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Comparison of Toxicological Models for Evaluation of Air Pollutants: Response of the Pulmonary Alveolar Macrophage to Hexavalent Chromium

Jennifer Baker Galvin
Utah State University

COMPARISON OF TOXICOLOGICAL MODELS FOR EVALUATION OF AIR POLLUTANTS: RESPONSE OF THE PULMONARY ALVEOLAR MACROPHAGE TO HEXAVALENT CHROMIUM

by

Jennifer Baker Galvin

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ABSTRACT

Comparison of Toxicological Models for Evaluation of Air Pollutants: Response of the Pulmonary Alveolar Macrophage to Hexavalent Chromium

by

Jennifer Baker Galvin, Master of Science

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Major Professor: Dr. S. G. Oberg
Department: Toxicology

This study was designed to accomplish two primary objectives: (1) to compare two test methods commonly used to evaluate toxicity of inhaled air pollutants, and (2) to observe the response as measured by each of the methods, of pulmonary alveolar macrophages exposed to 2 μg hexavalent chromium.

The first method evaluated featured use of intratracheal injections to simulate live inhalation exposures, and the second required exposure of macrophages cultured on petri plates. Pulmonary alveolar macrophages harvested from Long Evans rats were used. The two cell function parameters measured in the evaluations were chemiluminescence and oxygen consumption (which was determined for cells at rest and during phagocytosis). These two tests have been shown to be sensitive indicators of macrophage damage. Results of CL output and oxygen consumption revealed the two methods were significantly different.

Evaluation of macrophages from live animals treated with CrO_3 or $CaCrO_4$ showed no differences between their respective untreated controls

as determined by measurement of their chemiluminescence production or of oxygen consumption rates.

Alveolar macrophages that were cultured in media during treatment with the same two forms of hexavalent chromium showed statistically significant differences from untreated controls.

These comparisons indicate that choices of investigative toxicological models influence interpretation of data recorded.

(91 pages)

INTRODUCTION

Environmental exposure to airborne contaminants in the metal industry has been studied extensively by those concerned with occupational health (NIOSH 1975). Some of these airborne particles have been shown to contain significant concentrations of metals in both soluble and insoluble forms (Lee and Von Lehmden 1973). Many of the insoluble metal particles are small enough to be inhaled into the lung and deposited in the alveoli (Lee et al. 1968). Once in the lung, these metal particles may affect the normal function of the cells lining the alveoli. Alveolar macrophages are free lung cells located on the surface of small airways and alveoli (Brain 1970). These cells play an important role in the protection of the lung against airborne bacteria and particles. The response of alveolar macrophages to inspired bacteria and particles is complex (Johnston 1978). Upon contact with the particles, alveolar macrophages release superoxide anion which may be involved in the detoxification of these foreign substances (Sweeney et al. 1978). The release of superoxide anion from alveolar macrophages following exposure to foreign particles can be monitored by the measurement of chemiluminescence (Miles et al. 1978). Exposure to foreign particles also causes large increases in oxygen consumption. Chemiluminescence and oxygen consumption have been shown to be the cellular responses most sensitive to damage from toxic agents (Castranova et al. 1980).

Metals have been shown to affect a variety of properties of alveolar macrophages (Bingham et al. 1972, Waters et al. 1974). Airborne particles in the metal industry may be toxic to alveolar macrophages, and the normal defense mechanisms of the lung may be compromised. Therefore, an investigation of the effects of metallic ions on the function of alveolar macrophages is of great interest.

As will be detailed in the subsequent section, with a review of the literature it became apparent that there were conflicts in reported results of metal toxicity to the alveolar macrophage. The reports conflicted because the alveolar macrophages were exposed by different methods--in the live animal or after incubation in a petri dish. The objective of this study, then, was twofold. The first was to test the comparability of in vivo (live exposure) and in vitro techniques used to indicate alveolar macrophage injury caused by air pollutants. The second was to use those techniques to test the effect on rat alveolar macrophages of an acute low dose to either of two hexavalent chromium compounds.

LITERATURE REVIEW

Chromium

Chromium is a hard, blue-white metal that is not oxidized in moist air and oxidizes only slightly even when heated. With an atomic number of 24 and a mass of 52.01, chromium belongs to the first series of the transition elements. It is a member of group VI B of the periodic table and can exist in oxidation states ranging from Cr^{2-} to Cr^{6+}, but only the ground states 0, +2, +3, and +6 are common. Bivalent chromium compounds are basic; trivalent, amphoteric; and hexavalent, acidic (Patty 1963).

The trivalent is the most stable oxidation state. It has a strong tendency to form complexes and chelates. Free chronic ion does not exist in aqueous solution; it is always coordinated, either with water or with other ligands in the solution.

The hexavalent form is almost always linked with oxygen and is a strong oxidizing agent. Complexes in which hexavalent chromium would be stabilized against reduction by organic matter are not known (Mertz 1969).

Some of the chemical properties of trivalent chromium serve as a basis to delineate some elementary functions this element can be expected to have in a biological system. The differences in oxidation potential between Cr^{+2} and Cr^{+3}, for instance, and Cr^{+3} and Cr^{+6} are so great that a reversible transition between two oxidation states is extremely unlikely. Therefore, the role of chromium in biological systems will be different, for example, from iron in the cytochrome

system. In the body, chromium occurs in only one configuration and most probably one oxidation state, and is, therefore, not likely to participate in biochemical oxidation-reduction reactions. Chromium (III) has a slow rate of ligand exchange. This property would be compatible with a more structural function like the binding of hormones to receptor sites (Mertz 1969).

Chromium distribution

Chromium is found in the soil, water, and air. Soils deficient in chromium show improper plant growth, while large amounts of chromium are poisonous to plants. Concentrations of chromium in soil are rarely high, usually between a trace and 250 ppm, as chromic oxide. Higher concentrations of chromium are usually found in ultramafic igneous rock, in shales and clays, and in phosphorites. The chromium in phosphorites used as fertilizers is an important source of man-induced contamination of soils with chromium. The phosphorites from Idaho, Wyoming, and Utah contain approximately 1000 ppm, which is much higher than the national average (Lotspeich and Markward 1963).

A survey of chromium in 15 rivers of North America showed that chromium is significantly more concentrated in Atlantic coastal river waters than in Gulf or Pacific river waters (Durum et al. 1970). Both trivalent and hexavalent forms have been shown to exist in water. The hexavalent chromium is constantly being diminished by interaction with organic particles and slowly settles. Municipal drinking water in the U.S. may contain up to 35 ppb. Most mammals absorb chromium very poorly from the gastrointestinal tract

except when it is in the organic form of the glucose tolerance factor. The level of 35 ppb, therefore, would produce no recognizable biological effects.

Inhalation is the primary mode of chromium exposure in man (Jenkins 1981) and lung burdens increase with age while other organs become more depleted (Schroeder et al. 1963). Schroeder et al. (1963) have estimated a yearly intake of 11-110 μg/year from the air for people living in cities with adjacent chromium industries. This may well account for the continuous accumulation in the lung with age.

Essential biological interactions

Chromium in its hexavalent state (chromic oxide, chromates, or dichromates) is a strong oxidizing agent and readily reacts with organic matter in acidic solution, leading to reduction to the trivalent state. The known toxic action of chromates is to a large degree due to this oxidizing reaction. Another biologically important property of hexavalent chromium is the ease with which it penetrates biological membranes. These properties clearly distinguish hexavalent chromium from the much less toxic trivalent form (Committee on Biologic Effects of Atmospheric Pollutants 1974).

Chromium is an essential trace element of the human body, existing in the oxidation state of +3, and is found in the serum at a concentration of approximately 2.5 μg/ml. One of the first intentional biological applications of hexavalent chromium was to tag red blood cells. Injected intravenously, chromium penetrates the

red blood cell rapidly where it is reduced to the trivalent form and becomes bound to hemoglobin (Mertz 1969).

It was first discovered that chromium was an essential element when it was shown that impairment of glucose tolerance is the first symptom of chromium deficiency in experimental animals (Schwartz and Mertz 1959). Deficiency results in impaired glucose metabolism due to the poor effectiveness of insulin. The effectiveness of insulin can be restored by giving "glucose tolerance factor" (GTF). GTF is an organic, low molecular weight, dialyzable, naturally occurring chromium-containing compound (Hambridge 1974). Chromium-responsive forms of impaired glucose tolerance are known to exist in the U.S., particularly in elderly people (Levine et al. 1968). There are also data that show physiological amounts of chromium in diets of experimental animals increase their survival and growth rates (Schroeder et al. 1963).

Occupational exposure and toxic properties of chromic acid (CrO_3)

The toxic effects of exposure to chromium and its compounds have been well known since the early nineteenth century. The health problems associated with chromium are primarily related to hexavalent chromium.

Older epidemiological surveys use names that are intended to be synonymous: chromic acid anhydride, chromic trioxide, and "chromic acid." Most of the chromic acid commercially produced is used for chrome plating. This process results in chromic acid aerosols released by bubbles of hydrogen in the solution which are evolved into

the air. It has been found that industrial employees working with the following products or processes may have been exposed to chromic acid: tanning, primer paints, pigments, graphic arts, printing and reproducing, fungicides, wood preservatives and corrosion inhibitors (Committee on Biologic Effects of Atmospheric Pollutants 1974).

Ulcerative properties of hexavalent chromium were first described by Cumin in 1827. There have been many reports since that have described symptoms of workers exposed to mists of CrO_3 from plating tanks (Bloomfield and Blum 1928, Kleinfeld and Rosso 1965, Gomes 1972, Royle 1975). The investigators determined unequivocally that exposure to CrO_3 can cause ulcers of the nasal mucosa, perforations in the nasal septum, and ulcers of the skin known as "chrome holes." Hexavalent chromium is reduced within the skin by methionine, cystine, and cysteine, to promote ulceration (Committee on Biological Effects of Atmospheric Pollutants 1974). Zvaifler (1944) stated that only when the CrO_3 mist concentration became "negative" did the irritative effect subside. He did not state the level he considered to be negative.

Occupational exposure and toxic properties of calcium chromate ($CaCrO_4$)

Several authors have dealt with the effects of chromates other than chromic acid (Baetjer 1950, Mancuso 1951, Walsh 1953). Hexavalent chromates are formed when chromium III is oxidized by atmospheric oxygen at high temperatures. This occurs when chromium is being recovered from chromite ore. The actual form that hexavalent chromium will assume in aqueous solution depends on the pH and chromium

(VI) concentration. Nevertheless, chromium (VI) is always hydrated and appears as a monochromate, bichromate, or polychromate ion (NIOSH 1975).

The effects seen by those authors in workers at chromate plants were the same ulcerative lesions as seen with CrO_3. In addition, they noted squamous-cell carcinomas of the bronchi and a specific cutaneous sensitization.

Since chromium trioxide and chromates were used in the same plant investigators assumed that all chromium compounds produced lung carcinoma. It was determined by Baetjer (1950) that, in all probability, it was actually a chromate dust compound that produced the lung carcinomas. Kazantis (1972) later made the observation that lung carcinomas were produced in men involved in producing chromates from raw ore and not in workers using the chromates. He then hypothesized that the carcinogenic material must be in the ore or in a furnace product.

In the cement industry, chromium-containing cements have led to dermatitis. Contact dermatitis has also been reported from handling timber in which chromium salts were impregnated (Walsh 1953). Newhouse (1963) demonstrated that of a large number of dermatitis patients patch-tested for sensitivity to several common chemical substances, chromates caused positive reactions in the largest number of cases.

Experimental exposure to chromates and chromium trioxide

Several hexavalent chromates and chromic acids have been found to be mutagenic by Petrilli and Deflora (1977). The Ames back-mutation test was used with concentrations of 10-200 μg/ml for all chromium compounds. Venitt and Levy (1974) have found chromium to be mutagenic in other strains of bacteria.

Since the ulcerative properties of chromium compounds are reversible, many experimental investigators concentrated on chromium's carcinogenic potential (Hueper 1958, Payne 1960, Roe and Carter 1969, Laskin et al. 1970). Of the many implantation studies performed, the most successful were those that allowed for the continuous contact of the compound with the intended tissue via a suitable vehicle. Vehicles used were sheep fat, tricaprylin, or gelatin. The materials were implanted chiefly into the pleural cavity and thigh muscles and produced sarcomas, some of which were invasive. A few adenomas were also produced. Of far greater importance has been the production of squamous-cell carcinoma of the bronchi in rats by the local implantation of calcium chromate in cholesterol pellets (Laskin et al. 1970). To date, no carcinomas have been experimentally induced with chromium trioxide.

Administration of chromates to laboratory animals by intratracheal injection or by inhalation has been shown previously to result in significant pulmonary retention of these materials (Baetjer et al. 1955, Baetjer et al. 1959a, Baetjer et al. 1959b). A study by Steffee and Baetjer (1965) showed reactions to the inhalation of chromate dusts and chromic acid produced a high incidence

of alveolar and interstitial inflammation, with fibrosis in the lungs of guinea pigs, but no malignant tumors.

Systemic effects of hexavalent chromium compounds introduced intravenously or subcutaneously at less than 1 mg/kg usually leads to nephritis. Doses larger than this are generally fatal due to marked renal damage (Simonds and Hepler 1945).

Alveolar Macrophage

The lung, although its main purpose is for gas exchange, also serves nonrespiratory functions. Protecting the body from airborne bacteria is one of these important nonrespiratory functions. This is afforded through two mechanisms--the alveolar macrophage and the bronchociliary escalator which serves to rid the lung of these contaminants (Heinemann and Fishman 1969).

Alveolar macrophages are of variable size (15-30 μm) with faintly basophilic cytoplasm. The nuclei of the smaller cells are round but in larger cells they may be deeply indented. The cytoplasm may contain dust, erythrocytes, fragments of cell debris and bacteria. The cytoplasm is usually associated with phase dense granules and vacuoles which stain with fat soluble dyes. Staining with vital dyes is useful in the identification of macrophages, but the feature which most clearly demarcates macrophages from other cell types is the ease with which they adhere to a glass surface to form a monolayer (Hocking and Golde 1979). The mechanism of this adherence is due to the phagocytic nature of the cell (Van Oss et al. 1975).

Alveolar macrophages are phagocytic cells. They differ from other phagocytic cells, such as polymorphonuclear leukocytes, monocytes, and peritoneal macrophages, in many respects that include variations in their intermediary metabolism, lipid uptake (Karnovsky 1962) and enzyme activity (Leake et al. 1964). Alveolar macrophages are able to synthesize proteins, contain various hydrolases, and are rich in lysozyme, an enzymatic protein with bacteriolytic properties (Pearsall and Weiser 1970).

Source and clearance of the alveolar macrophage

The source of alveolar macrophages presents a question that has not to date been fully resolved. In recent years, attention has focused on the hematopoietic system and the great alveolar cell (type II pneumonocyte) as the primary origin of normal lung macrophages (Brain 1970, Van Furth 1970, Brunstetter et al. 1971). The immature macrophage follows a course that is thought to originate in the bone marrow, is then transported by the peripheral blood, and eventually becomes a mature tissue macrophage. Once macrophages reach the lungs there are also questions about what occurs there. Roser (1970) proposed that the pulmonary alveolar macrophages are removed from the lung intact. Evidence exists that these cells are moved up the trachea and are swallowed. Spritzer and co-workers (1964, 1968) have made attempts to measure the hourly clearance of alveolar macrophages. They estimated that 19.5 percent of the total lung macrophages were cleared every 24 hours. According to the Task Group on Lung Dynamics (1966) the biologic half-time for translocation

of insoluble particles from the alveolar surface to the ciliated surface is 24 hours. Once the insoluble particle is phagocytized there is little evidence that macrophages laden with particles can reenter the alveolar wall (Hatch and Gross 1964). Soluble material, however, can diffuse into the lymphatics, the bloodstream, or be taken up by macrophages. The process by which macrophages take up soluble material is called pinocytosis. At this point, the macrophage could be cleared as described above.

The kinetics of the alveolar macrophage is an important factor which determines the actual population being studied. Van Furth (1970) has established that alveolar macrophages divide very infrequently; approximately 1 percent per 10 hours. In a short term study, therefore, the number of new cells produced through cell division that have not been exposed to the test chemical would not be significant. When washing macrophages from the lung after exposure it is important to know if the cells recovered are representative of the population exposed, or through some mechanism they have left the lung. Spritzer and co-workers (1964, 1968) have shown the rate of migration of the macrophage out of the lung is fairly low. The last consideration is that through some immune response the phagocytic activities (release of chemotaxins) of alveolar macrophages cause the infiltration of more macrophages. These new cells may come from the bloodstream or lung interstitum into the air spaces, thereby making themselves available to the lavage procedure. Brain (1970) has reported that the intratracheal injection of a noninfectious insoluble material seems to elicit recruitment of peripheral macrophages.

These cells would also be exposed to the material injected. Based upon the criterion mentioned above, the turnover rate of alveolar macrophages is such that a constant cell pool size exists over reasonable lengths of time.

Immune function

The alveolar macrophage was first thought to be little more than a dust collector; purely a scavenger cell. Yet recent reports have pointed out the increased importance of the cell to immune defense systems (Askonas and Jaroskovâ 1970, Bowden 1971, Kölsch 1970). Phagocytosis is an important step in the induction of immune responses since the most powerful antigens such as bacteria, viruses and cells are particulate in nature.

Macrophages are thought to enhance the immunogenicity of ingested antigens and act as the immediate receptor of inhaled particulates. The alveolar macrophage is uniquely situated for this initial process. The precise mechanisms of the process and the sequential events that link phagocytosis of antigens to the production of antibodies by lymphoid cells are not known (Bowden 1971).

Macrophages contribute to the antibody response in several ways: they trap, process, and store antigens. In addition, they can present specific information to antibody-forming cells in the form of a fragment of antigen coupled to ribonucleic acid (RNA) (Pearsall and Weiser 1970).

Green and Kass (1964b) and Myrvik and Evans (1967) have reported that many agents ranging from alcohol and air pollutants to drugs can

directly impair the phagocytic mechanism of alveolar macrophages. The immunologic potential of these cells is, therefore, markedly reduced. The role of the alveolar macrophages in the immunological chain reaction may be poorly defined but there is no doubt that the need for active participation of this cell type to combat lung diseases and induce immune responses (Green and Kass 1964a).

Phagocytosis

The most important function of the alveolar macrophage is phagocytosis. Phagocytosis, by definition, is the ingestion of particles by single cells and is phylogenetically the oldest and most fundamental defense mechanism of the host against foreign particles and microorganisms. The process is comprised of a number of quite separate steps and activities: (1) initial recognition of a foreign particle to be phagocytized, followed by (2) pursuit, i.e., movement of the phagocyte toward the foreign particle, (3) surface recognition of the particle or bacterium and (4) engulfment and destruction of the invading organism (Van Oss et al. 1975).

The discriminatory capacity of mammalian phagocytes is extremely fine. They shun homologous cells but engulf some foreign ones; and they devour some microorganisms but tend to reject encapsulated ones. It is well known that the uptake of most particles is mediated by antibody or other factors from serum (Stossel 1975). Phagocytes differentiate between particles that bind antibody or complement and particles that do not. The specificity of the recognition is mainly governed by immune factors (Rabinovitch 1970). Alveolar macrophages have receptors for the crystallizable fragment (Fc) of IgG and the

third component of complement. These receptors are important in particle attachment and ingestion by alveolar macrophages (Daughaday and Douglas 1976).

Complement should be adequately defined. The term "complement" refers to a complex group of enzymes in normal blood serum that is heat labile. Antibodies and complement working together play an important role to mediate both immune and allergic responses. The antibody identifies the foreign organism, then activates the complement system against it. Complement causes lysis of some organisms, but certain enzymatic fragments are chemotactic. The third fragment of complement serves as an opsonin which is a term that has come to represent any agent in serum which acts on particles or organisms to increase their liklihood of engulfment by phagocytes (Mayer 1973).

The pursuit of the foreign particle by the macrophage is affected by chemotaxins. These are substances which initiate directed movement when presented to cells in a concentration gradient (Barrett 1974). The chemotactic agent used in this experiment is serum complement, yet many compounds are known which influence the chemotactic phase of the inflammatory response. It should be noted that certain substances that are chemotactic in vivo appear not to be chemotactic in vitro. Some of the chemotactic agents are released from various cell types, such as leukocytes, virus infected cells, stimulated lymphocytes, mast cells, tumors, and various types of dying cells such as erythrocytes or leukocytes (Keller et al. 1975). The chemotactic response of macrophages appears to be modulated through cell surface membranes (Allred and Hill 1973).

A particle with the proper surface configuration positioned adjacent to a phagocyte somehow results in movement of the cell so that the particle becomes internalized. Ingestion by mammalian macrophages is an energy-dependent process that has been described by Stossel (1975). Microfilaments and microtubules have been demonstrated in the cytoplasm of phagocytic cells in areas of pseudopod formation. Stossel (1975) has described a system of contractile protein and a co-factor required for activation of the myosin-associated Mg^{++} ATPase. These findings have prompted the proposal that particle contact with the plasma membrane activates the actin-binding protein. This activation leads to polymerization and cross-linking of actin into microfilaments. In the presence of co-factor, myosin causes contraction of the cytoplasm; this contraction provides the forces for pseudopod formation and engulfment of the particle. This is a hypotheses at present. The presence in mobile nonmuscle cells of actin and myosin with structural and functional similarities to muscle actin and myosin, lends weight to the hypothesis that locomotion of phagocytes, and ingestion itself, may be driven by contractile proteins. Also, similar to skeletal muscle, nonmuscle actin and myosin seem to show some regulatory effect by divalent cations (Stossel 1975).

Locomotion through pseudopod formation obviously demands the formation of new phospholipid membranes. Karnovsky and Wallach (1961) have reported that increased phospholipid synthesis accompanies high phagocytic activity by macrophages and this probably

reflects the formation of many phospholipid phagosomal membranes. Increased phospholipid synthesis is accompanied by an increase in oxygen uptake and glucose metabolism through the hexose monophosphate shunt (Day and Hokin 1967).

Phagocytosis has been shown by Ouchi et al. (1965) to be dependent on optimal temperature (37°C), pH (7.0), incubation time (60 minutes), and osmolarity (0.9 percent NaCl). It is noted that Bertalanffy (1964) reported that alveolar cells phagocytized material within 4 minutes in vivo and the peak phagocytic activity occurred from 6-16 minutes. Ouchi et al. (1965) claimed an optimal incubation time of 60 minutes in vitro for particle ingestion. Macrophages also require an optimal particle:phagocyte ratio and require serum, depending upon the nature of the particle used to induce phagocytosis.

Metabolism of the alveolar macrophage has shown differences when the cells were at rest compared to cells phagocytizing. The oxygen consumed during phagocytosis increases 20 percent over when the cell is at rest. It has been shown that alveolar macrophages depend on aerobic oxidative metabolism for energy needs during phagocytosis (Babior 1978).

Inactivation of ingested material

The mechanism through which alveolar macrophages inactivate ingested material is poorly understood. Until recently most studies have been performed on other phagocytic cell types (Allen et al. 1972, Baehner et al. 1975, Klebanoff 1975), and scientists

have drawn conclusions from such studies and have applied them to alveolar macrophages.

Bacterial killing involves a multiplicity of mechanisms, all of which are set into motion by two cellular events: degranulation and initiation of the respiratory burst. Degranulation describes the process of fusion between the primary phagosome and the granule present in the cytoplasm of the phagocyte. Granules contain materials that participate in bacterial killing and degradation. During degranulation, these materials are discharged into the vesicle containing the ingested micro-organism.

The respiratory burst describes a metabolic pathway, dormant in resting cells, whose function it is to produce a group of highly reactive microbial killing agents by the partial reduction of oxygen. Killing is accomplished through the actions of both the granule contents and the oxidizing agents provided by the respiratory burst (Babior 1978).

The first event in the respiratory burst metabolic pathway is the sharp increase in oxygen uptake that occurs upon stimulation of the phagocyte. It was originally thought that the rise in oxygen consumption was to provide energy for phagocytosis. It was soon reported, however, that part of the oxygen consumed was converted to hydrogen peroxide (H_2O_2) (Iyer et al. 1961). The mechanism of H_2O_2 production is thought to occur through an NADPH oxidase. There is some evidence that NADPH oxidase, either directly or indirectly via a glutathione-dependent peroxidative pathway, may provide a link between stimulation of respiration and

activation of the hexose monophosphate shunt (HMPS) (Johnston 1978).

An important aspect of the respiratory burst recently discovered is the production of superoxide (O_2^-) (Allen et al. 1972). This compound, formed by the one-electron reduction of oxygen, has attracted much attention since the discovery of superoxide dismutase, the enzyme that catalyzes the destruction of O_2^- (Babior et al. 1973). Boxer et al. (1979) have clearly demonstrated augmentation of superoxide anion production by alveolar macrophages after membrane perturbation or phagocytosis. Superoxide dismutase, the enzyme that catalyzes the formation of hydrogen peroxide and molecular oxygen from superoxide anion, is also present in the alveolar macrophage of several species (Gee and Khandwala 1976).

The respiratory burst features increases in oxygen uptake, O_2^- production, H_2O_2 production and hexose monophosphate shunt activity--all of which occur when phagocytes are exposed to a stimulus. The schematic shown below illustrates the metabolic concomitants of particle ingestion by macrophages (Hocking and Golde 1979).

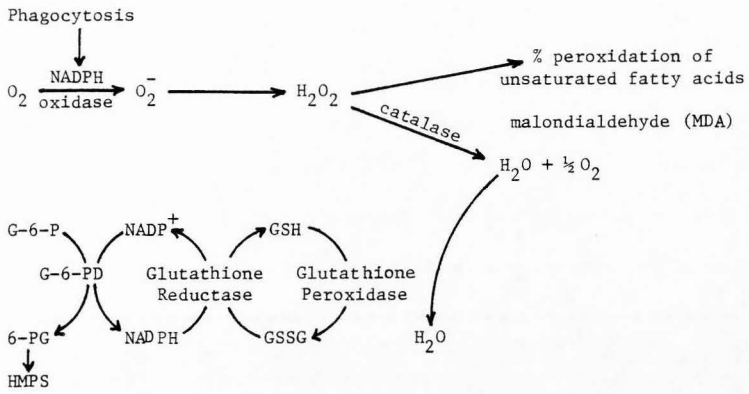

Illustration 1. Metabolic Concomitants of Particle Ingestion by Macrophages.

Mechanism for chemiluminescence production

A manifestation of macrophage function which has attracted considerable interest recently is the emission of light (chemiluminescence) by cells engaged in phagocytosis (Allen et al. 1972, Allen and Loose 1976, Babior 1977, Bryant and Hill 1980). This phenomenon, first reported by Allen et al. (1972), was found to be inhibited by superoxide dismutase and catalase. Chemiluminescence was not observed in cells from patients with chronic granulomatosus disease (CGD). The neutrophils from these patients do not exhibit a respiratory burst, i.e., no O_2^-. In the original report (Allen et al. 1972), chemiluminescence was taken to indicate that phagocytes were generating singlet oxygen during the respiratory burst and this chemical species in turn was relaxing to ground state (triplet) oxygen. This supposedly led to

the emission of three discrete light photons. Cheson et al. (1976) have examined the spectrum of light emitted from phagocytizing granulocytes and discovered that very little of the light emission could actually be attributed to the relaxation of singlet-to-triplet oxygen. This is due to the fact that the emissions represent broad spectra of light and no discrete photons are recognized. Hart and Cormen (1979) proposed that the singlet oxygen produced oxidized secondary molecules to form a light-emitting product. Barenboim et al. (1969) noted that many cell types thought to emit chemiluminescences actually derive their emissions from the oxidation of lipids. Emissions from lipids stretch from the blue part of the light spectrum to the infrared. The metabolic pathway suggested by Hocking and Golde (1979) implicates the peroxidation of unsaturated fatty acids as a possible part of that pathway. In a study regarding the possible mechanisms of paraquat toxicity, Bus et al. (1976) proposed a more refined model of lipid peroxidation due to singlet oxygen production. It is feasible, therefore, that the making and breaking of phospholipid membrane links (such as in the formation of phagocytic vesicles) could conceivably underlie the broad spectrum of light emission responsible for chemiluminescence. This hypothesis is further supported by the work of Mason et al. (1972) that shows malondialdehyde (MDA), a product of peroxidation of certain unsaturated fatty acids, is present in alveolar macrophages. The study also reported the presence of lipid peroxides in isolated phagocytic vesicles. The studies mentioned above infer that peroxidation of endogenous lipid can occur during phagocytosis.

Although the mechanism of chemiluminescence is still unproven its use is becoming more common. Chemiluminescence is used as a screening test in pediatric hospitals for patients suffering chronic granulanatosus disease (CGD). In this disease respiratory bursts after stimulation by particles in neutrophils of the blood do not occur. Consequently, lowered or absent light emissions indicate a positive response, in afflicted persons, to the test.

Chemiluminescence has also been used as a screening test for anti-inflamatory drugs; the more potent drugs inhibit chemiluminescence the most (Van Dyke et al. 1979). The test is simple enough to perform that rapidly increased clinical usage is expected.

Metals and Macrophages

The defensive capacity of the alveolar macrophage against airborne particles and microorganisms has led to an intense investigation of factors that control the function of these cells. Since the lungs of most persons, especially in the urban population, are subjected to a variety of gaseous contaminants and particulates, alterations in the functions of alveolar macrophages induced by these agents are evidently relevant to the study of pulmonary disease. The continuous disruption of these cells by low-level exposure to air pollutants may cause not only impaired defense against infectious agents (Green and Kass 1964b), but may also result in changes to the lung, such as fibrosis, which is thought to be due to overstimulation of lung defense mechanisms (Gee and Khandwala 1976).

Environmental exposure to airborne contaminants involving metals has been studied extensively. Many heavy metals have been found to be associated with airborne particles from combustion sources, such as fossil-fueled power plants, metallurgical smelter blast furnaces, and also from autos and municipal incinerators. Recent reports, notably those of Lee and Von Lehmden (1973), and Natusch et al. (1973) have confirmed that several metallic elements occur among the smallest particles collected from ambient air. Small particle size increases the probability of deposition in the alveolar region which, in turn, challenges the alveolar macrophage to rid the lung of the metallic contaminant.

In vivo studies

Many studies have been performed to predict the fate of metals in the lung. Bingham et al. (1972) subjected rats to soluble and insoluble aerosols of lead or nickel at concentrations near or below the levels proposed as acceptable for occupational exposures. Results reported made it evident that a nonspecific increase in the number of alveolar macrophages cannot be assumed after inhalation of metallic particulates. Henderson and co-workers (1978a, 1978b, and 1979) attempted to develop a rapid screening test for pulmonary toxicity to energy-related effluent material by examining the enzymatic and cytologic profiles of airway fluids. Animals were exposed by inhalation to $CdCl_2$ or $CrCl_3$ which resulted in a generalized increase in lavage fluid enzymes. Cytologically, the $CrCl_3$ exposed lungs were essentially normal, while the lungs exposed to $CdCl_2$ had significant histopathological alterations. Further cytological studies were performed by Johansson et al. (1980) on rabbit alveolar macrophages after

6 months inhalation exposure to metallic nickel dust. Most macrophages had a strikingly smooth surface and high amounts of electron-dense inclusions. This gave the impression of an inactive surface. Macrophages from exposed animals had an increased oxidative metabolism at rest, and the lack of significant increases in oxidative metabolism upon stimulation with bacteria suggested an impaired functional capacity. Macrophages from patients with pulmonary alveolar proteinosis demonstrated the same cytological and metabolic response (Golde et al. 1976).

Other in vivo studies performed have investigated animal exposures to large doses of oxides of iron, antimony, and silicon. In a study reported by Gross et al. (1969) the investigators administered the metal compounds either intratracheally (30 mg/lung) or via inhalation (4200-1200 mg/m^3). The study compared the two routes of administration and their effects on the number of macrophages harvested. Results in hamsters showed that, initially, the intratracheal administration elicited more macrophages in the wash-out. By day four, there was no difference between the number of cells harvested between the techniques of administration. Many other in vivo studies have been performed with various air pollutants where different parameters of the macrophage were measured (Coffin et al. 1968, Hurst et al. 1970, Hayes et al. 1977, Bergstrom and Rylander 1977, Sanders et al. 1977). All of these studies had two things in common: the exposure route and the parameters measured in vitro. The cells were, in each case, washed from the lungs to allow the different measurements of response.

In vitro studies

The expense of equipping an inhalation laboratory is great. Many researchers, consequently, have turned to in vitro studies, e.g., taking the cells from the body of the experimental animal and incubating them with media on petri plates. The alveolar macrophage is ideal for this type of preparation. These cells adhere to petri dishes by forming a monolayer and allow contaminating cell types to be poured off. Soderland and Naum (1973) perfected the technique of culturing alveolar macrophages for long periods of time. Many studies have been performed in the last decade to observe the effects of metallic pollutants dissolved in the media of the cell culture.

Cadmium is one of the more studied metal contaminants using this approach (Cross et al. 1970, Waters et al. 1975, Loose et al. 1977, Castranova et al. 1980). Cadmium chloride has been shown to decrease oxygen consumption and ATPase activity in intact cells and isolated mitochondria from a treatment of 0.5 mM to 5mM. Cadmium chloride, even at a level of 3.75 μg/ml in the media, has caused a decrease in cell number, viability, and bacterial killing enzyme. Cadmium acetate has also been shown to decrease oxygen consumption in phagocytizing cells.

The effect of metallic contaminates on phagocytosis is usually studied by testing the ability of macrophages to ingest the contaminants themselves (Casarett et al. 1971) or of Teflon particles coated with the metal of interest (Camner et al. 1974). Many interrelated variables such as the number of cells, the number of particles, the duration of exposure,

the ionic concentrations of the media, and the electrostatic potential of the particle can affect phagocytosis; interpretation of these results is difficult. Experiments in which attempts have been made to control these variables indicate that the rate of uptake of iron oxide particles by alveolar macrophages differs from that for chromium metal oxide particles. Similarly, particles coated with silver are ingested at slower rates than particles coated with aluminum or chromium (Goldstein 1977). Although the toxicologic importance of these differences is unclear, the recent observation that metallic ions (Ni^{2+}, Mn^{2+}, Cr^{3+}) are cytotoxic for alveolar macrophages suggests that the uptake of these particles is not innocuous (Waters et al. 1975). Because ingestion is important to phagocytic functions, it can be anticipated that the effects of airborne agents on this function will be evaluated more thoroughly in future toxicologic experiments using many different methods.

The experimental method of choice may be identified more clearly in the future along with determinations of the actual pulmonary effects of heavy metals. Waters et al. (1974) described shortcomings of *in vitro* testing when they reported on the solubility of several vanadium compounds. They enumerated these problems of *in vitro* testing: the cells being tested are out of their normal biochemical and physiological environment, the doses of test compounds dissolved in small amounts of media are usually large and the stress on the cells in the experimental preparation are significant.

To lend credence to the objective of this thesis study, comparisons of similar *in vivo* and *in vitro* studies were made.

The primary objective of this study was to elucidate any differences that might exist between two experimental models used in observing effects of air pollutants. The impetus for performing this study arose from recognition of discrepancies found in the literature. To present more clearly the problems involved in extrapolating in vitro data to human situations, a comparison of the experimental technique has been made (Table 1).

Henderson et al. (1979) measured the aerodynamic diameters of $CdCl_2$ aerosol particles to assure their deposition in the lower alveolar lung regions, and also confirmed that the particles were not hydrated or charged before entering the animal during inhalation. The electrostatic potential of the aerosol can affect phagocytosis (Casarett et al. 1971) as well as penetration depth. Combined with elemental ions in media to neutralize or enhance the electrostatic potential which in turn affects the phagocytic response, may cause results to vary widely as a function of the media time used for individual experiments. Many different medias have been used in macrophage function experiments. There are certain buffers in some media that very effectively chelate heavy metals and prevent them from dissolving properly (Good et al. 1966). Acid phosphatase, the enzyme studied in the first two experiments in Table 1, is a hydrolytic enzyme dumped into the phagocytic vesicle after a Ca^{++} ion influx (Casarett et al. 1971). The ionic concentration of Ca^{++} in media at an improper concentration could affect results involving this enzyme.

Table 1. Comparison of results obtained following live or in vitro exposure methods to metal compounds. Rodents were used as test animals in each study.

Metal Compound	Method of Exposure	Dose	Species	Parameter Measured	Result	Reference
$CrCl_3$	Inhalation	20 µg/lung	hamsters	Acid Phosphatase	Increased	Henderson et al. (1979)
$CrCl_3$	In vitro	916 µg/ml	rabbit	Acid Phosphatase	Decreased	Waters et al. (1975)
$CdCl_2$	Inhalation	4.4 µg/lung	hamsters	Number of cells counted	Increased	Henderson et al. (1979)
				Acid Phosphatase	Increased	
$CdCl_2$	In vitro	22_6µg/26x 10^6 cells	rabbits	Number of cells counted	Decreased	Waters et al. (1975)
				Acid Phosphatase	Decreased	
$NiCl_2$	Inhalation	109 µg/m^3	rats	Number of cells counted	Increased	Bingham et al. (1972)
$NiCl_2$	In vitro	129 µg/ml	rabbits	Number of cells counted	Decreased	Waters et al. (1975)
metallic Ni^{++}	Inhalation	.5-2 mg/m^3	rats	oxidative metabolism	Increased	Jarstrand et al. (1978)
ionic nickel	In vitro	.58 mg/ml	rats	glucose metabolism and O_2 consumption	Decreased	Castranova et al. (1980)

Comparison of the number of macrophages collected after exposure to $CdCl_2$ involves many factors. Waters et al. (1975) incubated macrophages for 20 hours with different metals. Twenty hours is sufficient time to turnover 20 percent of the live animal lung macrophage populations via cell division and translocation according to Spritzer and co-workers (1964, 1968). Inhalation studies involving lung irritants report an increase of polymorphonuclear neutrophils (PMN), eosinophils, and macrophages in lavage solutions. A proportionate increase in macrophages does not occur when cells are collected in petri dishes prior to exposure. Waters et al. (1974), in a previous study, reported a 30 percent spontaneous lysis rate of cultured macrophages after 20 hours in media. Therefore the decreases seen in macrophage number in studies involving cultured macrophages could be due to other influences in addition to the effects of the metals.

The inhalation study by Bingham et al. (1972) demonstrated that not every compound that the lung is exposed to can be expected to elicit an increase in macrophages recovered by lavage; however, $NiCl_2$ exposure did cause an increase in numbers of macrophages harvested when compared to controls. Waters et al. (1975) tried to draw conclusions about cytotoxicity of $NiCl_2$ to the macrophages. If the criterion used is macrophage death then a decrease in a closed *in vitro* system would be expected since recruitment of peripheral macrophages is not possible. This was the conclusion drawn from the *in vitro* experiment. In contrast, the inhalation study clearly showed an increase in the number of macrophages recovered after exposure to $NiCl_2$.

Jarstrand et al. (1978) exposed animals for 4 weeks to nickel dust in a manner that simulated a working person's exposure (7 hours/day, 5 days/week). The inhalation study showed that the oxidative metabolism of the macrophage actually increased after a 4 week exposure to low doses of nickel. In comparison, Castranova et al. (1980) exposed macrophages for 15 minutes to high concentrations of ionic nickel (Ni^{++}) which resulted in decreased glucose metabolism and oxygen consumption. Cross et al. (1970) have shown the impairment of membrane-bound ATPase by divalent cations in an in vitro study and implied that the cation may effect respiration; consequently, oxidative metabolism is impaired.

As in all aspects of toxicology, the dose and exposure method chosen clearly affect the results obtained in lung toxicity studies. The studies cited in the paragraphs above cannot be compared solely on the basis of dose, but they do represent some of the results of metal toxicity determinations to pulmonary alveolar macrophages. There are many physical factors in inhalation studies that determine the site at which toxic agent will act (which can drastically change the effective dose given). Consequently, the dose administered may not be the actual dose delivered to the expected site of action. For these reasons it makes direct comparison very difficult between these two methods--when the cell is isolated and treated compared to when the live animal is treated.

Ever increasing degrees of sensitivity are sought in toxicological tools in cases of very low exposure to elements. Methods that have been used routinely for granulocytes, but less so for

alveolar macrophages, is chemiluminescence and oxygen consumption. Miles et al. (1978) published a synopsis of these phenomena and reported that based on ED_{50} values chemiluminescence and oxygen consumption were found to be the alveolar macrophage responses most sensitive to metal ions (10^{-5} m). Sensitive indicators were necessary in this study due to the low exposure used; therefore, chemiluminescence and oxygen consumption were obvious choices for measuring toxicity.

MATERIALS AND METHODS

Animals

Long Evans male rats, 51 days old, 126-150 grams (Charles Rivers Breeding Laboratories, Inc., Wilmington, Massachusetts), were used for all experiments. The rats were acclimatized for 10 days, five animals per plastic cage. Housing conditions consisted of a 12-hour day-night cycle with food and water ad libitum.

The Long Evans rat (hooded or piebald coat) used for this experiment was selected due to the work of Stratman and Conejeros (1969), which revealed that this strain is particularly resistant to lung infections. Lung infections could produce considerable experimental error in the parameters measured in this experiment. Consequently, these test rats were visually observed for evidence of lung infection for 10 days prior to initiating the study.

Chemicals Used for Live Exposure and Their Preparation

Treatment chromium compounds

The two hexavalent chromium compounds used in the experiment, CrO_3 and $CaCrO_4$, were purchased in reagent grade, powdered form from Sigma Chemical Company (St. Louis, MO). Both compounds were mixed with physiological saline made from deionized water to produce 2 µg/0.3 ml doses.

Lavage solution

The alveolar macrophages were washed from the lungs by lavaging

with an ice-cold solution consisting of 145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.3 mM Na_2HPO_4, and 5 mM glucose (Myrvik et al. 1961). A tenfold concentrated lavage solution was made using deionized water mixed lavage solution and 90 mg glucose were added. The pH was adjusted to 7.4 using 1 N NaOH.

Buffer suspended macrophages to measure chemiluminescence and trypan blue

After the macrophages were pelleted by centrifugation, they were resuspended in a HEPES-buffered medium containing: 140 mM NaCl, 5 mM KCl, 10 mM Na - HEPES (N-2-hydroxyethlpiperazine-N'-2-ethane sulfonic acid) and 5 mM glucose (Castranova et al. 1980). The HEPES was purchased from Sigma Chemical Company (St. Louis, MO). This solution was also made in a tenfold concentrated solution in advance without glucose. Before beginning the experiment a 1:10 dilution was made of this solution and glucose added. The pH was adjusted to 7.4 using 1 N NaOH. All parameters determined were made with the cells suspended in this medium. HEPES buffer was used because it does not bind to heavy metals (Good et al. 1966).

Chemicals Used for Incubated Cell Exposure and Their Preparation

Treatment chromium compounds

The compounds, CrO_3 and $CaCrO_4$, were made using de-ionized sterile physiological saline in the same concentration as the live exposure method.

Lavage solution

The washing procedure was performed using millipore filtered lavage solution made in the same manner as the live exposure lavage solution.

Cell culture media

The cells when collected were centrifuged and then resuspended in Minimum Essential Medium (MEM) with 25 mM HEPES buffer. This media is made for monolayer cultures with Hanks' salts and without glutamine. The media was purchased from Grand Island Biological Company (GIBCO) (Grand Island, NY). The media was supplemented with 50 units/ml of Penicillin-Streptomycin also purchased from GIBCO. The fetal calf serum supplement was purchased from Sterile Systems, Inc. (Logan, UT) and heat-inactivated for 30 minutes at 56°C. The media contained 20 percent heat-inactivated fetal calf serum. The pH was adjusted to 7.4 using 1 N NaOH. The supplemented medium was then millipore filtered and stored at 4°C.

Trypsin solution

The 0.5 percent trypsin solution used for removing the cells from the plates was made with sterile HEPES buffers. It was purchased from Becton-Dickinson (Cockeysville, IN). The trypsin used had an activity of 1:300, as measured by casein degradation.

Zymosan preparation

The foreign particle used in all experiments was zymosan, an extract from the cell walls of Saccharomyces cerevisiae yeast. This

was purchased from Schwarz/Mann (Orangeburg, NY). The preparation for opsonizing the zymosan was taken from Hill et al. (1977) and N. Hogan (1980, personal communication). Thirty mg of zymosan was weighed into sterile plastic tubes (Falcon, Oxnard, CA). The particles were washed once in HEPES buffer, vortexed vigorously for 5 minutes to break up clumps, then centrifuged at 2,000 rpm for 10 minutes in Sorvall GLC-1 centrifuge (Newton, CT). The HEPES buffer was poured off and fresh human serum was added to a concentration of 10 mg/ml and mixed in a vortex mixer. The tubes were capped and rotated for 1 hour at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific Company, Inc., New Brunswick, NJ). Following centrifugation at 2000 rpm for 10 minutes, the serum was removed and replaced with an equal volume HEPES buffer. This was stored at -70°C in 1 ml aliquots which were individually thawed before each experiment.

Trypan blue stock solution

The exclusion of trypan blue was used in all experiments to determine cell viability. The powder was purchased from Fisher Scientific Company (Pittsburgh, PA). The stock solution was prepared by combining 0.4 grams trypan blue, 0.81 grams sodium chloride, and 0.06 grams potassium phosphate monobasic in 95 ml deionized water. This solution was brought to a boil, then allowed to cool. The pH was adjusted to 7.2-7.3 with 1 N NaOH. The final volume was brought up to 100 ml with deionized water.

Live Exposure Procedure

The rats in this group received an acute intratracheal dose of either 2 µg/0.3 ml $CaCrO_4$ or 2 µg/0.3 ml CrO_3. These doses represent the maximal average environmental exposure to the general population reported to date (Sullivan 1969).

The chromium lung dosing was accomplished with the use of an illuminated intratracheal speculum. Each of the rats was lightly anesthetized with ether and suspended dorsally on a slanted board by its upper incisors with the tongue depressed by the speculum, the light made the upper portion of the trachea visible. The injection was made with a blunted, curved, 8 cm, 18 gauge hypodermic needle. This method has been described previously (Oberg et al. 1978). A sample group of six rats for each exposure was sacrificed 9 hours post-injection. This time period was chosen to allow maximal cytotoxic effects in the live exposure and the incubated cell exposure.

Lavage procedure

Alveolar macrophages were obtained from male rats by tracheal lavage according to the method of Myrvik et al. 1961. Each animal was sacrificed by cervical dislocation. The thoracic cavity was opened and the upper part of the trachea dissected free and clamped shut with a hemostat to prevent blood from entering the lung when the trachea was transected. The trachea was cut above the point where it was clamped and the lungs, heart, and trachea were surgically

removed as a single unit. These organs were washed with warm, physiological saline (37°C) with the trachea closed. The heart was then dissected free while carefully avoiding any injury to the lungs or bronchi. The lungs were then sponged free of excess fluid with gauze pads, and suspended with the lumen of the trachea open by attaching a hemostat to the wall of the trachea. The lungs from each rat were lavaged 10 times with a total of 60 milliliters of ice cold 2+ buffer solution, described previously. This procedure was carried out insuring both lobes of the lungs were expanded and lavaged. The trachea was then clamped, the lungs massaged gently and then the fluid was drained into individual test tubes. This allowed washings that were contaminated with blood to be discarded.

The cells were separated from the lavage fluid by centrifugation at 200 x g for 5 minutes. The cells were resuspended in 1-2 ml of HEPES buffer, and then the number of cells per milliliter of this suspension was determined by use of a hemocytometer. The HEPES used to resuspend the cells, and in all remaining experimental proceedings, contained 5 mM glucose and was maintained at pH 7.4. This is the optimal pH for phagocytosis in these cells (Pearsall and Weiser 1970).

Viability of the macrophages was determined using exclusion of trypan blue dye (Phillips 1973).

Oxygen consumption was measured with a Gilson Medical oxygraph, with a 2.5 ml chamber using a Clark electrode.

Chemiluminescence was determined using a Packard Tri-Carb liquid scintillation counter.

Trypan blue dye exclusion test

An aliquot of 0.1 ml of cells was suspended in 0.9 ml fresh HEPES-buffered medium. Sufficient volumes of a 0.4 percent solution of trypan blue dye was added to this suspension so that the final concentration was 0.1 ml of dye per milliliter of suspension. After 4 minutes in the dye, the cells were observed with a light microscope. Viable cells were taken as those which excluded dye and were therefore not lysed (Phillips 1973). The number of viable cells was determined using the following equation:

$$\% \text{ viable Cells} = \frac{\text{nonstained cells}}{\text{total number counted}} \times 100.$$

Oxygen consumption procedure

To measure the amount of oxygen consumed by alveolar macrophages, the cells were preincubated 15 minutes at 37°C prior to measurement. An aliquot of the cells (3×10^6) in HEPES-buffered medium was then transferred to the chamber of the oxygraph which was also maintained at 37°C, and oxygen consumption in nmoles/10^7 cells/minute was determined for macrophages at rest for 10 minutes and while phagocytizing a foreign material. After this time 2 mg zymosan was injected into the chamber and oxygen consumption for macrophages that were phagocytizing was measured for 10 minutes.

Chemiluminescence procedure

Chemiluminescence is measured by the method used by Miles et al. (1978). Plastic vials (5 ml) were dark adapted for 24 hours prior to the study by wrapping in foil and placing in a dark place. Prior to the chemiluminescence measurement, 3×10^6 macrophages

were drawn into a syringe; enough fresh HEPES buffer to make up to 3.3 ml was also added. At that time, 2 mg of zymosan (0.2 ml) was drawn into a separate syringe. The macrophage-buffer suspension was then injected into a dark adapted vial while in the dark. The vial was returned to a light-tight container for transportation to where the actual measurement was made.

The Packard Tri-Carb 3000 liquid scintillation counter was used to determine chemiluminescence. The machine was switched to the out-of-coincidence mode with the discriminators set from zero to infinity. The entire machine was draped with black plastic so that no extraneous light would be counted in the measurement. At this time, while under the drape, the macrophage-buffer preparation was removed from the light-tight container and placed in a test tube warmer set at 37°C for 15 minutes. During this time, background readings were made. Once the machine was switched to out-of-coincidence the average machine background was recorded as 15,000 counts per minute (cpm). All reported results are net results, i.e., gross counts per minute minus average background.

At Time Zero, 2 mg zymosan were added to each vial and they were counted immediately for 1 minute, then returned to a test tube warmer for 1 minute at 37°C. This reaction is temperature dependent, therefore, this was done to keep the reaction at the proper temperature. Counting, alternated with warming, was repeated for 30 minutes for replicate samples. The CL data were recorded as counts per minute.

Live Exposure Controls

In the live exposure case, the test animals received intratracheal injections of the toxic compound; therefore, an animal that received an intratracheal injection of physiological saline served as a control animal.

Controls for chemiluminescence (CL)

Duplicate runs on both the untreated control and treated animals, when cell numbers allowed, served as controls for CL. Those controls consisted of:

1. Background count with the machine switched to out-of-coincidence.
2. HEPES-buffered medium alone
3. HEPES-buffered medium plus zymosan
4. Macrophages plus HEPES-buffered medium (background for experiment).
5. Macrophages, HEPES-buffered medium, plus zymosan (cells phagocytizing).

Controls for oxygen consumption

Duplicate runs for both control and treated animals served as controls for oxygen consumption. The baseline for oxygen consumption was determined using HEPES-buffer in which the oxygen content (nmoles/l) was determined using the Winkler titration method (Skoag and West 1976). This titration used a sodium thiosulfate titration to bind all the oxygen dissolved in the HEPES-buffer at 37°C, that is,

in the calculations involving consumption of oxygen by the macrophages this was an established baseline.

The cell suspension in HEPES buffer was added to the oxygraph chamber and oxygen consumption of the cells at rest was determined after 10 minutes. Then zymosan was injected into the chamber and the oxygen consumption determined for 10 minutes while the cells were phagocytising.

Plated Cell Procedure

Six animals per exposure group were sacrificed by cervical dislocation and lungs lavaged as with the live exposure procedure, with the exception sterile technique was used. The macrophages from two animals were pooled due to the number of cells that could be retrieved from the plates.

The macrophages harvested were separated by centrifugation and resuspended in 1.5 ml prewarmed (37°C) tissue culture media (MEM) with Hank's balanced salt solution; supplements added to the medium included heat-inactivated fetal calf serum (20 percent) and 50 units/ml penicillin-streptomycin. Cells were counted by means of a hemocytometer and viability determined via trypan blue exclusion as described above. The cells were then divided and transferred to 50x15 mm petri dishes. The macrophages were allowed to attach for 2 hours in a humidified atmosphere of 96 percent air and 4 percent CO_2 at 37°C. Cells that did not attach were carefully poured off and the chromium to be evaluated was added in 1.5 ml fresh supplemented medium.

After 9 hours macrophages were harvested from the petri dishes. This time was selected to permit maximum opportunity for the development of any toxic effects in both systems and to minimize the effects of spontaneous cell lysis (Waters et al. 1974), particularly in the macrophage preparation being described. The medium was poured off and cells that remained attached were removed with 1 ml prewarmed 0.5 percent trysin solution. The reaction was stopped after 10 minutes by adding 1 ml fresh media. This solution was poured off and petri dishes were rinsed twice with HEPES buffer. The cells plus rinses were centrifuged for 5 minutes at 2,000 rpm.

At this point the macrophages were resuspended in 1 ml HEPES buffer. The cells' viability and number were again ascertained as described previously. Chemiluminescence and oxygen consumption were carried out as described under live exposure.

Plated Cells Controls

This method requires that macrophages first be taken from the animal and exposed to chromium compounds after attachment to petri dishes. To achieve this the animals were sacrificed immediately and the macrophages harvested. The cell suspension was counted and viability determined. The suspensions were cultured in MEM with supplements for 2 hours with nonadherent cells poured off after this time. At this time 0.3 ml of physiological saline was added to the media. This preparation was allowed to incubate for 9 hours. At the end of that time the cells removed for analysis were again counted and viability was determined. At this point the

controls were identical to the live exposure procedure for the analysis of CL and oxygen consumption.

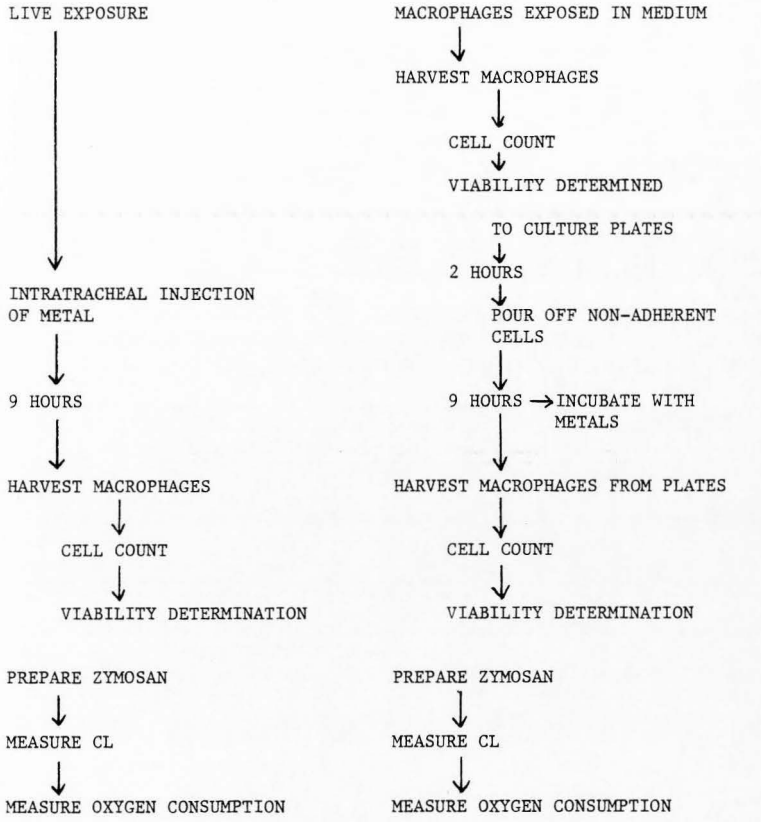

Illustration 2. Methods Flowchart

RESULTS

One objective of this study was to compare two similar toxicological techniques that have been used as models for studying the effects of air pollutants on alveolar macrophages. The two methods were also utilized to examine the effects of exposure to low doses of either of two hexavalent chromium conpounds. Fresh cells (in vivo) and incubated cells (in vitro) were evaluted for similarities and differences because of conflicting results reported in the literature (see Table 1 in LITERATURE REVIEW section). In efforts to extrapolate toxicological data to human situations, it is important to appreciate any differences that might exist between models analyzed. The cellular responses most sensitive to pulmonary alveolar macrophage (PAM) damage, O_2 consumption and chemiluminescence (CL), were used to determine the differences in the two methods and the two treatments.

An intratracheal injection was used to simulate inhalation with the live exposure (in vivo) method. Although this method results in a relatively greater proportion of the material being deposited in the lower respiratory tract than normal inspiration, it is thought that the lung deals with the contaminant in the same manner (Watson et al. 1969). Due to the possible stress this injection procedure could subject the test animals to, control rats received doses of equal volume of physiological saline in the same manner. Likewise, the petri dishes containing the incubated (in vitro) control cells also received 0.3 ml physiological saline.

Parameters measured in this experiment, oxygen consumption, CL, and trypan blue dye exclusion, involved in vitro techniques. In other words, whether the cells were exposed in live animals or in petri dishes, the study results were derived from tests performed on the cells in test tubes, i.e., in vitro. Cells from each source, harvested from animals or petri dishes, were subjected to the same tests which took place with the cells suspended in a HEPES buffer solution.

Another objective of the study was to observe the effects of chromium treatments on the alveolar macrophage as gauged by each of the two experimental methods indicated. The primary function of the macrophage, as has been explained, is to phagocytize (engulf) foreign particles and microorganisms. In order to activate this function, a foreign particle was added to the buffer-cell suspension to stimulate the cell to phagocytize. This foreign particle was zymosan. The nature and preparation of zymosan was explained in the MATERIALS AND METHODS section. This feature is an integral part of the experiment because researchers have shown that air pollutants may not disturb the metabolism of the resting macrophage, but can impair this functional property of the cell (Johansson et al. 1980).

Objective One: Comparison of the Two Experimental Methods

Chemiluminescence of untreated controls

It is imperative when discussing the two methods studied that they do not become confused in the mind of the reader. It is necessary, therefore, to clarify the terminology used to portray each method. The macrophages exposed to chromium compounds in the live

animal are variously referred to as fresh macrophages, macrophages harvested from live animals, and macrophages from live exposure animals--these terms are synonymous in this work and all relate to in vivo exposures.

Macrophages that were harvested first and then attached to petri plates before exposure are referred to as plated cells, macrophages that were incubated in media during exposure and incubated macrophages--all refer to the in vitro preparations. Also, controls are cells that have received no exposure to chromium, therefore, are called untreated controls. To keep understanding of the terminology clear periodic clarification will be given.

Figure 1 represents the CL time course for untreated controls from both methods. This graph shows that the macrophage-buffer reaction with zymosan produced CL that reached its maximum at 7 minutes after initiation in the untreated fresh cells, i.e., those macrophages harvested from live animals. After peaking CL levels subsided in approximately exponential fashion. By 29 minutes after initiation there were no significant differences between the final counts and the beginning 1 minute sample counts. Conversely, in the untreated plated cells, those that were exposed after attachment to petri plates, CL was high initally, decayed exponentially to its average value after 9 minutes, which was not significantly different from minute 7 values, and then began to rise slightly with time. The 29 minute value, however, was not different from the 7 or 9 minute results. The shaded area highlights the least significant difference (LSD) 1 way ANOVA of the points on the graph that are significantly different ($P<.005$).

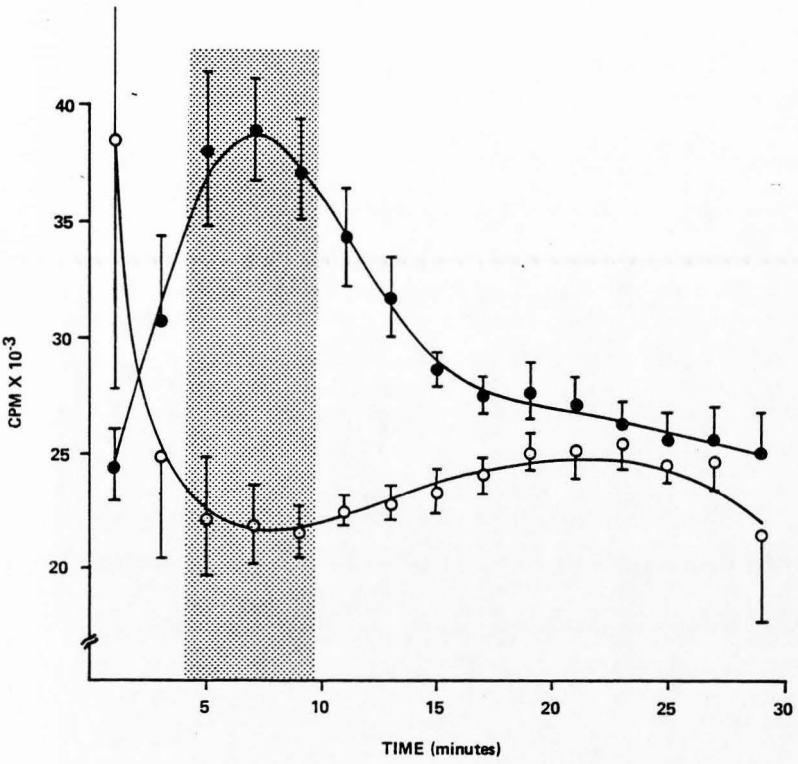

Figure 1. Chemiluminescence of untreated macrophages from live animals (●——●) and incubated in media (○——○). Shading represents points of significant difference ($P<.005$). All points on the graph have machine background (15,000 cpm) subtracted from them.

Comparison of two methods: CL after exposure to chromic trioxide

Figure 2 shows the CL values of cells prepared by each method after treatment with 2 μg of chromic trioxide. Cell chemiluminescence from the macrophages exposed in the live animal peaked at 7 minutes and showed little difference from the untreated control group shown previously. The macrophages that had been incubated in media during exposure, however, displayed considerable differences from their treated controls. The CL output started high at the outset of the experiment in this case and decayed in an approximately exponential fashion to its lowest point at minute 29.

Comparison of two methods: CL after exposure to calcium chromate

Figure 3 displays the CL values from macrophages derived by each test method after treatment with $CaCrO_4$. Light from the cells harvested from live exposed lungs again peaked at 7 minutes and then decayed toward the starting values. The light emission from the treated, incubated cells was considerably higher initially, and fell to its lowest point, again, at minute 29. This graph differs from Figure 2 mainly by the higher values of CL expressed for the $CaCrO_4$ exposed macrophages. It illustrates that the treated cells prepared after exposure on petri plates or exposure in intact animals responded differently when challenged with zymosan.

Differences between methods related to chromium treatments

Figure 4 emphasizes the differences in CL responses between cells that received exposure to chromium after attachment to a petri dish and

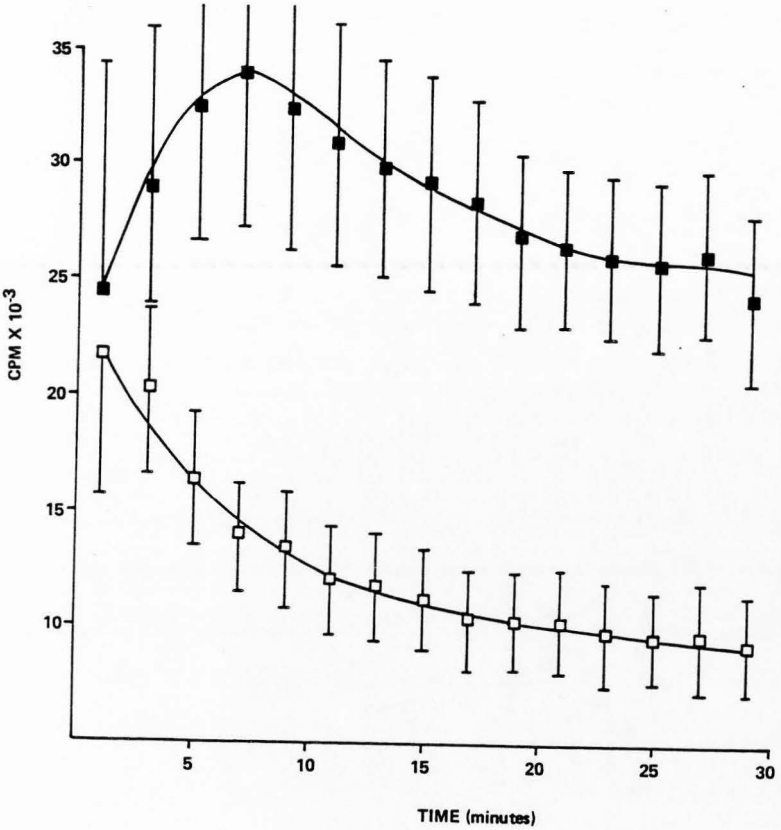

Figure 2. Chemiluminescence of macrophages treated with CrO_3 from live animals (■——■) and those incubated with CrO_3 in media (□——□).

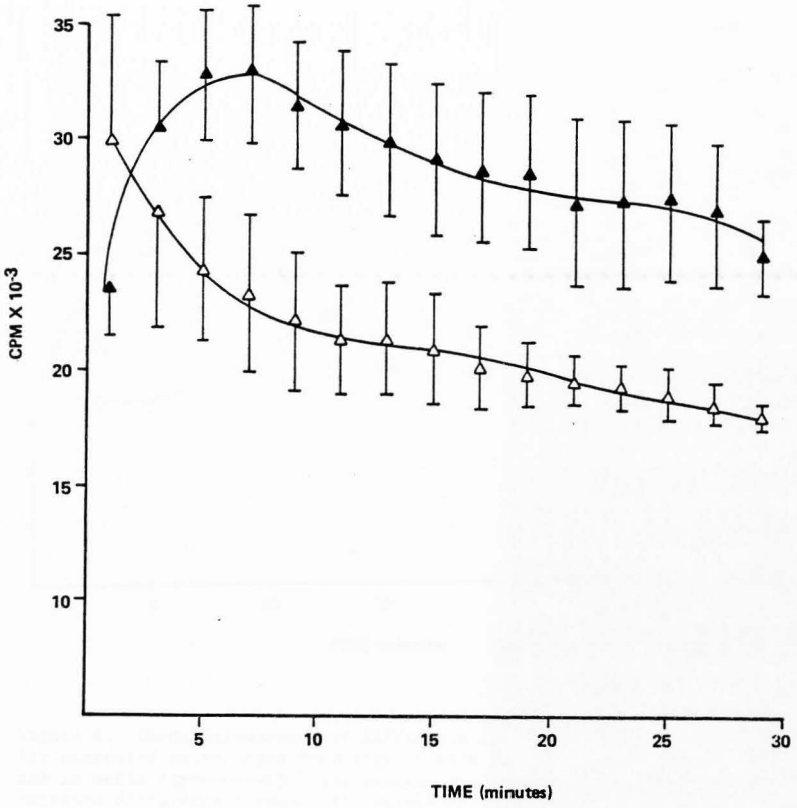

Figure 3. Chemiluminescence of macrophages treated with $CaCrO_4$ from live animals (▲——▲) and those incubated with $CaCrO_4$ (◁——▷).

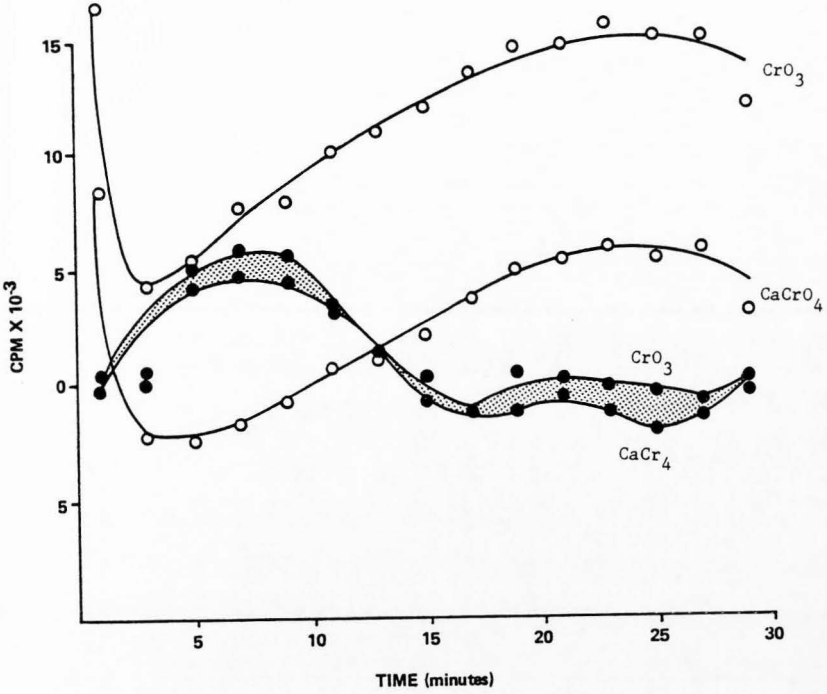

Figure 4. Chemiluminescence of difference curves (control - treatment) for untreated macrophages from live animals (●——●) and incubated in media (○——○). The shaded area represents the live exposure difference curves. All points on the graph have machine background (15,000 cpm) subtracted from them.

those that received exposure in the live animal. This is a difference curve of both experimental methods and both chromium treatments and represents the effect of the treatment subtracted from the CL yield of untreated controls.

This graph displays that macrophages prepared by different methods were variously affected by the two chromium treatments. It is emphasized by the shaded area that either treatment, CrO_3 or $CaCrO_4$, affected macrophages exposed in the live animal to the same degree as both curves bordering the shaded area displayed peak CL at 7 minutes, diminish from that point to minute 17; after that time there was some fluctuation. This shaded graph never rose over 5,000 counts, indicating that there was essentially no difference between the treatment groups and their controls.

As can be seen, the macrophages exposed on petri dishes displayed quite an opposite effect from the macrophages exposed in the animal. The two lines on the graph not related to the shaded areas represent the effect of the two chromium treatments on macrophages exposed after attachment to petri plates. The effect on CL production of CrO_3 or $CaCrO_4$ was very similar as is represented by the shape of the lines, but the yield values were different. One chromium compound, CrO_3, showed a much greater effect than the other.

Both treatments yielded the lowest CL output after 3-5 minutes and gently increased from that time to minute 23 when it dropped slightly through minute 29.

Comparison of the two methods by oxygen consumption rates

Oxygen consumption rates of the macrophages were compared in each test situation. Measurements of this parameter were compared in two different situations--while the cells were at rest and while phagocytizing zymosan (Table 2). Student's t test for the difference between means of the untreated controls (while the cells were at rest) was used and this proved that the two test methods were significantly different from each other ($P<.01$). A point of interest is the comparison when the cells are phagocytizing zymosan. As stated earlier there was an increase in oxygen consumption during phagocytosis. This was found with the fresh macrophages to be increased by approximately 75 percent over the cells at rest. When observing the cells prepared by the plated method, however, there was no increase in oxygen consumption when the cells were exposed to zymosan. This can be correlated with the CL rate (Figure 1) which was seen to decline over the first 9 minutes.

Table 2. Oxygen consumption of macrophages (Untreated Controls) harvested fresh from animals and those maintained in culture media.

	nmols O_2/min/10^7 cells		Change in oxygen consumption compared to cells at rest.
	Cells at Rest	Exposure to Zymosan	
Fresh macrophages	2.52 ± 0.440	4.43 ± 1.18	75%
Cultured macrophages	3.93 ± 0.743	3.10 ± 1.05	-21%

The trypan blue dye exclusion test was performed for both methods and cell viability remained high in each case; ranging from 92 to 97 percent.

Objective Two: Measured Effects of Hexavalent Chromium Exposure on Macrophages

The experiment was also designed to look at the effects of environmental levels of two chemical forms of hexavalent chromium on the alveolar macrophage using fresh cells (exposed in vivo) and plated cells (exposed in vitro). Calcium chromate is moderately water soluble (Handbook of Chemistry and Physics 1972) and has been shown to be carcinogenic and mutagenic. CrO_3 is water soluble (Handbook of Chemistry and Physics 1972). These compounds were dissolved in physiological saline before treatment of either live rats or cell cultures.

Live animal exposures to hexavalent chromium

Figures 5 and 6 illustrate the course of CL production for the two chromium compounds when administered to live animals as compared to untreated controls. It is seen by inspection of the standard deviations that there were no significant differences between either compound and their respective untreated control values. It can also be seen that there were no apparent differences between individual compounds. The two compounds produced maximum CL count rates after 7 minutes, as did the controls, and each rate decreased exponentially thereafter to minute 29.

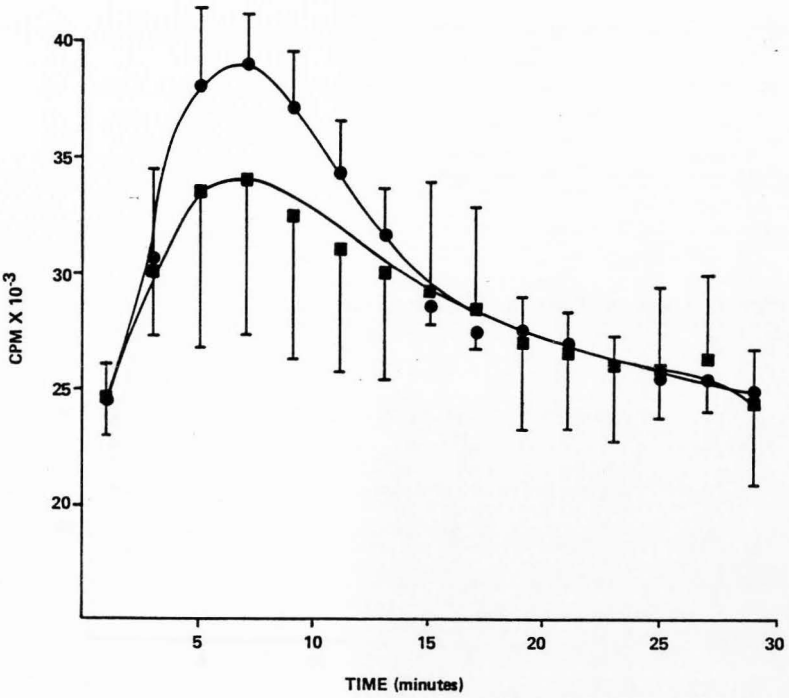

Figure 5. Chemiluminescence of untreated control (●——●) and CrO_3 treated (■——■) macrophages from live animals.

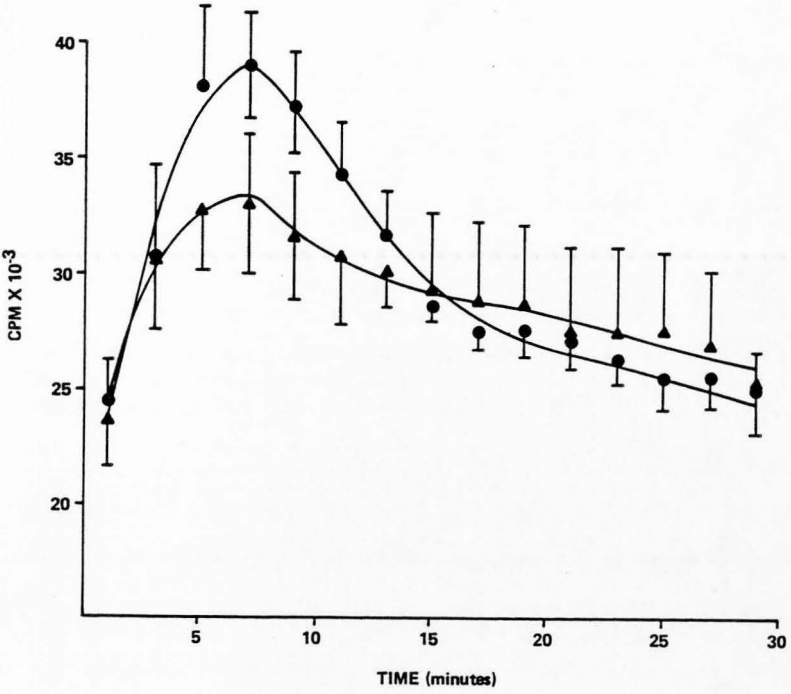

Figure 6. Chemiluminescence of untreated control (●——●) and $CaCrO_4$ treated (▲——▲) macrophages from live animals.

Table 3 shows the oxygen consumption rates of the cells prepared after live exposure. This reveals that the oxygen consumption rates of the chromium compounds-treated cells were not significantly different from the untreated control averages, or from each other. The untreated control cells, while in a resting state, consumed 2.52 nmoles $0_2/min/10^7$ cells. When the cells were stimulated into phagocytic action by adding zymosan, consumption increased approximately 75 percent over the resting rates in all cases.

Table 3. Oxygen consumption of rat alveolar macrophages following exposure of live animals: Hexavalent Chromium Treatment.

Condition	nmols $0_2/min/10^7$ cells		Change in oxygen consumption compared to cells at rest.
	Cells at Rest	Exposure to Zymosan	
Control	2.52 $\pm$ 0.440	4.43 $\pm$ 1.18	75%
$CaCrO_4$	2.13 $\pm$ 0.435	3.56 $\pm$ 0.685	67%
CrO_3	2.40 $\pm$ 0.483	4.13 $\pm$ 0.756	72%

In summary, the CL production between controls and treated macrophages did not differ significantly. The oxygen was substantiated by an F test analysis of variance over time. The oxygen consumption data corresponded to this by showing no differences between the means of oxygen consumption values (cells at rest) for controls or for treated macrophages. Further, macrophages challenged by zymosan showed very

similar increases in oxygen consumption whether the macrophages were held as controls or were treated with chromium compounds.

Macrophages attached to petri plates exposed to hexavalent chromium

Cells from each preparation method were resuspended in fresh HEPES buffer (pH = 7.4) to determine cell number, viability, CL, and oxygen consumption. Because there were some concerns as to how long the cells would remain viable in this buffer, viability (trypan blue dye exclusion) was again determined after the last cell function indicator was measured. After 2 hours in this buffer the viability remained greater than 85 percent.

Figure 7 depicts the CL production of the untreated control and the macrophages exposed to CrO_3 in culture media. An F test for analysis of variance determined over time showed that the CL control was significantly different from the treated macrophages' CL output. A point-by-point comparison for least significant differences also revealed points at minutes 23 to 27, specifically, were significantly different from the control. The control values fell to their lowest point at minute 9 then rose gently to a peak at minute 23. The CL of the treated macrophages showed a continuous decay without subsequent increase after time. The later time periods of the graph display where significant difference in values occur due to the different amounts of CL measured from controls and treated macrophages.

Figure 8 displays the CL production rates from untreated controls and macrophages exposed to $CaCrO_4$. This graph is very similar to Figure 7. The primary difference is that the CL from the treated

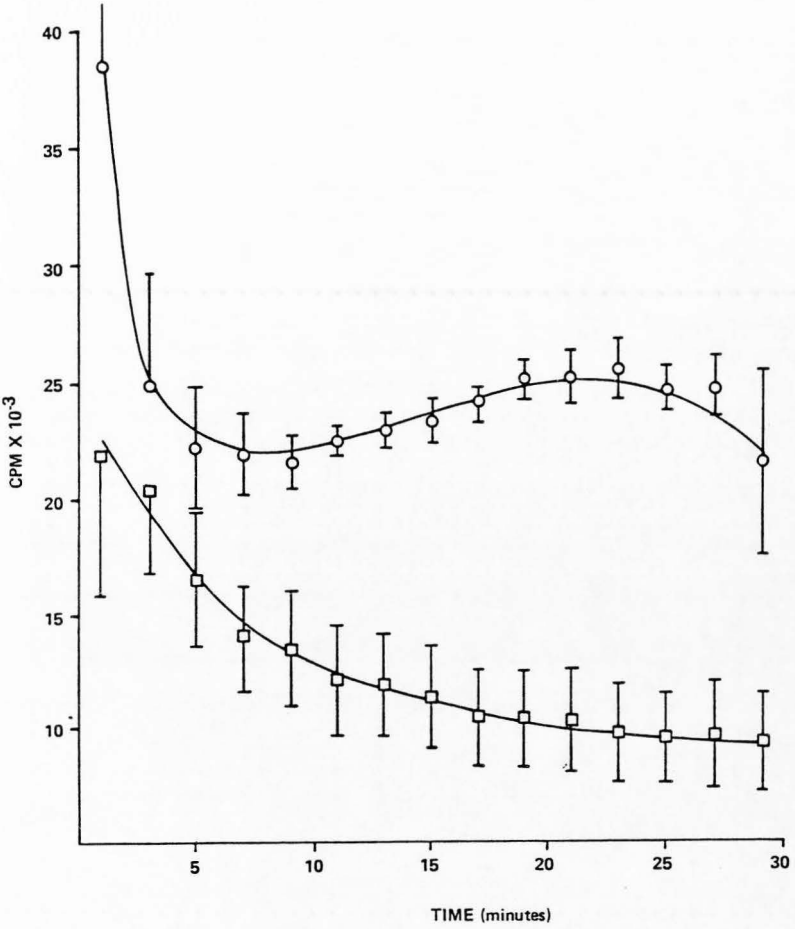

Figure 7. Chemiluminescence of untreated control (○——○) and CrO_3 treated (□——□) macrophages after incubation in media.

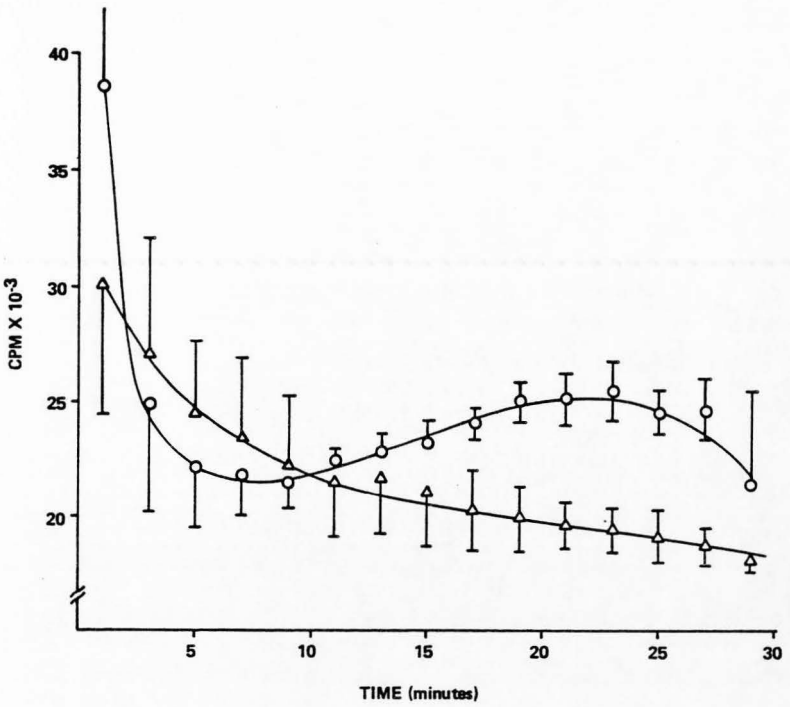

Figure 8. Chemiluminescence of untreated control (○——○) and $CaCrO_4$ treated (△——△) macrophages after incubation in media.

macrophages was not depressed as much at any point in time. The F test significance indicated that the CL reaction from treated cells over time was different from the control. This is not reflected, however, when a point-to-point comparison for least significant difference was performed. This method of comparison showed that at any time there were no significant differences for CL production for the treated macrophages when compared with the control. This is in contrast to the oxygen data listed in Table 4, where there were significant differences ($P<.001$) noted between the untreated control and both chromium treated macrophage preparations when the cells were in the resting state. It is seen that when the macrophages were challenged with zymosan the macrophages treated with the two compounds reacted as did the untreated controls, i.e., by showing no increase in oxygen consumption. This evidence does not represent a discrepancy between the two parameters measured, however, because the oxygen consumption was measured over a 10-minute period, and the CL production rates were not different between untreated controls and treatments over the initial 10-minute period. The viability of the macrophages after being trypsinized from the petri plates was high, with a mean of 96 percent.

In summary, the data revealed that the macrophages treated with CrO_3 during attachment to petri plates produced significantly different CL output than the control. The CL output of calcium chromate treated macrophages did not, however, differ significantly from the control values. The oxygen consumption data (cells at rest) revealed that cultured macrophages treated with each chromium compound reacted significantly different from the untreated controls. The oxygen

Table 4. Oxygen consumption of rat alveolar macrophages after exposure in petri dishes: Hexavalent Chromium Treatment.

Condition	nmols O_2/min/10^7 cells		Change in oxygen consumption compared to cells at rest.
	Cells at Rest	Exposure to Zymosan	
Control	3.93 ± 0.743	3.10 ± 1.05	-21%
$CaCrO_4$	1.39 ± 0.205	1.44 ± 0.582	3.6%
CrO_3	1.33 ± 0.232	1.33 ± 0.711	0

consumption of the treated macrophages during phagocytosis reacted similarly to the control by showing essentially no increase in O_2 consumption. The CL determinations and oxygen consumption correlated well with each other. The one exception was that calcium chromate treated macrophages did not show significantly different CL outputs from their treated controls, while the oxygen consumption data (cells at rest) depicted the treatment effects as being different.

DISCUSSION

Comparison of test results for untreated macrophage controls from the live animal treatment group and those controls cultured after attachment to petri dishes (the *in vitro* test group) indicate that there are significant differences between the two methods of treatment. These control macrophages were subjected to equal 0.3 ml volumes of physiological saline to simulate the exposure methods employed for chromium treatment. Cell viability of both macrophage preparations remained high, therefore, the variances seen could not be attributed to changes of viability. Also, the animals used in each preparation were of the same sex, age, and weight range, so the differences seen either related to inherent differences between test methods, or were due to other unrecognized variables.

Cells Obtained from Live Exposure Rats Compared to Plated Cell Exposure Groups

Castranova et al. (1980) have reported that CL production peaked within 5 minutes after untreated macrophages harvested from rats were exposed to zymosan. In the present study the macrophages harvested from control animals appeared to yield maximum CL at 7 minutes. This value was not, however, significantly different from the minute 5 measurement. Since CL is purported to represent recognition and ingestion of foreign particles, this peak time course corresponds with Bertalanffy's study (1964) that reported phagocytosis *in vivo* occurred between 4 to 16 minutes after stimulation. Conversely, the

untreated control macrophages that were harvested after attacment to petri dishes showed no discrete CL peak but rather a gradual rise to minute 23. Ouchi et al. (1965) reported the optimal incubation time for plated cells to be 60 minutes to allow for maximal phagocytosis, and even then only 70 percent of the macrophages had recognizable particles ingested. This implies there are stresses exerted on the cells from preparative procedures of this type that do not allow one of the steps of phagocytosis to occur as rapidly as the natural process would dictate; whether it is recognition, pursuit, or engulfment of the particle.

Further evidence that the two methods are different are contained in the results from the oxygen consumption tests. Not only were the means for the untreated control macrophages different while at rest, but an equally noticeable difference occurred when the cells were challenged with zymosan. The cells of the untreated controls from the live animal showed a 75 percent increase in oxygen consumption when challenged with zymosan. Literature reports vary greatly on the actual amount of the increase; Oren et al. (1963) reported 20 percent while Castranova et al. (1980) reported 272 percent. These macrophages allowed to attach to petri dishes actually showed a decrease in oxygen consumption when comparing average values of the cells at rest to the cells that phagocytized zymosan. Although this phenomenon has also been reported in the literature by Loose et al. (1977) with cells that were incubated in petri dishes, the fact that untreated control cells from the two preparation responded so differently to stimuli suggest

that the methods alone exert an effect on cell functions and/or reaction rates.

Cells treated with hexavalent chromium compounds in live animals

It was shown in Figures 5 and 6 that CL rates of the treated macrophages showed peak CL at the same time as the controls. In the case of both treatments, peak CL was slightly depressed, although not significantly. This could suggest some slight effect of the compounds on the cells' ability to release active forms of oxygen. This effect is supported by the results listed in Table 2 where only slight depressions in the resting oxygen consumption rates of the treated macrophages were noted. None of these differences was statistically significant and the increase in oxygen consumption during phagocytosis seems to substantiate this observation by revealing very similar rates for both treated macrophages and untreated controls.

Effects of hexavalent chromium compounds on plated macrophages

It was shown in Figure 7 that a significant difference existed between the treated cells and the control. This may reflect the effects of chromic trioxide on the cell membrane. Macrophage respiration has been shown to decrease from metals binding to the membrane linked to ATPase (Cross et al. 1970). The continuing exponential decay of CL for the treated cells implies these cells never recognized or engulfed any of the particles they were challenged with. This type of curve is characteristic of zymosan and HEPES buffer alone, i.e., without cells. Consequently, the zymosan did not perturb the cell membrane or

stimulate the macrophages to phagocytize. This is confirmed by the oxygen consumption data. When the macrophages were challenged with zymosan, no increase in oxygen consumption was observed. The oxygen consumption data also bear out the observation that the treated cells differ in activity from their untreated controls while at rest. There was a significant decrease in average values for oxygen consumption by the treated macrophages at rest as shown in Table 4.

Figure 8 displays a similar trend as seen in Figure 7. However, much more overlap between CL values of treated cells and the untreated controls is recorded. Although the F test for analysis of variance over time proves these curves are significantly different, a point by point comparison between means for least significant differences indicates that there are no differences. The effect on the release of active forms of oxygen (CL), however, is evident by the exponential decay curve of the treated macrophages. This once again implies that the cells were not actively phagocytizing the particles over the 30-minute period in which CL was measured. This evidence is supported by the oxygen consumption data for the $CaCrO_4$ treated macrophages exposed to zymosan; a very slight increase in oxygen consumed compared to the cells at rest is reported. This also is not significant, however, when the standard deviations for both the cells at rest and cells phagocytizing zymosan are examined.

In summary, there appear to be stresses imposed upon the plated macrophages that are not exerted upon the macrophages harvested from live animals. These stresses appear to be independent of exposure to the hexavalent chromium compounds. This conclusion is derived from

the contrasting responses provided by the two experimental methods. The alveolar macrophage is a very facultative cell type. It is accustomed to a high oxygen tension environment and submerging this cell in a liquid medium may alter its metabolism which could then lead to abberant test results following exposure to chemical stresses.

CONCLUSION

If the ultimate purpose of performing toxicological research on test animals is to allow comment on human systems, it seems the responsibility of investigators is to fully evaluate animal models for discrepancies prior to data extrapolation to other mammals.

The literature reviewed revealed that different authors used different models to study the same metals by the same test endpoints--a broad range of doses and animal species were evaluated. The discrepancies noted were that measured parameters were greatly different in value from model to model; the differences in dose or animal species studied could account for some variation. Hoidal et al. (1978), however, has studied the differences between human, rat, and rabbit alveolar macrophages. The study revealed that when samples were corrected for protein concentration the parameters measured were very similar.

In the present study, it is obvious that contradictory conclusions could be drawn from each of the two experimental methods even though very similar doses were administered. In this study the two techniques, live exposure via intratracheal injection and exposure while cells were attached to petri dishes, were employed. In each exposure case the same endpoints were examined, i.e., the endpoints that are credited with being the most sensitive indicators of cellular damage from inhalation exposure to metal compounds. These results imply that there are apparent differences between these two experi-

mental methods as determined by comparison of oxygen consumption rates and CL output of macrophages (as compared to controls) for each method tested.

The two methods were also compared after each test system was exposed to chromium compounds. This revealed statistical differences between methods even when the cells were at rest. This is highly significant in that exposing macrophages to a toxicant may have a synergistic effect with the method via which they are exposed.

The second objective must be considered individually from the standpoint of each test method. The exposure of live animals to 2 μg of hexavalent chromium revealed essentially no effect as compared to controls by determing the CL output and oxygen consumption.

Results from CL output and oxygen consumption determined from macrophages exposed to 2 μg hexavalent chromium after attachment to petri plates revealed significant differences from controls.

The results seem to point out that the level of chromium used (2 μg) may have approached the level of sensitivity for CL in the in vivo method. Different doses, therefore, need to be investigated to see if the discrepancies between the two models persist at higher doses.

In vivo testing may be more appropriate for evaluating actions of potential toxicants on dynamic biologic systems. Yet, all influencing variables in life processes cannot be controlled in the laboratory. On the other hand, for evaluation of toxic properties of insulting agents over a short term, efficient and convenient in vitro procedures are desirable. If those procedures do not reflect "real

life" responses accurately enough, then their use should be limited or their results fully qualified.

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