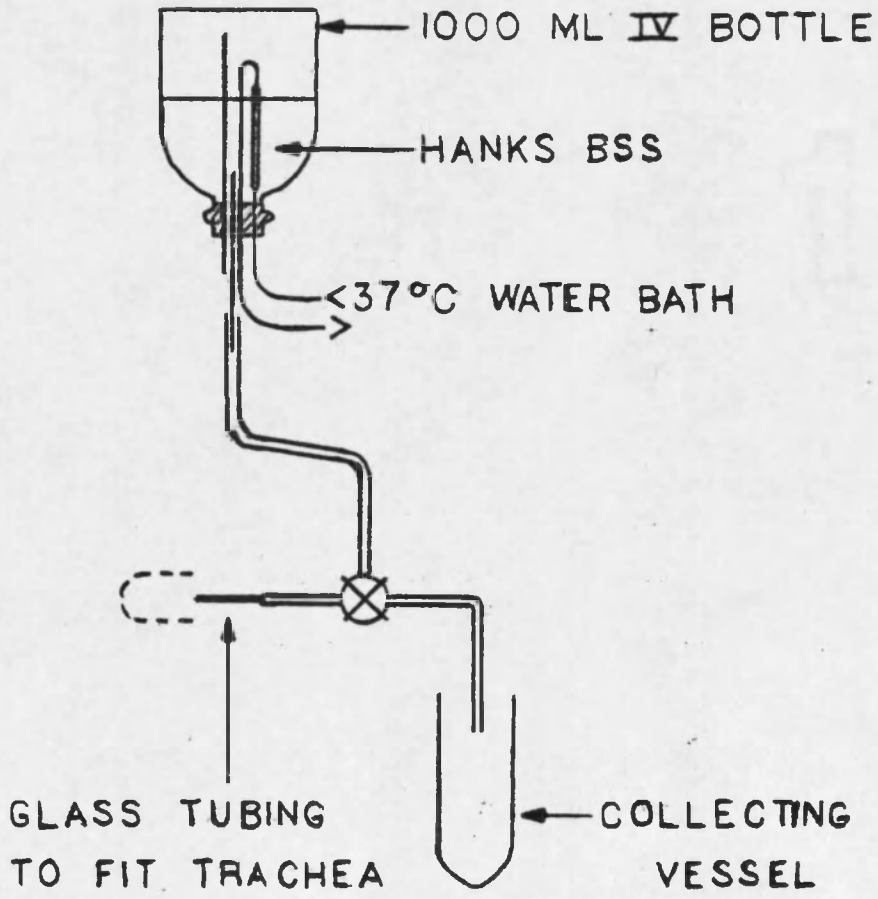

Figure 8. Constant pressure lung lavage.

Note: Gravity flow, at a 75 cm height, results in a slow uptake by the isolated lung of 30-40 ml of lavage fluid. Hanks BBS--Hanks Balanced Salt Solution (available from Grand Island Biological Company).

approximately 10 cells/ml of lavage fluid. Volumes of 0.1 ml, 1.0 ml, or 2.0 ml of the metal solutions were added and all tubes were brought to 22 ml final volume with HBSS. The test tubes were incubated at 37°C and samples removed as a function of time.

The tubes were prepared for the cell count by addition of 0.5 ml of 0.2% Trypsin (available from Sigma) to each tube upon removal from the incubator, vortexing them to mix, allowing the tubes to stand for 10 minutes to permit release of cells from tube walls and then vortexing again to resuspend settled cells. The cells were counted as rapidly as possible because exposure to the metal solutions would continue during the count and percent viability determinations, and some damaged cells might be destroyed by the trypsin. Therefore, cell concentration was determined by Coulter count electronically, rather than microscopically by hemacytometer count (Clinical Diagnosis by Laboratory Methods, 1974). The manganese and nickel exposures were counted by hemacytometer. However, the Coulter count proved to be as good as, or better than, the hemacytometer count due to the greater rapidity of performance.

Lung macrophage viability can be determined by several methods. Dye exclusion of 1% Trypan Blue according to the method of Rabinowitz (1964) has the advantages of simplicity, quick performance, reliability and relatively

little sample volume loss. By this method, viable cells actively exclude the dye; non-viable cells take up the dye and appear blue by light microscopy. One drop of 1% Trypan Blue solution per 3 drops of cell culture suspension were placed on a microscope slide, mixed, allowed to stand no more than 5 minutes and observed for morphology and percent viability on the basis of a 200-cell count of several microscopic fields.

Results

The very simplicity of this study created some difficulty in drawing valid conclusions from the data obtained. A few general observations were possible:

1) The most obvious effect of the metal sulfates seemed to be on the cell membrane, visible as cell aggregation under the light microscope. In a few cases, e.g., copper and cadmium sulfate, after longer (24-48 hour) exposures to the highest concentrations ($CuSO_4 \cdot 5H_2O$--42.96 µg/ml and $3CdSO_4 \cdot 8H_2O$--93.18 µg/ml) cell membranes appeared to disintegrate.

2) Metal oxides were phagocytosed to the point that the cell nucleus was obscured upon light microscope observation. However, no loss in viability could be determined.

3) Giant or oversize cells, 3-10 times larger than the normal cell size, were noted in the manganese and zinc sulfate exposures at high concentrations. However, from this data

it is not known whether or not this phenomena represents enhanced, normal, macrophage activity or a toxic effect.

4) Loss of cell viability was noted only (Table B.1) in the 2 ml stock solution/20 ml cell suspension, manganese sulfate tubes from 7 hours to the end of the 72-hour exposure and in the 2 ml stock solution/20 ml cell suspension, copper, cobalt, and cadmium sulfate tubes at 24 and 48 hours. Because no loss of viability could be determined in the corresponding metal oxide exposures, this would seem to indicate an added toxic effect due to the presence of the sulfate anion. The complete data are presented in Appendix B.

Comment

No reliable decrease in cell numbers could be determined for any of the exposures. But this may have been due to the use of a trypsin solution to remove adherent macrophages from the test tube walls. Trypsin is a potent protease and even in dilute solutions, it could easily destroy weakened cell membranes. This may account for the uneven distribution of exposures with few or no cells.

The cell aggregates observed could be due to a "bridging" effect by the metal ions somewhat like the "bridging" effect of antibody molecules to cause hemagglutination (lattice formation to create visible clumps of erythrocytes). If so, it would be an extremely complex reaction and proving

this theory would require much more sophisticated methods than those employed here. Also, the use of a static, round bottomed tube may have contributed to the aggregation observed, merely by allowing the macrophages to settle and stack-up on each other.

The loss of viability noted in several of the soluble (e.g., $MnSO_4 \cdot H_2O$) metal sulfate exposures could have been due to membrane effects of the metal ions (Mustafa et al., 1971) or possibly to an acid pH effect of the sulfates (Charles et al., 1977). Any attempt to distinguish between these mechanisms of cytotoxicity would require further experimentation with metal sulfates and, in addition, with metal compounds containing an "inert" anion. Possibly metal chlorides (Charles et al., 1977) would be useful because chloride anions are a normal constituent of the extracellular environment.

The acid pH observed in some exposures after longer incubation periods (48-72 hours) was attributed to bacterial contamination. This problem could be resolved by use of a 0.45 um Millipore filter and sterilization of glassware prior to use.

Several other difficulties with the experimental technique detract from the validity of the results. These include:

1) The use of a standing test tube. This allows the cells to settle and, in so doing, causes uneven exposure characteristics, e.g., those cells on the bottom receive little exposure to the test solution, while those on top receive a full exposure. Roller tube culture might alleviate the problem (Burrell and Anderson, 1973).

2) Cell counts were performed post-exposure only and compared to control cell counts. This procedure did not allow for the variability in number of cells originally inoculated. A better procedure would be to perform cell counts on each test tube prior to addition of the test solutions.

3) Too few controls were included. A media control and media plus test solution control should have been included. Then any change from optimal pH as indicated by the phenol red could have been accounted for by media breakdown or bacterial contamination.

4) Particle suspensions were not sized to a uniform range, even though Hahn et al. (1974) reported decreasing phagocytosis for particles more than 3 um in size. However, all particle suspensions were sonicated to give an even suspension. In addition to this, Casarett (1971) suggests that particles be allowed to settle until all aggregates larger than 1 um have settled out. This could be confirmed by hemacytometer to determine not only size, but also concentrations.

Experimental Modifications

Animal sacrifice via air bolus is an extremely painful procedure. Therefore, injection of sodium pentobarbital into a peripheral ear vein to anesthesize the animal would be a more humane procedure with no apparent effect on the cell population obtained. Further modifications in the lavage technique such as gas-freeing the lungs and allowing the lavage solution to remain in the lungs will maximize precision (Brain, 1970; Brain and Frank, 1968; Coffin, 1968), but are unnecessary unless an attempt to determine total numbers of lung macrophages is being made. Use of the viability index described by Waters et al. (1975), where percent viability is multiplied by the ratio of total intact cells of the test to controls, would increase the reliability of viability as a measure of cytotoxicity. Another factor that might generate some useful information would be the determination of the relative amounts of pinocytosis.

More precise data could be obtained by use of a macrophage monolayer culture system which is in general use in inhalation toxicology (Casarett, 1971: Cohn, 1966; Davies, Page and Allison, 1974; Dannenberg and Burstone et al., 1963; Virolainen, 1968). This procedure has several advantages over a cell suspension procedure:

1) No necessity for centrifugation and multiple steps in cell preparation.

2) Cell purification based on the binding properties of macrophages.

3) Even cell exposure to the test system.

4) Availability of cells for morphologic observation without mechanical disturbance.

However, a monolayer culture system is not without some disadvantages and certain precautions must be exercised:

1) Changing the media after allowing for macrophages adherence not only removes contaminating cellular debris, erythrocytes and lymphocytes, but also limits the macrophage population to the most healthy cells.

2) Axline (1970) noted morphologic alterations occurring in the cells within a few hours of culture. These changes included cell spreading, increase in size and protein content, and a progressive increase in lysosomal activity. However, Axline suggests that these changes may correlate with <u>in vivo</u> activation to become "angry" macrophages, instead of a reaction to the glass or plastic surface of the culture chamber.

3) Culture media must be strictly controlled. Cohn and Benson (1965b) demonstrated that too little serum (1%) results in decreased phagocytosis and too much (50%) results in greatly increased pinocytosis and production of increased numbers of lysosomes as well as hydrolytic enzymes. Most

researchers use 10% fetal calf serum or homolygous serum. If media is not used within 5 days of preparation, more glutamine must be added. In addition, more sodium bicarbonate may be added if the pH begins to drop (phenol red indicator is red at pH 7.2-7.4 and yellow at any pH acid to that).

4) In vitro methodology places little dependence on physical movement of macrophages for phagocytic activity but depends on membrane properties of charge or stickiness and plasticity. This may not be the case, in vivo, because of active, rather than passive movements by the cell itself (Dannenberg, Walter and Kapral, 1963).

5) Lung macrophage concentrations greater than 1-1.5 x 10^6 cells/ml in culture may suffer a loss in viability, due to inadequate nutrition, or may form an uneven monolayer.

6) The atmosphere should be humidified to prevent media concentration. Also, most researchers use a 5-10% CO_2 and air atmosphere to more closely mimic lung gas concentrations at the alveolar level.

Another in vitro method of interest is the hanging drop technique Weissbecker et al. (1969) used to expose lung macrophages to oxidant gases, including SO_2. The exposure concentration is readily controlled by this method. However, because of the small number of macrophages exposed, the sensitive enzyme indicators described below are not applicable.

Lung Macrophage-Enzyme Basis of Pulmonary Disease

The diversity of cellular defense systems in the lung and the central role of the macrophage in the response of the lung to environmental exposures suggests that these systems are of key significance in the mediation of pathologic responses to air pollution exposure. In fact, Casarett (1972, p. 26) states: "Early changes in lung macrophages may be considered as initial events related to the etiology of ultimate pulmonary effects;" therefore, the mechanisms of these responses bear significantly on our understanding of lung disease. The complex physical and biochemical processes involved in particle adherence, particle digestion, and intracellular processing make this system particularly vulnerable to a multiplicity of genetic defects and to disturbance or suppression by environmental agents such as acid sulfate aerosols. Katz and Laskin (1975) demonstrated increased phagocytosis of latex particles by lung macrophages harvested from rats exposed to 5-20 ppm SO_2 for 24 hours, whereas a 24-hour exposure to 25 ppm NO_2 depressed phagocytosis. A dose response was demonstrated because the increased phagocytic activity was noted within 24 hours of culture at 10 and 20 ppm SO_2, but was delayed 3 days in the 5 ppm exposure. A similar dose-response relationship was obtained for NO_2: exposure to 10 ppm NO_2 did not impair phagocytosis as the 25 ppm exposure did. The numbers of lung

macrophages constantly increased during the sulfur dioxide exposures suggesting that sulfur dioxide reaches the alveoli. However, the response noted could not be sustained in the majority of animals if the exposure stopped. Environmental exposure is usually much lower, but continuous.

If lung macrophages are overwhelmed either by the magnitude of inhaled aerosols or solid particles or by their inherent toxicity, the consequences to the lung and to the entire organism may be serious. Air pollutants both initiate and aggravate a variety of respiratory diseases, including the pneumoconiosis and emphysema. Patients with an inherited tendency to emphysema exhibit clinical symptoms of the condition at a much earlier age when exposed to air pollution (Stokinger, 1969). Air pollution likewise adversely affects antibacterial defense mechanisms of the lung. Patients with bronchial illnesses, such as ordinary respiratory viral infections, show increased symptoms when exposed to even customary levels of air pollution (Environmental Protection Agency, 1974; French et al., 1973). As mentioned previously, sulfur dioxide diminishes the rate of bacterial clearance by interfering with the ciliary elevator. Also, Weissbecker et al. (1969) showed that sulfur dioxide (317-19,100 ppm for 1 hour) has acute toxic effects on macrophages which could interfere with their phagocytic efficiency. Hurst, Gardner and Coffin (1970) demonstrated that 0.25-7.0

ppm *in vivo* exposures of New Zealand white rabbits for 1 hour significantly decreased acid hydrolases of lung macrophages, including lysozyme, which is one of the important bacterial substances according to Goldstein et al. (1976). These effects are logical because macrophages ingest deposited matter or droplets, so they are often exposed to higher concentrations of toxic materials than other lung cells. The consequences of chronic inhalation of substances toxic to macrophages would be expected to impair the phagocytic effectiveness of macrophages also, but it is dangerous to extrapolate from acute to chronic exposures.

The phagocytic efficiency of the lung macrophage system is related to both the numbers of resident macrophages and their functional status. A depression in quantitative efficiency can result in (a) undesirable proliferation of infectious agents in lung tissue, or (b) persistence of nonviable particles on the alveolar surface where they may exert toxic effects or penetrate beneath the epithelium where clearance mechanisms operate more slowly (Goldstein, 1976; Kavet and Brain, 1977). Despite the fact that deposition of particles in the lungs has been demonstrated to be followed by increased numbers of lung macrophages and increased levels of phagocytosis (Fairchild et al., 1975; Kavet and Brain, 1977; Labelle and Brieger, 1959) showed that sulfur dioxide and sulfuric acid decreased the clearance rates of

streptococci. Thus, decreased clearance rates and competition for phagocytosis upon simultaneous inhalation of particles and contaminant bacteria could result in greatly increased rates of lower respiratory infections as has been demonstrated in children in many large, polluted cities in the United States such as Chicago and New York (French et al., 1973).

The cytotoxic effects of air pollutants, especially particulate pollutants, is also mediated through injury to the lysosomal digestive apparatus of the lung macrophage. However, the relation between endocytosis by macrophages and increased lysosomal enzyme production remains obscure in spite of intensive investigation. Significant increases in levels of acid hydrolases extracellularly have been shown for phagocytosis of non-digestible particles, mucopolysaccharides, bacillus Calmette-Geurin (BCG), digestible proteins and polyamino acids (Axline, 1970; Bowden, 1973; Heise, Myrvik and Leake, 1965). However, Axline (1970) showed that phagocytosis of non-digestible particles such as polyvinyl toluene, polystyrene and starch and D-amino acid homopolymers does not stimulate elevation of enzyme levels. Based on these observations, it has been suggested by Axline (1970) that the quantity and digestibility of endocytosed material may control acid hydrolase production. This does not account for the various non-digestible materials, including

dust, latex particles and PPG (polysaccharide and peptidoglycan--streptococcal cell wall substance), which have been shown to result in increased acid hydrolase levels (Ackerman and Beebe, 1974; Dannenberg, Walter and Kapral, 1963; Davies et al., 1974; Weissman, Dukor, and Zurier, 1972). Also, Cohn and Wiener (1963b) demonstrated that phagocytosis of dead E. coli, compared to live E. coli, resulted in lower levels of increased production of acid hydrolases. Seasonal variations and previous animal history may account for some of the inconsistencies.

Certain particulates such as silica are known to injure the phagolysosomal structures leading to leakage of digestive enzymes into the cytoplasm and into the surrounding medium (Allison, Harington, and Birbeck, 1966; Burrell and Anderson, 1973). Three different mechanisms, all of which are considered to be indicative of cell damage or abnormal function, have been described to account for the release of lysosomal acid hydrolases from cells (Cohn and Benson, 1965a; Weissman et al., 1971; Weissman, Dukor and Zurier, 1972). These include:

1) Loss of integrity of the plasma and lysosomal membranes resulting in enzyme leakage and cell death.

2) Endocytosis of substances which perturb lysosomal and plasma membranes leading to fusion and selective release.

3) "Regurgitation" of acid hydrolases during aborted or incomplete phagocytosis.

The effects of lysosomal injuries may be mediated through the resultant inflammatory responses leading to collagen production and through injury to supportive collagen and elastin fibers leading to emphysema. Although studies to date have focused principally on fibrogenic inorganic particulates, it is not unlikely that the components in sulfate air pollution might also operate to induce lysosomal injuries. These air pollutant effects are probably accentuated where genetic deficiencies exist.

Because of the possibility of acid sulfate injury to lung macrophages directly or mediated through lysosomal injury, a worthwhile addition to the previously described metal sulfate and oxide comparative experiment would be the determination of selected acid hydrolases, e.g., lysozyme, acid phosphatase, and β-glucuronidase. In addition, lactate dehydrogenase (LDH) and glutamic oxalacetic transaminase (GOT) determinations would indicate not only cell viability, but also the possible mechanism of cell death. LDH is a cytoplasmic enzyme that leaks to the extracellular environment from dead and dying cells (Ackerman and Beebe, 1974) without cell lysis. GOT is an enzyme released to the extracellular environment upon cell lysis (Clinical Diagnosis by Laboratory Methods, 1974). Lung macrophages also release

substantial amounts of elastase when stimulated Allison, Harington and Birbeck, 1966). Several reviews (Ackerman and Beebe, 1974; Gross et al., 1969) emphasize the importance of neutral proteases, collagenase and elastase secretion by macrophages in the pathogenesis of emphysema. Therefore, elastase might also be assayed.

Determination of the time course of enzyme level and distribution would be most important. These particular enzyme assays are suggested as valuable adjuncts to *in vitro* sulfate research for several reasons:

1) Ease of performance and availability of reliable methodology (Osserman and Lawlor, 1966; Schill and Schumacher, 1972; Schumacher and Schill, 1972; Sigma Chemical Company, 1977, Worthington Chemical Company, 1977).

2) Reliability for quantitative determination and comparisons.

3) Sensitivity to change in normal cellular activity or viability.

4) Implication in etiology of sulfate pollution-related pulmonary diseases.

Enzyme assays are of particular importance in determining long-term total secretion because the ability to support steady state enzyme production will determine the results of long-term exposure *in vivo*.

There is a need to conduct comparative toxicologic investigations of enzyme levels in lung macrophages following sulfate exposure. Elvick (1978) reports some depression of acid hydrolases without loss of cell viability as determined by LDH and GOT. However, his determinations did not differentiate between extracellular and intracellular levels. The primary concern of previous sulfate research, as discussed in Chapter 2, has been epidemiologic studies of morbidity and mortality due to sulfur dioxide, in vivo animal exposures to various sulfates with determinations of the effects on pulmonary histology and physiology, and delayed studies of acute exposure episodes such as Donora. These studies are important to alert people to pollution-related disease and point out possible agents or combinations of agents responsible for such disease; however, they do little to clarify the biochemical basis of pulmonary disease. A note of warning is in order here. Lack of research connecting the malfunctioning macrophage with lung disease makes it premature to assume that results from in vitro lung macrophage experiments constitute the basis of pollution-related respiratory disease.

APPENDIX A

ZINC AMMONIUM SULFATE AEROSOL PRODUCTION

This Appendix contains the basic data from experimental work performed to produce a range of zinc ammonium sulfate aerosol concentrations and particle sizes using a 5.00 g/100 ml H_2O solution.

The following footnotes are identical for the tables:

a. Chamber equilibrated with the aerosol for 15 minutes prior to MMD determination.

b. Refer to the graph on the following page.

Table A.1. Zinc Ammonium Sulfate Aerosol Concentrations from a 5.00 g/100 ml H_2O Solution.

Exposure	Sample Time (Minutes)	Diluting Air Setting[a] (l/min.)	Generating Air Flow (ml/min.)	Aerosol Concentration (mg/m^3)
1	0-5	6.78	80	6.25
	15-20	6.78	80	8.75
	30-35	6.78	80	8.33
	45-50	6.78	80	11.25
2[b]	0-5	3.28	230	50.83
	15-20	3.28	230	102.71
	30-35	3.28	230	110.42
	45-50	3.28	230	112.71
3[c]	0-5	0	423	99.79
	15-20	0	423	185.62
	30-35	0	423	175.42
	45-50	0	423	175.42

[a]Kennecott Aerosol Generator flowmeter (1/4" steel ball).

[b]Faint haze appeared in chamber at 15 minutes and remained throughout 60-minute exposure time.

[c]Faint haze appeared at 3 minutes, became a fine fog at 10 minutes and thickened at 18 minutes to remain throughout 60-minute exposure.

Table A.2. Zinc Ammonium Sulfate (ZAS) Aerosol[a] Mass Median Diameter Determination for Lowest ZAS Concentration.

Cascade Impactor Effective Cut-Off Diameter (micron)	ZAS Concentration[b] mg/m^3)	Percentage of ZAS on Each Stage	Cumulative[c] Percentage
3.51	0.412	2.00	2.00
2.72	5.595	27.13	29.13
1.83	8.571	41.57	70.70
1.25	2.676	12.98	83.68
0.73	1.850	8.97	92.65
0.26	1.137	5.51	98.16
0	0.379	1.84	100.00

[a]Generating air flow = 80 ml/minute. Diluting air flow = 6.78 l/minute.

[b]Chamber equilibrated with aerosol for 15 minutes prior to MMD determination.

[c]Refer to graph on the following page.

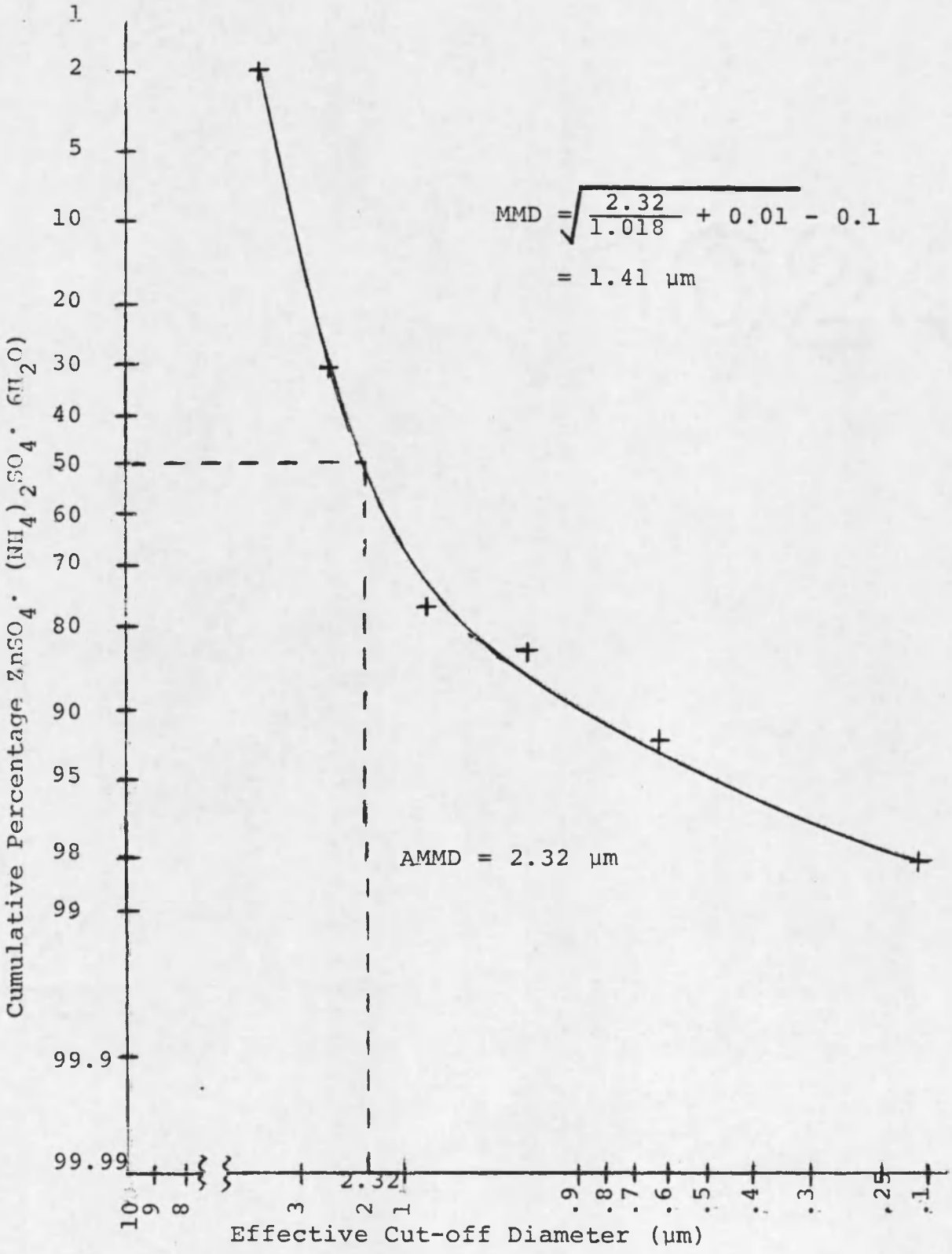

Figure A.1. Particle Size Determination (MMD) for 8.65 µg/cum ZAS Aerosol.

Table A.3. Zinc Ammonium Sulfate Aerosol[a] Mass Median Diameter Determination for the Mid-range Concentration.

Cascade Impactor Effective Cut-Off Diameter (micron)	ZAS Concentration[b] mg/M^3)	Percentage of ZAS on Each Stage	Cumulative[c] Percentage
3.51	0.07	11.86	11.86
2.72	0.14	23.73	35.59
1.83	0.06	10.17	45.76
1.25	0.07	11.86	57.63
0.73	0.02	3.40	61.02
0.26	0.10	16.95	77.97
0	0.13	22.03	100.00

[a]Generating air flow = 230 ml/minute. Diluting air flow = 3.28 l/minute.

[b]Blockage of the last hole, on Stage 6, of the cascade impactor caused a pressure drop; therefore, the run was terminated at 9.5 minutes.

[c]Refer to graph on following page.

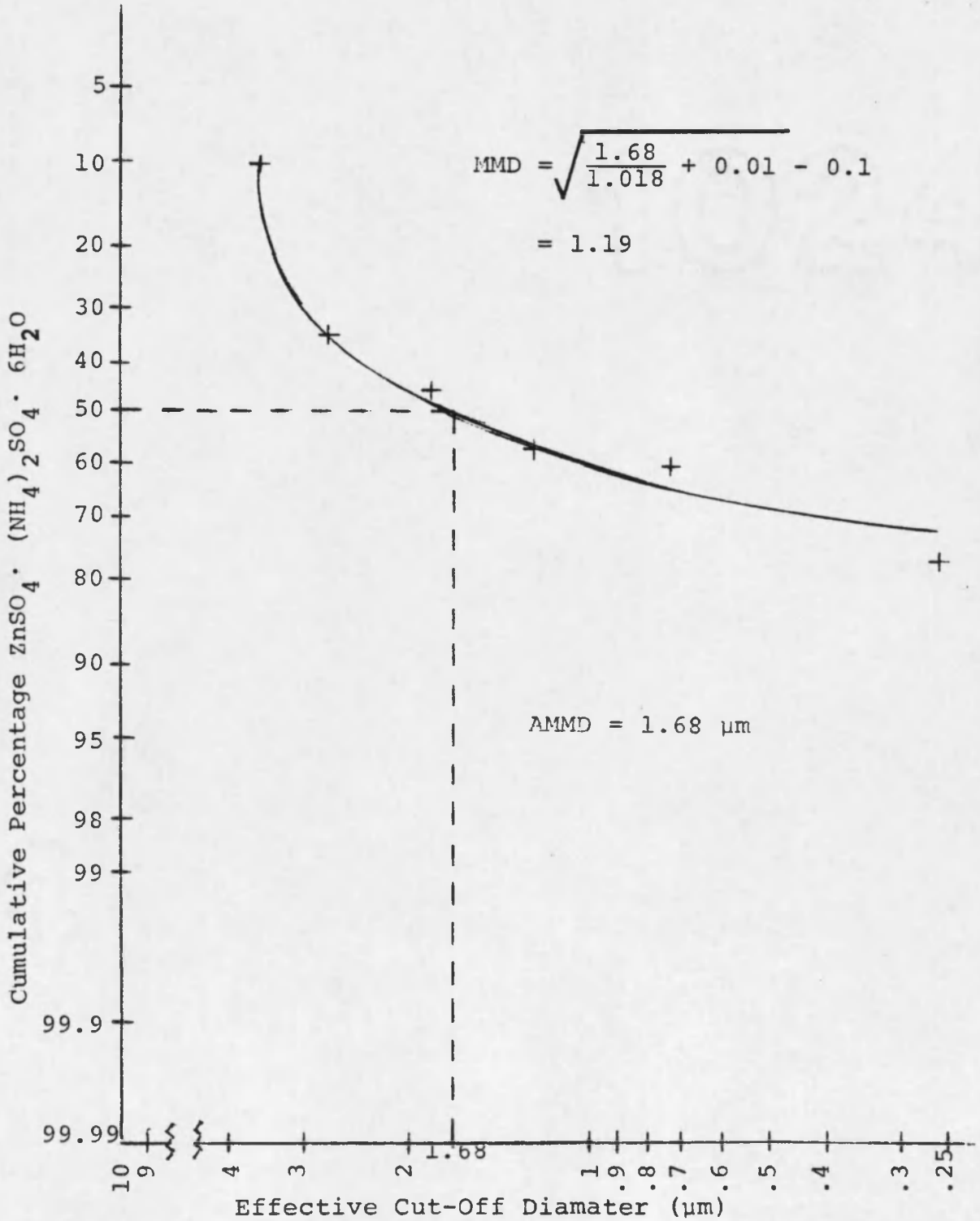

Figure A.2. Particle Size Determination (MMD) for 94.17 µg/cum ZAS Aerosol.

APPENDIX B

SHORT-TERM CULTURE COMPARISON OF METAL SULFATES VS. METAL OXIDES

The following footnotes are identical for the tables in the appendix:

a. Slight amount of turbidity present in stock solution, but not present in test samples.

b. All numerical superscripts refer to morphologic observations on the following page.

c. All cell counts performed with a Coulter Model Z_F at settings of I = 1, Amperage = 4, and Threshold = 25, except where noted otherwise.

Table B.1. $MnSO_4 \cdot H_2O$ vs. MnO_2[a] (Mn^{2+}).

Time 0 Hour 12:00 AM	Control Final Volume 22 ml	$MnSO_4 \cdot H_2O$ (3.82 g/l)			MnO_2 (0.5514 g/l)		
		0.1 ml[2]	1.0 ml[4]	2.0 ml[4]	0.1 ml	1.0 ml	2.0 ml[8]
Macrophage Percent Viability:[a]							
0 hr.	--	--	--	100	--	--	100
1½ hr.	--	--	--	--	--	--	100
3½ hr.	97[1]	--	--	--[3]	--	98	90
7 hr.	99[1]	100	78	9[9]	100[5]	95[6]	94
11½ hr.	98	100	55	15	--	--	100
25 hr.	100	99	40	20	90	90	95
47½ hr.	100	--	72	75	--	100[7]	98
72 hr.	98	--	50	24	--[b]	--	92

Table B.1--Continued.

Time 0 Hour 12:00 AM	Control Final Volume 22 ml	$MnSO_4 \cdot H_2O$ (3.82 g/l) 0.1 ml[2]	1.0 ml[4]	2.0 ml[4]	MnO_2 (0.5514 g/l) 0.1 ml	1.0 ml	2.0 ml[8]
Macrophage Concentration[c] (cells/ml):							
0 hr.	185,000	161,250	153,750	167,500	178,750	163,750	168,750
1½ hr.	173,750	155,000	157,500	157,500	173,750	175,000	141,250
3½ hr.	156,250	106,250	97,500	136,250	120,000	131,250	185,000
7 hr.	192,500	138,750	118,750	203,750	132,500	145,000	128,750
11½ hr.	120,000	72,500	107,500	173,750	108,750	115,000	102,500
25 hr.	132,500	71,250	105,000	176,250	90,000	96,250	78,750
47½ hr.	117,500	96,250	156,250	208,750	112,500	146,250	118,750
72 hr.	55,000	70,000	101,250	160,000	--[b]	75,000	31,250

[a]See notes a and b, page 101.

[b]Sample lost.

[c]Hemacytometer Count.

Morphologic Observations (Table B.1):

$MnSO_4 \cdot H_2O$ vs. MnO_2 (Mn^{2+})

1. A few cells were adhering to the test tube wall at 3½ hours. Occasional small aggregates (2-8 cells) were observed at 7 hours.
2. Many cells were adhering to the test tube wall at 3½ hours. A few small aggregates (4-20 cells) were observed at 7½ hours. Slightly oversize cells were observed at 11½ hours. A slight precipitate was visible macroscopically at 47½ and 72 hours.
3. Approximately 13% of cells were 3x normal size and dead.
4. A large precipitate seen. Microscopically appears as some aggregates containing many dead, oversize cells in the 7, 11½, 25 and 72 hour, 1.0 and 2.0 ml manganese sulfate tubes. At 47½ hours the giant cells were missing. Giant cells have apparently "normal" morphology, other than size and lack of viability.
5. Occasional small (2-4 cells) groupings of cells. Phagocytosis 94%.
6. Occasional small (2-4 cells) groupings of cells. Phagocytosis 100%.
7. Approximately 20% larger cells, 2-3x normal size observed.

8. One hundred percent phagocytosis from 3½ hours throughout entire exposure. Several larger cells observed at 3½ hours. Occasional small groupings, lacking aggregate appearance, seen. At 25 hours swollen, empty cells, only, observed.
9. One huge, dead cell, approximately 20x normal size, seen.

Table B.2. $CuSO_4 \cdot 5H_2O$ vs. CuO (Cu^{2+}).

Time 0 Hour 12:00 PM	Control Final Volume 22 ml	$CuSO_4 \cdot 5H_2O$[1] (1.8906 g/l)			CuO (1.816 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml[4]
Macrophage Percent Viability:[a]							
0 hr.	100	--	--	100	--	--	--
1 hr.	100	--	--	100	--	--	100
2 hr.	100	--	--	100	--	--	100
4 hr.	100	--	--	100	--	--	100
8 hr.	--	--	--	--	--	--	--
12 hr.	100	--	--	80	--	--	100
24 hr.	--	--	40[2]	20[3]	--	--	High
48 hr.	100[5]	--	--	20	70[6]	25[7]	3

Table B.2--Continued.

Time 0 Hour 12:00 PM	Control Final Volume 22 ml	$CuSO_4 \cdot 5H_2O$[1] (1.8906 g/l) 0.1 ml	1.0 ml	2.0 ml	CuO (1.816 g/l) 0.1 ml	1.0 ml	2.0 ml[4]
Macrophage Concentration (Cells/ml):							
0 hr.	39,886	43,751	46,762	47,235	48,359	44,965	45,356
1 hr.	38,362	42,536	42,139	47,068	37,913	44,341	44,123
2 hr.	48,543	42,282	43,804	46,525	43,735	24,619	44,788
4 hr.	53,917	45,623	44,746	44,810	43,191	45,391	50,602
8 hr.	46,475	45,642	44,814	44,374	41,030	45,861	48,416
12 hr.	48,368	44,678	50,461	46,356	44,900	50,057	48,060
24 hr.	37,213	43,256	50,979	46,813	46,893	48,648	42,646
48 hr.	35,773	40,293	55,028	53,708	42,752	41,811	41,070

[a]Refer to notes a, b, and c, page

Morphologic Observations (Table B.2):

$CuSO_4 \cdot 5H_2O$ vs. CuO (Cu^{2+})

1. No precipitates or cell aggregates seen in copper sulfate tubes at any time.
2. Blister cells, dead, comprised 10% of the cell population.
3. Cell membrane missing on 50% of dead cells and many of the live cells were atypical.
4. Phagocytic index rose steadily from 50% at 1 hour, through 80% at 2 hours, to 100% by 4 hours, where it remained for duration of exposure.
5. Some swollen cells present.
6. Very few cells found.
7. Phagocytosis 100%.

Table B.3. $CoSO_4 \cdot 7H_2O$ vs. Co_2O_3[a] (Co^{3+}).

Time 0 Hour 11:00 AM	Control Final Volume 22 ml	$CoSO_4 \cdot 7H_2O$[2] (4.032 g/l)			Co_2O_3[1] (0.7944 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml[3]
Macrophage Percent Viability:[b]							
0 hr.	100	--	--	100	--	--	100
1 hr.	100	--	--	92	--	--	98
2 hr.	98	--	--	96	--	--	100
4½ hr.	97	--	--	93	--	--	100
8 hr.	--	--	--	96	--	--	96
12 hr.	--	--	--	90	--	--	88[4]
24 hr.	--	--	--	Low[c]	--	--	60[5]
51 hr.	Viable[d]	--	--	0[c, 6]	--	--	75

Table B.3--Continued

Time 0 Hour 11:00 AM	Control Final Volume 22 ml	$CoSO_4 \cdot 7H_2O$[2] (4.032 g/l) 0.1 ml	1.0 ml	2.0 ml	Co_2O_3[1] (0.7944 g/l) 0.1 ml	1.0 ml	2.0 ml[3]
Macrophage Concentration (Cells/ml):							
0 hr.	55,174	58,236	56,177	57,923	56,763	56.404	58,457
1 hr.	57,083	48,917	43,711	44,859	52,305	53,225	51,860
2 hr.	60,762	51,132	40,976	41,403	48,973	50,193	52,158
4½ hr.	62,166	47,805	43,467	29,328	48,531	50,240	55,882
8 hr.	81,275	62,073	48,519	33,096	57,348	57,894	59,950
12 hr.	73,365	67,893	51,906	33,566	67,164	61,199	57,735
24 hr.	76,496	71,762	57,033	34,122	65,544	64,563	65,969
51 hr.	62,031	58,690	42,432	43,670	69,203	60,398	60,519

[a]Refer to notes a, b, and c, page 101.

[b]Animal only 6 weeks old. Therefore, trachea was fragile and some erythrocyte contamination of lavage solution resulted.

[c]Only 5 live cells/slide.

[d]Very few cells seen.

Morphologic Observations (Table B.3):

$CoSO_4 \cdot 7H_2O$ vs. Co_2O_3 (Cu^{3+})

1. Particles of Co_2O_3 are larger than those of previous dispersions.
2. No precipitate or cell aggregates seen.
3. Phagocytic index slowly increased: 1 hour--20%, 2 hours --35%, 4½ hours--85%, 8 hours--93%, 12 hours--90%+, 51 hours--100%.
4. Some cell distortion seen; bacteria in excess present.
5. Few undistorted cells seen.
6. Those cells seen had disintegrating membranes.

Table B.4. 3 $CdSO_4 \cdot 8H_2O$ vs. CdO[a] (Cd^{2+}).

Time 0 Hour 3:30 PM	Control Final Volume 22 ml	$3CdSO_4 \cdot 8H_2O$ (4.10 g/l) 0.1 ml	1.0 ml	2.0 ml	CdO (0.9124 g/l) 0.1 ml	1.0 ml	2.0 ml
Macrophage Percent Viability:[a]							
0 hr.	100[b]	--[1]	--[1]	97[1]	--	--	100
1 hr.	100	--	--	100[1]	--	--	100[5]
4 hr.	100	--[1]	--[1]	100[1, 2]	--	--	100
6 hr.	100	--	--	98[3]	--	--	100[6]
9 hr.	93	--	--	80[4]	--	--	100[6]
13 hr.	97	--	--[4]	90[4]	--	--	High[6]
24 hr.	93	20	15[4]	10[4]	--	--	90
48 hr.	--	70	0[4]	0[4]	10[7]	--[5]	--[8]

Table B.4--Continued.

Time 0 Hour 3:30 PM	Control Final Volume 22 ml	$3CdSO_4 \cdot 8H_2O$ (4.10 g/l)			CdO (0.9124 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml
Macrophage Concentration (Cells/ml):							
0 hr.	26,059	35,889	37,211	36,300	31,984	33,656	32,777
1 hr.	30,103	32,722	35,788	34,070	33,207	32,439	33,377
4 hr.	31,847	33,536	31,540	30,077	32,851	34,523	33,517
6 hr.	20,540	34,607	31,640	33,207	34,379	34,875	38,699
9 hr.	36,970	36,398	33,561	35,411	33,217	34,135	33,423
13 hr.	36,769	34,518	33,987	34,714	34,359	35,596	36,186
24 hr.	55,762	28,821	30,055	32,321	43,730	45,280	30,083
48 hr.	44,705	39,778	26,531	26,886	37,200	36,118	31,320

[a]Refer to notes a, b, and c, page 101.

[b]Volume low, only 12.5 ml compared to other controls and test standard volume of 22 ml.

Morphologic Observations (Table B.4):

$3CdSO_4 \cdot 8H_2O$ vs. CdO (Cd^{2+})

1. Solutions cloudy.
2. Some cells observed that seem to be in the process of combining--viz. a dumbbell shape with an indistinguishable membrane.
3. Some clumping and distorted cells, but no enlargement.
4. Large amount of precipitate observed. Many clumped cells, red-brown under phase contrast, present in 9 hr., 2.0 ml cadmium sulfate tube. Both 1.0 and 2.0 ml cadmium sulfate tubes contain cells appearing red-brown under phase. Stock cadmium sulfate solution red-brown. Cells are partly disintegrated in 48 hr., 1.0 and 2.0 ml cadmium sulfate tubes.
5. Some cell enlargement observed.
6. Phagocytic index low--10% in 6-hour sample and 15% in 9-hour sample.
7. Very few cells present.
8. Phagocytosis 100%.

Table B.5. $Cr_2(SO_4)_3 \cdot x\ H_2O$ vs. Cr_2O_3 (Cr^{3+}).

Time 0 Hour 3:30 PM	Control Final Volume 22 ml	$Cr_2(SO_4)_3 \cdot x\ H_2O$ (2.390 g/l)			Cr_2O_3 (0.8368 g/l)		
		0.01 ml	0.1 ml	1.0 ml	0.01 ml	0.1 ml	1.0 ml
Macrophage Percent Viability (%V):[a]							
0 hr.	--	--	--	96	--	--	94
½ hr.	--	96	97	92	--	95	93
1½ hr.	--	--	96	100	--	--	100
3½ hr.	100	100	100	100[1]	100	100	100
6½ hr.	--	--	--	100[2]	--	--	--
10½ hr.	--	--	--	--[3]	--	--	94
24 hr.[4]	95	100	--	--	--	--	--

Table B.5--Continued.

Time 0 Hour 3:30 PM	Control Final Volume 22 ml	$CR_2(SO_4)_3 \cdot x H_2O$ (2.390 g/l)			CR_2O_3 (0.8368 g/l)		
		0.01 ml	0.1 ml	1.0 ml	0.01 ml	0.1 ml	1.0 ml
Macrophage Concentration[a] (Cells/ml):							
0 hr.[c]	78,058	94,288	89,812	48,612	84,404	95,644	89,348
½ hr.	74,650	88,324	90,308	77,416	70,582	91,332	86,542
1½ hr.	83,076	85,124	82,710	43,130	80,780	86,996	76,784
3½ hr.	95,692	87,144	86,284	66,676	104,462	91,112	80,858
6½ hr.	75,014	88,796	82,118	62,604	86,540	104,528	77,358
10½ hr.	69,420	87,536	79,400	61,992	77,616	87,718	75,236
24 hr.	31,560	39,382	35,792	33,650	62,204	62,664	47,098

[a]Refer to notes a, b, and c, page 101.

[b]Instead of the test system described under methods and materials, substitute: final volume of combined and diluted lavage solutions equal 120 ml, and 2 ml of this cell suspension plus 0.01, 0.1 or 2.0 ml of the chromium solution. This set-up used only for chromium exposures.

[c]0.2% trypsin not added to the ½ and 1½ hour exposures; therefore, see indications of strong binding to tube walls by control macrophages.

Morphologic Observations (Table B.5):

$$Cr_2(SO_4)_3 \cdot x\ H_2O \text{ vs. } Cr_2O_3$$

1. Viability of 100% is probably due to dead cell membrane disruption from the Trypsin remaining with the cells too long. A slight precipitate was observed in the 1.0 ml chromic sulfate tube at 3½ hr.
2. A larger precipitate visible in same tube at 6½ hr. Microscopically the precipitate resolved into a large cell aggregate with approximately 100% V. Some question as to the viability since cannot be sure dye is reaching all cells.
3. Large precipitate that could be partially disrupted with 2 minutes vortexing (at high). Some cells in the aggregate have an indistinct membrane.
4. Aggregates were visible, increasing in size from 2-10 cells in the control to 50-100+ cells in the 1.0 ml chromium sulfate tube. Cell walls were not always distinct in the chromium sulfate tubes and % V difficult to determine.
5. No cell aggregates or precipitate visible at any time or concentration in the chromic oxide test tube.

Table B.6. $Fe(NH_4)_6SO_4 \cdot 12\ H_2O$ vs. Fe_2O_3[a] (Fe^{3+}).

Time 0 Hour 12:20 PM	Control Final Volume 22 ml	$Fe(NH_4)_6SO_4 \cdot 12\ H_2O$ (2.1034 g/l)			Fe_2O_3 (0.5909 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml
Macrophage Percent Viability:							
0 hr.	92	--	--	100	--	--[1]	96[1]
1 hr.	100	--	--	100	100	--	80
2 hr.	100	--	--	98	--	--	100
4' 40"	100	--	--	92	--	--	100[5]
8 hr.	100	--	--	100	--	--	100[6]
12 hr.	--	--	--	100	--	--	90
24 hr.	70	60	--	--	--	--	0[7]
48 hr.[9]	75	70	--[b, 3]	--	--[8]	Low[c]	Low[c]

Table B.6--Continued

Time 0 Hour 12:20 PM	Control Final Volume 22 ml	$Fe(NH_4)_6SO_4 \cdot 12\ H_2O$ (2.1034 g/l)			Fe_2O_3 (p.5909 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml
Macrophage Concentration (Cells/ml):							
0 hr.	35,567	38,589	39,233	37,507	34,343	35,638	37,558
1 hr.	32,694	34,608	36,091	38,655	37,766	33,921	35,902
2 hr.	39,504	34,136	36,270	37,648	33,935	31,514	31,944
4' 40"	35,944	35,497	38,389	39,134	34,850	33,448	37,172
8 hr.	37,368	36,385	38,741	40,809	38,393	34,494	36,475
12 hr.	57,678	59,324	57,650	53,535	50,163	45,435	47,202
24 hr.	47,937	27,016	64,348	52,400	40,608	28,705	32,799
48 hr.	65,144	74,198	101,464	96,361	36,569	25,245	35,372

[a]Refer to notes a, b, and c, page 101.

[b]Viable.

[c]Approximately 5 viable cells/slide.

Morphologic Observations (Table B.6):

$Fe(NH_4)_6SO_4 \cdot 12\ H_2O$ vs. Fe_2O_3 (Fe^{3+})

1. Cloudy solution.
2. Macrophages began showing some distorted shapes at 2 hours and this continued and worsened throughout the exposure so that by 12 hours the cells were so distorted as to be almost unrecognizable, although viability continued to be high. A few tenuous surface projections were noted in the 4' 4" and 8-hour samples.
3. Heavy precipitate. Rare free cells. Gelatinous masses of cytoplasm with no visible cell walls present.
4. Phagacytic index 80% at 1 hour and 100% for duration of exposure.
5. Viability difficult to determine because cells were so full of particulate.
6. Some cells swollen, but not dead.
7. Many clumps of particulate seen, but no distinguishable cell material.
8. Could not find cells.
9. All test tubes, except control and 0.1 ml iron ammonium sulfate, had become acid (yellow indicator color).

Table B.7. $NiSO_4 \cdot 6H_2O$ vs. $NiO \cdot Ni_2O_3$ (Ni^{2+}).

Time 0 Hour 3:00 PM	Control Final Volume 22 ml	$NiSO_4 \cdot 6H_2O$ (4.04 g/l)			$NiO \cdot Ni_2O_3$ (1.6414 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml
0 hr.	--	--	--	99	--	--	100
1 hr.	100	--	--	94	--	--	100
4 hr.	100	--	100	81	--	--	94[2]
6¼ hr.	100	--	--	100	--	96	96[2]
9¼ hr.	99	93	--	98	--	--	100[2]
24 hr.	--	--[1]	--	97	--	--	100[4]
48 hr.[3]	97[4]	--	--	96[4]	--	--	98[4]
69 hr.[3]	100	--	--	94[4]	--	--[5]	100

Table B.7--Continued.

Time 0 Hour 3:00 PM	Control Final Volume 22 ml	$NiSO_4 \cdot 6H_2O$ (4.04 g/l)			$NiO \cdot Ni_2O_3$ (1.6414 g/l)		
		0.1 ml	1.0 ml	2.0 ml	0.1 ml	1.0 ml	2.0 ml
Macrophage Concentration[b] (Cells/ml):							
0 hr.	116,250	123,750	121,250	133,750	140,000	103,750	116,250
1 hr.	98,750	120,000	148,750	122,500	82,500	111,250	135,000
4 hr.	110,000	102,500	156,250	135,000	87,500	101,250	90,000
6¼ hr.	167,500	156,250	115,000	177,500	130,000	162,500	155,000
9¼ hr.	138,750	160,000	140,000	121,250	126,250	107,500	105,000
24 hr.	56,250	103,750	108,750	91,250	71,250	63,750	53,750
48 hr.	57,500	60,000	98,500	121,250	97,500	52,500	58,750
69 hr.	27,500	112,500	91,250	121,250	98,750	81,250	80,000

[a]Refer to notes a and b, page 101.

[b]Hemacytometer Count.

Morphologic Observations (Table B.7):

$NiSO_4 \cdot 6H_2O$ vs. $NiO \cdot Ni_2O_3$ (Ni^{2+})

1. One aggregate of 6 cells seen. This was the only tube in this exposure to show any aggregation.
2. Percent phagocytosis ranged from 80% at 4 hours to nearly 100% by 9¼ hours with extremely full cells seen at 9¼ hours.
3. All tubes, except 2.0 ml nickel sulfate and 2.0 ml nickel oxide at 48 hours and 0.1 ml nickel oxide at 69 hours, had become acid as indicated by the phenol red color change from red to yellow.
4. Some swollen, viable cells seen. Very few cells overall were seen in the 48-hour control tube.
5. A precipitate formed when trypsin was added to the test. This was probably due to bacterial contamination.

Table B.8. $ZnSO_4 \cdot 7H_2O$ vs. ZnO[a] (Zn^{2+}).

Time 0 Hour 2:15 PM	Control Final Volume 22 ml	$ZnSO_4 \cdot 7H_2O$ (4.04 g/l) 0.1 ml[1]	1.0 ml[1]	2.0 ml[1]	ZnO (1.066 g/l) 0.1 ml	1.0 ml	2.0 ml
Macrophage Percent Viability:							
0 hr.	97	99	100	100	100	100	100
1 hr.	100	100	98	100	100	100	100
2 hr.	100	--	99	99	--	--	100[7]
5 hr.	--	--	--	93[2]	--	--	99[7]
8 hr.	92	--	--	98	--	--	100[7]
24 hr.	100	--	100[3]	84[4]	--	--	100[8]
47½ hr.	100[5]	--	--	100	--	90[9]	30[9]
72 hr.	100	95	90	20[6]	--	--	95[7,9]

Table B.8--Continued.

Time 0 Hour 2:15 PM	Control Final Volume 22 ml	$ZnSO_4 \cdot 7H_2O$ (4.04 g/l) 0.1 ml[1]	$ZnSO_4 \cdot 7H_2O$ (4.04 g/l) 1.0 ml[1]	$ZnSO_4 \cdot 7H_2O$ (4.04 g/l) 2.0 ml[1]	ZnO (1.066 g/l) 0.1 ml	ZnO (1.066 g/l) 1.0 ml	ZnO (1.066 g/l) 2.0 ml
Macrophage Concentration (Cells/ml):							
0 hr.	92,175	95,707	109,092	101,959	99,726	99,671	101,028
1 hr.	92,275	101,213	91,945	98,271	97,381	101,542	96,270
2 hr.	93,861	101,877	92,035	96,623	99,641	102,687	--[b]
5 hr.	97,185	106,215	89,593	98,696	103,511	97,053	86,503
8 hr.	102,622	106,652	90,056	103,841	103,609	99,888	91,914
24 hr.	73,171	93,858	66,572	103,646	102,736	76,932	40,367
47½ hr.	73,247	59,502	68,606	86,032	64,153	56,845	41,162
72 hr.	118,291	47,829	64,004	80,248	97,998	110,308	54,403

[a]Refer to notes a, b and c, page 101.

[b]Sample lost.

Morphologic Observations (Table B.8):

$ZnSO_4 \cdot 7H_2O$ vs. ZnO (Zn^{2+})

1. Slight precipitate, appearing microscopically as few to many small (3-4 cell) aggregates, present in 1.0 and 2.0 ml zinc sulfate tubes at 1 and 2 hours; and in the 0.1 ml zinc sulfate tube from 24-72 hrs. Precipitate visible and aggregates large enough to block counting orifice of Coulter present in 1.0 and 2.0 ml zinc sulfate tubes from 5-72 hrs.
2. Five to ten percent of large (100+ cells) aggregate dead cells.
3. Flocculent precipitate with blister cells.
4. Flocculent precipitate containing many large cells (3-10x normal). Many deformed cells, expanded cytoplasm. Cell walls blurred in aggregates.
5. Occasional deformed cell.
6. Aggregates very large (1000 cells), many dead cells present and few free cells.
7. High phagocytic index (95+%). Some distorted cells at 2 hours and many at 5 hours with few round cells.
8. Masses of cells, difficult to count.
9. Some aggregates and many deformed cells in the 1.0 and 2.0 ml zinc oxide tubes at 47½ hours. No aggregates, however, at 72 hours.

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