

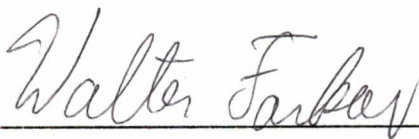
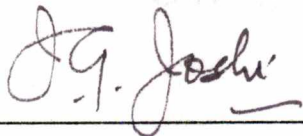
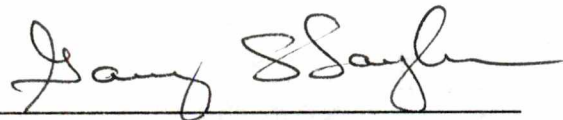
To the Graduate Council:

I am submitting herewith a dissertation written by Fay Maureen Martin entitled "Pathogenesis and Modification of Cadmium-Induced Lung Injury in Mice." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.



H. P. Witschi, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



The Graduate School

**PATHOGENESIS AND MODIFICATION OF CADMIUM-INDUCED
LUNG INJURY IN MICE**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Fay Maureen Martin

March 1984

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ABSTRACT

The effects of hyperoxia on Cd-damaged mouse lungs were studied to determine if there was potentiation of the injury by O₂. Female Balb/c mice were exposed to aerosols of 0.65% CdCl₂ for one hour by the nose-only method while control animals were exposed to water aerosols. Immediately after, some animals were placed in 70 to 80% O₂ for 5 days, while others were left in room air. Lung injury was determined by two biochemical methods, the hydroxyproline assay and ¹⁴C-thymidine uptake by DNA, as well as by histopathological examinations and lung function studied. Cell kinetic studies of parenchymal and bronchiolar labelling index (LI) and cell differentiation following autoradiography were also done. The O₂ caused a delay in cell proliferation, but following removal from the O₂, cell proliferation increased and remained higher than controls for 15 days. Cd exposed animals had significantly increased levels of hydroxyproline/lung, indicative of increased collagen secretion and hence fibrosis. The Cd + O₂ exposed animals had even higher levels showing the synergistic effects of O₂. The histopathological studies at 3 weeks for Cd + air animals showed multifocal areas of interstitial and peribronchiolar fibrosis. For Cd + O₂ animals, the lesions were similar except for greater consolidation. The parenchymal LI in the Cd + O₂ group peaked at a later time than the Cd + air group. The cell differentiation studies with Cd showed a delay in the peak of Type II epithelial and endothelial cell division when followed by the O₂ treatment. Experiments on the effects of prednisolone and X radiation in modifying the Cd-induced injury were also done but neither showed any alteration of the

Cd-induced lesion. Pretreatment with 1 mg/kg of Cd i.p. for 4 days before Cd inhalation was also shown not to alter fibrosis. Investigations were made of the long term effects of Cd + O₂ on the fibrotic lesion. Mechanisms of the action of Cd, and a literature survey are included. The study provides an experimental model for the interactions of two inhalants, Cd and O₂, and elucidates the behavior of different cell populations following injury.

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INTRODUCTION

Cadmium, described as a dissipated element (Fulkerson and Goeller, 1973), has been the focus of great attention over the last few decades, and exhaustive research has been carried out on every aspect of its study. Cadmium was isolated by F. Strohmeyer and Hermann (independently) in 1817 and soon after the discovery of its acute toxic effects in the gastrointestinal tract (after ingestion) and in the lung (after inhalation) were made (Sovet, 1858; Wheeler, 1876). Although these local effects were known, it was not until recent years that the systemic effects became elucidated. In 1867, Marmé's toxicity studies in animals showed lethal doses for Cd by iv injection in dogs (30 mg), cats (16 mg), and rabbits (10–20 mg).

With the increasing production and utilization of Cd, it was inevitable that some industrial workers would become exposed to CdO fumes, and suffer acute ill effects such as pulmonary edema. However the long term effects on industrial workers were not studied until 1948 when Friberg (1948; 1950) conducted some biochemical and toxicological studies.

Soon it became recognized that the exposure of the general population to Cd could produce adverse health effects, especially after the discovery of Itai-itai disease in Japan. In the United States, studies of autopsies have shown that the Cd content of the human fetus is about 1 μ g, but for the "standard human" the normal Cd burden had increased to 20 mg by the age of 50 years (Chisholm, 1974).

It is essential to have an awareness of the fate of Cd in mammalian systems, therefore the factors influencing absorption, distribution, and

excretion should be examined. In the disposition and action of Cd in mammals some of the influencing factors include levels of exposure, duration of exposure, route of administration, and species and strain dependent responses.

In this study, the lung is the organ of interest, and it should be noted that the lung plays many roles in the functioning of the body. Its main function is the maintenance of O₂ supply and the excretion of CO₂, but it also takes an active part in several body defense mechanisms, including immunological responses and complex interrelations with hemodynamic and electrolyte homeostases (Casarett, 1975). The viable and functional elements of the lung are in direct contact with the environmental air and thus can be quite vulnerable to toxic compounds.

A metal such as Cd, when taken into the respiratory system in the form of an aerosol will follow the general laws governing the deposition of particulate matter. To what extent Cd reaches the deep lung depends not only on such processes in the lung as deposition, mucociliary clearance and alveolar clearance, but also on the duration of exposure, the concentration of Cd in the inspired air, the Cd compound itself, and its water and lipid solubility.

Figure 1 shows a model for Cd kinetics in mammalian systems. At first the Cd in plasma is found combined with albumin (which is a reversible reaction) but after 24 hours metallothionein is the dominant Cd-binding protein. There are many studies of Cd distribution in organs (Friberg et al., 1974; Nordberg, 1972; and a report by Forssen, 1972) but through the actual values in the organs vary, the relative distribution among organs is quite similar. The kidneys and liver account for the major portion of an injected dose with 70% going to the liver and 1-4% to the kidneys. The route of administration can also have an effect on Cd distribution in tissues.

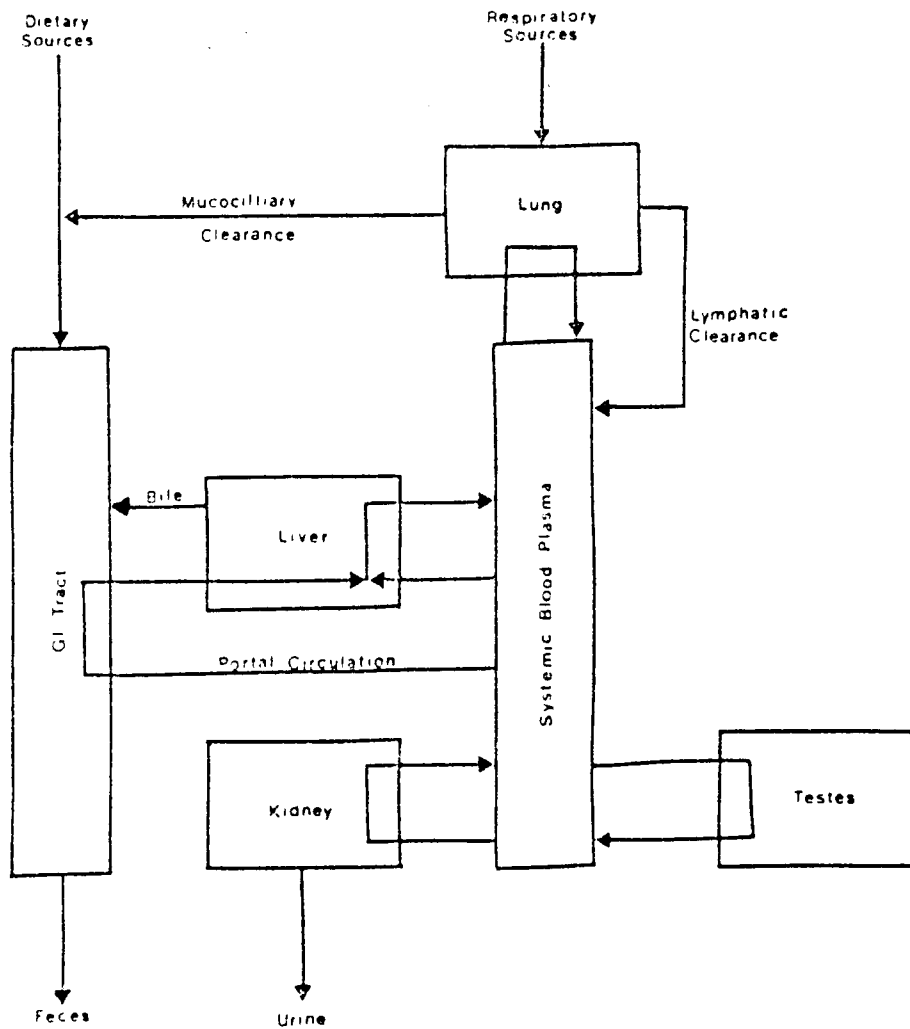


Figure 1. Compartmental model for analyzing cadmium kinetics in mammalian systems. Reprinted by permission of the publisher and author from: *The Role of Metallothionein in the Systemic Distribution of Cadmium* by John M. Frazier in *Biological Roles of Metallothionein, Developments in Toxicology and Environmental Science, Volume 9*, E. C. Foulkes, ed., 327 pp., Copyright 1982 by Elsevier Science Publishing Co., Inc.

Cd is excreted primarily in urine and feces, with minor amounts possibly excreted in hair, sweat, skin, milk, and saliva (Friberg et al., 1974; Hise and Fulkerson, 1973). Excretion is slow and the biological half-life of Cd in humans is very long, of the order of several decades. It is this long half-life that adds to the concern about Cd toxicity in man. Cd effects almost every organ system in the body, including pulmonary, renal, skeletal, hematopoietic, hepatic, and cardiovascular, and is also associated with carcinogenicity, mutagenicity, sterility, anosmia, dental caries, changes in pancreatic and adrenal functions, and immunosuppression. These widespread effects have led to the intensity of research in elucidating the nature and mechanism of its physiological and toxicological reactions.

On examining the voluminous literature it was discovered what a monumental task it would be to cover all aspects of the toxicology of Cd, as well as the studies on the lung and its many diseases, so it was decided in the literature review and background information to touch on selected aspects of Cd such as its chemical properties, presence in the environment, and some of the effects on selected organ systems of man and experimental animals.

Since the study employed inhalation techniques, some background on inhalation toxicology was also presented. This included inhalation techniques, common toxicants that effect the respiratory system, and a description of some of the cells that would be exposed to the toxicants. Two of the agents, other than Cd, employed in this study, namely O₂ and prednisolone, are also discussed briefly, as well as some related studies on cell kinetics in lung after treatment of animals with various toxicants.

Chapter II states the problem and purpose of the study which briefly is to explore the possible interactions between inhaled Cd and another inhalant, namely O₂, in the lungs of mice, from biochemical, physiological, histopathological, and cytokinetic aspects, to study the effects of other agents which may modify the changes, and an investigation of the changes present after a prolonged period of time. The other chapters are self explanatory. No data are available in the literature on the cell kinetics of Cd induced lung injury or the interaction between Cd and a second toxic agent. The experiments described herein have provided quantitative and qualitative information on the development of a lung lesion caused by the interaction between two toxic inhalants, and the modifying actions of some other agents

CHAPTER I

STATEMENT OF THE PROBLEM AND PURPOSE OF THE STUDY

Lung diseases constitute a major group of human diseases, and millions of dollars are spent in the United States each year to rehabilitate persons suffering from such illnesses. To know some of the general pathogenetic principles underlying acute and chronic lung disease is a challenging task. The work reported in this thesis describes attempts to study how lung injury, brought about by one toxic agent, can be aggravated by a second toxic agent.

The model chosen was oxygen therapy of adult respiratory distress syndrome. Adult respiratory distress syndrome usually begins with a pulmonary, but often extrapulmonary pathological condition, e.g., viral pneumonitis, massive trauma as in battle wounds and drowning. Initially there may be signs of improvement, which may continue for several hours to four days, but then there may be sudden and unexpected respiratory impairment. Oxygen therapy is widely used at this point and some patients may recover. Others however continue to show diminishing compliance and increasing hypoxemia despite administration of increasing concentrations of O_2 , until death occurs from respiratory failure, usually within 10–40 days. The O_2 , rather than being beneficial, actually may aggravate cell damage and lead to the development of edema and hyaline membrane formation. Extensive fibrosis also develops and may be the main factor responsible for death from respiratory failure (Rinaldo and Rogers, 1982). In one experimental model, butylated hydroxytoluene- (BHT)-induced lung damage in mice, the

administration of O_2 is deleterious to an already damaged lung and actually potentiates the alveolar damage (Witschi et al., 1981).

In an attempt to broaden the implications of previous studies done with BHT, the effects of Cd, a common environmental pollutant were examined. Moreover acute Cd exposure has been shown by several workers to cause lung damage (see reviews by Mennear, 1979; Friberg et al., 1974; Webb, 1979). Oxygen, which is commonly used in therapy, was chosen as the second agent to test whether it would potentiate Cd-induced lung damage in a manner similar to which it potentiates BHT-induced lung damage.

It is certainly paradoxical that oxygen, which is considered essential for most forms of life on earth, should be investigated for its toxic effects. However, as Paracelsus (1493–1541) noted, "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy."

To quantitate the damage done by the agents Cd and O_2 , we initially chose a biochemical method. As shown by Oehlert (1973), Bullough and Laurence (1965), and Hennings and Elgjo (1970), epithelial cell renewal appears to be controlled by a negative feedback mechanism. According to this theory, when there is a decrease in the number of differentiated cells, an increased proliferation of progenitor cells follows until the differentiated cells are replaced. Thus examination of cell proliferation gives valuable insights into the lungs response to injury. DNA synthesis in the proliferating cells can be assessed by determining the incorporation of ^{14}C -thymidine into DNA (which takes place at the S-phase of the cell-cycle). This can in turn be related to the amount of cell proliferation taking place and may serve as a secondary index to

the severity of the initial damage induced. Thus the first experiments were designed to document that Cd would produce lung damage and to check the potentiation of damage by oxygen by measuring the ^{14}C -thymidine incorporation into DNA.

Another end point for measuring tissue damage is to determine the amount of fibrosis that develops. Fibrosis, which is the abnormal deposition of collagen following tissue damage, can be determined by measuring the lung collagen content. This is done by measuring total lung hydroxyproline. Hydroxyproline is an amino acid present virtually only in collagen. This was the second method chosen to assess the lung damage by Cd and the potentiation of damage by oxygen.

Thirdly, the changes in the lung parenchyma taking place can be studied by histopathological methods. Accordingly, lungs from both treated and control animals were studied with the light microscope at the same time when the hydroxyproline determinations were carried out.

In order to have a better understanding of the disease process, one has to get down to the cellular level and find how various cell populations react under stressful conditions. This was done by undertaking a cell kinetic study. One interesting question is how does the behavior of one cell population affect the behavior of other populations. A better understanding of cell-cell interactions could lead to means to hasten repair or to find a method to suppress proliferation of unwanted cell populations, such as those producing excessive collagen. It has been shown for example that when some noxious agents damage Type I alveolar cells the Type II cells divide, and some differentiate into Type I cells to repair the damage done (Adamson and

Bowden, 1974). Moreover, it has been postulated by Haschek and Witschi (1979) that if these Type II cells were damaged when at the peak of proliferation, that the interstitial cells would grow comparatively undisturbed, producing excessive collagen which would lead to lung fibrosis.

Haschek and Witschi (1979) and Witschi et al. (1980) speculated that proliferation of interstitial cells in the alveolar zone is controlled directly or indirectly, by the continuous presence of an intact epithelial layer. This hypothesis is supported by the experiments of Terzaghi et al. (1978) who showed that if an excised trachea is stripped of its epithelium and implanted s.c. into a syngeneic host, the tracheal lumen will become obliterated with connective tissue within one week. However, if isolated epithelial cells are reintroduced into the tracheal lumen, reepithelialization take place and the trachea remains open. These studies show the interdependence of different cell populations. In the present investigation with Cd and O₂, special emphasis was placed therefore on the cell kinetics so that a better understanding could be gained of how the cell populations in this system behaved.

For the cell kinetic study, autoradiographic methods were employed. The cell kinetic study was broken down into three main parts. The first task was to obtain the labelling index of the parenchymal cells of the alveoli. The lung can be thought of as the only organ which is in direct contact between the contents of environmental air and the vital, viable functional elements of the organ. Because of this proximity of the alveolar cells to the noxious agents introduced, namely Cd and oxygen, it is important to examine what changes are occurring in the cells. As mentioned before, when cells are damaged, cell proliferation takes place to repair the injury. When ³H-thymidine is injected

into animals with damaged lungs, DNA of the proliferating lung cells will incorporate the ^3H -thymidine. Preparation of histological lung sections for autoradiography, allows one to determine the percentage of cells with labelled nuclei and to express the data as labelling index.

Next the labelling index of the bronchiolar epithelium was determined. The bronchiolar cells come in contact with the noxious agents before they arrive at the alveoli and it is of interest to see how much damage is done, whether the cells are protected from the toxicants by mucus secretions or surfactants for example, and how much repair takes place.

Finally the relative proportions of different cell populations is determined to answer the intriguing question of what happens to cell populations when repair is taking place and another toxic agent is introduced. Many studies have been done relating to repair after one toxic agent. For example, Adamson et al. (1977) in their study with BHT in mice showed the times at which the various cell populations reached their peak of proliferation, with the epithelial cells showing a peak at two to three days and the endothelial cells reaching their peak on day 6. This is the reverse of the regenerative pattern after oxygen poisoning shown by Bowden and Adamson (1974) and Adamson and Bowden (1974). It will therefore be interesting to see how the cell population behaves after treatment with Cd and than what the effects are of exposing the lungs to oxygen while repair is taking place.

If O_2 proves to be deleterious to the lung damaged by Cd, then the question can be asked, what other agents would have a similar effect on the Cd damaged lung. In addressing this question, two other agents were chosen for investigation, namely, X radiation and prednisolone. It has been shown for

example that a combination of bleomycin or cyclophosphamide treatment with thorax irradiation enhanced pulmonary toxicity (Phillips and Fu, 1976; Catane et al., 1979). Work by Haschek et al. (1980) has also shown that mice exposed to 200 rad of thoracic X radiation at a crucial time after lung injury induced by butylated hydroxytoluene, developed pulmonary fibrosis after 2 weeks. However, Hakkinen et al. (1982) did not find a potentiating effecting of thoracic X irradiation in mice in which lung damage had been induced by bleomycin and cyclophosphamide.

Following lung damage by a noxious agent, the cell populations, which proliferate to repair the damage do so at different rates, and one type of cell population may be more susceptible to damage by the second toxic agent that is applied. Thus the time of application of this second agent may be crucial to the eventual outcome, depending on which population is predominant at the time of application. By treating the Cd exposed animals with X radiation at various time intervals, not only can the results of the interaction of these two agents be compared with the results of similar studies but one hopes to learn more of the mechanism of action of these toxic substances.

Steroids have been used for a long time for the treatment of chronic inflammatory diseases. This is possibly due to the fact that they have been shown both *in vivo* and in cell culture to selectively decrease collagen polypeptide synthesis. Shubin (1965) has also shown that steroids can ameliorate the symptoms of pulmonary fibrosis and other lung diseases in man. On the other hand, Smith and Brody (1981) found that methylprednisolone inhibited Type II cell proliferation when lung injury was mild, but the result was different when lung injury was severe—then there was a failure to inhibit

proliferation. Thus, it would be interesting to compare the interaction of Cd and a steroid such as prednisolone with the results of such studies, and if lung damage is potentiated by steroids it would suggest that one should apply caution in applying steroid therapy to human patients with primary lung damage.

Another important question that comes to mind is how else can the damaging effects of Cd be modified? Is it possible for example to "immunize" the animals by pretreating them with small amounts of Cd before the major assault. It was shown by Ito and Sawauchi (1966) that the injection of 1/2 to 1/4 of a testis-destructive dose of CdCl₂ (0.1 ml of 0.1% CdCl₂ solution per mouse) two days prior to the injection of the normally testis-destructive dose, prevented testicle destruction in more than half of the animals. Studies of the protective effect of Cd by pretreating with a small dose has also been shown by Nordberg (1971) and Gunn and Gould (1970). Terhaar (1965) in a study on pretreatment by oral administration found that oral doses of 200 and 400 mg/kg of CdCl₂ killed 9/12 and 9/10 rats respectively, but when 20 mg/kg was given prior to the high doses only 1/11 and 0/9 were killed. He also showed that doses as low as 0.01 mg/kg given 24 hr prior to 100 mg/kg protected against massive testicular atrophy.

Theories have been advanced to explain the protective effects of pretreatment and one is that the pretreatment with small amounts of Cd induces larger quantities of methallothionein. Thus when the larger dose of Cd is presented it combines with this metallothionein and is therefore not available to cause the damage. The next study carried out was therefore one where the mice were pretreated with small doses of Cd prior to exposing them to a known damaging dose of Cd by inhalation.

Finally, it was interesting to find out how the animal with an acutely damaged lung fared in the long run? Does the lung return to its normal state? Does fibrosis persist? Or is emphysema the ultimate end? This question was answered by doing long-term studies. Animals were treated with Cd + O₂ and surveys were done at 3 months, 6 months, and 1 year to discover what processes were taking place, and what was the outcome of these treatments. Both fibrosis and emphysema are thought to represent a late healing stage after lung injury, but it is not known what determines in what direction the lung injury will evolve. The determining factor may be the nature of the initial injury, but differences in the repair processes may be just as important.

One method of investigating what processes are taking place is a histopathological examination. Thus for the various time points, slides of control and treated animals were prepared and examined by light microscopy. Special care was taken to fix the lungs under constant pressure since in evaluating emphysematous changes microscopically, the size of the alveolar spaces is taken into account and should not be influenced by improper fixation procedures.

Another method for detecting pathologic changes in the lung is lung function tests. It is the elastic properties of the lung tissue that is important in these tests. During expiration, the elastic tissue within the lung retracts, and exhalation occurs passively. The lung has to comply with normal movements of the chest wall, but if the elastic properties of the lung tissue are affected this may become difficult. An index of the functional stiffness of the respiratory system is referred to as compliance. It shows the relationship between the pressure required to overcome "elastic recoil" and the volume of air that moves

into the lung as a result of this pressure change. Compliance can be determined by plotting changes in lung volume during inspiration and expiration against pleural pressure. As the normal lung becomes more inflated it becomes less compliant and the pressure-volume curve becomes more horizontal. Pathologic processes which diminish the elastic properties of the lung, such as fibrosis will cause a decrease in compliance, whereas emphysema will lead to an increase in pulmonary compliance.

Lung function tests were therefore carried out on the treated and control animals to provide an answer to questions of whether emphysema or fibrosis is the result of healing after Cd and O₂ treatments.

The third and final method chosen to investigate the long-term effects of Cd plus O₂ inhalation was that of lung hydroxyproline determination. In these long-term studies however, the age factor and condition of the animals may have some bearing on the results.

The purposes of this work can thus be summarized as follows:

(a) To explore in a systematic way the possible interactions between inhaled Cd and another potentially harmful inhalant, oxygen. Three methods were employed: (i) Biochemistry, by C¹⁴-thymidine-incorporation into DNA and by hydroxyproline determination. (ii) Histopathology. (iii) Cell kinetics, including parenchymal and bronchiolar labelling index, and cell differentiation.

(b) To investigate the ameliorating or potentiating effects of two other agents, X radiation and prednisolone.

(c) To determine a method for modifying the development of the Cd lesion by pretreatment with small amounts of Cd.

(d) To investigate the long term effects of the interaction of cadmium and oxygen by histopathology, lung function tests and a biochemical test (hydroxyproline determination).

CHAPTER II

LITERATURE REVIEW AND BACKGROUND INFORMATION

A. Physical, Chemical, and Biological Properties Information

Cadmium is a soft, bluish-silver white metal, harder than tin and softer than zinc. The melting and boiling points of Cd are low compared with those of other metals (321°C and 765°C respectively). The vapor pressure is 1.4 mm at 400°C but it rises rapidly to 16 mm at 500°C, when an orange-yellow vapor is emitted. It is this vapor which may be discharged during metal refining or during combustion of fossil fuels or other materials, and it represents a major source of atmospheric Cd (Goeller, Hise and Flora, 1973; Hise and Fulkerson, 1973).

In the periodic table of elements, the elements Zn, Cd, and Hg form Group IIB; the inner electron shells are filled and the outermost shell has 2 electrons. (The outer-electron configuration is $[nd^{10} (n + 1)s^2]$). Cd thus has an electrovalence of 2 as the only stable form.

Organic compounds of Cd (R_2Cd and $RCdCl$ where R is an aliphatic hydrocarbon) can be synthesized in the laboratory and also by bacteria (Summers and Silver, 1978) but are unstable when exposed to air or water. Unlike the stable alkyl Hg compounds, it is unlikely that alkyl Cd compounds are hazardous to biological systems. One exception was the study of Chang and Sprecher (1976) who showed that the nephrotoxicity of methylcadmium chloride after i.p. injection into cats was greater than that of inorganic Cd.

In order to understand metal-ion behavior in biological systems at the macromolecular level, one needs to know certain properties such as the ionic radius, hydration, polarizability, and charge density of the metal ion, since these properties provide a unique profile for the metal ion and help in predicting what reactions will take place with other ions. So that these parameters do not have to be evaluated individually, another predictive method can be used, based on the hard-soft classification of metal ions by Ahrland et al. (1958) and Pearson (1963). By this classification the hard acids and bases form their most stable complexes with ligands that have electron donor atoms from the first short period (N, O, F) while the soft acids and bases form their most stable complexes with donor atoms from the second (P, S, Cl) or later period. Cadmium is a soft acceptor and prefers to bind with soft donors. Cadmium, because of the easily deformable outer electron shells, has a high degree of polarizability and this behavior is consistent with predominantly covalent bonding between soft ligands (in contrast to ionic bonding that is characteristic of the association of hard donors and acceptors). The parameter of softness has been shown to correlate well with the LD₅₀ of metal ions (Nieboer and Richardson, 1980; Williams et al, 1982) and these toxic metal ions because of their high degree of softness may form more stable complexes with cellular components than the essential ions, thus preventing normal metal-mediated functions. The reactions of Cd with DNA, proteins and amino acids have been discussed by several authors, among them Jacobson and Turner (1980). Studies of the action of Cd in cells and tissues indicate that Cd²⁺ is the active form (Berry, Osgood, and St. John, 1974). Metal ions are linked to biological molecules mainly through O, N, and S. For example in the

crystal of a cadmium derivative of cytidine 5'monophosphate, $[\text{Cd}(5'\text{-CMP})(\text{H}_2\text{O})]_n$, Cd^{2+} bonds to the N(3) of the pyrimidine, to a phosphate oxygen from each of three neighboring 5'-CMP molecules, and to a water molecule (Jacobson and Turner, 1980).

Cd will bind to a large number of soluble or structural proteins, as well as to compounds of lower molecular weight. Its binding to metallothionein deserves special mention and will be discussed later.

B. Cadmium in the Environment

Cadmium is ubiquitous in the environment. An estimated 5 million pounds of Cd is dissipated into the U.S. biosphere annually from industrial operations, combustion of fuels and of solid waste, phosphate fertilizers and pesticides (Fulkerson and Goeller, 1973).

The occurrence of Itai-itai disease in Japan (1950–1960), stressed the importance of environmental contamination by Cd. The disease became apparent through several painful health effects, such as osteoporosis and osteomalacia. In 1969, 223 cases had been registered of which 119, mainly elder multiparae, died. Metabolism was disturbed, proteinuria occurred, and in autopsies high concentrations of Cd were found in bone. It was determined that the disease was caused by discharges of a zinc mine into the Jintsu River, its water being used to irrigate the paddy soil in that area. Vitamin D and Ca deficiency also play an important role in the development of the Itai-itai, literally 'ouch-ouch', disease (Tsuchiya, 1978).

A cause for concern is the increased use of fossil fuels for energy since the large quantities that are consumed can lead to considerable Cd emissions.

Some measurements of the Cd content of coal and fuel oil show them to be in the range of 0.25 to 2.0 ppm and 0.1 to 0.5 ppm respectively. If the annual U.S. consumption of coal is about 500 million short tons, the associated flow of Cd would be 125 to 1000 tons (250,000–2,000,000 lb). It is not known what percentage of this Cd gets into the environment from the stacks or what is retained in ash and furnaces, or removed by scrubbers or precipitators. Similarly the annual U.S. consumption of diesel and heating oil can be taken to be about 200,000,000 tons in 1968, and with a Cd concentration of 0.3 ppm this would give a Cd flow of 120,000 lb. Another 50,000 to 500,000 lb/yr of Cd is released to the environment from the use of superphosphate fertilizers in agriculture. Figure 2 shows the flow of Cd in U.S. society in 1968. The magnitude of the various streams is expressed as a percentage of the U.S. annual demand of Cd, which in 1968 was 13.3 million pounds.

One large source of contamination comes about because Cd is always present in zinc ores and thus is a byproduct in zinc refining. Some Cd contamination also results from the refining of lead and copper. The highest concentrations of cadmium are therefore most likely to be found in the regions of smelters or in industrial urban areas.

The background levels of atmospheric cadmium are usually quite low but in urban areas they may vary from 0 to $0.150 \mu\text{g}/\text{m}^3$ with means of 0.005 to $0.015 \mu\text{g}/\text{m}^3$ (Hammons et al., 1978). Uncontaminated soil contains about 0.4 ppm cadmium whereas soil in the vicinity of smelters would have levels of about 10 to 100 ppm, and the soil in industrial urban areas would have about 0.1 to 1 ppm cadmium.

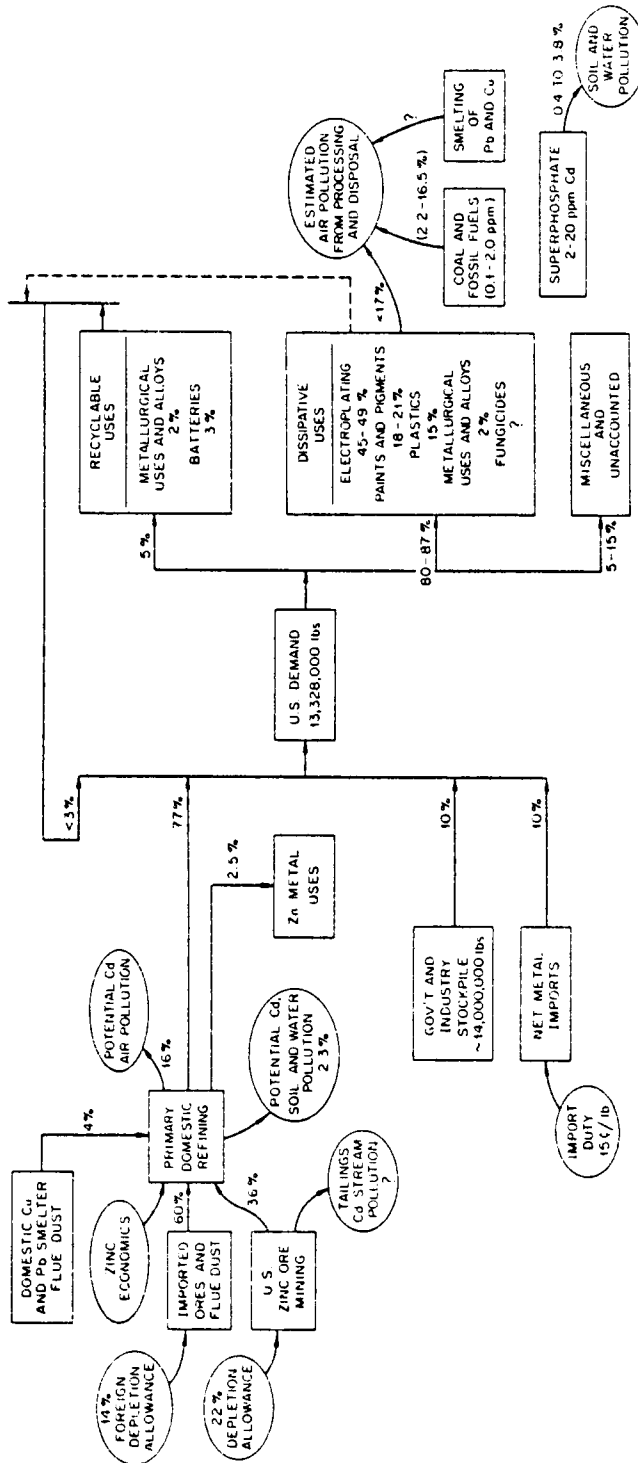


Figure 2. Flow of cadmium in U.S. Society, 1968. From: Cadmium, The Dissipated Element. W. Fulkerson and H. E. Goeller, eds., 1973. ORNL/NSF/EP-21, Oak Ridge National Laboratory, Oak Ridge, Tenn. 473 pp.

The cadmium content of natural waters is highly variable, near industrial areas values vary from 1 to 100 ppb, while in open rural waters the content is less than 1 ppm (Hammons et al., 1978).

The source of Cd in the soil is the rock fragments from which the soil developed, and many studies have been done on the uptake of Cd by plants and its subsequent transfer to animals and man in the food chain. Cd because of its ubiquitous nature—its presence in food, air, soil, and water—may present a threat to the welfare of man. In the industrialized countries of the world—U.S.A., Japan, and Europe—the daily intake of Cd of 30–60 μg Cd/day approaches or equals the maximum tolerable daily intake of 57.1 to 71.4 μg per individual proposed by the FAO/WHO Committee (WHO, 1972). Because of the many toxic effects of Cd, maximum permissible exposures have been established. The maximum allowable concentration in the United States is 0.05 mg/m^3 for Cd oxide fumes and a time weighted average (TLV or TWA) of 0.05 mg/m^3 for Cd dust, both proposed by the American Council of Governmental Industrial Hygienists (1976) (CEC, 1976).

C. Toxic Effects of Cadmium

There are several good reviews of cadmium toxicity (Friberg et al., 1974; Fulkerson and Goeller, 1973; Flick, Kraybil and Dimitroff, 1971). The toxic effects can be classified as acute or chronic and can occur through ingestion, inhalation, or both. Some effects on organ systems will be considered below.

1. Effects on lungs

There are several reports describing workers chronically exposed to high cadmium levels in cadmium related industries who developed ill health effects. In most of these cases Cd exposure was by inhalation. The most prominent symptoms exhibited were chronic bronchitis, emphysema and proteinuria (Friberg et al., 1974) and resulted from exposure to cadmium oxide fumes, cadmium oxide dust, and cadmium pigment dust. Usually the symptoms did not appear until a few years after exposure. One such example was presented in a study by Friberg (1950) on workers in Sweden. The first group of 43 men exposed to Cd oxide dust for an average of 20 yr had impaired lung function, while another group of 15 workers with similar exposure but only one to four years of employment had normal lung function.

Acute Cd poisoning was first reported by Sovet in 1858 in Belgium. The poisoning involved three persons who were polishing silverware with cadmium carbonate and were exposed to a cloud of Cd dust. With acute poisoning there is usually only slight discomfort at the time of exposure but several hours later there is severe bronchial and pulmonary irritation. This includes irritation and dryness of the nose and throat, cough, headache, dizziness, weakness, chills, fever, severe chest pain, and breathlessness. Sometimes nausea, vomiting, and diarrhea can occur. There is a delayed onset of pulmonary edema which is responsible for the fatal outcome. Several studies on the effects of Cd on the lungs of experimental animals have been reported in the literature, and many of these will be mentioned later in the Chapter V (Discussion).

A few that will be mentioned here include the study on biochemical changes in rats after exposure to aerosols of 0.005M CdCl₂ by Hayes et al.,

1976. Here the total lipid content, lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities showed approximate doubling by the fourth day after exposure. These changes coincided with doubling of wet lung weight and total lung DNA content.

A fibrotic lesion was induced in hamsters in a study by Snider and Karlinsky (1977). They treated the animals with a single endotracheal dose of CdCl_2 (0.025% in 0.5 ml 0.15M NaCl/100g body wt) and noted at 21 days that there was focal but widespread areas of interstitial fibrosis with cellular infiltration and areas of epithelial cell hyperplasia.

Cellular proliferation in rat lung was also noted by Palmer et al. (1975) following treatment of rats with aerosols of 0.1% CdCl_2 , while Snider et al. (1973) showed that centrilobular emphysema was induced by aerosols of 0.1% CdCl_2 in rats exposed for one hour on 5, 10, or 15 occasions. The severity of the emphysema was related to the number of exposures to Cd aerosol.

CdCl_2 inhalation studies in dogs were reported by Harrison et al. (1947) and in guinea pigs intratracheal injection of CdCl_2 solution containing 0.9 mg Cd^{2+} /ml (Thurlbeck and Foley, 1963) caused massive pulmonary congestion, hemorrhage of the lungs and dilation of the pulmonary lymphatics. There was also acute bronchitis and bronchiolitis and some hyaline membrane formation.

2. Effects on the kidneys

The kidney is the critical organ for Cd damage and exhibits the first adverse effects following long term excessive exposure to Cd by inhalation or ingestion (Nordberg, 1976). Smith and Kench (1957) reported an incidence of

proteinuria in workers exposed to cadmium oxide fume and dust from 8 to 30 yr in an alkaline accumulator factory. They showed that there was increased proteinuria in the workers exposed to fume, 60%, and 20% in those inhaling dust, compared to 5% in the control group. Lauwreys et al. (1974) showed that there was renal damage in man exposed for over 20 yr to a concentration of $21 \mu\text{g}/\text{m}^3$ Cd, with particles in the respirable range.

3. Effects on the vascular system

The effects of Cd on the vascular system include both anemia and hypertension. Anemia is considered one of the most sensitive parameters of oral cadmium intoxication (Cousins et al., 1972; Freeland and Cousins, 1973). It was shown that the route of administration of Cd determined the development of anemia, since in experiments of Prigge et al. (1977), after oral uptake of Cd there was a reduction of hemoglobin and hematocrit, while after Cd inhalation with comparable Cd organ levels there was no anemia.

Cd administration has a striking influence on the vasculature and especially on the structure of arterioles and of small blood vessels. A biphasic effect has been shown for Cd; at high concentrations it produces vasodilation, whereas at low concentrations it is vasoconstrictive. (Perry and Erlanger, 1974; Nechay et al., 1978).

4. Hypertension

There is no general agreement to the question of whether cadmium causes hypertension in man. Schroeder (1965) reported a correlation between Cd concentration in the kidneys and the incidence of hypertension for about

400 persons in various cities of the world. Other studies of this kind are reported by Friberg et al. (1974) and Hise and Fulkerson (1973), but certain important factors related to hypertension, for example population density and smoking were not discussed. Several studies reported on a lack of relationship between kidney Cd levels and hypertension (Hunt et al., 1971; Lewis et al., 1972a; Morgan 1969). In addition, patients in Japan with Itai-Itai disease, and persons in the surrounding districts did not have an unusually high incidence of hypertension (Friberg et al., 1974).

It is known that hard water contains less cadmium than soft water and there are studies showing an inverse correlation between hypertension and the hardness of drinking water (Crawford et al., 1968).

There are several reports of Cd-induced hypertension in animals. For example, Thind et al. (1970), induced hypertension in rabbits by repeated small doses of i.p. injection of Cd acetate (2 mg/kg) over 9 months, and Schroeder et al. (1966) showed that 1 or 2 mg Cd/kg body weight injected i.p. as Cd citrate produced hypertension in rats within a month. The blood pressure in these rats however returned to normal within 300 days following the cessation of Cd exposure. Revis et al. (1981) who exposed white Carneau pigeons to drinking water containing 0.6 ppm Cd, found that the Cd induced aortic atherosclerosis and hypertension in the pigeons. They also found that when calcium was present the cardiovascular effects of Cd were significantly reduced. These results may be compared to epidemiological studies on the incidence of cardiovascular mortality in man dwelling in areas with soft water containing relatively high concentrations of Cd.

5. Carcinogenic effects

Parenteral administration of Cd has been shown to be carcinogenic in rats, for example Gunn et al. (1964) found sarcomas at injection sites and interstitial cell tumors in rat testis, following a single injection of 0.03 mmole/kg of CdCl_2 . There are other studies of the development of sarcomas at injection sites (Haddow et al., 1964; Lucis et al., 1972; Reddi et al., 1973) but the first report of a potential cancer risk to man was given by Potts (1965). In a group of 74 workers exposed for greater than 10 yr to CdO dust, there were eight deaths, three due to carcinoma of the prostate, one to carcinoma of the bronchus and one to carcinomatosis. However, because of the small number, no conclusions were drawn. In another study of 248 workers with a minimum of 2 yr of exposure, 12 deaths due to respiratory malignancies were reported versus 5.11 expected, and 4 deaths due to prostatic cancer were reported versus 1.15 expected (Leman et al., 1975). Kolonel (1976) studied 64 patients who were admitted from 1957 to 1964 to an American hospital with a final diagnosis of malignant renal tumor. Control groups comprised of nonmalignant cases of the digestive system and of colonic cancer were compared. The results suggested a significant association of renal cancer with exposure to Cd and there was also a synergistic effect between occupational exposure and smoking.

One recent study, the first of its kind to be reported, has shown a high incidence of lung cancer in rats following the prolonged (18 months) inhalation of CdCl_2 (Takenaka, 1983). A distinct dose-response effect was seen, 71.4%, 52.6%, and 15.4% incidence of lung cancer in the 50, 25, and $12.5 \mu\text{g}/\text{m}^3$ CdCl_2 aerosol groups respectively. Histopathologically the lung

tumors observed were adenocarcinomas, epidermoid (squamous cell) carcinomas, combined epidermoid carcinomas and adenocarcinomas, and mucoepidermoid carcinomas.

6. Teratogenicity

A number of investigators have shown that Cd is a potent teratogenic agent in the golden hamster and rat. Most studies were done with relatively large doses given i.v. or s.c. Chernoff (1973) found that s.c. injection of 12 mg/kg dose of CdCl_2 induced up to 50% fetal mortality and 70% abnormalities in rats. Among these abnormalities were micrognathia, cleft palate, club foot, and small lungs. Ferm and Carpenter (1968) showed the development of abnormal facial formation (unilateral and bilateral cleft lips and palates) in golden hamsters following i.v. injection of 2–4 mg/kg CdSO_4 on the eighth day of gestation.

There is no direct evidence of teratogenic effects of Cd in humans, but children of women (aged 18 to 48 yr) working in cadmium industries had significantly lower birth weights than the children of control women (Cvetkova, 1970 in Friberg et al., 1974). Also four of 27 children born to women working in a zinc smelter in the above study, showed signs of rickets and delayed development of teeth.

7. Mutagenesis

It has not been definitely stated that Cd is mutagenic, but several studies of the effects of Cd on chromosomes have been reported. Shiraishi and Yosida (1972) found an increased number of chromosomal aberrations in the

peripheral leukocytes of Itai-itai patients, and these two investigators along with Kurahashi (1972) showed that chromosomal abnormalities were induced in cultured human leukocytes by Cd sulfide at 0.062 $\mu\text{g/ml}$ of culture medium.

D. Metallothioneins

Metallothioneins are a group of low molecular weight cytoplasmic metalloproteins (MW 6800 daltons) with a high affinity for Hg, Cd, Zn, Ag, and Cu and they are thought to function in heavy-metal detoxification and in zinc and copper homeostasis. Metallothioneins contain about 6 to 11% metal and 30% cysteine and are completely devoid of aromatic amino acids. In metallothionein the metal ions are bound to the protein moiety through mercaptide linkages and three cysteinyl residues are involved in binding with a metal ion (Cherian and Goyer, 1978).

When the animal is exposed to metals (e.g., Cd, Zn, Hg) the synthesis of thionein is induced in the liver and to a lesser extent in the kidney. Induction proceeds until most, if not all, of the exogenous metal has been sequestered by metallothionein (Brady, 1982).

Several works have been done on the role of metallothionein in the development of resistance to Cd (Gick et al., 1981; Rugstad and Norseth, 1975). These studies have shown that selection for Cd resistance is associated with a Cd-mediated increase in the synthesis of metallothionein. The development of resistance is associated with an amplification of the metallothionein gene, as well as an increase in the rate of transcription of this gene in a variety of cell lines, such as mouse sarcoma cells (S-180) (Beach et

al., 1981). These studies lend support to the theory of the mechanisms of Cd detoxification based on the chelation of the metal by metallothionein. In addition, studies in mice have shown that Cd is one of a number of metals that causes the induction of mRNA for metallothionein in liver and kidney (Durnam and Palmiter, 1981).

Unusual results were obtained by Nordberg (1972) and Nordberg et al. (1975) who found that Cd bound metallothionein injected into mice was more toxic to the kidney than Cd salts injected as such, which suggested that metallothionein played a role in eliciting renal tubular impairment at chronic exposure. It has been shown by Nordberg et al. (1975) that the Cd complexes are readily taken up by the kidney but inorganic Cd in contrast is neither filtered nor accumulated by the kidney (Foulkes, 1974). One theory, therefore for explaining the high nephrotoxicity of the metallothionein complex is that in its passage through the kidney the metallothionein complex is degraded, and the Cd released will bind to high molecular weight renal proteins. Alternatively, the Cd could exchange with Zn in certain enzymes necessary for the reabsorption of proteins. It appears then that in some situations metallothionein will reduce Cd toxicity, while under other circumstances, toxicity is enhanced.

E. Inhalation Techniques in Toxicological Studies

One widely used method for the study of the effects of toxic agents on the respiratory tract, is the exposure to gases or aerosols of experimental animals in an enclosed space called an inhalation chamber. Within this chamber the test agent is maintained at the desired concentration. The two

main types of chambers are the whole body chambers and ones for nose-only exposure. Good reports of inhalation techniques can be found in MacFarland, 1976 and Fraser et al., 1959.

Whole body chambers may vary in size from a few liters up to several cubic meters, depending on the number and size of the animals to be exposed. In order to maintain animals comfortably within a closed chamber, the chamber loading rule should be followed, that is, that the total body volume of the animals should not exceed 5% of the total chamber volume. When there are several animals in a cage they tend to bury their noses into each other's fur and this may produce a filter effect and reduce the amount of material that is actually inhaled. Another disadvantage is that the location of the animals in the chamber may determine what concentration of the test agent the animal receives, that is the ones closest to the inlet source may receive a higher concentration. Also the test agent may be deposited in the fur of the animals and when they groom themselves, it will be absorbed by the gastrointestinal tract instead of the lung. The main advantage of whole body exposure is that the animals are relatively comfortable.

There are two modes of operating inhalation chambers, namely static and dynamic. In the static mode, the desired amount of test agent is introduced into the chamber and uniformly distributed by mixing it with air. The animals are then kept in this atmosphere for the experimental time. The disadvantages of this system is that heat and humidity may build up rather rapidly and there may be problems with oxygen depletion and CO₂ accumulation. Also the chamber concentration of the test agent will decrease with time as it is used up by the animals.

In the dynamically operated system, there is a constant flow of air into the inhalation chamber at a flow rate of 1/10 to 1/2 of the total chamber volume per minute. The test agent is delivered into the air of the chamber at the desired concentration. In this system there is a constant concentration of the test agent and no CO₂ accumulation or O₂ depletion. A simple equation can be used to calculate the time t , it takes for the test agent to reach 99% of the desired chamber concentration.

$$t = k \times \frac{a}{b}$$

where a equals chamber volume, b equals flow rate, and k is a constant = 4.605 (MacFarland, 1976).

The nose only method will be described in Chapter III. The advantage of this technique are that the exposure chamber can be quite small, thus requiring less of the test material. One disadvantage is the stress placed on the animals by restraining them in the tubes, and the time it takes to fit them into the tube and to remove them at the end of the experiment.

Other methods of inhalation exposure include the use of a face mask, and intratracheal injections (MacFarland, 1976).

F. Factors Influencing Results of Inhalation Studies

Not only is the method of inhalation important in evaluating the toxic response, but several other factors have to be considered. Brain et al. (1976) compared aerosol inhalation versus intratracheal instillation and found that with the latter method there were nonuniform distribution patterns, with preferential deposition in the dependent portions of the lung, whereas with

aerosol inhalation there was more even distribution of particles, with preferential deposition in the apical lobes.

The site of deposition of particles in the respiratory tract is another important factor and is determined by the anatomy of the respiratory tract as well as a combination of physical forces that govern the removal of particles from an airstream. The site of deposition will have an effect on the severity of tissue damage done, the degree of absorption of the toxicant and the availability of clearance mechanisms for the removal of the particles. There are three main methods of deposition (Figure 3) namely inertial impaction, which takes place in the nasopharyngeal region and involves particles with an aerodynamic diameter of 5–30 μm , sedimentation, which takes part in the trachea, bronchus and bronchiole and is the method for particles in the 1–5 μm range, and diffusion, which takes place in the alveolar region and involves the smallest particles 1 μm and under.

As particles traverse the respiratory tract, the directional changes imposed on the airflow become less abrupt, and also the air velocity decreases (Casarett, 1975). Other factors that should be considered are particle size of the inhalant, concentration and solubility of the solution used to generate the aerosols, health status of the experimental animals, which might affect such physiological functions as tidal volume and functional residual capacity, and pattern of breathing, such as breath holding which increases deposition from sedimentation and diffusion.

G. Cells of the Lung

The complexity of the lung can be appreciated when it is known that over 40 distinctive cell types make up the tissues of normal mammalian

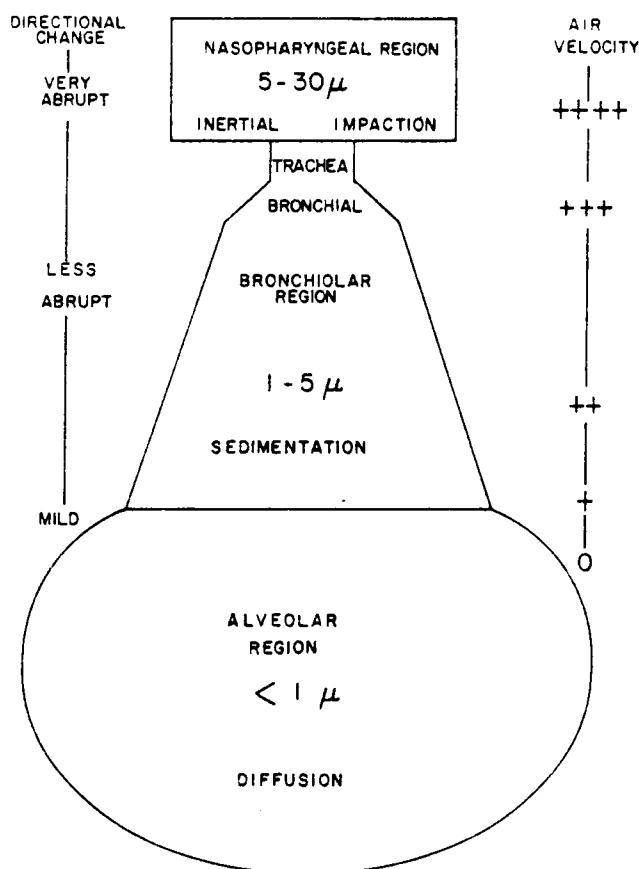


Figure 3. Parameters influencing particle deposition. From: Casarett, L. J., The vital sacs: Alveolar clearance mechanism in inhalation toxicology: In Blood, F. R. [ed.]: Essays in Toxicology, Vol. 3, Academic Press, Inc., New York, 1972, Permission granted.

lungs. Most of these types occur also in other tissues of the body, but a few are unique to the lungs. The cell types include 17 types of epithelium, nine types of unspecialized connective tissue, two types of bone and one cartilage, seven types of cells related to blood vessels, two types of muscle cells and one pleural type, and five types associated with nervous tissue elements (Sorokin, 1970).

In order to give a clearer picture of cellular interactions in this work, the cells of the terminal bronchioles and the alveolar region will be briefly described, since it is in these regions that most of the action described in this work takes place.

The principal role of the cells in the alveolo-capillary region is formation and maintenance of an efficient gas exchange apparatus. This is done by maintaining an extremely thin tissue barrier between air and blood while there is still a complete lining of the blood and air spaces. This air-blood barrier consists of endothelium, basement membrane and alveolar epithelium with a total thickness of 0.36 to 2.5 μm . Between adjacent alveoli, the tissue thickness is made up of the thickness of the alveolar wall, basement membrane, interstitium and any interposed capillary to give a mean thickness of 9 μm (Figure 4).

The morphogenetic processes which succeed in minimizing the tissue barrier while at the same time maintaining the protective cell mechanisms has been described by Weibel et al. (1976) as "a formidable bioengineering achievement."

The alveolar epithelium is made up essentially of two cell types whose cytoplasm completely line the alveolus. The junction between the Type I and Type II cells is "tight," forming a *zonulae occludens*. The surface area of the

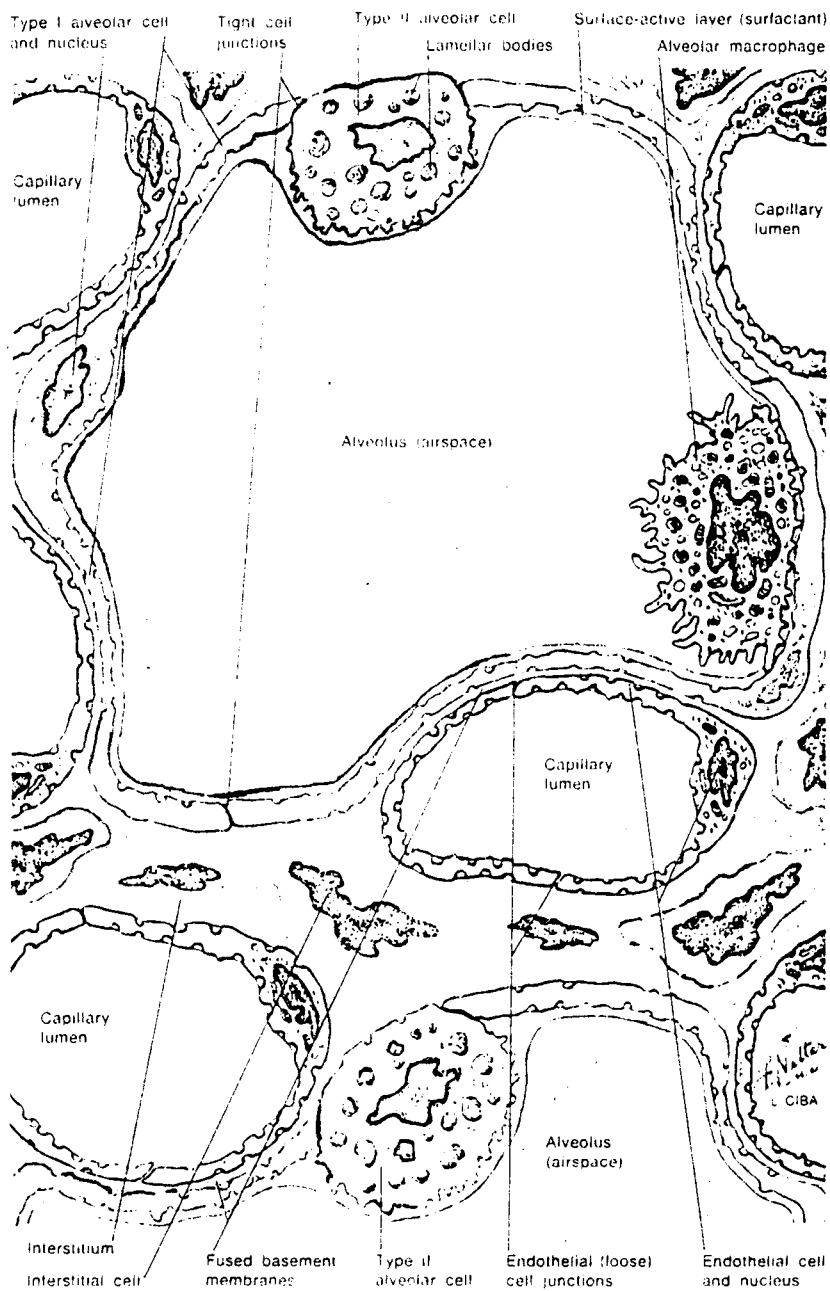


Figure 4. Ultrastructure of pulmonary alveoli and capillaries. Copyright 1979, CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation. Reprinted with permission from The CIBA Collection of Medical Illustrations illustrated by Frank H. Netter, M.D. All rights reserved.

Type I cell is $2290 \mu\text{m}^2$ and that of the Type II is $63 \mu\text{m}^2$, but although the ratio of Type I to Type II is 2:3, the Type I makes up most of the blood-air barrier. The Type I alveolar cells are barely visible with the light-microscope because of their organelle-poor cytoplasm which may extend up to 50μ from the nucleus. The Type I cells are not simple squamous cells, but rather complex branched cells, each branch being able to form an "apical" cytoplasmic plate on either side of the alveolar septum (Weibel, 1971). This complex structure of the Type I cells may restrict their proliferative capacity, since they appear to be incapable of mitotic division, and when injured they are replaced by the division of Type II cells.

Type II cells, or granular pneumocytes, are of cuboidal shape and often lie in the corners of the alveoli. The nucleus is spherical and the cytoplasm abundant with vacuoles. The surface is covered with microvilli and the cytoplasm has a granular endoplasmic reticulum, extensive Golgi apparatus, numerous multivesicular bodies, and prominent osmophilic multilamellated inclusions, called lamellar bodies, which are the intracellular storage sites of surfactant.

The capillary endothelial cells are squamous cell with an organelle-poor cytoplasm. They have small vesicles which are capable of engulfing blood-bone particles through pinocytosis and transporting them across the cells.

The interstitium is composed of three-fourths cells and one-fourth actual interstitial space and fibers.

The alveolar macrophages are an important non-sessile cell population found in the lung. They are originally formed in the bone marrow and carried via the blood stream to the lung where they migrate into the alveolar

interstitium. There they lie dormant until activated by the presence of foreign bodies such as bacteria or inhaled particles. The macrophages then move into the alveolar space and begin to ingest the foreign material by phagocytosis. Alveolar macrophages contain lysosomal enzymes which can digest some foreign particles like the bacteria, while the non-digestible particles such as inorganic dusts will remain within the macrophages. Macrophages eventually leave the alveolar regions via the airways, through the pulmonary lymphatics or through pulmonary blood vessels.

In the small bronchioles, the principal epithelial cells are the ciliated cells and the Clara cells (see Figure 5). The Clara cells are typified by their bulging into the bronchiolar lumen, by the absence of cilia, and the presence of apical cytoplasmic granules. They produce granules containing a basic protein. These granules are formed in the smooth endoplasmic reticulum and in the Golgi apparatus and are secreted by exocytosis into the bronchiolar lumen where they contribute to the bronchiolar lining layer (Kuhn, 1976).

The ciliated cells are columnar cells with a centrally placed nucleus, moderate amount of endoplasmic reticulum, a few lysosomes, a well developed Golgi apparatus and scattered mitochondria, some concentrated in the apex beneath the basal bodies of the cilia (Kuhn, 1976).

H. Effects of Other Toxicants on the Lung

A wide variety of substances can cause damage to the lung. Among the excellent reviews on this topic are Doull et al. (1980), Witschi and Côté (1976), and Boyd (1980). The toxic substances can be grouped into six classes, namely: gases, pollens and dusts, metals, drugs, other organic compounds, and

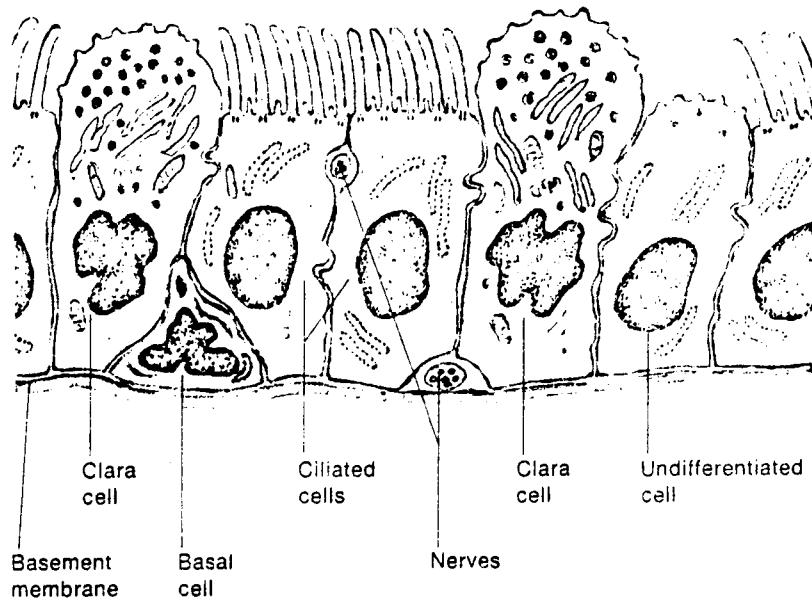


Figure 5. Ultrastructure of the bronchiolar epithelium. Copyright 1979, CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation. Reprinted with permission from The CIBA Collection of Medical Illustrations illustrated by Frank H. Netter, M.D. All rights reserved.

cigarette smoke. Among the gases are included ozone, sulfur dioxide, nitrogen oxides, and oxygen. They produce different effects and may have different mechanisms of action. For example ozone causes chest pain and irritation of the upper airways, and in greater concentration may produce fatal acute pulmonary edema in man. Nitrogen dioxide, in some cases of high accidental exposure caused respiratory distress followed by obliteration of the small airways through proliferation of connective tissue (*bronchiolitis obliterans*), which proved fatal. In some animal studies with NO_2 , damage was seen in the terminal bronchioles and proximal portions of the alveolar ducts. It was characterized by necrosis of the ciliated bronchiolar and alveolar epithelial cell accompanied by exudation of fibrin and macrophage aggregation (Stephens et al., 1972).

In the case of chronic exposure to SO_2 , there was increased susceptibility to acute upper respiratory tract infections, chronic bronchitis, bronchial asthma, and chronic degenerative lung disease (Rall, 1974). In animal studies, damage was found mostly in the upper respiratory tract down to the level of bronchi where there was airway constriction and increased secretion of mucus. In guinea pigs exposed continuously to 1000 ppm SO_2 death occurred within 20 hr and within 6–7 days when exposure was to 130 ppm. The most prominent pathologic findings in these cases were edema and hemorrhage. The effects of O_2 will be described later.

The second group, dusts and pollens, can lead to occupational asthma in specially susceptible individuals. Examples are farmer's lung which is caused by the spores of certain thermophilic actinomycetes growing in moldy hay, bagassosis—caused by exposure to moldy bagasse (sugar cane) and

byssinosis—caused by inhalation of cotton dust fibers. Silicosis, asbestosis and coal miners lung are the best known examples of a chronic form of lung damage from industrial dusts.

In the third class, the metals, the most common offenders are beryllium, cadmium, nickel, and chromium. Exposure to beryllium may cause a chronic, often progressive form of lung disease characterized by the development of granulomatous lesions. The effects of cadmium will be dealt with in this work. Chromium and nickel have been identified as human carcinogens, for instance workers in nickel mining and nickel processing industries have a higher risk for the development of cancer in the nasal sinuses and the lung than has the normal population (Witschi, unpublished).

Among the drugs which cause lung damage are bleomycin, a polypeptide antibiotic first isolated from *Streptomyces verticillatus*, and the antineoplastic agents, bisulphan, melphalan, cyclophosphamide and methotrexate. Some patients treated with bleomycin develop lung fibrosis and in the treatment of cancer using the antineoplastic agents, often fatal lung complications occur. In animals, bleomycin produces lung toxicity in mice, rats, hamsters, dogs and birds. The primary site of bleomycin toxicity is the pulmonary endothelium, while necrosis of Type I epithelial cells develops later, followed by interstitial fibrosis (Adamson and Bowden, 1977).

Some other organic compounds that cause lung damage are paraquat, a bipyridylium salt widely used as a herbicide, and two furans, 4-ipomeanol, and 3-methylfuran. In man, paraquat poisoning leads to congestion and edema of the lungs, followed by fibrosis. In animals there is damage to both Type I and Type II alveolar cells, as well as edema, necrosis of capillary endothelial cells and fibrosis.

The toxicity of 4-ipomeanol was first noticed in cattle that had been fed moldy potatoes, and they showed severe hemorrhagic and edematous lesions in the lung. Special target cells of 4-ipomeanol are the Clara cells of the small airways (Boyd, 1980) and these cells are also affected by inhalation or parenteral administration of 3-methylfuran.

I. Cigarette Smoke

Cigarette smoke has been put in a class by itself, but it is really the Cd content of the smoke that is of interest in this study.

Not only Cd but several of the constituents of cigarette smoke are considered as health hazards. These include tar, nicotine, phenolic compounds, aromatic hydrocarbons, and nickel compounds. Nandi et al. (1969) tested six brands of cigarettes and found a range of 18–28.5 and a mean of 22.7 μg Cd per 20 cigarettes.

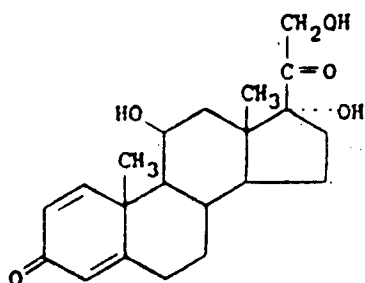
Tobacco smoke contains as many as several billion particles/ cm^3 (Carter and Hasegawa, 1975) with most of these particles being in the range of 0.2 to 0.6 μm in diameter, so they can penetrate deep into the lung during inhalation. Hoegg (1972) reports that approximately 50 to 80% of inhaled smoke is deposited in the lungs which results in approximately 25 mg of particulate matter per unfiltered cigarette being deposited. If the average cigarette is taken to contain 1 to 2 μg Cd and if about 10% of this Cd is inhaled, and one-half of the inhaled load is retained in the body, then a smoker may add as much as 2 μg Cd to his body burden with each pack of cigarettes.

The total Cd intake of the average adult human in the United States is presently about 50 to 75 $\mu\text{g}/\text{day}$, and this is made up of <1 μg of airborne Cd, 2–20 μg from water and about 50 μg from food. However because the efficiency of intestinal absorption Cd is low, the daily exposure only amounts to a daily retention of about 1 to 2 μg Cd. Thus with heavy smoking the daily dose may be doubled. Studies done by Hirst et al. (1973) have shown elevated levels of Cd in the lung of smokers with emphysematous lungs compared with nonsmokers with normal lungs, so these findings have led to the hypothesis that Cd in cigarette smoke may be one of the causes of emphysema in man.

Several animal experiments have been carried out to investigate the effects of cigarette smoking. For example, Auerbach et al. (1967) exposed tracheotomized dogs to twice-daily sessions of smoking up to six cigarettes. Among the adverse effects, they found areas of fibrosis thickening of alveolar septa, and dilated air spaces beneath the pleura, and these animals were believed to have developed widespread emphysema.

J. Prednisolone

Much research has been done on the controlling of the development of pulmonary fibrosis by various drugs. These agents fall into two groups, namely, those that affect the inflammatory response prior to fibrosis, and those that affect the synthesis or processing of collagen. Prednisolone, which has the following structure, has been used in this study to ascertain its effect on lung collagen synthesis.



The results of experiments with the administration of corticosteroids to various animals with lung injury have shown that the corticosteroid may be either beneficial, have no effect, or be detrimental. For example, Phan et al. (1981) found that daily treatment of rats with methylprednisolone for two weeks following intratracheal instillation of bleomycin resulted in significant decreases in lung collagen synthesis and lung hydroxyproline content. Cheney et al. (1979) also found that methylprednisolone aided in the repair of oleic acid-induced lung injury in dogs.

Smith and Brody (1981) showed that when methylprednisolone was administered on days 1 and 2 to mice with mild BHT-induced lung injury it inhibited Type II alveolar epithelial cell proliferation, but prolonged administration (days 1–5) resulted in enhanced Type II cell proliferation, greater lung injury and mortality. They also found that methylprednisolone failed to inhibit Type II cell proliferation when lung injury was severe, while Aronin et al. (1980) in their studies with the antineoplastic agent, carmustine (BCNU) found that patients receiving this compound, along with steroids (dexamethasone, prednisone or prednisolone) tended to develop more cases of pulmonary toxicity than patients receiving BCNU and no steroid.

K. Oxygen

Oxygen is the choice of a second agent in this study to determine its interactions with Cd in the development of a lung lesion.

Shortly after the discovery of O₂ some 200 years ago, its potential toxicity was recognized. In mammals during hyperbaric exposure to pure O₂, death occurs as a result of central nervous system toxicity. However when animals are continuously exposed to one atmosphere of 100% O₂, the principal organ that is damaged is the lung (Jenkinson, 1982). High tensions of O₂ are shown to produce diffuse alveolar damage (Clark and Lambertson, 1971). In the early stages of O₂ toxicity, the pathologic features include endothelial swelling, interstitial edema, and an increase in the predominance of Type II alveolar cells (Kapanci et al., 1969; Kistler et al., 1967). When there is only minor damage and the exposure is discontinued, the pathologic changes may resolve and leave no permanent damage. However, when the exposure is continued beyond a certain point of reversibility, the pathologic changes may evolve into interstitial fibrosis (Clark and Lambertson, 1971). Pratt and Currie (1977) have also noted airspace enlargement and Brooksby and Staley (1966) noted a decreased elastic recoil pressure in rat lung several weeks after recovery from O₂ toxicity.

A lucid account of the mechanisms of O₂ damage involving the superoxide anion, hydrogen peroxide, and the hydroxyl radical is given by Jenkinson (1982), discussing lipid peroxidation, the effects of oxygen radicals on nucleic acid, and protein sulhydryl oxidation. Protective mechanisms involving cytochrome oxidase, superoxide dismutase, catalase, peroxidase, Vitamins E and C, are also discussed.

L. Cell Kinetic Studies

Cell kinetic studies in lungs of animals exposed to a toxic agent have been reported by several researchers but usually only after one toxic substance, not a combination of agents. Studies of cell kinetics are designed to provide a dynamic picture of cell renewal in a damaged organ and comparison with normal cell turnover. Adamson et al. (1977) in studying the cytodynamic changes in mouse lung after treatment with butylated hydroxytoluene found the peak of DNA synthesis was reached on day 3, remained at this value until the 5th day and fell to normal levels by day 9. The differential percentages showed that the epithelial cells peaked at 2 to 3 days, then there was a plateau of interstitial labelling at days 5 to 9 and the endothelial labeling showed a late peak at 6 days. Adamson and Bowden (1974) and Bowden and Adamson (1974) found a reverse in the regenerative pattern in animals exposed to oxygen, where the DNA synthesis in the endothelium preceded that in the epithelium.

Evans (1973) studied the kinetics of the Type II cell division in rat lung following exposure to NO_2 . In this work, the alveolar cells were studied by light and electron microscopy using autoradiographic techniques. One interesting aspect of the study was the estimation of the kinetics of Type II cell division from a curve constructed from the percentage of labelled mitotic figures.

One study using two toxic agents is that of Hackney et al. (1981), where they showed that O_2 concentrations of 61% and higher depressed Type II labelling indexes throughout four days of recovery in ozone-damaged alveoli of mice.

A similar study by Haschek et al. (1983) was done on cell proliferation and collagen synthesis in mice treated with BHT and then exposed to 70% O₂. They found that the proliferating cell population after removal from O₂ was primarily interstitial and not epithelial and there was a drop in the percentage of newly synthesized Type III collagen.

CHAPTER III

MATERIALS AND METHODS

A. Materials

1. Animals

The animals for the experiments were male BALB/c mice, 6–10 weeks old, bred in the barrier facilities at Oak Ridge National Laboratory. They were housed on hardwood bedding, 10 to a cage, and kept in a controlled atmosphere where the temperature was $72 \pm 2^\circ\text{F}$, humidity was $50 \pm 5\%$ and the light to dark cycle was 12:12 hr. They had access to food and water *ad libitum*. The food consisted of Rodent Laboratory Chow 5001 (Ralston Purina Company) and the water was chlorinated at a concentration of 16–18 ppm.

2. Chemicals

Thymidine (methyl- ^3H), specific activity 17 Ci/mM was obtained from Schwarz Mann (Orangeburg, NY). Methyl- ^{14}C thymidine, specific activity 55.5 mCi/mmol was obtained from Amersham Corporation, a subsidiary of the Radiochemical Centre. Aquasol was from New England Nuclear, Boston, MA, Cadmium Chloride ($\text{CdCl}_2 \cdot 2\frac{1}{2} \text{H}_2\text{O}$) was from Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH, Dithizone (Diphenylthiocarbazone) was from Fisher Scientific Company, Fair Lawn, NJ, p-Dimethylamino-benzaldehyde was from Sigma Chemical Company, St. Louis, MO. All other chemicals were reagent grade.

B. Methods

1. General

(a) Exposure to cadmium chloride aerosols

(1) Inhalation chamber

The inhalation chamber used for exposing the animals to CdCl_2 aerosols was a 28 liter nose-only type, similar in design to one developed by Raabe et al. (1973) and modified by Dalbey (Figure 6). Each large side of the chamber had 20 exposure ports, placed in three tiers. There were no compartments or internal baffle plates between the point of aerosol entry and the exposure ports. The rectangular exposure area measured 50 cm in width, 10 cm in depth and 40 cm in height. The incoming air flowed down the upper cone (60° angle), through the rectangular area, through a baffle plate across the bottom of the exposure area and out a series of holes to a common exhaust tube. The upper cone and baffle system were designed to assure an even distribution of the air flow. Simultaneous sampling at multiple exposure ports showed that the aerosol was distributed uniformly throughout the chamber.

The mouse holding tubes (Figure 7) are plastic test tubes, 10.16 cm long, 2.86 cm OD, 2.54 cm ID and a wall thickness of 0.16 cm. The tubes are open at one end to insert the animal and then sealed with a rubber plunger to hold the animal firmly in place.

The whole exposure unit was housed in a large fume hood, with the face velocity of air in the hood being 190 L/min. The CdCl_2 aerosol was generated with a Collison nebulizer using a solution of 0.65% CdCl_2 . For a diagram of the apparatus see Figure 8. The aerosols generated in the nebulizer are mixed with the diluting air in the dilution flask and then passed through a

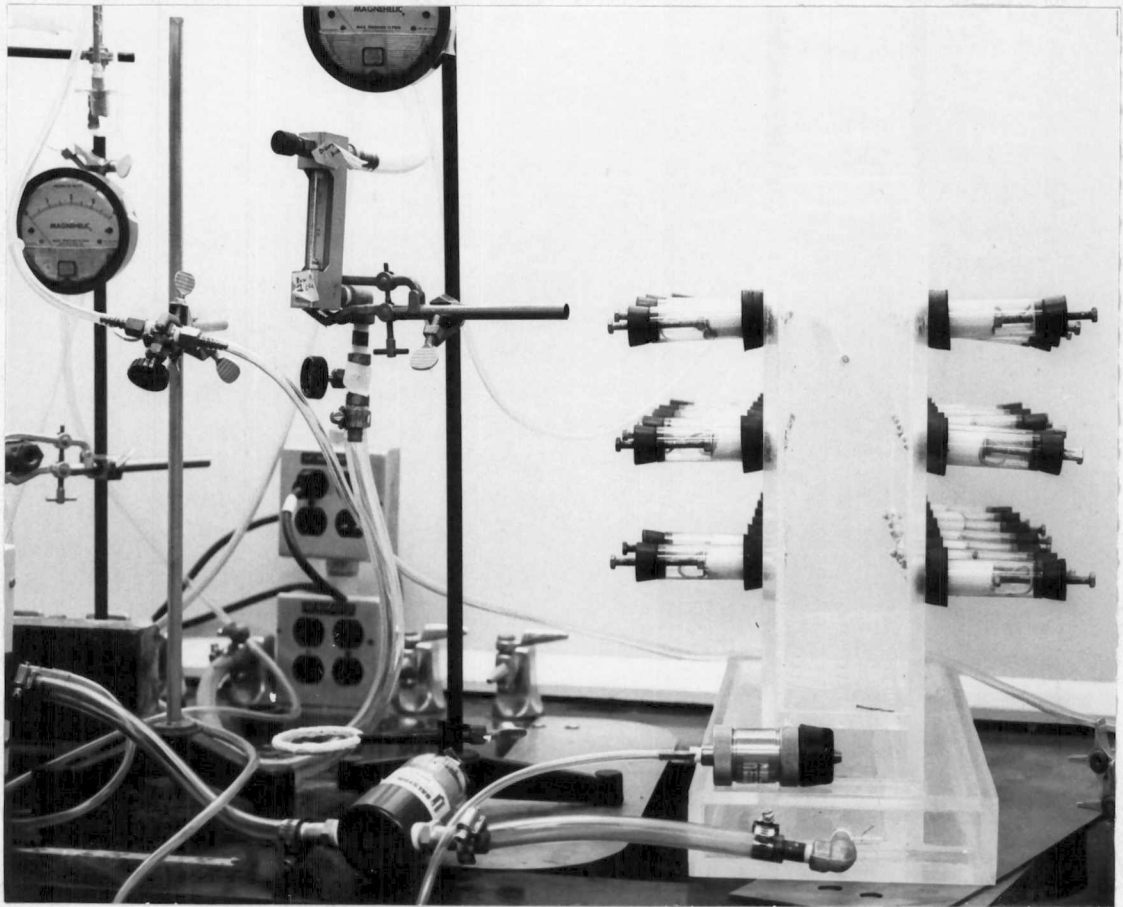


Figure 6. Inhalation chamber for nose-only exposure.

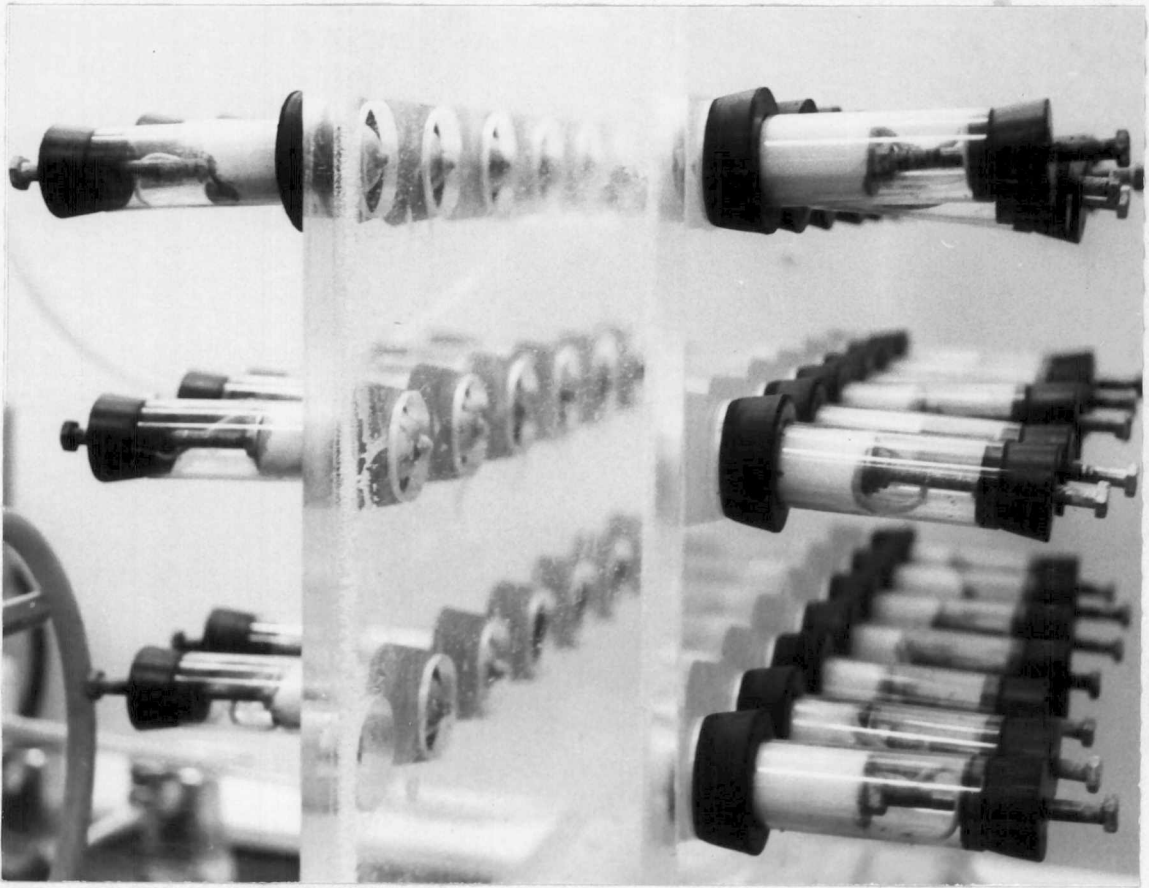


Figure 7. Mouse holding tubes during inhalation exposure.

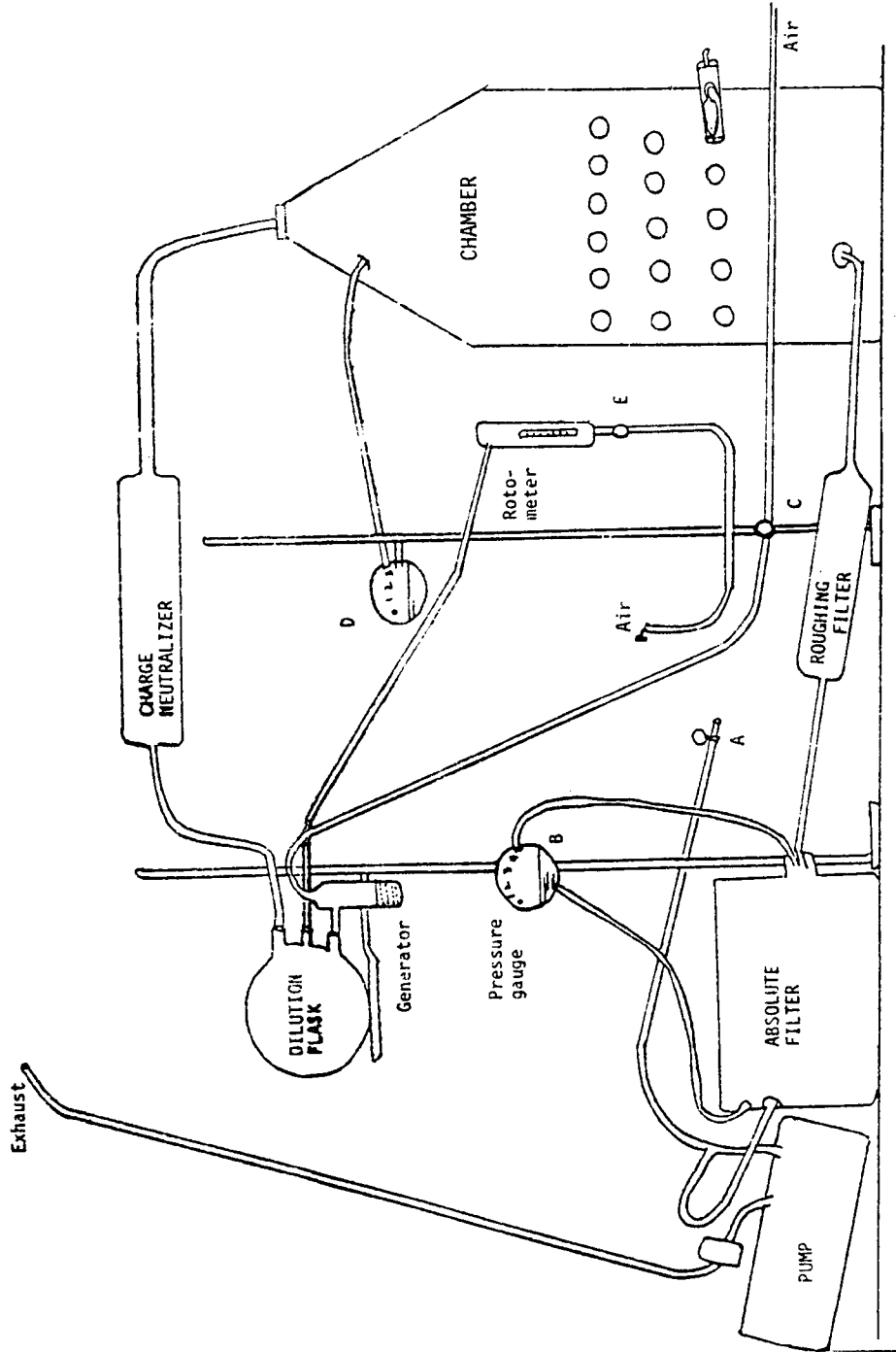


Figure 8. Diagram of the cadmium inhalation apparatus.

charge neutralizer containing Kr 85 which ionizes the air and prevents electrostatic loss of particles in the system.

To avoid inadvertent releases of Cd into the atmosphere, the exposure unit was operated under slightly negative pressure (1.27 cm H₂O). Operation was always done with all ports closed. If some ports were not in use they were plugged with rubber stoppers. The exposure time was one hour.

Chamber aerosol concentration and mass median diameter were determined using a cascade impactor by Aries, Inc., Aerosol Research Instruments, Equipment and Services, Davis, CA (Model D7)—Figure 9. Round coverslips 2.5 cm in diameter were cleaned in 70% alcohol dipped in a saturated solution of Apiezon grease in xylene, and air dried. One coverslip was used for each impactor stage and the backup filter was a nucleopore 0.2 μ m polycarbonate membrane by Nucleopore Corporation, Pleasanton, CA. The cascade impactor was fitted into one of the openings in the chamber and sampling was done during the middle 20 min of the one hour cadmium run. The average Cd concentration in the chamber was $4.9 \pm 0.3 \mu\text{g/L}$ (mean $\pm$ SD from 5 independent observations); mass median diameter of the aerosol was $0.88 \pm 0.11 \mu$, and the geometric standard deviation, σ_g , was 1.875 ± 0.27 . Airflow within the chamber was 30 L/min. Control animals were exposed under identical conditions to H₂O aerosols.

(2) Dithizone method

The cadmium deposited on the coverslips from each impactor stage and from the backup filter was measured colorimetrically by the dithizone method (Sandell, 1950). This method is based on the reaction of Cd with dithizone to

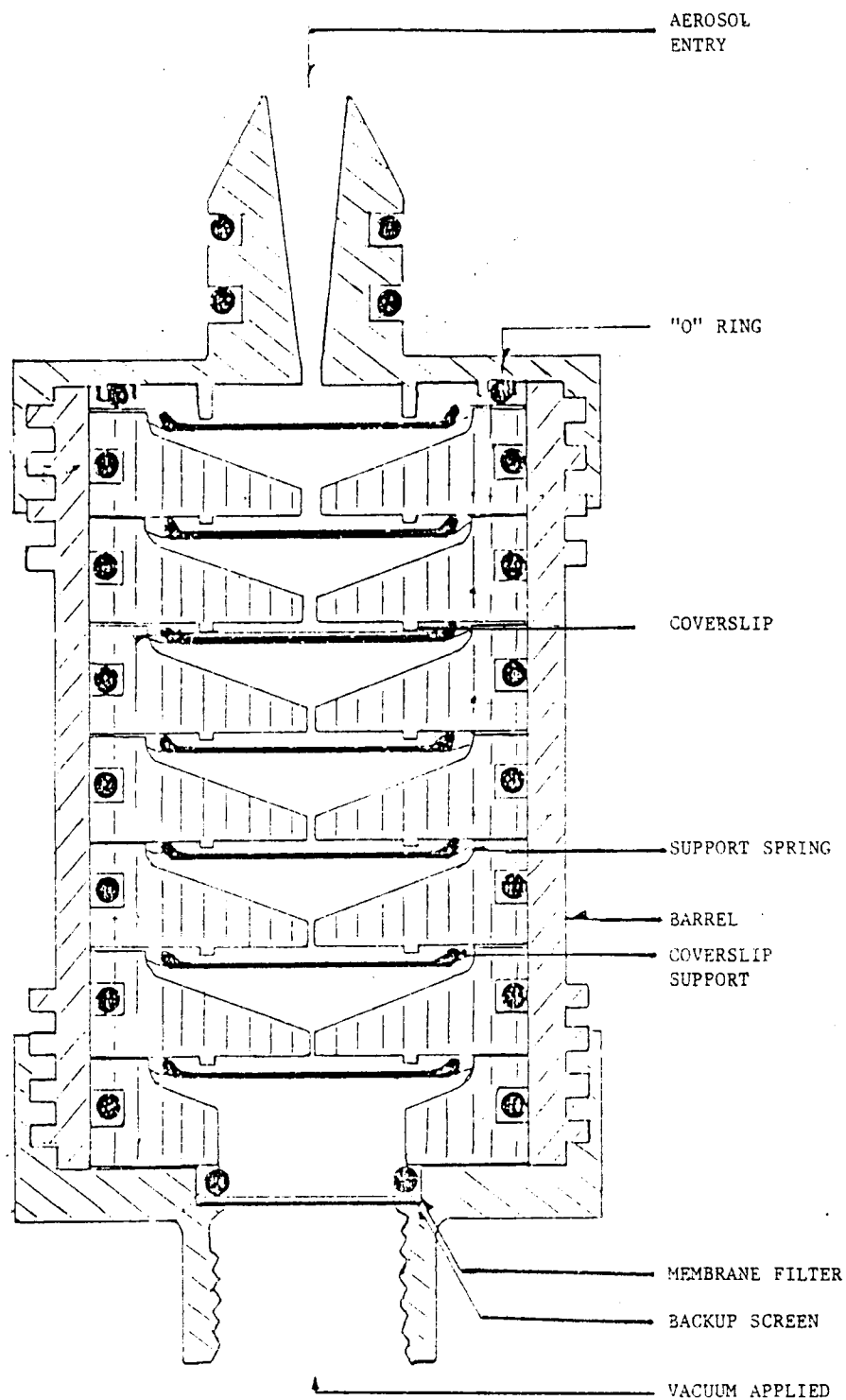


Figure 9. Cascade impactor. From Aries, Inc., Aerosol Research Instruments, Equipment and Services, Davis, CA.

form a red complex which is soluble in chloroform, stable in strong base and unstable in dilute acid. The stock solution was 20 μg $\text{CdCl}_2 \cdot 2.5 \text{ H}_2\text{O}/\text{L}$ of water. The cadmium on the filters was dissolved in 5 ml water, and to 5 ml sample or standard were added 3.0 ml of 20 g% sodium tartrate, 0.6 ml 20 g% hydroxylamine HCl, 3.0 ml KOH (25.0 g%), and 6.0 ml dithizone (20 mg/L in chloroform). The mixture was vortexed and the chloroform phase was transferred by Pasteur pipettes to glass containers to eliminate the water phase in the curvette. The absorbance was read at 518 nm against the reagent blank using a Gilford or Perkins-Elmer Spectrophotometer. By regression analysis, the concentration of Cd in each sample, that is, the amount from each impactor stage, was determined, and the total divided by the air flow times sampling time gave the chamber concentration of Cd.

For each sample, the percentage of the total chamber concentration of Cd was calculated and then the percentage less than the effective cutoff diameter (ECD) was determined. This value was plotted against the ECD for the various impactor stages at the particular air flow (numbers supplied by the Cascade Impactor manufacturer) on log probability paper.

The mass median diameter (MMD) was found where the 50% size bisects the curve, and the geometric standard deviation, σ_g , was calculated as:

$$\sigma_g = \frac{84.1\% \text{ size}}{50\% \text{ size}}$$

The σ_g of the particle size distribution is a measure of the heterogeneity of the aerosol, with $\sigma_g < 1.2$ being considered as monodisperse (Doull et al, 1980).

(b) Oxygen exposure

The animals in their cages were placed inside a 450 L plastic chamber which was continuously ventilated with 70 or 80% O₂ at an average flow rate of 15 L/min (Figure 10). The temperature within the chamber was 24–25°C and the relative humidity was 55 to 65%. O₂ concentration within the chamber was monitored daily with an oxygen meter and remained within $\pm 3\%$ of the desired concentration. After 6 days the animals were removed from the O₂ chamber and kept in room air until used for the experiments, or in certain experiments, the animals were removed at the desired times. Controls were left in room air.

(c) Hydroxyproline analysis

Lung collagen content was determined by measuring the amount of hydroxyproline, an amino acid present virtually only in collagen. Measuring the lung collagen content is a means of quantitating the degree of lung fibrosis. For this analysis, the animals were killed by cervical dislocation on day 21 after Cd exposure and the lungs were perfused *in situ* via the right ventricle with 0.9% NaCl. The lungs were excised, rinsed, blotted dry, weighed, and placed in test tubes kept on dry ice. The lungs were then lyophilized, reweighed, and hydrolyzed with 6 N HCl in sealed tubes for 18 hr at 107°C. An internal standard of DL-hydroxy [2-¹⁴C] proline was added to the hydrolysate, which was then filtered through activated charcoal, and reconstituted to a known volume with distilled water. Recovery, generally 80–90%, was calculated using an internal standard, and appropriate corrections were made. Hydroxyproline concentrations were determined by a colorimetric method

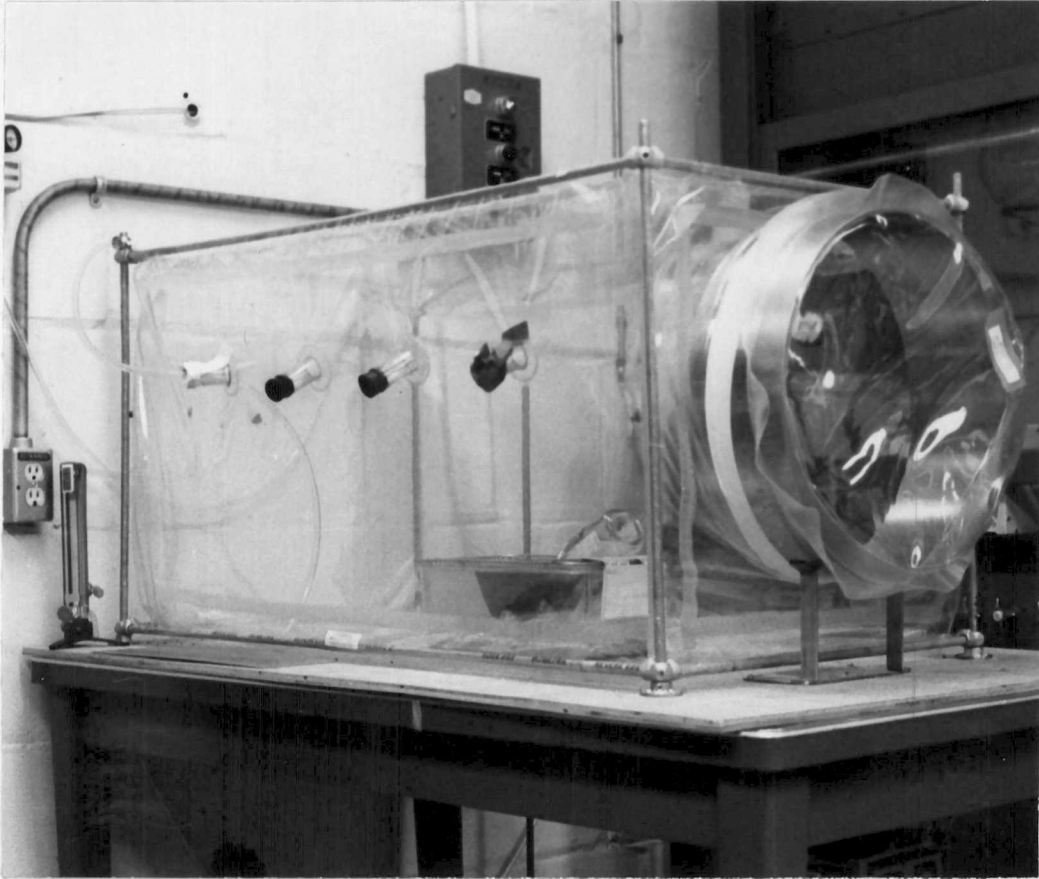
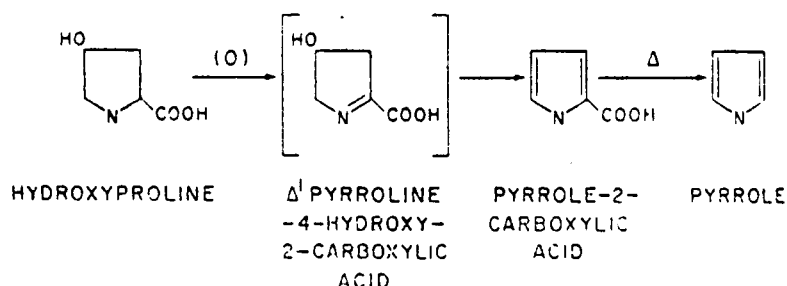


Figure 10. Oxygen chamber.

using Ehrlich's reagent (p-dimethyl amino benzaldehyde) (Prockop and Udenfriend, 1980). The hydroxyproline was reduced to the pyrrole form by oxidizing agents such as Chloramine T, and heat (100°C in a water bath), and the pyrrole formed reacted with the reagent (see Equation below).



The results were calculated as g hydroxyproline per total lung. Expressing the data per total lung rather than dry or wet weight, seems to be the most satisfactory way since the figures will not be affected by changes in the chemical composition of the lung (edema, hemorrhage, enzymes, etc.) brought about by the toxic agent (Witschi, 1975).

(d) Histopathology

The effects of Cd and O₂ were studied microscopically in mice at four different time periods, namely 3 weeks, 3 months, 6 months, and 1 year. Animals were given an overdose of sodium pentobarbital. The abdominal aorta was cut to exsanguinate the animal. The lungs were inflated *in situ* through the trachea with 10% neutral buffered formalin at 20 cm constant pressure. Sections of lung lobes were then embedded in paraffin, sectioned at 3–4 μ m and stained with hematoxylin and eosin. Duplicate sections of lung were dehydrated and embedded in glycol methacrylate. Large methacrylate sections were

cut at 1 μm on a Sorvall JB4 microtome and stained with Lee's methylene blue-basic fuchsin.

(e) Measuring cell proliferation

(1) Incorporation of [^{14}C] thymidine into DNA

When lung is damaged, cell proliferation takes place to repair the damage. DNA synthesis in the proliferative cells can be assessed by determining the incorporation of ^{14}C -thymidine into DNA.

Four to six animals from each group were injected i.p. with 0.5 μCi of ^{14}C -thymidine and sacrificed by cervical dislocation 90 min later, on days 1, 3, 5, 7, 9, 11, 13, and 15.

The lungs were removed, weighed, and homogenized in 5 ml distilled water. 2.5 ml 0.6 N HClO_4 were added and the mixture was centrifuged for 15 min at 2000 rpm at 15°C . The supernatant was discarded and the pellets were washed three times with 0.2 N HClO_4 and extracted for 20 min with 4 ml of 0.5 N HClO_4 at $70\text{--}80^\circ\text{C}$. Two ml of extract were mixed with 10 ml of Aquasol and 0.2 ml of extract were analyzed by Burton's modification of the diphenylamine method for the colorimetric determination of DNA (Burton, 1956). Corrections for quenching were made by automatic external standardization. Results were calculated as dpm/mg of DNA.

(2) Autoradiography

Another method of measuring cell proliferation is by determining the number of labelled cells after the uptake of ^3H thymidine during the the autoradiographic process.

Animals were injected i.p. with [methyl- ^3H]thymidine ($2\ \mu\text{Ci/g}$ body weight) 1, 3, 5, 7, 9, 11, 13, 15 days after Cd exposure. Ninety minutes later, the animals were anesthetized with pentobarbital and their lungs fixed by intratracheal instillation of 10% neutral buffered formalin. Sections were taken from three lung lobes of each animal, dehydrated and embedded in glycol methacrylate. Large methacrylate sections were cut at $1\ \mu\text{m}$ on a Sorvall JB4 microtome and mounted on glass slides. The slides were coated with NTB₂ emulsion (Eastman Kodak Co., Rochester, NY) and stored in light-tight boxes at 0 to 4°C. After 10 days the slides were developed in Kodak D-19 developer, rinsed in distilled water, fixed in Kodak fixer, and stained with Lee's methylene blue basic fuchsin.

(3) Labelling index

The sections of lung were examined under a dissecting microscope and about eight of the most distinct terminal bronchioles were selected and marked. From these 2000 cells were counted for each lung using the 45X power and a reticule in the eye piece. Only the regions around the terminal bronchiole were counted. This was decided on because particles <1 micron are deposited in the alveolar region by the process of diffusion during aerosol inhalation, and this was the size of Cd aerosols generated in this study.

In performing the counts, the reticule was moved parallel to the bronchiolar wall, then around the alveolar region and again parallel to the bronchiolar wall on the other side. Only cells with >5 grains per nucleus were considered as labelled. To be included in the counts, the cells had to be alveolar, that is, epithelial, fibroblast, endothelial or macrophages, and a nucleus

had to be present. The labelling index (LI) was defined as the percentage of labelled cells per total number of cells counted. The bronchiolar cells were not included in this part of the study. Airways, large vessels, necrotic nuclei, and inflammatory cells were also excluded.

(4) Differentiation

Differential counts were performed by counting and identifying 100 to 200 labelled cells per animal using oil immersion. The cells were categorized as Type II epithelial, endothelial, interstitial/fibroblast, and alveolar macrophages. Cells that were difficult to identify were put in the "other" category. The percentage of individual cell types per total number of labelled cells was determined. This procedure gives the relative distribution of labelled cells but does not take into account differences in overall cell proliferation as determined by the labelling index. The data was further analyzed by calculating from the labelling index the number of labelled cells per 10,000 parenchymal cells, and using the differential count to find the corresponding number of labelled macrophages, Type II, endothelial and interstitial/fibroblasts per 10,000 lung cells for the various time points.

(5) Bronchiolar labelling index

For each lung 900 cells of the terminal bronchioles were counted using the 45X power. Only cells which protruded into the lumen were counted and the regions chosen were ones where the basal muscle layer was <1 layer thick. Labelled cells had to have >5 grains per nucleus, and the labelling index was defined as the percentage of labelled cells per total number of cells counted.

(f) Lung function tests

The apparatus shown in Figure 11 was designed to measure quasi-static lung deflation volume-pressure curves in anesthetized mice. The curves were used to compare compliance and inspiratory capacity at 3 weeks, 3 months, 6 months, and 1 year timepoints in groups of Cd-treated and control mice.

Compliance can be defined as the volume change produced by a unit of pressure change, or in other words, the relationship between the pressure required to overcome "elastic recoil" and the volume of air that moves into the lung as a result of the pressure change. For this test, the anesthetized mouse is placed in a plethysmograph, the lungs are inflated with a syringe and as they are deflated, the transpulmonary pressure is measured to give the pressure required to overcome the "elastic recoil." The changes in pressure in the plethysmograph are proportional to the volume changes in the lungs. The slope of the line that results from plotting the pressure against the increase in volume serves as a measure of the distensibility of the lungs. A slope that is closer to a horizontal line denotes "stiffer" lungs, such as result from fibrosis.

The apparatus (Figure 11) shows a 350 ml plethysmograph, 16 cm long with an inner diameter of 5.5 cm which was constructed of plexiglass and inside was a plexiglass shelf built to support an anesthetized mouse in the supine position. Some copper wire was placed in the far end of the plethysmograph as a heat sink. This end was closed by a rubber stopper which had one centrally placed outlet consisting of a 14 gauge needle. Pressure changes occurring within the plethysmograph were monitored through the outlet, which was connected through a 3-way stopcock and tygon tubing (I.D. 4 mm) to a differential pressure transducer (Validyne DP-45-26). Signals



Figure 11. Apparatus for lung function studies. P = plethysmograph, C = carrier demodulator, M = mouse in supine position, R = reference volume flask with copper wire, and insulated to provide constant reference pressure, F = flask, to prevent pressure fluctuations, and TR = transducer.

generated by the transducer were conditioned by a carrier demodulator (Vali-dyne Model MC-1-3) connected to a strip chart recorder (Lineac model 285/EJ). The tracings were used to calculate the volume changes occurring inside the plethysmograph.

The chart recorder also recorded information from a second transducer which monitored transpulmonary pressure. Transpulmonary pressure was defined as the difference between tracheal pressure and pleural pressure. Pleural pressure was measured through a water-filled esophageal catheter made of polyethylene tubing (P.E. 160) with 3 to 5 small lateral holes placed near its tapered end.

Airway pressure changes passed through polyethylene tubing (P.E. 60) and two 3-way stopcocks into the transducer. At these stopcocks was the connecting point for the magnahelic pressure gauge (Dwyer 2000-30 cm) used in calibration. To calibrate the system for changes in transpulmonary pressure, the connection leading to the tracheal outlet was closed and air introduced through the stopcocks in increments equal to 5 cm H₂O as measured by the magnahelic up to a maximum of 25 cm H₂O, followed by a stepwise return to a reading of 0 cm H₂O. The corresponding chart recording was used to determine the conversion factor necessary to translate chart deflections into transpulmonary pressure changes.

To calibrate the plethysmograph for changes in volume, 0.5 ml increments of air were added until reaching a total input of 2.5 ml of air followed by the removal of 0.5 ml increments in similar stepwise fashion. The corresponding chart recording was then used to determine the constant necessary to convert chart deflections into volume changes.

Each treatment or control group tested was represented by a minimum of three mice. Each mouse was anesthetized with 0.2 ml/10 g body weight of 6 mg/ml pentobarbital solution given intraperitoneally. A tracheostomy was made just distal to the larynx, and a 31 mm long endotracheal tube (P.E. 60) was tied into place with 4.0 silk. As the mouse was positioned in dorsal recumbency on the plexiglass holder, the esophageal catheter was put in place and the mouse was placed into the plethysmograph. The chart recorder reflected changes in plethysmographic volume as well as the esophageal component of transpulmonary pressure.

A series of three inflations and deflations were performed for each mouse. A 3 ml syringe was used to introduce air to the airway to cause a forced lung inflation to 25 cm H₂O. This was followed without hesitation by a forced deflation to -5 cm H₂O. Inspiratory capacity was obtained by subtracting the value for 0 cm H₂O from that for 25 cm H₂O.

For individual mice, there was a high degree of reproducibility between trials. The mean of the 3 P-V curves was calculated and recorded for each mouse.

2. Experimental protocols

(a) Development of fibrosis

Fibrosis is the abnormal deposition of collagen following tissue damage. The amount of collagen can be estimated by measuring hydroxyproline, an amino acid present virtually only in collagen. Hyperoxia has been shown to cause severe damage to lung tissue, and it was of interest to determine the combined effects of O₂ on the Cd-damaged lung.

The animals in this experiment were divided into four groups. Two groups were exposed to H₂O aerosols for 1 hr in the inhalation chamber previously described. The other two groups were exposed to CdCl₂ aerosols for 1 hr. During this time the Cd content of the chamber and MMD were measured using the Cascade impactor. One H₂O group plus one Cd group were then exposed to 80% O₂ in the oxygen chamber for six days while the other two groups were left in room air. At the end of three weeks all animals were sacrificed for hydroxyproline analysis (previously described).

(b) [¹⁴C]-thymidine incorporation into DNA

In this experiment there were four groups of animals: H₂O aerosol + room air, H₂O aerosol + 70% O₂, Cd aerosols + room air and Cd aerosols + 70% H₂O. Four to six animals from each group were injected i.p. with 0.5 μ Ci of [¹⁴C]-thymidine and sacrificed by cervical dislocation 90 min later, on days 1, 3, 5, 7, 9, 11, 13, and 15. The lungs were removed and the procedure described previously was followed. The extract was analyzed by Burton's modification of the diphenylamine method (Burton, 1956) and the results calculated as dpm/mg of DNA.

(c) Autoradiographic studies

These studies were carried out on four different groups of animals namely H₂O aerosol + air, H₂O aerosol + 80% oxygen, Cd aerosol + air, and Cd aerosol + 80% oxygen. Four to five animals from each group were injected i.p. with [methyl-³H]thymidine (2 μ Ci/g body weight) 1, 3, 5, 7, 9, 11, 13, 15 days after Cd exposure. Ninety minutes later

they were sacrificed and their lungs fixed, sectioned, dehydrated and embedded. Sections were cut and prepared for autoradiography as previously described.

(d) X radiation

It has been shown that exposure of mice to thoracic X-radiation potentiates the damage done by butylated hydroxytoluene but not by bleomycin and cyclophosphamide (Hakkinen, et al., 1982). This experiment was carried out to investigate what effects X radiation would have on the Cd-damaged lung.

Four groups of animals were designated. Two were exposed to Cd aerosols and two to water aerosols. One each of these groups was exposed to X radiation and the other two groups were sham irradiated. For exposure, unanesthetized animals were placed head first into plastic conical tubes with air holes. The mouse was held in place by a cork placed at the end of the tube. Irradiation was done on a rotating 3 cm thick lucite plate with 10 depressions for the tubes. A 1-cm thick lead shield located 2.5 cm above the lucite plate had ten 2-cm² holes placed so that only the thoracic region of the mice were exposed. The tubes were placed so that the ventral surface of the mouse faced the radiation source.

Radiation was delivered by a G.E. Maxitron 300 X-ray machine operated at 300 KVP and 20 mA with 0.5 mm Cu added filtration (HVL = 1.29 mm Cu). The average dose rate at 35 cm target to specimen distance was 222 rad/min and the total dose per animal was 200 rad. The dose to the rest of the body was less than 1% of the thorax dose. Control

animals were loaded into the chamber and kept there for a time corresponding to that received by the X-irradiated ones.

The experiments were carried out at three different time intervals after exposure to Cd and water aerosols, namely 2 hr, 1 day, and 2 days. All animals were sacrificed a 3 weeks after Cd exposure for hydroxyproline determination.

(e) Prednisolone experiments

These experiments were carried out to determine whether the administration of prednisolone would mitigate or potentiate the lung damage done by Cd inhalation.

(1) Experiment 1

Animals were divided into eight groups. Four of these groups were exposed to water aerosols and four to Cd aerosols. For two of the water groups and two of the Cd groups, one each was given prednisolone twice daily for days 1-6 and the others for days 7-12. For the other two water groups and two Cd groups, one each was given saline twice daily for days 1-6 and the others for days 7-12. The prednisolone dose was 30 mg/kg and was made up in methanol-saline (1:9). The injections were done subcutaneously and the suspension was stirred constantly during the injection period. All groups were sacrificed on day 21 and hydroxyproline determination was done.

(2) Experiment 2

Animals were divided into four groups. Two of these groups were exposed to water aerosols and two to Cd aerosols. One water group and 1 Cd

group were given prednisolone (30 mg/kg) twice daily for 12 days. The other water group and Cd group were given saline twice daily for 12 days. Half of the prednisolone-treated and half of the saline-treated animals were sacrificed after 2 weeks for hydroxyproline analysis and the other half were analyzed after 3 weeks.

(f) Cd i.p. + Cd inhalation

The experiment was to determine if Cd injected i.p. for 4 days prior to Cd inhalation would alter the deleterious effects of inhaled Cd. Two groups received 0.2 ml of 0.9% saline and two other groups received 0.2 ml of $\text{CdCl}_2 \cdot 2.5 \text{ H}_2\text{O}$ daily at a dose of 1 mg/kg body weight. After 4 days, one saline and one Cd i.p. group were exposed to H_2O aerosols while the other saline group and Cd i.p. group were exposed to Cd aerosols. All groups were sacrificed on day 21 for hydroxyproline determination.

(g) Long term study

An investigation was carried out to determine the long term effects of inhalation of $\text{CdCl}_2 + 80\% \text{ O}_2$, whether repair was complete, or whether emphysema or fibrosis persisted in the lung. This study encompassed three different experimental protocols, namely hydroxyproline determination, lung function tests and histopathological examinations. Over 500 animals were used in these experiments and they were divided into the four basic groups for each experiment, that is H_2O aerosol + air, H_2O aerosol + 80% O_2 , Cd aerosol + air, and Cd aerosol + 80% O_2 . The above mentioned tests were carried out at 3 weeks, 3 months, 6 months, and 1 year after CdCl_2 exposure. The procedures have previously been described.

CHAPTER IV

RESULTS

A. The Development of Fibrosis after Cd and Cd + O₂

1. Biochemistry effects of Cd and O₂ on mouse lung hydroxyproline levels

Two groups of animals were exposed to H₂O aerosols for 1 hr in the inhalation chamber and two other groups were exposed to CdCl₂ aerosols for 1 hr. One H₂O group plus one Cd group were then exposed to 80% O₂ in the oxygen chamber for six days while the other two groups were left in room air. At the end of three weeks hydroxyproline values were determined. Table 1 shows the hydroxyproline concentration expressed as both per wet and dry lung weight, and as total lung hydroxyproline. With the former method of expressing the results, when comparing the H₂O + air group with the Cd + air group, the values are seen to be lower for the Cd + air group. This is not surprising when one considers the significant increase in lung dry weight in the Cd + air group. Similarly the Cd + 80% O₂ group shows an apparent decrease when compared with the H₂O + 80% group. Again here the lung dry weight is significantly larger for the Cd + 80% O₂. It is known that the Cd treatment produces a recruitment of cells (PMN, macrophages) into the alveolar spaces, and also stimulates proliferation of the lung cells. This accounts for the increased lung dry weight, giving a large denominator for the calculation of μg hydroxyproline per mg lung dry weight.

Table 1. Effect of Cd and Cd + 80% O₂ on lung weight, total lung hydroxyproline, and hydroxyproline concentration per wet and dry lung weight

Group	Results are expressed as means $\pm$ S.E.				μg Hydroxyproline/lung
	Lung wt		Hydroxyproline ($\mu\text{g}/\text{mg}$)		
	Wet (g)	Dry (mg)	Wet	Dry	
H ₂ O + air (A)	.20 $\pm$.04	28.5 $\pm$.77	12.40 $\pm$ 1.33	77.37 $\pm$ 1.80	220 $\pm$ 4
H ₂ O + 80% O ₂ (B)	.15 $\pm$.01	27.7 $\pm$.48	15.15 $\pm$ 0.72	83.74 $\pm$ 4.12	231 $\pm$ 11
Cd + air (C)	.23 $\pm$.02	38.53 $\pm$ 2.68 ^a	11.89 $\pm$ 0.62	70.40 $\pm$ 2.32	270 $\pm$ 20
Cd + 80% O ₂ (D)	.29 $\pm$.04 ^b	54.0 $\pm$ 4.99 ^{b,c}	14.77 $\pm$ 0.52 ^c	79.02 $\pm$ 2.60 ^c	432 $\pm$ 57 ^{b,c}

^ap < 0.05 comparing groups A and C.

^bp < 0.05 comparing groups B and D.

^cp < 0.05 comparing groups C and D.

For the wet weight calculation, the presence of edema also has to be considered. However when hydroxyproline per total lung was calculated, it was immediately seen that there was a true net increase in the treated groups when compared to the control groups. To give a true picture of the biochemical changes taking place in the lung, it was therefore decided to express all future experiments as μg hydroxyproline per lung.

Figure 12 is a histogram showing the Cd and oxygen effects on lung hydroxyproline levels expressed per total lung. It can be seen that there was no difference in lung content of hydroxyproline between the animals treated with water aerosols and left in air and those exposed to water aerosols and 80% O_2 . However the animals treated with CdCl_2 aerosols and left in air showed a slight increase in hydroxyproline values above control levels. The animals that were treated with CdCl_2 aerosols and then exposed to 80% O_2 showed a dramatic increase above the control values ($P < 0.05$). The difference in hydroxyproline content between the Cd and air group and the Cd and 80% O_2 group was also significant ($P < 0.05$), showing that the 80% O_2 augmented the lung damage done by the CdCl_2 aerosols.

2. Histopathology

Figure 13 shows the lungs of control mice that inhaled H_2O aerosols, were kept in room air, and killed 3 weeks later. The alveolar structure was normal in these animals and also in those that had inhaled H_2O aerosols, were kept in 80% O_2 for 6 days and killed 3 weeks later. On the other hand, the animals that were exposed to cadmium aerosols, kept in room air and killed after 3 weeks showed multifocal areas of interstitial, peribronchiolar and

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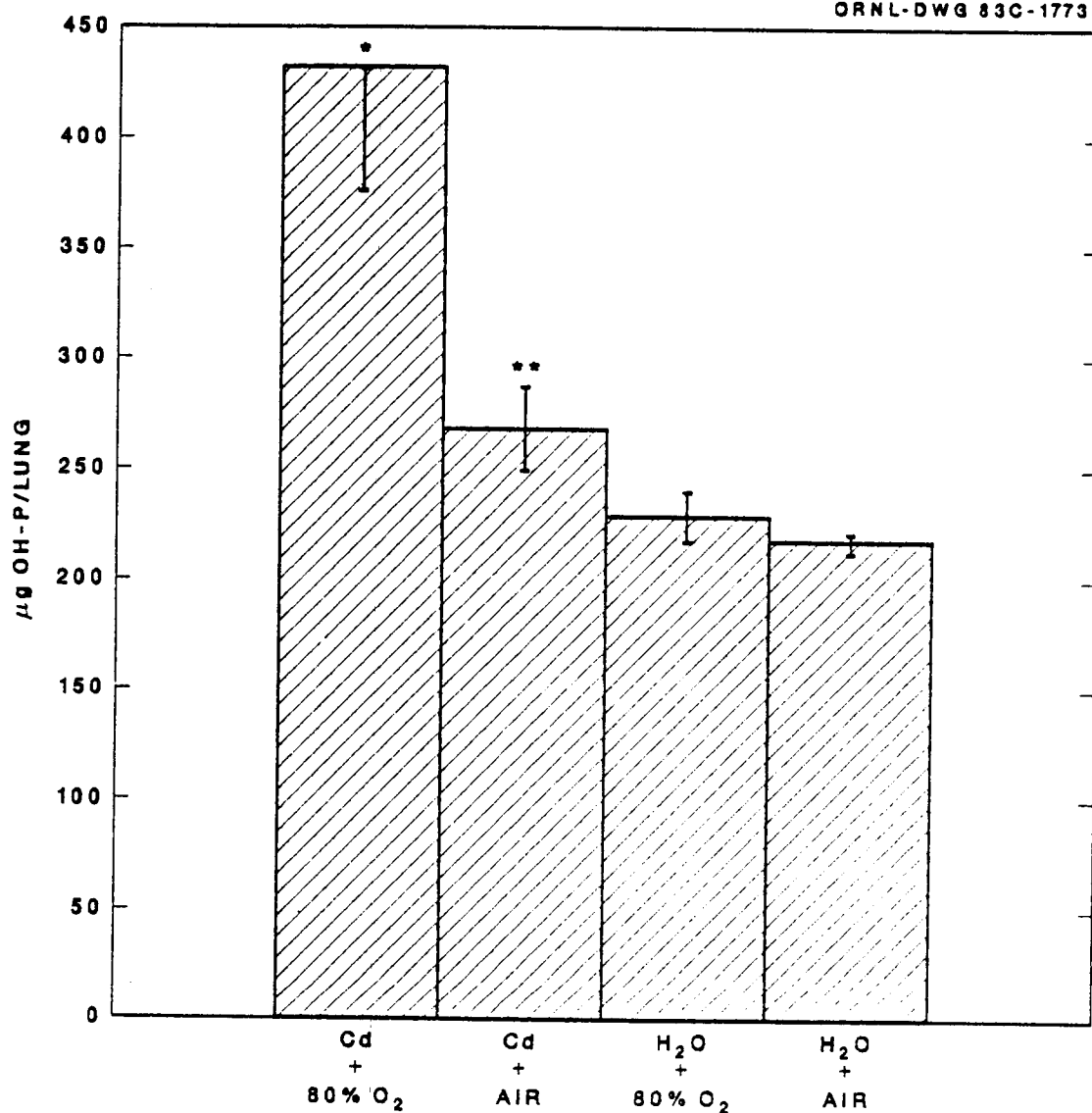


Figure 12. Cadmium and oxygen effects on mouse lung hydroxyproline levels. Lung hydroxyproline values measured 3 weeks after the initial treatment. All values given as mean $\pm$ SE ($n = 6-11$ mice). * denotes $P < 0.05$ compared to H₂O + 80% O₂. ** denotes $P < 0.05$ compared to Cd + 80% O₂.

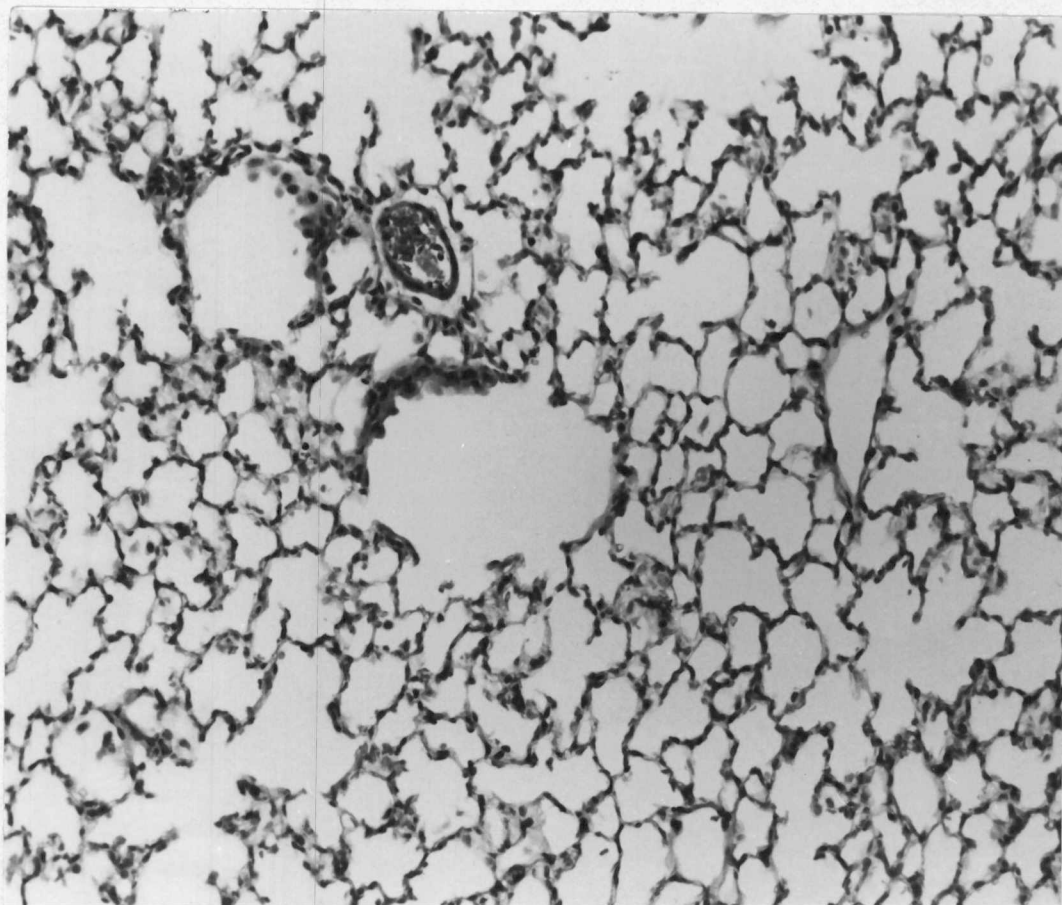


Figure 13. Lungs from mice that inhaled H₂O aerosols, were kept in room air and killed 3 weeks later (control). Normal alveolar structure is present. Hematoxylin and Eosin x675.

alveolar ductal fibrosis (Figure 14). In some areas, the fibrosis was quite extensive with complete loss of pulmonary architecture and atelectasis peripheral to the lesion. The fibrotic lesions were characterized by the presence of large numbers of interstitial fibroblasts, some mononuclear inflammatory cells, and increased numbers of alveolar macrophages. Some macrophages contained a brown-green pigment and multinucleated giant cells were occasionally seen. Dilated bronchioles were seen amid the severely affected areas.

In animals that were exposed to Cd + 80% O₂ and killed after 3 weeks (Figure 15) the lesions were similar to those of the Cd + air animals, except there was more extensive consolidation of the peribronchiolar lung tissue and there was residual mild neutrophilic infiltration as well as perivascular edema present. Occasional alveolar polyps were seen. In many areas the lung consisted of bands of atelectasis surrounded by areas of emphysema.

B. Other Attempts to Modify Development of Lesion

1. Cadmium and X-radiation

Two groups were exposed to Cd aerosols and two groups to H₂O aerosols. One Cd group and one H₂O group were exposed to X radiation at three different time intervals, namely 2 hr, 1 day, and 2 days, and the other two groups were sham irradiated.

For the animals exposed to X radiation 2 hr after Cd inhalation there was no significant difference in hydroxyproline levels between that group and the Cd + sham exposed group although both groups had elevated levels of

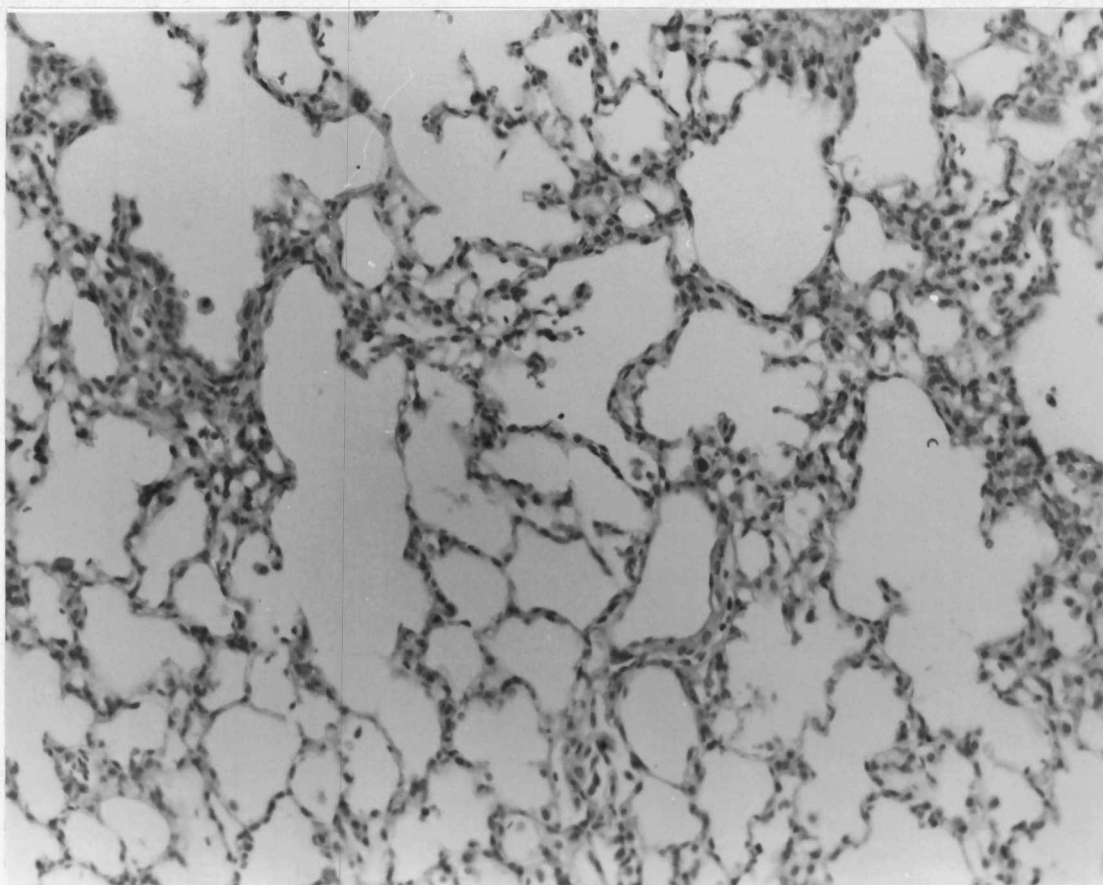


Figure 14. Lungs from mice that inhaled cadmium aerosols, were kept in room air and killed 3 weeks later. There are multifocal areas of interstitial and alveolar ductal fibrosis. Hematoxylin and Eosin x675.

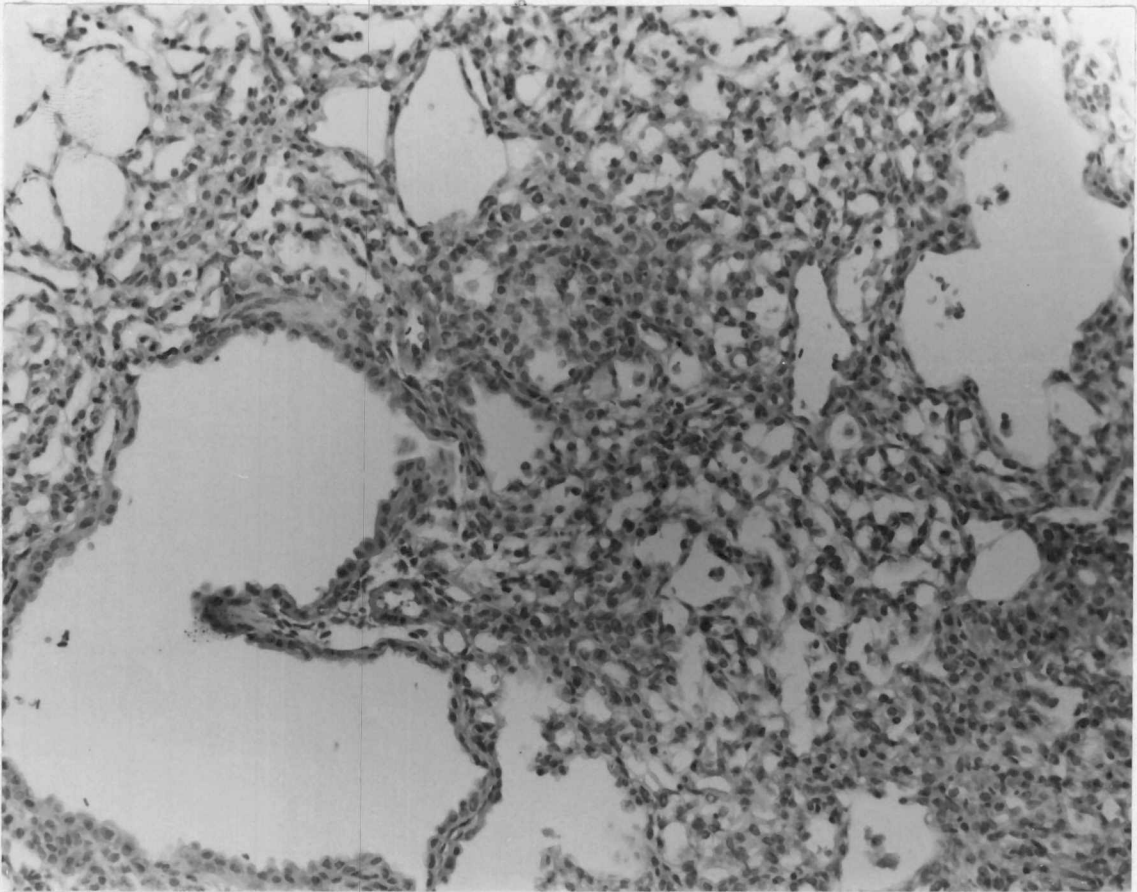


Figure 15. Lungs from mice that inhaled cadmium aerosols, were kept in 80% O₂ for 6 days and killed 3 weeks later. Note disrupted parenchymal architecture with extensive consolidation of the peribronchiolar lung tissue. Hematoxylin and eosin x675.

hydroxyproline (Figure 16). For the other 2 groups, that is, water aerosol + sham radiation and water aerosol + 200 rad X radiation there was a low level of hydroxyproline per lung and no significant difference between these groups. The difference between water + sham group and Cd + sham group was significant at the 95% level ($P < 0.05$) which shows that the high hydroxyproline values were caused by the Cd but there was no potentiation by the X radiation since the Cd + sham group was not significantly different from the Cd + X radiation group. The experiment was duplicated at the day 1 time period and on both occasions there was no difference between the Cd + sham group and the Cd + X ray group.

The day 2 results were similar to the other two time intervals (2 hr and 1 day) in that there was no potentiation by X radiation of the Cd damage to the lung. Here there was a significant difference in hydroxyproline levels between the H_2O + 200 rad group and the Cd + 200 rad group ($P < 0.05$), which would mean that the increased lung collagen content was caused by the Cd.

2. Combined effects of Cd and prednisolone

Various protocols were followed in the experiments investigating the modifying effects of prednisolone treatment on Cd-induced lung damage. In one experiment prednisolone was given on days 1-6 or days 7-12 following Cd inhalation, and hydroxyproline determinations were done at 3 week after Cd inhalation. As seen in Figure 17, at day 1-6 there was a significant difference ($P < 0.05$) in hydroxyproline levels between the H_2O + saline and Cd + saline groups as well as between the H_2O + prednisolone and

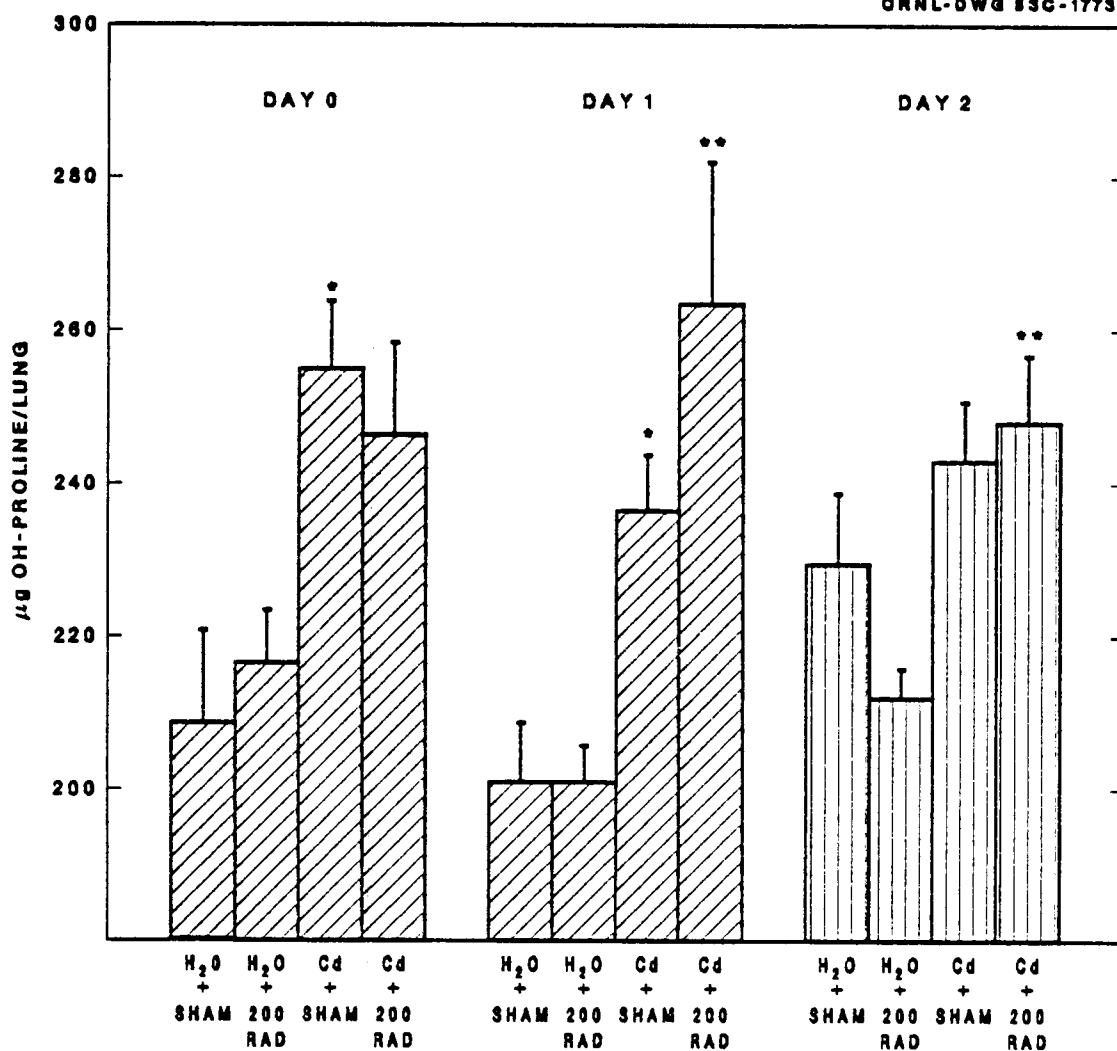


Figure 16. Effects of 200 rad thoracic X radiation on cadmium-induced pulmonary fibrosis in mice on days 0, 1, and 2. Mean $\pm$ SE, $n = 5-9$ mice. * Significantly different from H₂O + sham $P < 0.05$. ** Significantly different from H₂O + 200 rad $P < 0.05$.

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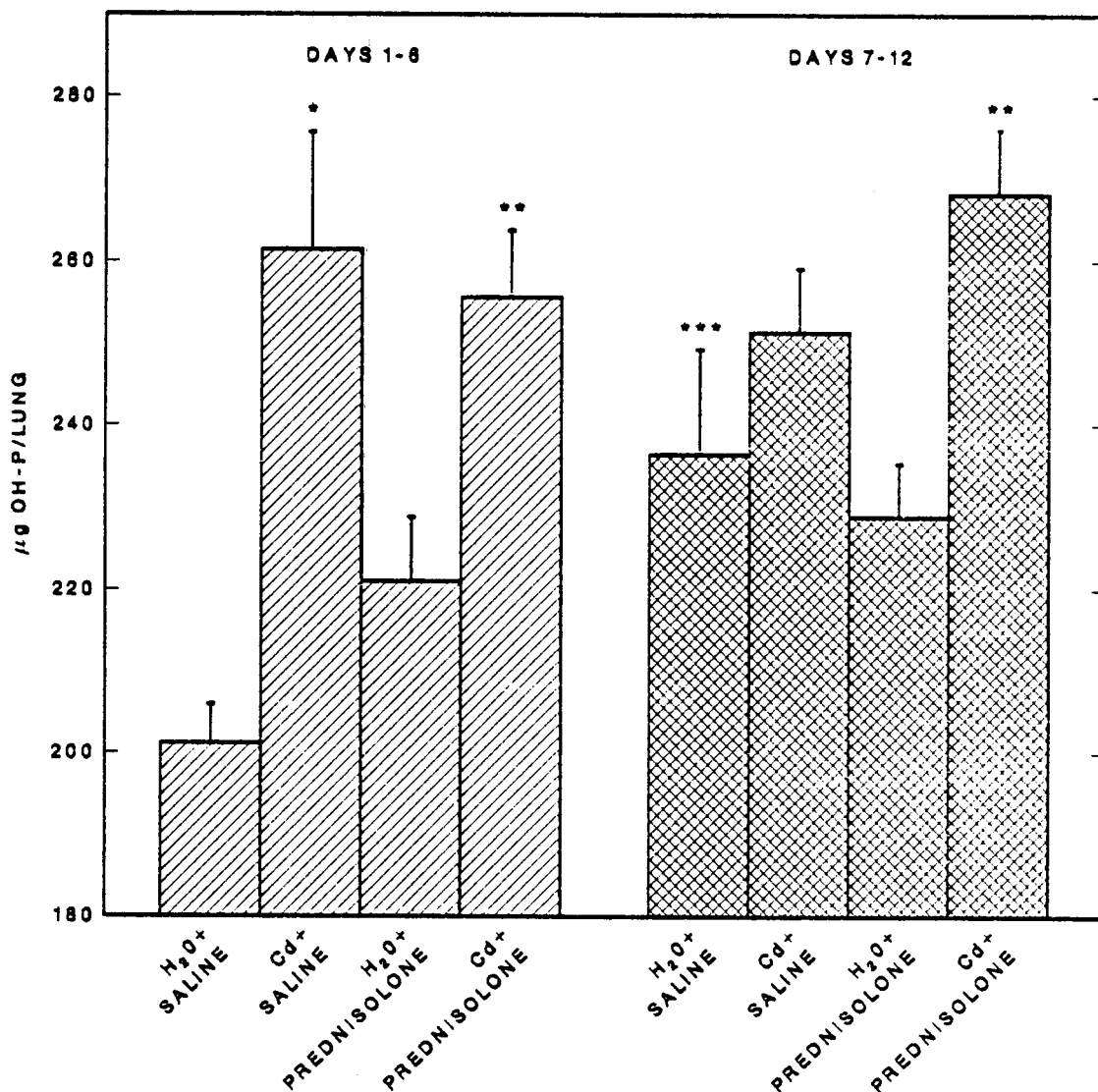


Figure 17. Hydroxyproline levels after prednisolone treatment on days 1-6 and 7-12 following cadmium inhalation. Lung hydroxyproline values measured 3 weeks after Cd inhalation. All values given as mean $\pm$ SE ($n = 5-10$ mice). * Denotes significantly different from H₂O + saline ($P < 0.05$). ** Denotes significantly different from H₂O + prednisolone ($P < 0.05$). *** Only 4 animals in this group.

Cd + prednisolone groups showing that the Cd did cause lung damage in these animals. The results were similar for the day 7-12 animals except that in the H₂O + saline group there were only four animals and this control group gave unusually high results. In both of these treatment regimes however, there was no significant difference between the Cd + saline group and the Cd + prednisolone group, showing that the prednisolone did not augment the damage done by Cd.

In another experimental protocol, the animals were injected twice per day for 12 days with prednisolone and hydroxyproline determination was carried out on half the animals at 2 weeks and on the other half at 3 weeks. Here again for both regimes there was a significant difference in hydroxyproline values between H₂O and saline and Cd + saline groups and between H₂O + prednisolone and Cd + prednisolone group ($P < 0.05$) (Figure 18), showing beyond doubt the damaging effect of Cd on the lung.

However, although there was a difference for both regimes between the Cd + saline and Cd + prednisolone groups, this difference was not significant at the 95% level, so the prednisolone could not be said to augment the damage done by Cd. There were large differences in individual values for hydroxyproline determination in the Cd + prednisolone groups showing the varied responses of these animals. Values ranged from 251.9 to 527.61 μg OHP/lung and accounted for the large standard error. Also greater weight loss was observed in the prednisolone-treated animals than in the saline treated animals whether they had inhaled H₂O or Cd previously.

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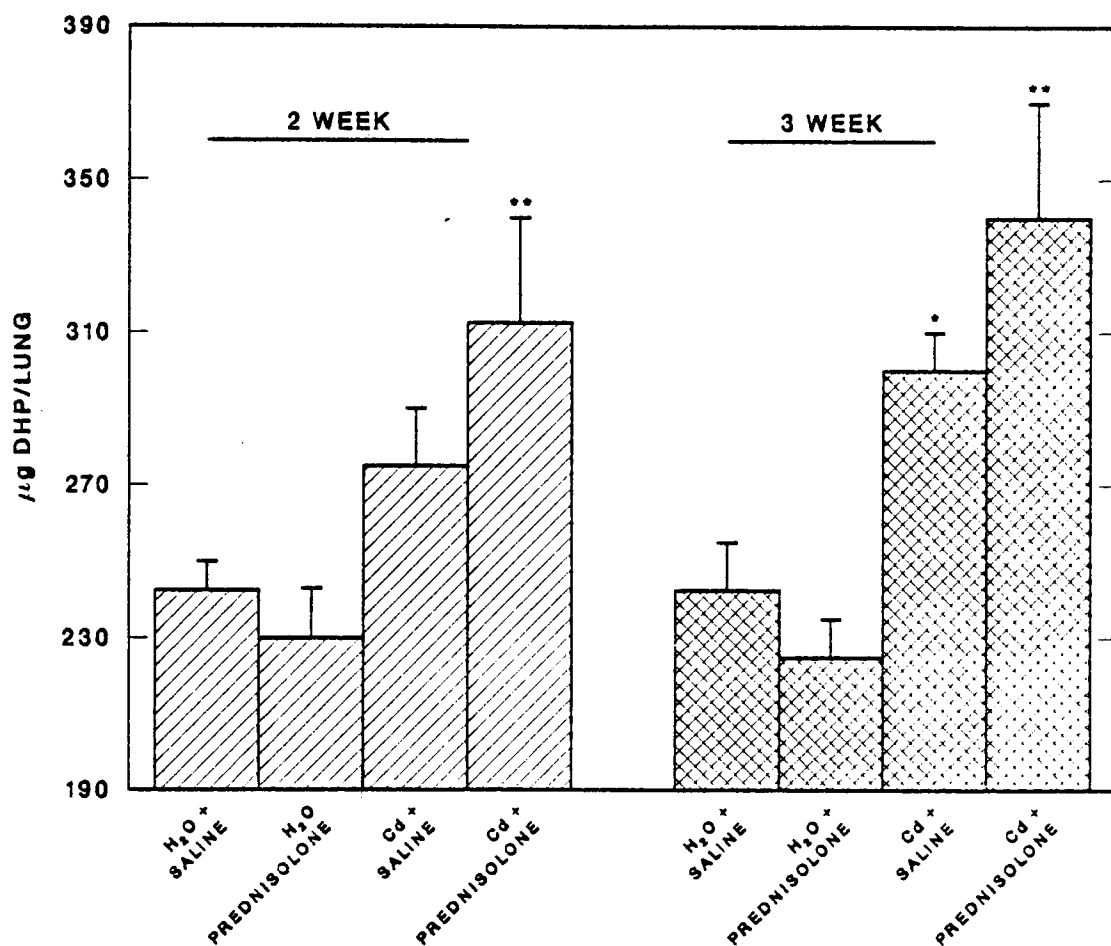


Figure 18. Hydroxyproline levels after 2 and 3 weeks after prednisolone treatment on days 1-12 following cadmium inhalation. All values given as mean $\pm$ SE ($n = 7-8$ mice). * Denotes significantly different from H₂O + saline ($P < 0.05$). ** Denotes significantly different from H₂O + prednisolone ($P < 0.05$). No significance between cadmium treated groups.

3. Cadmium pretreatment

Two groups of mice received 1 mg/kg CdCl₂ intraperitoneally for four days and two groups received saline injections. One saline group and one Cd group were exposed to H₂O aerosols, and the other groups were exposed to Cd aerosols.

There was no significant difference in hydroxyproline levels between the group given saline i.p. + Cd aerosol and that given Cd both i.p. and as aerosols (Figure 19) therefore one could say the Cd i.p. did not "immunize" the animals towards the dose of Cd aerosols that followed after 4 days. In both groups that received Cd aerosols there was an increase in hydroxyproline content over the corresponding control group, confirming the previous results of fibrosis following Cd aerosol inhalation. For example the saline i.p. + H₂O aerosol group and the saline i.p. + Cd aerosol group was significantly different ($P < 0.05$) and the Cd i.p. + H₂O aerosol group was significantly different from the Cd i.p. + Cd aerosol group, ($P < 0.05$) showing that the Cd aerosols had been effective in increasing lung collagen content.

C. Long-term Effects of Interactions of Cd and Oxygen

1. Biochemistry: Mouse lung hydroxyproline levels at 3, 6, and 12 months

For the long-term study investigating the development of fibrosis or emphysema after combined treatment of mice with Cd and 80% O₂, there were four groups of animals, namely, H₂O aerosols + air, H₂O aerosols + 80% O₂, Cd aerosols + air, and Cd aerosols + 80% O₂. The hydroxyproline levels for animals sacrificed at 3, 6, and 12 months are

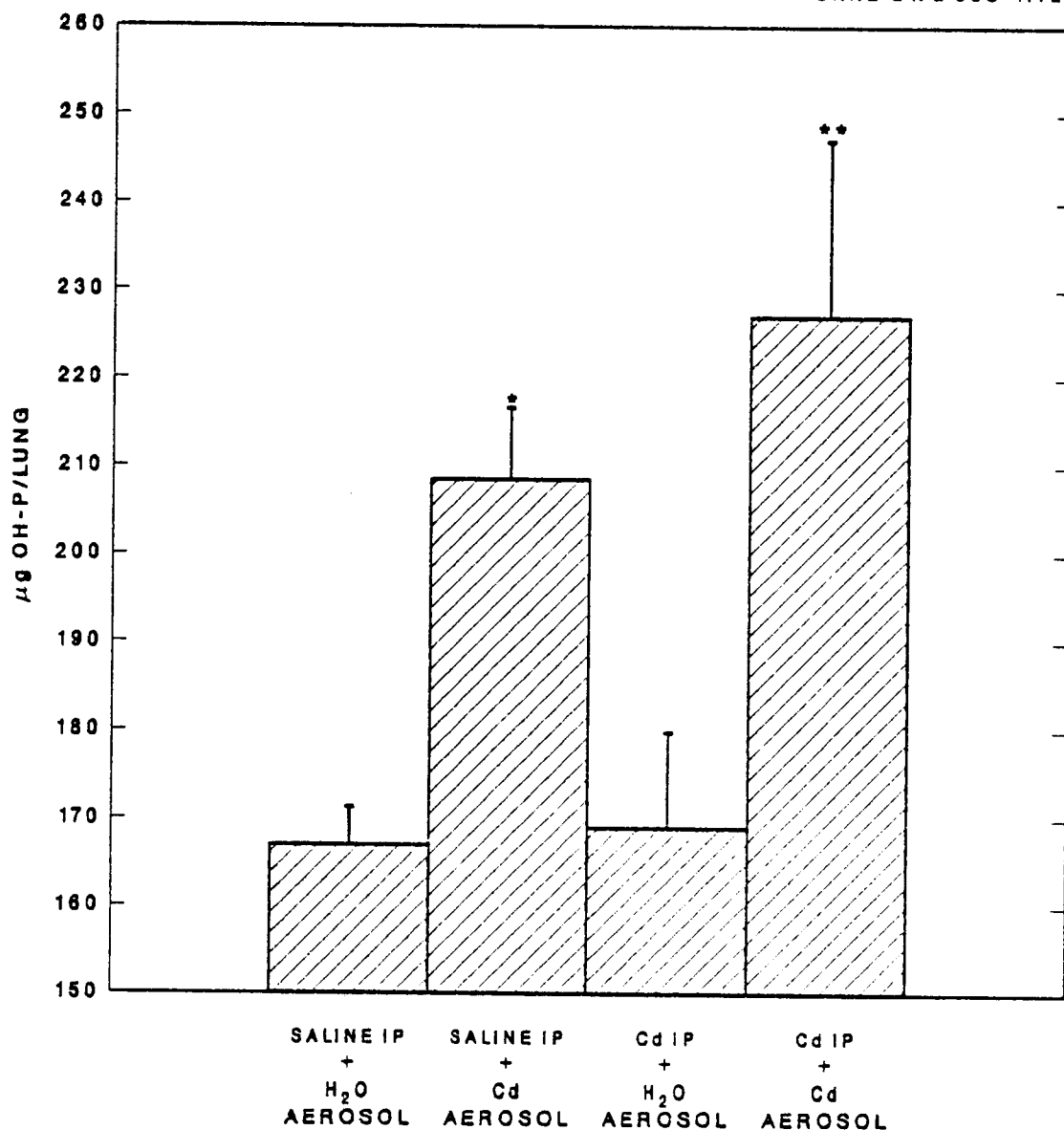


Figure 19. Hydroxyproline levels in mice pretreated with cadmium intraperitoneally before cadmium inhalation. Values given as mean $\pm$ SE ($n = 4-6$ mice). Lung hydroxyproline values measured 3 weeks after last dose of Cd i.p. * Denotes significantly different from saline i.p. + H₂O aerosol ($P < 0.05$). ** Denotes significantly different from Cd i.p. + H₂O aerosol ($P < 0.05$). No significance between saline i.p. + Cd aerosol and Cd i.p. + aerosol.

shown in Figure 20. In the case of the animals tested at three months, the H₂O and O₂ groups, Cd + air group and Cd + O₂ group were all significantly different from the H₂O + air group ($P < 0.05$). All the hydroxyproline values including the controls are higher than those found at 3 weeks which would suggest that more collagen is laid down in the lungs of older mice. The values for the H₂O + O₂ group are unexpectedly high at 3 months resulting in no significance between the H₂O and O₂ and Cd + O₂ groups. There is a significant difference between the Cd + air group and the Cd + O₂ group even at the 3 month time period, showing that the potentiating effect of O₂ on Cd-damage persists at this time.

At the 6 month time period both Cd groups again had significantly more hydroxyproline than their controls and all values were elevated when compared with 3 weeks values, showing the effects of age on the hydroxyproline levels.

The 1 yr hydroxyproline values were all elevated but there was still a significant difference between the H₂O and air group when compared with the Cd + air ($P < 0.05$). The hydroxyproline values of the H₂O and O₂ group however was now as high as the values for the Cd treatment groups, $436 \pm 45 \mu\text{g OH-P/lung}$ for the H₂O + O₂ group compared with $457 \pm 28 \mu\text{g OH-P/level}$ for the Cd + O₂ group.

2. Lung function studies

Figure 21 shows the pressure-volume curves for Cd-treated and control mice at intervals of 3 weeks, 3 months, 6 months, and 1 year. This method of study would not be considered ideal for measuring lung damage in

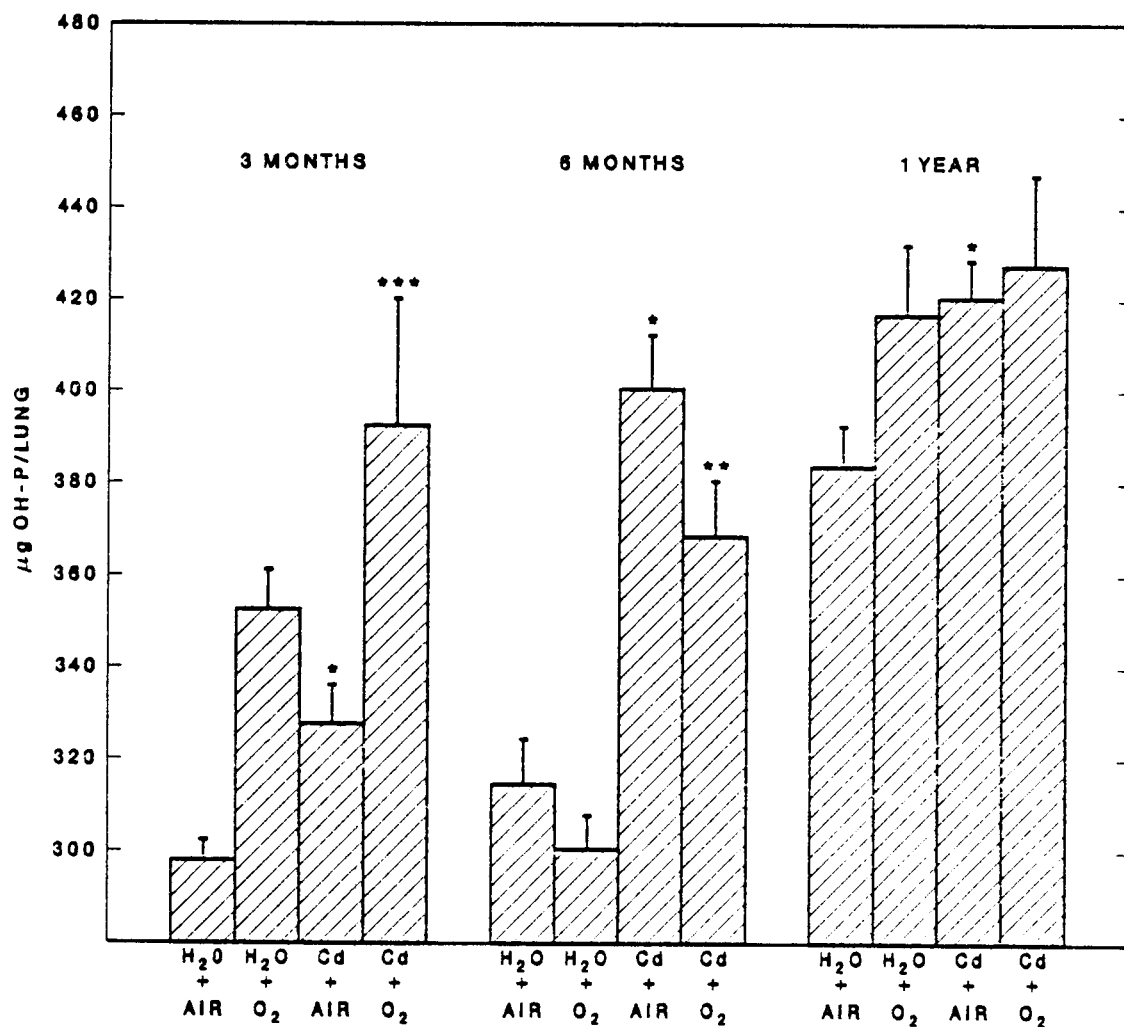


Figure 20. Hydroxyproline levels after 3, 6, and 12 months following Cd and Cd + O₂ treatment. Values given as mean $\pm$ SE ($n = 5$ mice). * Denotes significantly different from H₂O + air. ** Denotes significantly different from H₂O + O₂. *** Denotes significantly different from Cd + air.

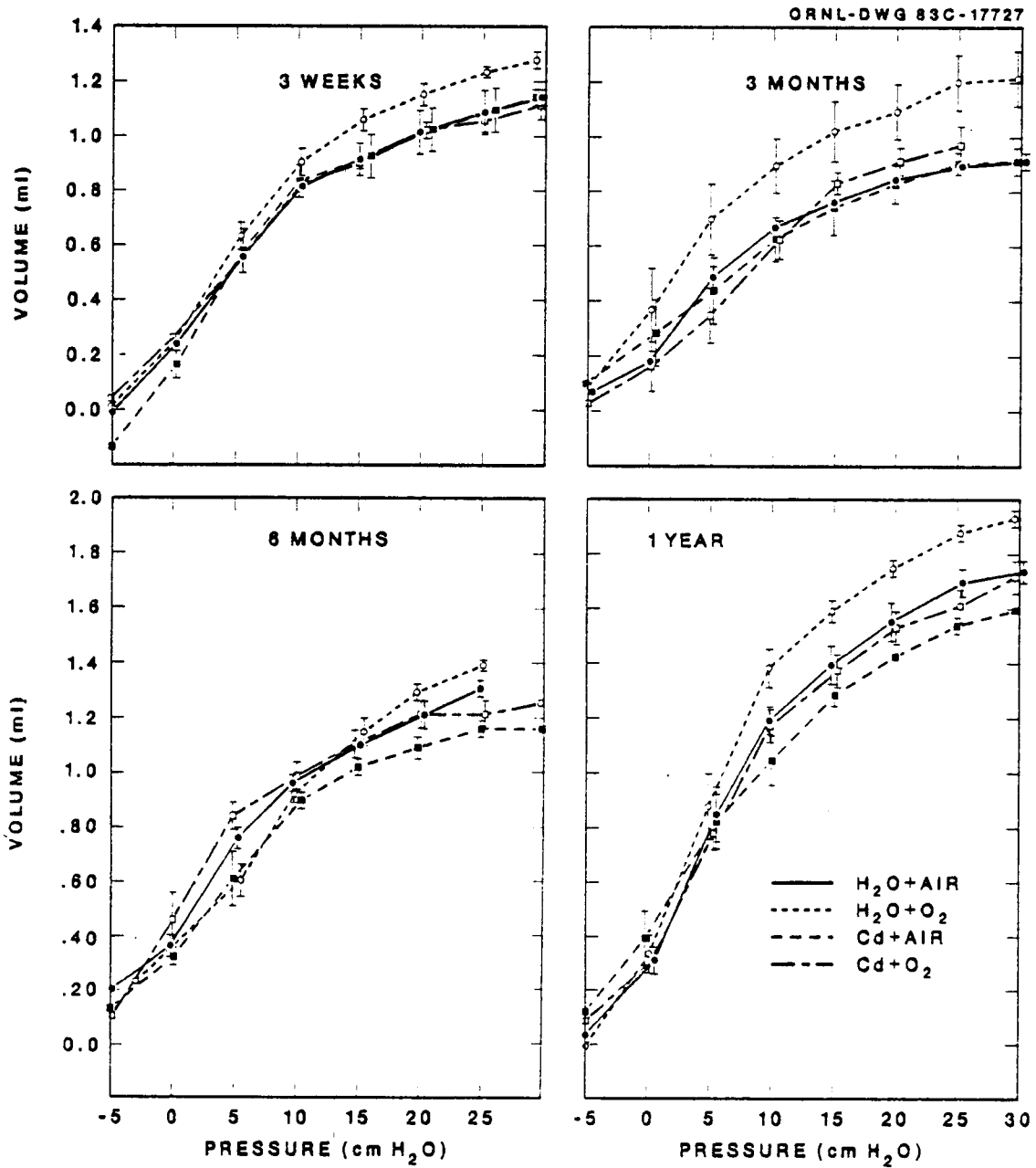


Figure 21. Pressure-volume curves for cadmium-treated and control mice.

mice, judging from the fact that all the curves were close together and showed no difference between treated and control groups. At 3 weeks, 3 months, and 1 year the curve for the animals treated with $\text{H}_2\text{O} + \text{O}_2$ show a slight shift to the right, denoting an increase in lung volume, in comparison with the lung volumes of the other groups. The reasons for these variations will be discussed in the next chapter.

The values for chord compliance (Table 2) which is the slope of the line connecting two points in the VP curve (in this case the point from 0 to 5 cm H_2O) were similar for the Cd treated and control groups at the 3 weeks, 6 months, and 1 year time points. At 3 months the chord compliance for the Cd treated groups was slightly lower than the control groups, which is the expected result in cases of lung fibrosis.

Table 3 shows values for the Inspiratory Capacity (IC) which was taken as the volume from 0 to 25 cm H_2O . At the 3 month, 6 month, and 1 year time points the values for the animals treated with Cd + O_2 were slightly lower than those for the animals treated with $\text{H}_2\text{O} + \text{O}_2$ and at the 6 month and 1 year time points the values for the animals treated with Cd + air were slightly lower than those for the animals treated with $\text{H}_2\text{O} + \text{air}$.

The highest values for IC_{25} are noted at 1 year in all groups, which could be related to the growth of the lungs, and decreased elastic recoil in the treated groups could account for lower values in those groups.

3. Histopathology after 3, 6, and 12 months

Four groups of animals, namely, $\text{H}_2\text{O} + \text{air}$, $\text{H}_2\text{O} + 80\% \text{O}_2$, Cd + air, and Cd + $80\% \text{O}_2$ were examined at the above time points.

Table 2. Chord compliance in cadmium and oxygen treated and control mice

Group	Compliance (0-5) (ml/cm H ₂ O)			
	3 week	3 month	6 month	1 year
H ₂ O + air	0.07	0.06	0.07	0.09
H ₂ O + 80% O ₂	0.07	0.07	0.05	0.1
Cd + air	0.08	0.04	0.06	0.08
Cd + 80% O ₂	0.06	0.04	0.08	0.09

Table 3. Inspiratory capacity in cadmium and oxygen treated and control mice

Group	Inspiratory capacity IC ₂₅ (ml)			
	3 week	3 month	6 month	1 year
H ₂ O + air	0.88 ± .08	0.71 ± .06	0.91 ± .09	1.25 ± .13
H ₂ O + 80% O ₂	0.96 ± .05	0.84 ± .22	1.02 ± .07	1.51 ± .09
Cd + air	0.92 ± .11	0.63 ± .13	0.83 ± .07	1.11 ± .08
Cd + 80% O ₂	0.83 ± .02	0.79 ± .13	0.75 ± .11	1.27 ± .08

Mean ± SE of 3-6 mice. Each mouse had 3 trials.

At all time points, the lungs of the mice from the control groups, that is $\text{H}_2\text{O} + \text{air}$ and $\text{H}_2\text{O} + 80\% \text{O}_2$, showed normal alveolar structure similar to controls at the 3 week time point shown in Figure 13.

After 3 months the lungs of the animals that were treated with Cd and left in room air were of almost normal appearance. In three of the 5 animals examined there were small pockets of emphysema at the tips of one lobe. One animal showed mild residual fibrosis around the bronchioles, with an increased number of foamy macrophages, thickened alveolar wall, and foci of lymphocytes. There was some Type II pneumocyte hyperplasia and metaplasia around the alveoli. This animal showed more residual fibrosis than all others in both the Cd and air and Cd and O_2 groups at this time period.

Overall, there was not much difference in the changes seen in the Cd and air and Cd and O_2 groups. With the scoring system used, the Cd + O_2 animals showed a little more fibrosis and emphysema than the animals in the Cd + air group.

At the 6 month time point, five animals were examined from each group. There were more severe changes in the Cd and O_2 groups as compared to the Cd + air group. In both groups, there were sections of the lung that appeared quite normal and, in most cases the pockets of emphysema were present at the margins of a lung section. In one animal treated with Cd + O_2 there was one lobe that showed intermixed areas of emphysema and of atelectasis accompanied by increased numbers of interstitial fibroblasts. Figure 22 shows the residual fibrosis seen in an animal from this group.

In some cases the fibrotic tissue produced contracture and collapse of the pulmonary tissue at the periphery of the lobe. Tissue from Cd + air

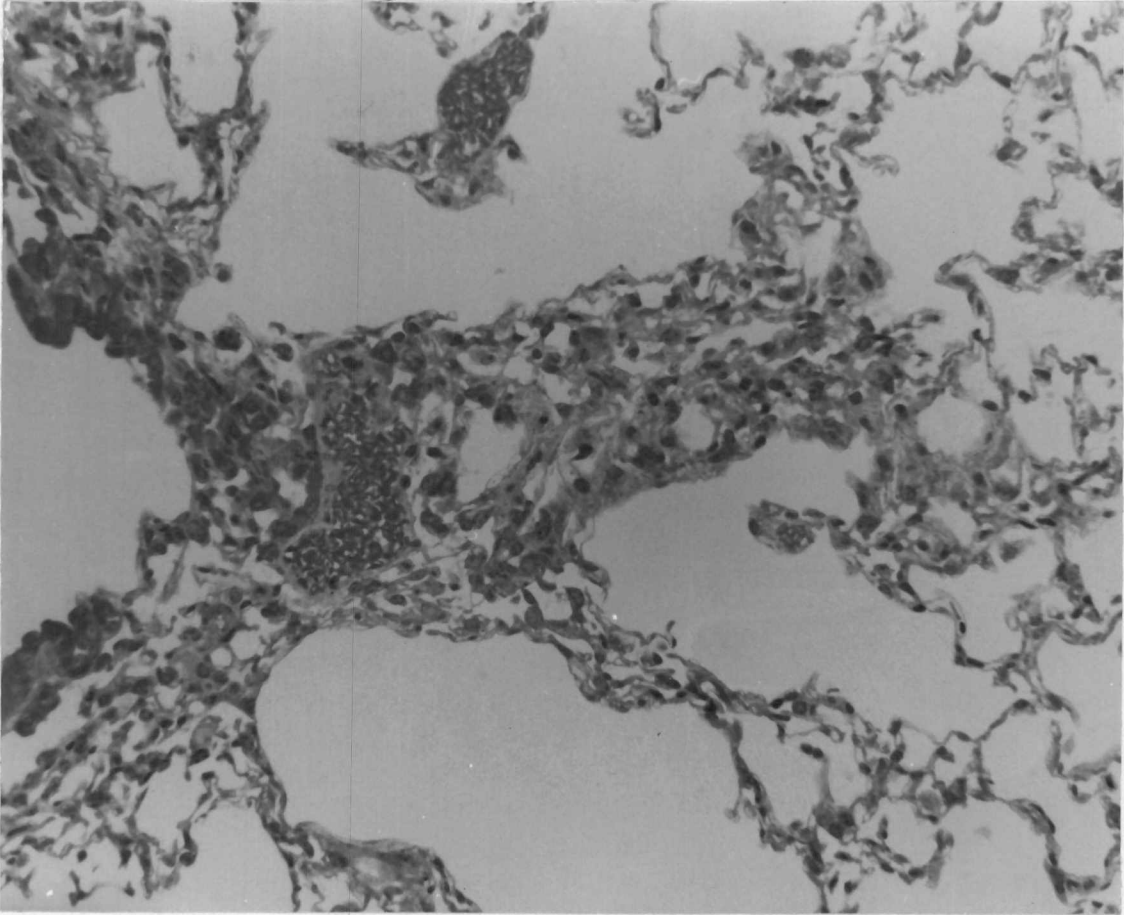


Figure 22. Lungs from mice that inhaled cadmium aerosols, were kept in 80% O₂ for 6 days and killed 6 months later. Residual fibrosis is present around terminal bronchiole, and there is irregular dilation of the alveoli. Hematoxylin and eosin x650.

treated mice showed similar but milder changes, except in one animal which had thickened alveolar walls, lymphocytic infiltration around the vessels and some peribronchiolar inflammation.

Figure 23 shows lungs from mice that were killed at the one year time period. Figure 23A is from the control group of animals that inhaled H₂O aerosols and were kept in room air. The terminal bronchioles, alveolar duct and alveolar structure all appeared normal. Five animals were examined in the group exposed to Cd and air and four animals in the group exposed to Cd + O₂. Both groups showed a high degree of resolution of the pulmonary lesions but some mice had mild residual fibrosis around the periphery of the lobes (Figure 23B). With the scoring system used, the Cd + O₂ group showed a little more residual fibrosis than did the Cd + air group. The latter group had two animals whose lungs had an almost normal appearance except for a few pockets of emphysema, while in both groups, there was one animal whose lungs were more severely affected with emphysematous changes not only at the tips but all over the lobe. The emphysema affected primarily alveolar ducts and there was periductal alveolar collapse. Likewise, the tips of several lung lobes were collapsed due to fibrosis of the subpleural tissue. There was peribronchiolar, periductal and occasional mild interstitial fibrosis. In fibrotic areas, there were increased numbers of macrophages and thickened alveolar walls. One lung section from a Cd + air treated mouse showed some alveolar collapse and in that animal as well as in one Cd + O₂ section there was some focal atelectasis.

All animals including the controls had peribronchiolar lymphocytic aggregates. These are typical of endemic mycoplasmosis. The bronchi and

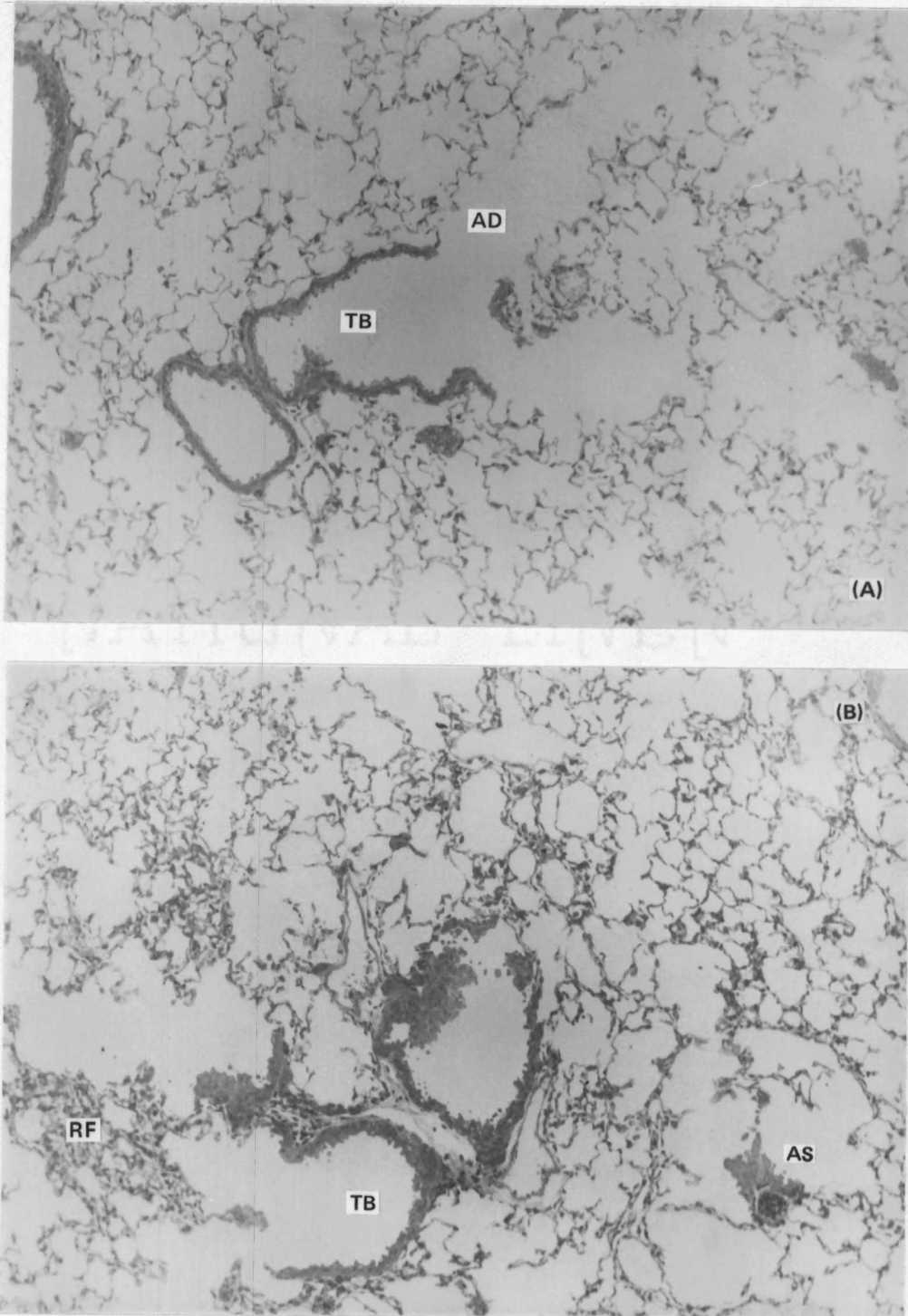


Figure 23. Lungs from mice killed 1 year after $\text{H}_2\text{O} + \text{air}$ or $\text{Cd} + 80\% \text{O}_2$. A. $\text{H}_2\text{O} + \text{air}$. Terminal bronchiole (TB), alveolar duct (AD), and alveolar structure appear normal. B. $\text{Cd} + 80\% \text{O}_2$. Note residual fibrosis (RF) and distention of air spaces (AS). Hematoxylin and Eosin x650.

bronchioles were back to normal and the smooth muscle around the airways was normal.

Some calcified nodules were present in the animals treated with Cd and O₂. These are similar to the large (10 μ m in diameter) calcopherites with concentric apposition lines seen in animals treated with BHT and O₂ (Haschek et al., 1982).

Special stains were performed to confirm the presence of pulmonary collagen deposition. Snook's silver stain showed brilliantly the extent of the collagen deposition by staining the reticulin fibers, which contain Type III collagen in association with argyrophilic glycoprotein (Minor, 1978). In the controls, Figure 24A there were small numbers of uniformly thick, wavy black reticulin fibers in the alveolar septa, while in the treated groups the fibers were more numerous, thinner and more irregular in shape and length in the thickened alveolar septa and areas of consolidation (Figure 24B). The fibers associated with the areas of atelataxis were more thickened and more condensed, but as the lungs returned to normal, the numbers of fibers tended to decrease.

The fibrillar material present in the fibrotic areas also stained positive for collagen with Masson's trichrome stain.

4. Miscellaneous findings

Tumors were present in a few animals, both controls and treated. There were both bronchiolar papillary tumors and alveolar tumors, similar to the ones described by Kauffman et al., 1979 and discussed later in Chapter V.

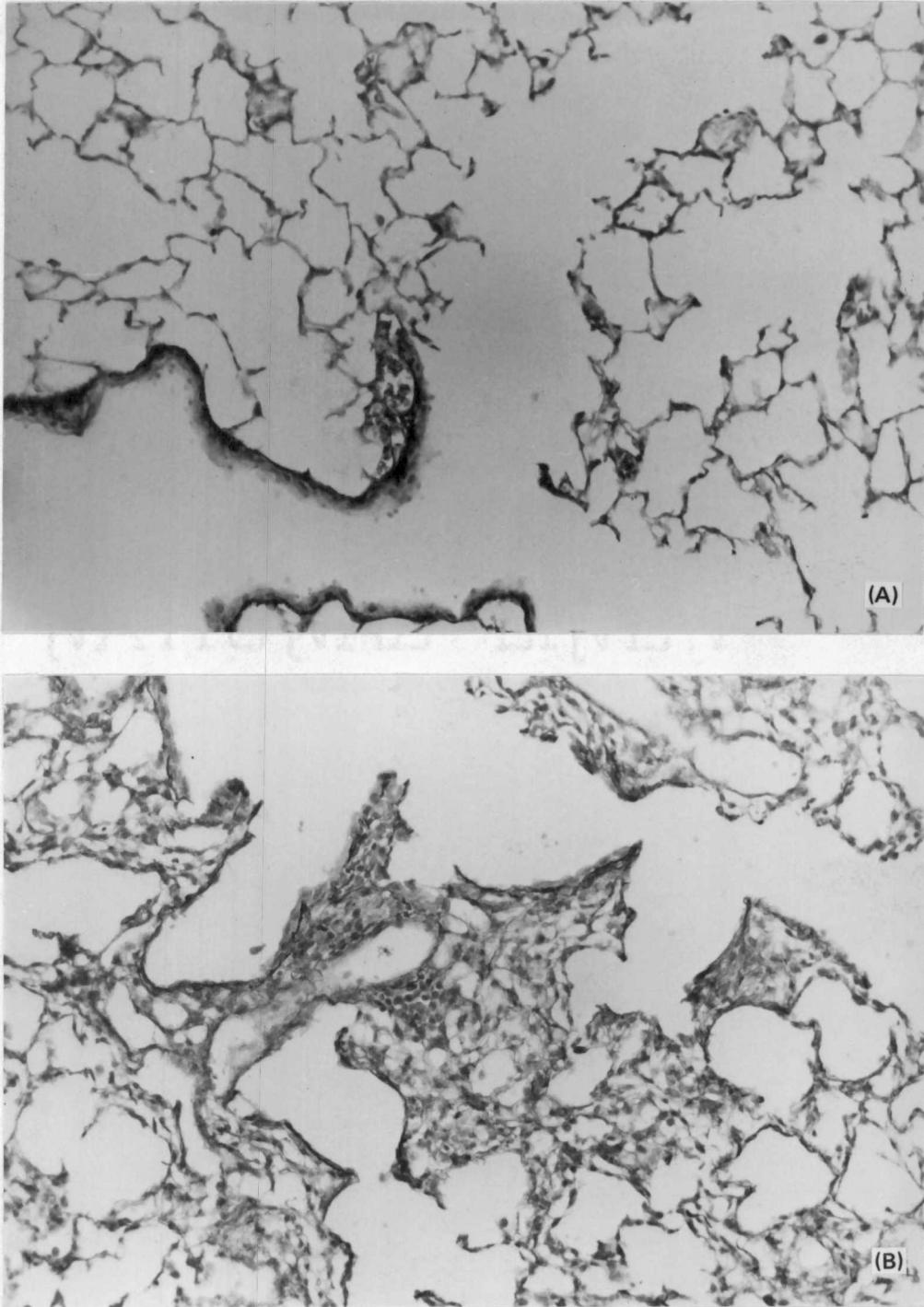


Figure 24. Reticulin fibers in lungs from mice killed 3 months after H₂O + air or Cd + 80% O₂. A. H₂O + air. There are small numbers of uniformly thick, wavy reticulin fibers in the alveolar septa. B. Cd + 80% O₂. The reticulin fibers are more numerous, thinner and more irregular in shape and length. Note consolidated areas are still present. Snook's silver stain x650.

D. Cell Kinetics

1. Overall repair after Cd and Cd + O₂. ¹⁴C-thymidine incorporation

In this experiment there were four groups of animals given the following treatments: H₂O aerosol + room air, H₂O aerosol + 70% O₂, Cd aerosols + room air, and Cd aerosols + 70% O₂.

The animals treated with Cd and left in air showed an increase in *in vivo* incorporation of thymidine into pulmonary DNA with the maximum mean specific activity of pulmonary DNA being 10,161 dpm/mg DNA on day 5 (Figure 25). The values fell to near control levels by day 11. For the animals treated with Cd and then exposed to 70% O₂ for 6 days, the peak of DNA synthesis was not reached until the animals were taken out of O₂. Then the DNA synthesis continued to rise until the peak was seen at day 7, (13,083 dpm/mg DNA) which was higher than that for Cd + air. The specific activity of pulmonary DNA fell throughout the remaining days, but by day 15 when the experiment was terminated, it still was not down to the level of the controls. For the animals treated with water aerosols followed by 70% O₂ there was also a slight peak at day 7 (1322 dpm/mg DNA) but then the values fell to control levels on day 9 (534 dpm/mg DNA) and remained there until the termination of the experiment.

2. Parenchymal labelling index

The labelling index was determined in four different groups of animals namely H₂O aerosol + air, H₂O aerosol + 80% oxygen, Cd aerosol + air, and Cd aerosol + 80% oxygen. Animals were injected with [methyl-³H]thymidine on the selected days, and the lung sections

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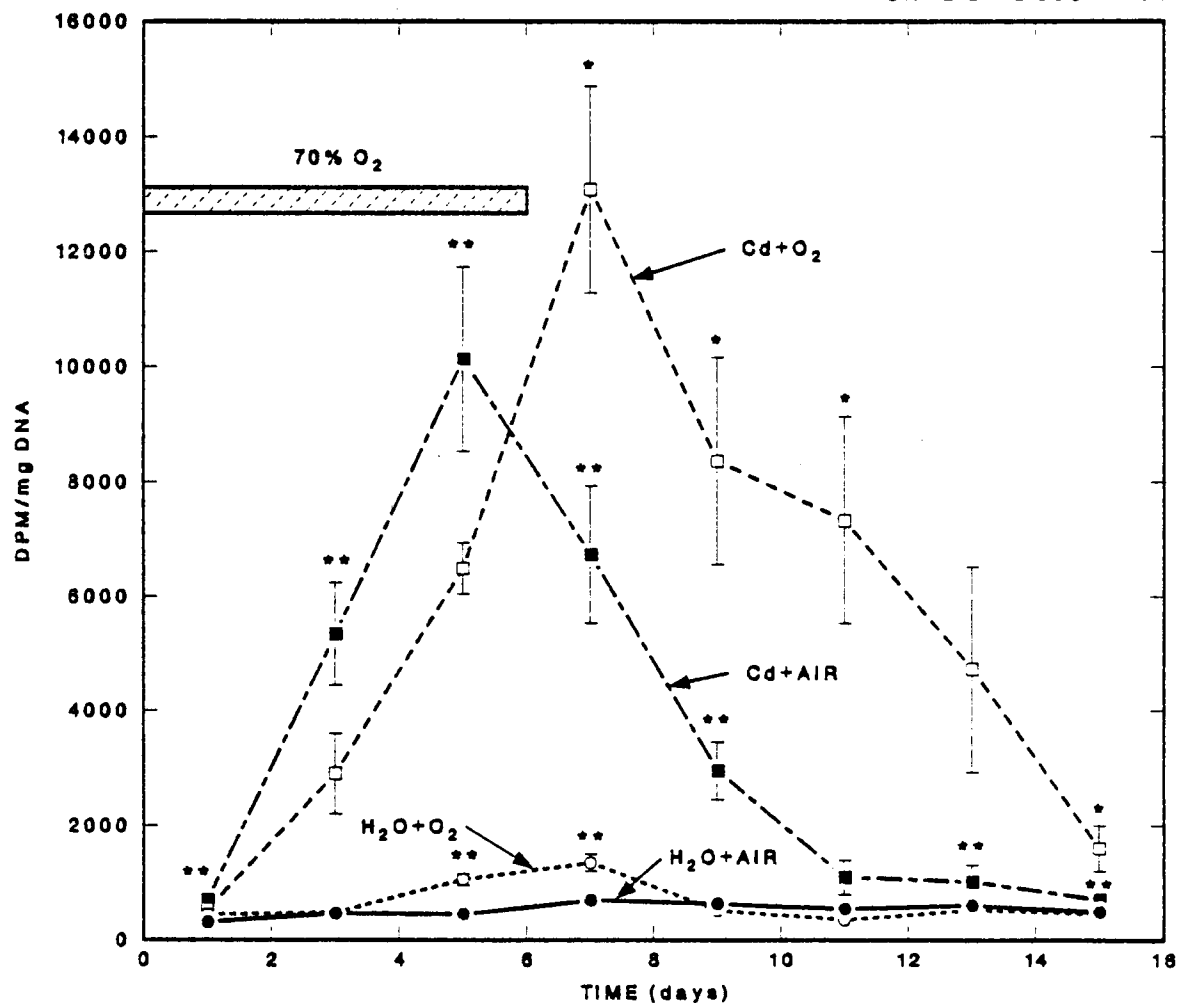


Figure 25. Overall repair after Cd and Cd + O₂. ¹⁴C-thymidine incorporation. Each point represents the mean value ± SE (n = 4–6 mice). * Denotes significantly different from Cd + air (P < 0.05). ** Denotes significantly different from H₂O + air (P < 0.05).

prepared for autoradiography. The labelling index (LI) was defined as the percentage of labelled cells per total number of cells counted.

These results (Figure 26) on the LI of pulmonary parenchymal cells closely paralleled the ones for the ^{14}C -thymidine incorporation seen in Figure 25. In the control animals, that is, those treated with H_2O aerosols and left in room air, the LI was less than 1%.

For the animals exposed to CdCl_2 aerosols and left in air, the peak of labelling was seen at day 5 and fell to control values on day 11. For animals exposed to both CdCl_2 and 80% O_2 , the peak of labelling occurred after the animals were taken out of O_2 , that is, on day 9 and was much higher than the peak for $\text{Cd} + \text{air}$. On day 15 when the experiment was terminated, the values were almost down to control values (1.85 ± 0.7 for $\text{Cd} + \text{O}_2$ and 0.5 ± 0.1 for $\text{H}_2\text{O} + \text{O}_2$ animals). Again the animals treated with H_2O aerosols and 80% O_2 showed a slight peak between days 5 and 11.

3. Correlation of ^{14}C -thymidine incorporation with LI

(^3H -thymidine autoradiography)

Figure 27 shows the graphs that were obtained when the labelling indices (LI) at the various time points for the two treated groups (Cd and $\text{Cd} + \text{O}_2$) seen in Figure 26 were plotted against the DPM/mg DNA for the various time points of the same treated groups shown in Figure 25. For the $\text{Cd} + \text{air}$ groups the correlation coefficient was 0.9877 and for the $\text{Cd} + \text{O}_2$ groups it was 0.8738, both signifying good agreement. This finding is particularly valuable because the determination of the LI is a time-consuming process where individual labelled cells are counted and it shows that

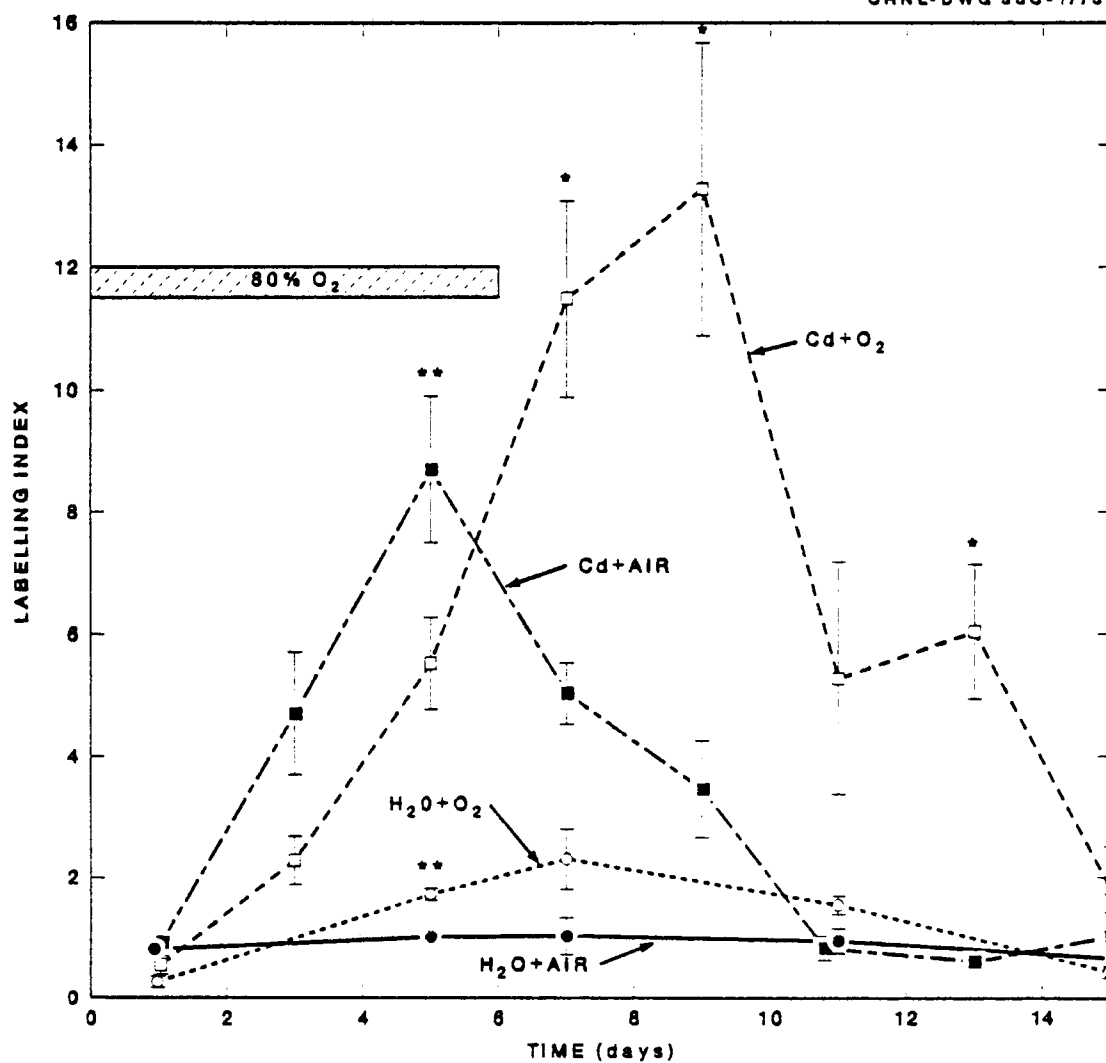


Figure 26. Labelling indices of pulmonary parenchymal cells after Cd and Cd + O₂. ³H-thymidine autoradiography. Each point represents the mean value ± SE (n = 4-5 mice). * Denotes values significantly different from Cd + air (P < 0.05). ** Denotes values significantly different from H₂O + air (P < 0.05).

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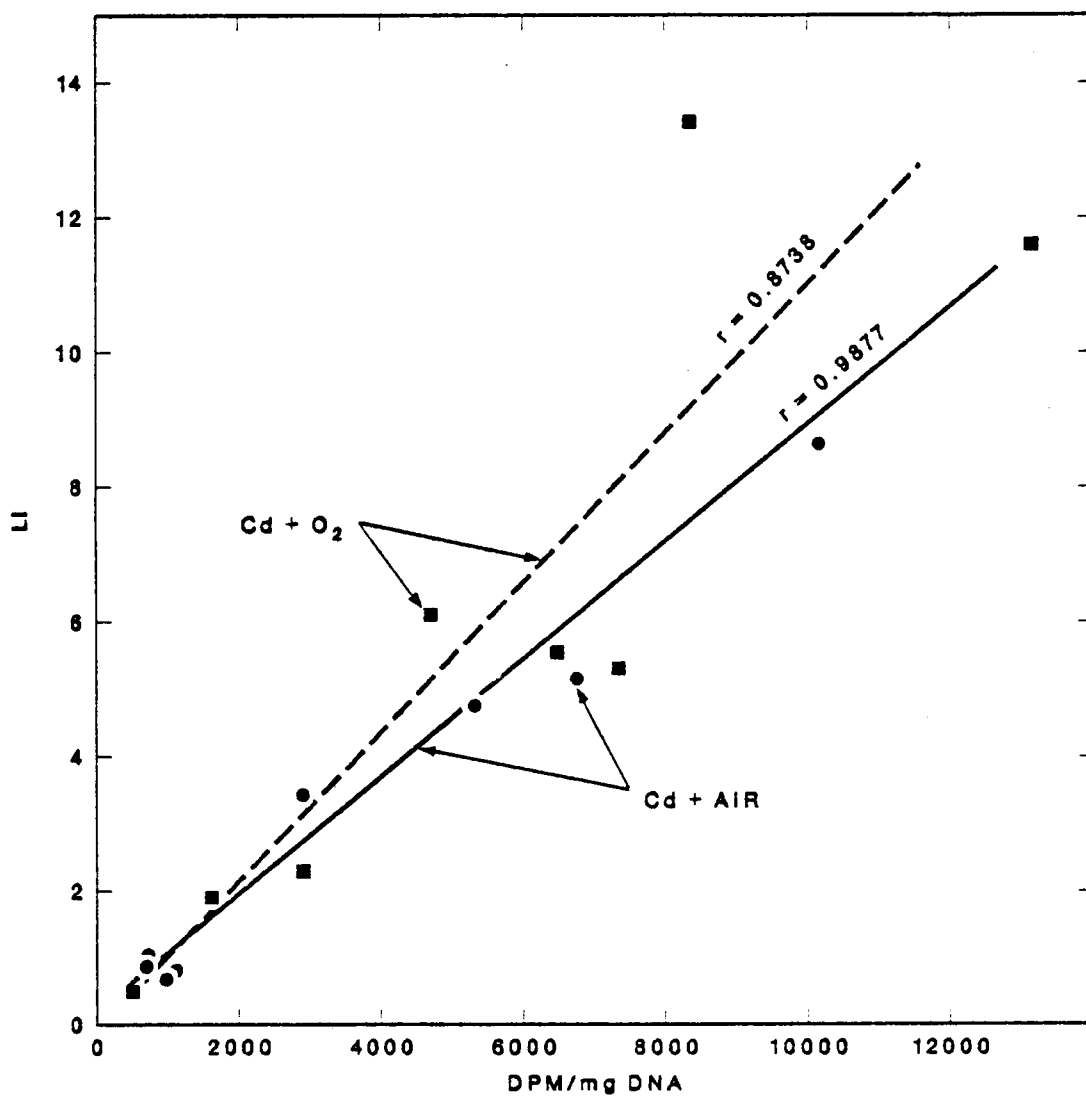


Figure 27. Correlation of ^{14}C -thymidine incorporation with labelling indices from ^3H -thymidine autoradiograph in Cd and Cd + O₂ treated mice.

the same results can be obtained with less effort by using the homogenized lung in the ^{14}C -thymidine incorporation into DNA method. Other factors involved in the correlation are discussed later in Chapter V.

4. Cell differentiation

Differential counts of the labelled cells from the autoradiographic study were performed using light microscopy with the oil immersion lens. The four categories of cells identified in this work, namely Type II pneumocytes, interstitial cells, endothelial cells and macrophages, are shown in Figure 28. Table 4 shows the differential cell count for the two treated groups, namely Cd + air and Cd + O₂, at the selected time points and Figure 29 gives a graphical representation.

In Table 4 it can be seen that in the animals treated with Cd + air, the highest percentage of labelled cells on day 3 are the Type II. At day 5 predominantly Type II and interstitial cells are labelled, while on day 9 the endothelial cells are prominent and the Type II are continuing to decline in number. At the termination of the experiment on day 15 the interstitial cells still show a high percentage of labelled cells.

In the animals treated with Cd + O₂ the highest percentage of Type II cells is not seen until day 7 (after the animals are taken out of O₂ on day 6) and the number gradually declines, similar to the animals in the Cd + air group. The percentage of endothelial cells was generally lower than in the Cd + air group. It was suppressed up to day 9 and then reached a peak at day 11. For the Cd + O₂ treated animals, the interstitial cells are the ones in greatest number (56%) at day 3 and then

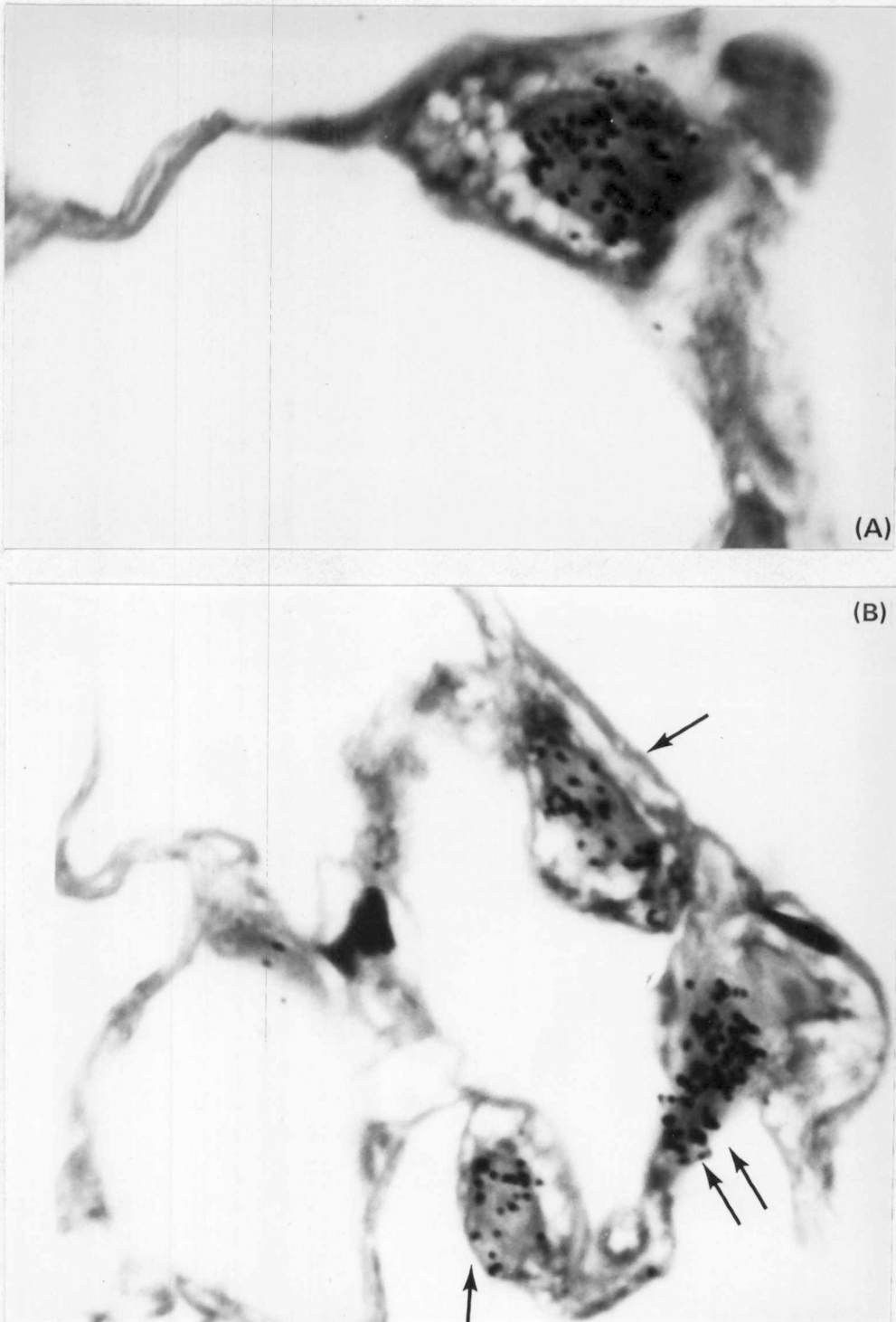


Figure 28. ³H-thymidine labelled cells of the alveolar capillary unit. A. Type II pneumocyte. B. Type II (single arrow), interstitial (double arrows). C. Endothelial cells (arrow) bulging into the capillary lumen (CL). D. Macrophage. Lee's methylene blue basic fuchsin x7000.

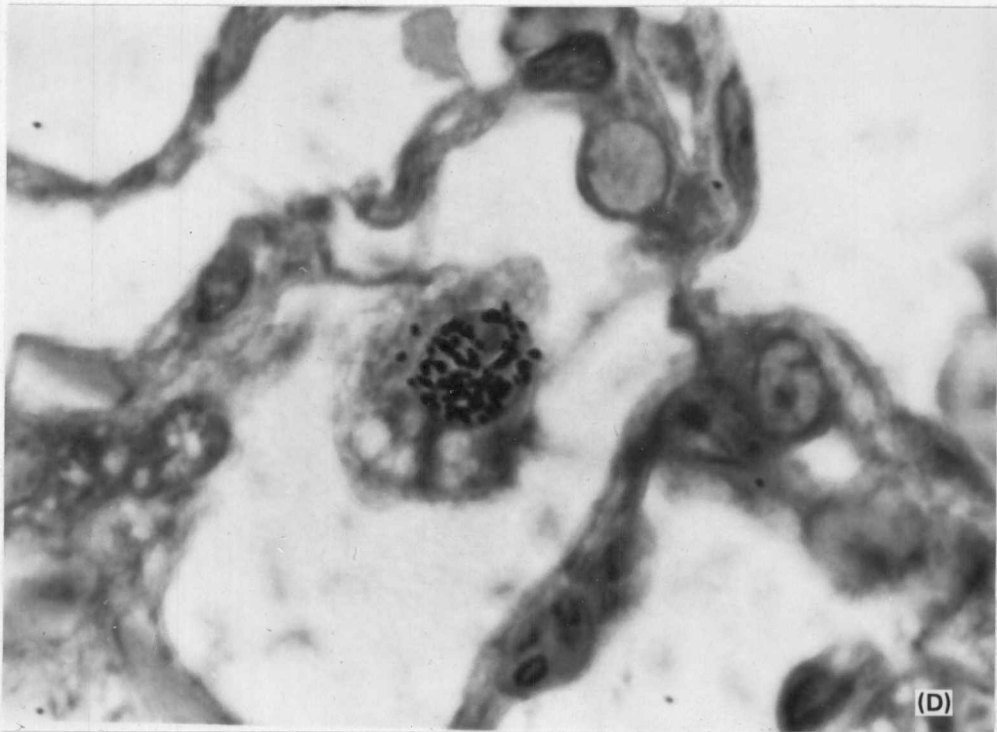
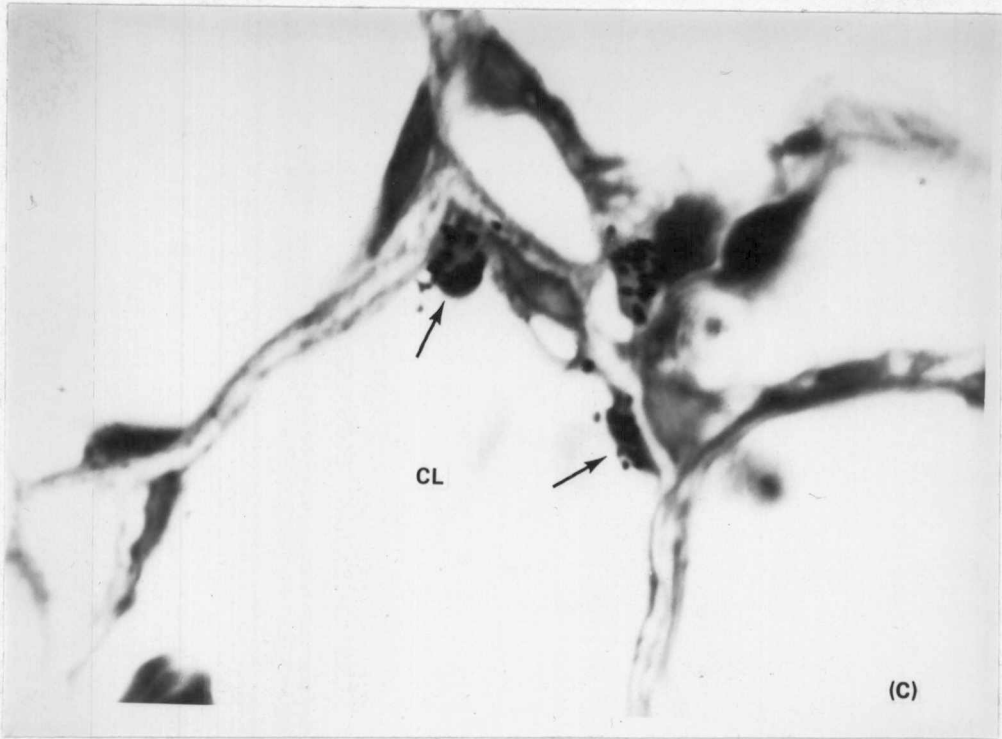


Figure 28. (continued)

Table 4. Differential cell count after cadmium exposure

Treatment	No. of days after Cd	Differential count (%)			
		Macrophages	Type II	Endothelial	Interstitial ^a
Cd + air	1	5 ± 3	49 ± 1	5 ± 2	41 ± 2
	3	7 ± 1	62 ± 3	4 ± 2	27 ± 2
	5	9 ± 1	48 ± 1	4 ± 0	42 ± 1
	7	8 ± 1	33 ± 3	18 ± 0	42 ± 2
	9	6 ± 1	22 ± 3	34 ± 3	40 ± 3
	11	5 ± 2	13 ± 0	25 ± 4	56 ± 5
	13	5 ± 2	15 ± 2	15 ± 2	66 ± 5
	15	8 ± 5	15 ± 4	15 ± 2	62 ± 9
Cd + O ₂	3	10 ± 3	33 ± 6	1 ± 1	56 ± 4
	5	12 ± 2	33 ± 2	5 ± 1	45 ± 1
	7	8 ± 1	50 ± 3	4 ± 1	38 ± 2
	9	13 ± 6	38 ± 2	7 ± 0	42 ± 5
	11	8 ± 3	25 ± 4	21 ± 9	46 ± 1
	13	6 ± 2	24 ± 3	17 ± 4	54 ± 1
	15	2 ± 1	12 ± 3	18 ± 5	68 ± 3

^aIncludes fibroblast + other.

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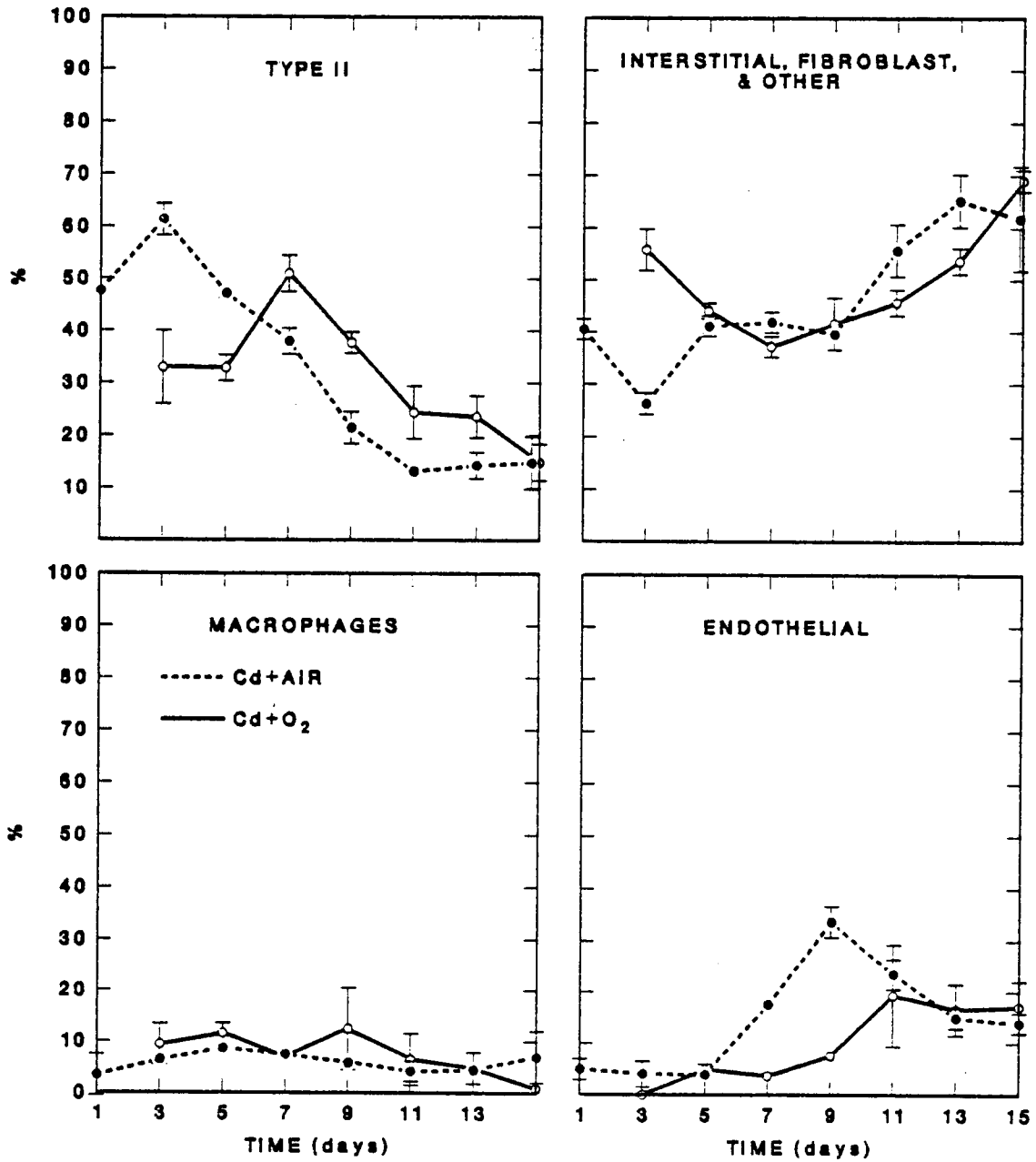


Figure 29. Differential percentages of labelled Type II, interstitial, macrophages, and endothelial cells after Cd and Cd + O₂.

they fluctuate between 56–38%, to their highest percentage (68%) on day 15. The percent of macrophages remains fairly constant throughout the experiment with the Cd + O₂ group showing slightly higher percentages, 2–13% (mean = 8) compared with 5–9% (mean = 7) for the Cd + air group.

When one relates the percentage of the various cell types to the labelling index and a calculation is made of the labelled cells/10⁻⁴ lung cells, a slightly different picture emerges (see Figure 30). For the Cd + air animals, the peaks of both Type II and interstitial cells occur at day 5 and the numbers are close (511 Type II vs 452 interstitial). However the pattern is different with the Type II starting out in high numbers, presumably to replace the injured Type I cells.

For the Cd + O₂ animals both Type II and interstitial cells are in relatively low numbers in the early days (suppressed by being in O₂ until day 6) then they increase in number and attain their peaks at day 9. The Type II cells decrease in number once their work of repair is done, while the interstitial cells are still fairly high in number at day 13.

The endothelial cells in both groups are low in number compared to Type II and interstitial cells. For the Cd and air group the endothelial cells reach a peak on day 9 and then there is a drop at day 11, remaining at that value until day 15. For the Cd + O₂ group there is a gradual rise until the peak is reached at day 11 and then a slow falling off. There are more macrophages in the Cd + O₂ group than in the Cd + air group, and in the former group the number varies greatly for different animals at each timepoint, as seen by the large standard errors.

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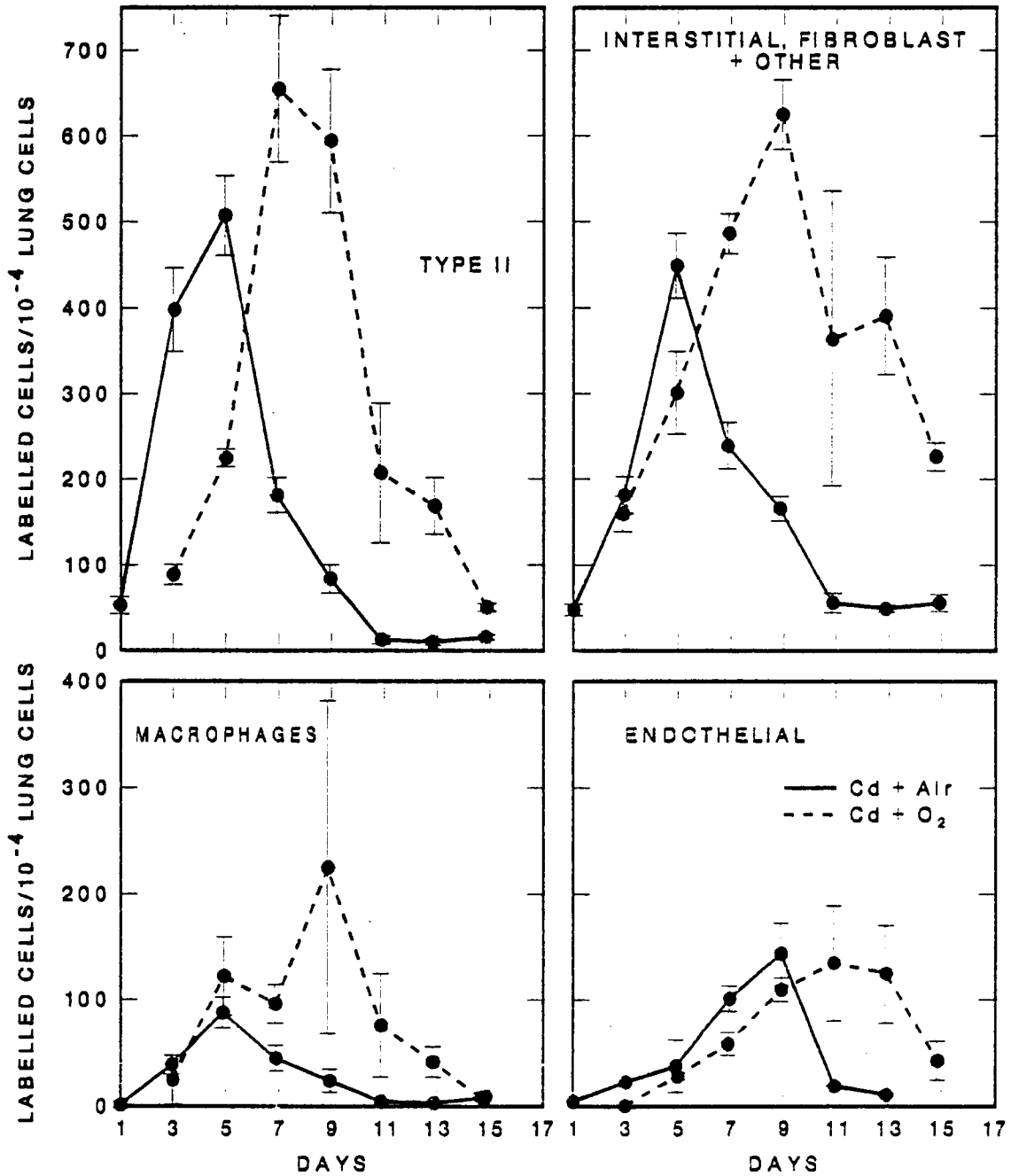


Figure 30. Number of labelled cells per 10,000 cells on days 1-15 after Cd and Cd + O₂.

5. Bronchiolar labelling index

Figure 31 shows an autoradiograph of cells of the bronchiolar epithelium. For this experiment these cells were not differentiated into ciliated cells, Clara cells, etc., but all labelled bronchiolar cells were counted to obtain the labelling indices shown in Figure 32.

With Cd and air, the peak in bronchiolar labelling occurred on or before the first day and values fell continuously until the 11th day when they reached the control level. For the animals treated with Cd and then exposed to 80% oxygen, there was a suppression of cell division at the early time period but by day 3 there was a slight peak, with the LI of 5 corresponding to that of the Cd + air animals (LI 5). The values fell slightly but by day 7 they were back to the same level as on day 3. They then continued to fall but at all times the values remained higher than the other three groups. For the animals treated with H₂O + 80% O₂ there was a slight peak at day 5 and then values fell to the control level by day 11.

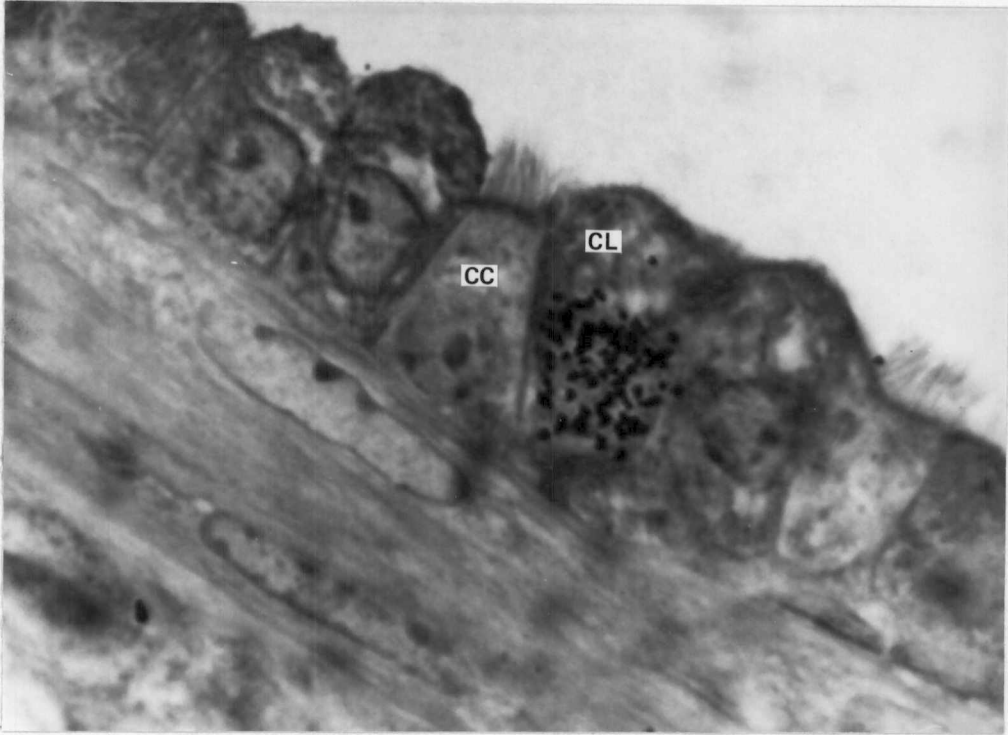


Figure 31. ^3H -thymidine labelled cells of the bronchiolar epithelium. CC = Ciliated cell, CL = Labelled Clara cell. Lee's methylene blue basic fuchsin x7500.

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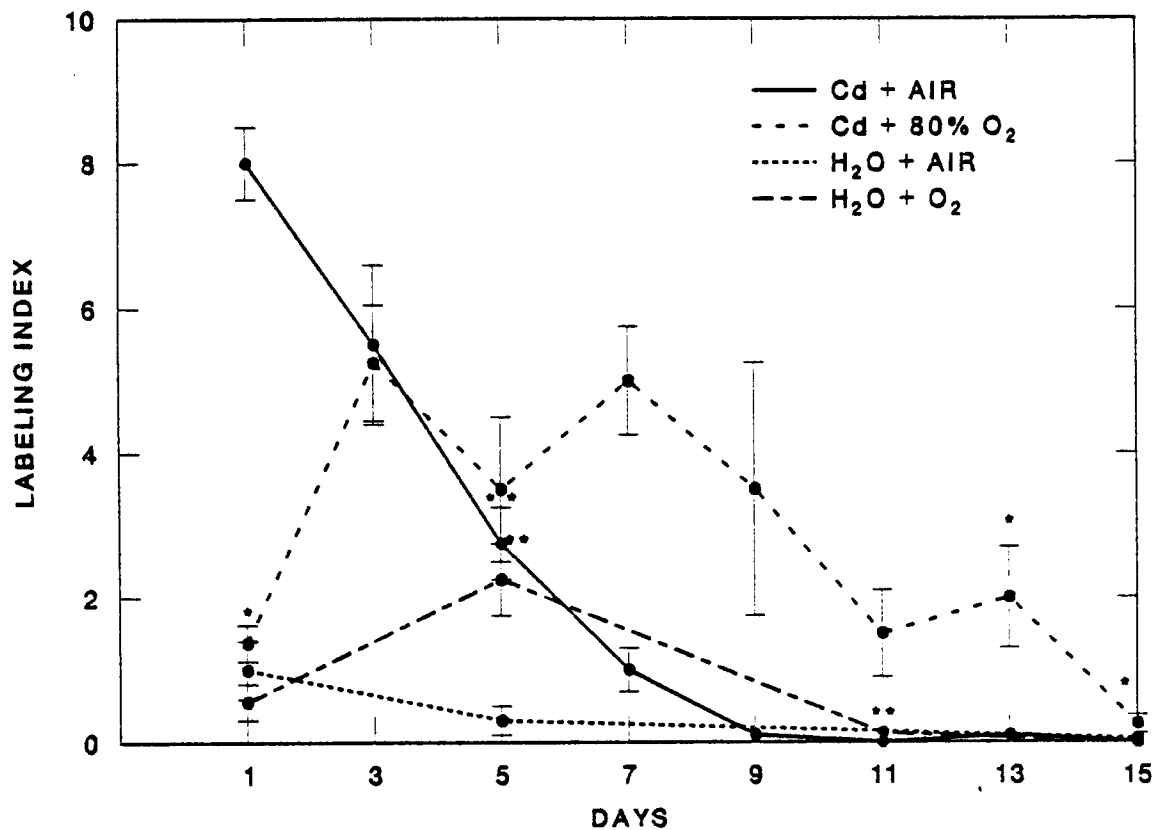


Figure 32. Labelling indices of the bronchiolar epithelium after Cd and Cd + O₂. ³H-thymidine autoradiography. Each point represents the mean value $\pm$ SE (n = 4-5 mice). * Denotes values significantly different from Cd + air (P < 0.05). ** Denotes values significantly different from H₂O + air (P < 0.05).

CHAPTER V

DISCUSSION

A. Fibrosis

One of the findings of the treatment of mice with Cd or Cd + O₂ was the development of interstitial pulmonary fibrosis. Pulmonary fibrosis is a disease which affects mainly the alveolar or respiratory zone. Interstitial pulmonary fibrosis may be caused by anticancer drugs, such as bleomycin, cyclophosphamide, and actinomycin D or by ionizing radiation, viral infections, or chronic exposure to organic or inorganic dusts. Pulmonary fibrosis may also be part of a multisystem disorder whose cause is unknown, such as rheumatoid disease or systemic lupus erythematosus. When there is no associated condition or specific agent, the cases are described as idiopathic.

Some of the distinguishing features of pulmonary fibrosis are disorganization of the normal alveolar structures, altered cellular population, disordered interstitial collagen, and an increase in total lung collagen. The mechanisms by which fibrosis occurs have not been fully elucidated, but one possibility is that it may result from the failure of the normal repair processes following injury by a toxic agent such as Cd.

In this study one of the methods of demonstrating lung fibrosis was by biochemical tests, whereby lung hydroxyproline levels were determined at three weeks following Cd inhalation. The measurement of hydroxyproline levels gives a qualitative measurement for collagen. Hydroxyproline is found almost exclusively in collagen, and, as one of the principal components of collagen, it

is present in a fixed amount namely 9.4 mole percent. (Other principal components are glycine, 33.0 mole percent, proline, 12.2 mole percent, and alanine, 10.7 mol percent.) (Dickerson and Geis, 1969).

The hydroxyproline levels for the animals that were treated with CdCl_2 aerosols and then left in room air showed a significant increase above the control levels, but for the animals that were placed in 80% O_2 for six days after Cd inhalation, there was a more pronounced incidence of fibrosis, that is, there was a synergistic reaction.

The results of the biochemical tests are supported by the histopathological examinations done at the three week interval. Our results are in agreement with those of Snider and Karlinsky (1977) who noted focal but widespread areas of interstitial fibrosis as well as cellular infiltration and epithelial cell hyperplasia at three weeks in Cd-treated hamsters. Our light microscopy examinations showed multifocal areas of interstitial fibrosis, located mainly around the bronchi and bronchioles, as well as hyperplasia and hypertrophy of the pneumocytes. There was some crenation of the lung surface due to the fibers contracting to form scar tissue, and infiltration of mononuclear cells, mostly lymphoid, and some foamy macrophages. There were also a few foci of acute inflammation remaining at this three week interval. The changes seen at this time represent the chronic response to an insult by a toxic agent. In such a case where the inciting agent is removed, the normal reaction is for healing to take place and the lung returns to normal with no signs of the disease being present.

It is typical of the lesions with Cd that they are focal instead of diffuse and this selective localization of damage to the lung has been done by other

workers. Three possible reasons for this localization have been suggested by Dervan and Hayes (1979), namely aerodynamic diffusion factors, or a breakdown of clearance mechanisms or a breakdown in the ability to inactivate the Cd.

The factors influencing the deposition and localization of particles (such as size, shape and density of particles, respiratory anatomy, and breathing pattern) are discussed by Brain and Valberg (1979). In a study with particles of Yb¹⁶⁹ ranging from 0.05 μm to 3 μm in aerodynamic equivalent diameter, Raabe et al. (1977) found that in rats and hamsters pulmonary deposition tended to increase as particle size decreased, while bronchial deposition was minimal for the 0.52 μm aerodynamic diameter particles. They also noted that deposition varied within the lung lobes, for example there was greater deposition of the larger particles in those lobes having shorter average path lengths from trachea to terminal bronchioles.

Another consideration in particle localization is the presence of "hot spots" and Shaw et al. (1978) have shown that these develop at lung bifurcations, where particle deposition can be greater than average by a factor of 5 to 30.

The peribronchiolar fibrosis which results from the Cd damage is one of the intriguing aspects of the study. The subsequent contraction of these scars can lead to a narrowing of the bronchiole, and this combined with some alveolar destruction results in a cystic, partially obstructed lesion which resembles centriacinar emphysema in man. Since the lesion produced by industrial cadmium fume poisoning in man has also been described as centrilobular (Gough, 1968) the Cd lesion in mice could provide a useful experimental model for studying this type of emphysema.

One of the features of fibrosis mentioned earlier is the overaccumulation of collagen. It is interesting to speculate on the source of this increased collagen. Presumably it is produced by interstitial fibroblasts. The origin of these cells is not certain. They may have arisen from recruitment and induced mitogenesis of cells committed to fibroblastic activity, or from the transformation of the normal population of cells to a population with enriched fibroblastic activity, or stimulation of a 'dormant' population of cells or undifferentiated cells. This increased collagen seen after the treatment with Cd is therefore thought to be due to increased synthesis by the increased number of interstitial fibroblasts.

Dervan and Hayes (1979) give supporting evidence for this. They observed the presence of increased numbers of interstitial "activated" fibroblasts with prominent rough endoplasmic reticulum in the lungs of Cd-treated animals. The cells were found in close association with small individual collagen fibrils close to the plasma cell membranes (suggesting that the collagen was being newly synthesized by these fibroblasts).

The behavior of other cell types seen in the fibrotic lesion deserves some attention. It is known that normal alveoli contain approximately 1.7 type Type II epithelial cells for every Type I, but in fibrotic alveoli there is a marked increase in the relative numbers of Type II cells (Crystal et al., 1978). This is understandable since the Type II cells are the ones that proliferate to repair the damage and replace destroyed Type I cells. Kawanami et al. (1982) have also described a third type of epithelial cell found in fibrotic alveoli, and these are called "cuboidal epithelial cells." They found that these cells, which are of bronchiolar origin, were the main source of epithelial renewal in areas of

very severe lung damage, while the Type II cells proliferated mainly in areas of less severe fibrosis.

Another cell type that is increased in fibrosis is the alveolar macrophage. Bitterman and Crystal (1980) have suggested that these cells release a factor which stimulates fibroblast replication. One important scientific work would be the prevention of fibrosis, and one thought is that if one could control the rate of synthesis of collagen then one would be able perhaps to control the development of fibrosis. It is not certain how the synthesis of collagen is regulated, but several mechanisms may contribute.

Some mechanisms to be considered are related to the control of the cells which synthesize the collagen, namely the fibroblasts. Fibroblast replication may be stimulated by factors released by macrophages, as mentioned above (Bitterman and Crystal, 1980) or as shown by Wahl et al. (1978) antigen triggered T lymphocytes may release a factor that can promote fibroblast growth and stimulate them into elevated collagen synthesis. Chemotactic stimuli for fibroblasts may be provided by collagen breakdown products and cause migration of the fibroblast to the site of tissue damage (Postlethwaite et al., 1978). Other mechanisms relate to controlling various steps in the synthesis of the collagen molecule (which will be described later).

From both the histopathology and biochemical results we have shown an increase in the connective tissue components but some authors, such as Kelley et al. (1980) point out that the absolute amount of connective tissue components may not be as important as how they are organized in relation to the pre-existing tissue architecture. The rearrangement of interstitial collagen in fibrotic lesions suggests that the collagen is laid down in abnormal locations

and this suggests that the cells that produce this collagen are themselves in abnormal locations. One general theory states that cell-cell and cell-matrix interaction play an important part in the normal topologic arrangements of tissue and according to the theory these processes involve large macromolecules that cause the cells to adhere to each other and to their extracellular matrix. Now if these macromolecules are destroyed, then cellular order is placed in chaos, and this has been shown to occur by proteases released by inflammatory cells (McDonald et al., 1979). Thus in the case of Cd-treated animals there is an example of cellular chaos leading to the deposition of collagen in abnormal areas. In the early acute phase of Cd injury several inflammatory cells are present and these are capable of producing proteases to cause the cellular disorder. Some investigators have concentrated on the proportion in the 2 main types of collagen found in lung parenchyma. Type I collagen contains three α chains, two of which are identical and designated $\alpha_1(1)$, and one that is different and designated as α_2 to give the tropocollagen molecule the designation of $[\alpha_1(1)_2\alpha_2]$. The Type III is represented by $[\alpha_1(\text{III})_3]$, showing that the three α chains are identical. In normal lung Type III collagen constitutes 33% of the total collagen, the remainder being Type I. In lung fibrosis there is a relative increase in the amount of Type I collagen in relation to Type III, but it has not been decided whether this is due to a preferential increase in the degradation of Type III collagen (Seyer et al., 1976). In rat lung organ cultures when exposed to high O_2 concentrations, there is a shift in the ratio of collagen types synthesized, from 70% Type I and 30% Type III to 30% Type I and 70% Type III (Bhatnager et al., 1978). Just the opposite is seen in the case of bleomycin when added to cultured lung or skin cells, in that there is an increase in Type I procollagen synthesis (Clark et al., 1979).

Crystal et al. (1976) have summarized that relatively compliant tissue like lung and intestine have a low Type I:Type III ratio, while tissue with less compliance like adult skin have a higher ratio of Type I to Type III collagen. Tissues that are very rigid, such as bone and tendon, have only Type I and no Type III collagen. In the case of the fibrosis caused by Cd the lung would be expected to be less compliant so a shift toward Type I would be expected. We have not conducted investigations of the collagen types involved in Cd-induced fibrosis but this would certainly be one of the interesting points to pursue in future research.

It can be concluded that there are many possible mechanisms involved in the development of the fibrotic lesion. It may be in the regulation of collagen synthesis, collagen deposition or matrix formation, or a disturbance in the degradation steps responsible for matrix turnover. In relation to collagen synthesis there could be changes in mRNA levels, species or efficiency of translation which could result in increased collagen production or could produce inappropriate ratios of specific gene products. Other mechanisms that could lead to an increase in the amount of collagen secreted are increased cellular processing of collagen, increases in the rate of maturation (the rate of extracellular cross link formation) and increased resistance of fibrils to proteolytic extracellular processing. Finally the control of degradation may be of importance in the fibrotic process either by regulating collagenase levels or by altering the levels of collagenase inhibitors.

Increased collagen production may also be due to the stimulation of collagen synthesis by endogenous factors, and several of these factors, for example ones from macrophages and platelets, have been reported in Fuller

and Mann (1981). It has been suggested that the inflammatory response contains a stimulating signal for the initiation of fibroblast proliferation and collagen accumulation, and when repair is complete, there are inhibitory factors for terminating the repair responses. If however there is a failure in the inhibitory signal, then collagen would continue to be produced and this would lead to a fibrotic lesion. As mentioned earlier, several toxic agents can cause lung injury and it is possible that there are different mechanisms for the development of fibrosis by the different agents. One general observation is that the development of fibrosis in severely damaged lungs is related primarily to the differential cellular responses to injury and with this in mind, we have devoted much attention to the cell differentiation studies following the Cd injury.

It has been shown that Type I epithelial cells are destroyed after treatment with some toxic agents such as BHT (Hirai et al., 1977) and our study has shown that repopulation (by proliferation of Type II cells) is delayed when O₂ is applied after Cd treatment. Fibroblasts are thought to be less susceptible to injury than Type I cells so they survive and many secrete collagen during the period of epithelial deficit. One early prefibrotic change which would result in the fibrotic lesion could also be the shift in the cell population (whatever cells are left undamaged) of the prefibrotic lung towards cells that are fibroblastic in nature. After this, the epithelial cells regenerate and the lung now has a thick band of collagen beneath an alveolus lined by cuboidal cells. This picture is similar to that described in long-term survivors of acute respiratory distress syndrome (Gould et al., 1972).

In summary, the factors which play important roles in the fibrogenic process are: epithelial cell-cell interaction (which controls the proliferation of interstitial cells in the alveolar zone), differential cellular response to toxic agents, alterations in collagen synthesis, and the expression of collagen gene products controlling synthesis, deposition and degradation of collagen.

It would seem that one obvious way to prevent fibrosis would be to prevent the excessive formation and deposition of collagen. Two main approaches can be taken, the first is specific inhibition of collagen based on characteristic features of collagen synthesis and metabolism. The second approach deals with the fact that abnormal collagen deposition is the final step of fibro-proliferative inflammation, and stresses the control of the inflammatory chain reactions by beginning with the early stages before the fibroblast becomes stimulated.

In the first approach there are two steps in collagen metabolism toward which most research has been directed for controlling collagen deposition, that is, the hydroxylation and polymerization steps. Blocking the hydroxylation step is very effective in that it also inhibits glycosylation and secretion, and consequently stops the synthesis of the whole collagen molecule (Chvapil, 1975). The inhibition of collagen hydroxylation can be achieved by interfering with individual cofactors or cosubstrates of prolyl hydroxylase, which in order to function requires ascorbic acid, ferrous iron, α -ketoglutarate and oxygen. Some fascinating studies have been done with proline analogs, which are erroneously picked up by the proline-tRNA and incorporated into the collagen polypeptide chain. However they cannot be hydroxylated because of the substrate specificity of proline or lysine hydroxylase and because of the

unavailability of the group to be hydroxylated. Thus there is inhibition of glycosylation and collagen secretion. One such compound, *cis*-4-hydroxy-L-proline was found by Riley et al. (1980) to decrease excess collagen deposition in lung tissue and prevented both the structural changes in airspaces and decreased tissue elastic recoil pressure in rats recovering from O₂ toxicity.

Another proline analogue, L,3,4-dehydroproline was shown by Kelley et al. (1980) to prevent the increase in lung collagen in bleomycin-treated rats and also improved volume-pressure relationships and lung volume in animals with pulmonary fibrosis. In the other step of collagen metabolism mentioned, namely polymerization, the compounds used for interference in this step are ones which are competitive inhibitors of lysyl oxidase. This enzyme initiates intra- and intermolecular crosslinking between collagen molecules. First there is oxidative deamination of lysine and hydroxylysine amino groups to form aldehydes, which react with the COH of another collagen chain to form stable aldol condensates or the ϵ -NH₂ to produce an unstable Schiff base. Reduction of the Schiff base establishes a stable covalent crosslink. It has been found that some compounds such as β -aminopropionitrile will inhibit lysyl oxidase by forming an irreversible complex with it and this prevents the polymerization of the collagen (Narayanan et al., 1972).

The second approach, concerning the events before the fibroblast becomes activated, deals with all the cellular events and cell-cell interactions in the course of inflammation, and it is here that treatment of fibrosis by antiinflammatory steroids come into play. Mention has been made of the invasion of PMN which relay humoral messages to macrophages which in turn

produce fibroblast stimulating factors, which then enhance collagen synthesis by fibroblasts. Relating to this is a study by Ross (1972) where he showed that depletion of macrophages in skin wound granulation tissue by antimacrophage serum resulted in inhibition of collagen accumulation in the healing wound. This points to the important role of the 'macrophage factor' in fibrosis. Other cells such as platelets may also produce a fibroblast growth-promoting factor (Kohler and Lipton, 1974) so this aspect of the control of fibrosis has great possibilities for future research.

B. Modification of the Fibrotic Lesion by X Radiation

Thoracic X radiation has been shown to have a potentiating effect on lung injury (Haschek et al., 1980) but our study of treating the Cd-damaged lung with X radiation did not show a potentiation of fibrosis at any of the time points tested, that is, 2 hr, one day or two days. It is possible that a later time point should have been chosen, perhaps three to five days when the Type II cells would have been at their peak of proliferation. Studies by Penney and Rubin (1977) have suggested that radiation damage to Type II cells may have some bearing on subsequent atelectasis. Similar results were obtained by Haschek et al. (1980) who treated BHT damaged mice with X radiation. There was an increase in fibrosis presumably related to the damage to the Type II cells during their proliferation. However in their experiments, exposure to X rays during proliferation of capillary endothelial cells or interstitial cells did not enhance the development of fibrosis. Moosavi et al. (1977) held the opposite view, attributing damage to the pulmonary endothelial cells as the cause of radiation injury in the lungs.

In our Cd study since the Type II cells were at their peak of DNA synthesis of days 3–5 and the endothelial cells at day 9, these days would have been more suitable to test the hypothesis of which cell population is involved in the radioinduced fibrosis. At day 2 there would have been about 55% of proliferating Type II cells, so one would expect a difference between day 2 and day 0 (before the start of Type II (cell proliferation) if the Type II cells were involved, but since there was not, some other explanation must be found.

One possible explanation could be that not enough time was allowed to elapse between the Cd and X radiation treatment and the killing of the animals. Mansfield et al. (1981) in an experiment with rats given bleomycin ip and 1200 rad of 25 MV thoracic X radiation, showed that there was histologic evidence of lung damage 200 days after treatment. This damage was greater than that found with either agent alone. Thus it is possible that if hydroxyproline assays had been done at a later time, they may have shown some potentiation of fibrosis, since radiation may require more time to exert a deleterious effect.

The fact that O_2 potentiated the damage done by Cd when X radiation did not, could mean that these two agents affect different populations of cells or that they have a different mechanism of action. The work of Witschi and Côté (1977a) implies that Type II cells are susceptible to the cytotoxic action of O_2 while the dividing interstitial cells are not, but X radiation may be non-selective and cause damage to all cell types. Thus if the interstitial cells are not susceptible they can proliferate and secrete collagen which will cause the increased fibrosis seen in the treatment with Cd + O_2 , whereas if these

interstitial cells are also destroyed by X radiation, there will not be an increase in collagen. Studies by Hakkinen et al. (1982) had similar results in that there was a potentiation of lung damage by hyperoxia but not by thoracic X irradiation in bleomycin and cyclophosphamide treated mice. In considering the mechanism of action of O_2 and X radiation in producing cellular damage, some similarities have been noted. In the case of O_2 , the agents responsible for cellular damage are the free radicals or intermediates which are produced when electrons are added to oxygen. These free radicals are formed in large amounts in situations like hyperoxia and they will attack polyunsaturated fatty acid side-chains of membrane lipids to initiate the process of lipid peroxidation. The lipid peroxides are powerful inhibitors of many cellular enzymes, or they may decompose to yield products which can damage proteins and membranes. X radiation also exerts its effects on cells by the production of free radicals. Here it is the radiation effects on the water molecules which are important (since living material is 70–90% water), and the absorbed energy is taken up by the water molecules. The free radicals formed may react with other molecules to cause a vast spectrum of chemical and biological damage. This is the “indirect” action of radiation, as opposed to the direct action when the ionizing radiation reacts with critical biological molecules, also forming free radicals. The reactions of these free radicals with biological material may lead to biological harmful products or to the initiation of damaging chain reactions (Coggle, 1971).

Specific enzyme defenses have evolved which protect the cell by eliminating the free radicals, for example, superoxide dismutase (SOD) eliminates the superoxide anion, and catalase and peroxidases eliminate H_2O_2

and lipid peroxides. Substances such as endotoxin which protect against oxygen-induced lung damage are thought to do so by causing an increase in the pulmonary superoxide dismutase, catalase, and glutathione peroxidase (Frank et al., 1980).

Thus though free radicals are produced by both O_2 and X radiation, they may be formed at different times or different rates, or require more time or greater concentration to exert their damage. Also different cell populations may be affected depending on the time of production of the free radicals. There may even be increased SOD, catalase or glutathione peroxidase produced, or other protective mechanisms brought into play when X radiation is applied.

It has been shown that isolated Type II cells under hyperoxic conditions will show increased SOD activity (Forman et al., 1979) thus with Cd damage and hyperoxia there would be increased proliferation of Type II cells and increased SOD activity, but there may be an overwhelming amount of free radicals produced because of the O_2 exposure, therefore there would not be enough SOD available for protection, and fibrosis would result.

The differing results obtained with these two agents, O_2 and X radiation, following the Cd damage, present a challenge for further research to be done in this area.

C. Modification of the Fibrotic Lesion by Prednisolone

In our experiments of Cd-induced lung damage in mice, treatment with prednisolone produced no significant modification in the fibrotic lesion. Several reports have shown that glucocorticoids both *in vivo* and in cell culture

selectively decrease collagen polypeptide synthesis. It is generally agreed that the steroid mediated alteration of collagen metabolism is mainly antianabolic in nature, but the molecular levels are still unknown.

During the biosynthesis of collagen, the hydroxylation of proline residues occurs before helix formation of the peptide chains. The extent of hydroxylation of proline residues of collagen is directly related to the structural stability of the molecule, thus an inhibited hydroxylating system could influence the quality and quantity of newly synthesized collagen. Glucocorticoids could either decrease the rate of collagen polypeptide synthesis and/or the rate of prolyl hydroxylation.

The determination of the degree of hydroxylation of collagen nascent polypeptides was not a part of this study. However, Newman and Cutroneo (1978), after multiple injections of glucocorticoid to newborn rats, showed a decrease of prolyl hydroxylase and a decrease in collagen synthesis, though there was no synthesis of underhydroxylated collagen. The decreases in collagen synthesis and in prolyl and lysyl hydroxylase activities brought about by the glucocorticoid treatment suggest that there is coordinate regulation of several key steps in collagen biosynthesis at the translation or transcriptional level. Although several reports show that glucocorticoids decrease collagen synthesis, a few do show that there is an increase, for example, Kehrer and Witschi (1981) found that administering prednisolone (60 mg/kg/day) on days 1-6 to BHT treated mice produced significantly more pulmonary fibrosis than in BHT-treated mice given saline. It appears that timing is an important factor in the production of fibrosis since only treatment of days 1-6 enhanced the accumulation of collagen.

Smith and Brody (1981) also found that methylprednisolone administered on day 1-5 after BHT resulted in greater Type II cell proliferation, lung injury and mortality, and again they found that the timing and duration of methylprednisolone administration as well as the severity of the lung injury were important determinants of the outcome.

The elucidation of the mechanisms by which corticosteroids exert their effects is an exciting challenge. A good review is presented by Thompson and Lippman (1974) in which they cover such hypotheses as direct interactions with enzymes, nucleic acids, lysosomes, and receptor proteins. One point agreed on by most authors was that steroids were capable of altering cellular phenotype through alteration in gene expression. A popular model for glucocorticoid action (Thompson and Lippman, 1974) states that initially the steroid binds to a cytoplasmic receptor protein. This complex undergoes modification and then enters the cell nucleus. In the nucleus, the steroid may interchange with a nuclear receptor or may still associate with the cytoplasmic receptor, but it then interacts with DNA, resulting in an altered rate of synthesis of certain species of RNA. For example, ribosomal and tRNA synthesis may be altered or there may be quantitative or qualitative alteration in the rate of mRNA synthesis. These alterations manifest themselves in the altered rates of synthesis of specific proteins and this leads to expression of the phenotypic effect.

Our study with all the various protocols definitely did not show that prednisolone caused a decrease in collagen synthesis. In the experiment where prednisolone was given for 12 days after Cd it appeared more as if prednisolone caused an increase in collagen, but this increase was not

significant. The results can be related to the amount and type of damage done by the Cd. In our studies with Cd there were occasions when the cell damage was immense and others when it was only mild as noted in the histological examinations. These varying results in the same group of animals would account for the variation also in hydroxyproline determinations.

Smith and Brody (1981) for example found that methylprednisolone inhibited Type II cell proliferation when lung injury was mild but failed to inhibit proliferation when lung injury was severe and they suggested that a large initial lesion with very extensive Type I cell damage could possibly override the inhibitory effect of the methylprednisolone.

How does the Type II cell "know" when it should proliferate? Again Smith and Brody (1981) suggest that the injured Type I cells release a signal that results in Type II cell proliferation. The signal could either directly stimulate cell proliferation or block the action of a mitosis inhibitory substance, for example, a chalone. The administration of corticosteroids may interfere with either recognition or expression of the signal. It would be interesting to test this hypothesis in experiments following the treatment with Cd^{+} + prednisolone. However, from all results noted, it would be wise to take a cautious approach to steroid therapy for human patients with primary lung damage, no matter the severity.

D. Cadmium Pretreatment

Several experiments have been described where tolerance to CdCl_2 was seen in experimental animals. Gunn et al. (1966) described a case of the tolerance of the testis to a second dose of CdCl_2 and they concluded that "the

biochemical systems of regenerated blood vessels differ from the original vasculature." However, Gabbiani et al. (1967) suggested that since the spermatogenic elements were permanently damaged by the first CdCl_2 administration, then the receptors for Cd in the testicular parenchyma could have been destroyed by the first CdCl_2 administration, which would result in tolerance to the next dose of Cd.

In Schnell's laboratory it was found that, following administration of subthreshold doses of Cd (0.21 or 0.42 mg Cd/kg i.p.) prior to the threshold dose (0.84 mg/kg i.p.), the Cd-induced alterations in the response to hexobarbital hypnosis and hepatic microsomal metabolism were no longer observed. This Cd-induced tolerance was thought to be related to elevated levels on metallothionein produced by the first dose of Cd, which would bind the second Cd dose and prevent the Cd from interacting with vital cellular constituents. They found that the minimum time period required for the subthreshold Cd dose to produce a tolerance to the threshold Cd dose was 14 hr and the phenomenon lasted for 336 hr (Schnell, 1978).

In our experiment there were four subthreshold doses given i.p. and it is possible that this "overloaded" the system and used up all the metallothionein induced so there was not as much metallothionein present when the large Cd dose by inhalation was administered. There is another important consideration in that the pretreatment was given i.p. so the metallothionein induced may be present in the liver, but the toxic dose was by inhalation and affected the lungs where there was no metallothionein induced to exert a protective effect.

Webb and Verschoyle (1976) in their investigation on the role of metallothionein in Cd-toxicity found that protection was maximum at

1–3 days, but was diminished 10 days after Cd pretreatment, whereas both the increased content and the capacity for the synthesis of metallothionein were maintained. They therefore concluded that preinduced metallothionein did not play a significant role in protection against acute Cd toxicity.

Since we did not perform an assay of the amount of metallothionein present at any time in the lung (or liver) it is not possible to come to any conclusions on the role of metallothionein in Cd toxicity from this experiment, but future experiments that would be of value would be perhaps to give just one small pretreatment dose endotracheally (so it would get to the lung) and then to give the larger inhalation dose when protection by metallothionein was at its maximum, say one to three days.

E. Long-term Effects of Interaction of Cd and Oxygen

1. Biochemistry

The results of the hydroxyproline determination showed that the levels became higher as the animals got older, and were markedly elevated at one year. The results of the Cd-treated groups showing increased collagen agree with the histological picture of residual fibrosis present at the later time points (3 months, 6 months, and 1 year).

At three months, the Cd and O₂ group had significantly higher hydroxyproline values than the Cd and air group, showing that the potentiating effects of O₂ could still be seen up to that time. However at six months there was no significant differences between the two treated groups, although both

The greatly increased hydroxyproline levels of the animals sacrificed at one year show that the fibrosis has persisted up to this time. However, the age

factor comes into play because even the control animals have a high level of hydroxyproline, 383 $\mu\text{g}/\text{lung}$ at one year (Figure 20) (page 85) compared with 220 $\mu\text{g}/\text{lung}$ at 3 weeks (Figure 12) (page 72). The Cd and air group was significantly different from the control group, so even after a year there is still some residual fibrosis of the lungs due to Cd (although all values are higher due to the age factor).

Several of the animals in both the control and treated groups had tumors, and this may have caused some of the elevated hydroxyproline values, for example, one animal not included in this study but possessing a huge tumor (7 mm diameter) showed hydroxyproline values of 1047 μg hydroxyproline per lung when sacrificed at 1 year.

Long term fibrosis studies have also been reported by Haschek et al. (1982) who determined hydroxyproline values in mice treated with BHT and O_2 . They showed that the maximum relative increase in lung hydroxyproline content compared with the controls occurred in the first 2 weeks after BHT treatment but the elevated levels persisted up to one year. The effect of age on elevating the hydroxyproline values was also seen, in that the animals treated with corn oil alone (controls), at 2 weeks had a hydroxyproline value of $192 \pm 3 \mu\text{g}/\text{lung}$ compared with $327 \pm 11 \mu\text{g}$ at 52 weeks.

2. Lung function studies

Lung function studies would not be considered the method of choice to determine the deleterious effects of Cd on the lungs. Because of the nature of the Cd lesion which is noted as focal areas of fibrosis interspersed with areas of almost normal lung tissue, and also some areas of emphysematous tissue mixed

with normal tissue, it would appear that one effect would cancel out the other and thus the volume-pressure curves would not be very meaningful.

One parameter that is measured in the lung function studies is compliance which indicates the loss of distensibility of the diseased lungs. These changes may be due to a loss of alveolar units or to an increase in the modulus of elasticity of the tissues comprising the lungs (Snider et al., 1978).

The breakdown of the walls of the alveoli may be attributed to the action of the proteases and it is possible that they could be released from macrophages by the action of Cd.

In considering the modulus of elasticity one thinks of the collagen fibers which have the highest modulus of elasticity of any of the materials which comprise the response of the lungs to injury. As stated before, the Cd causes an increase in collagen and this would be one of the factors influencing the compliance.

Compliance varies directly with the size of the lungs and is related to such indices of lung size as vital capacity, functional residual capacity, and predicted lung weight (Cook et al., 1958). The elastic recoil properties of the lungs are due not only to the mechanical properties of the connective tissue fibers but also to surface tension forces generated at the air fluid interface of the alveoli (Snider and Karlinsky, 1977). This is related to the amount of surfactant which is produced by the Type II cells. After Cd damage there is increased proliferation of the Type II cells as noted before, thus there may be an increase of surfactant which would contribute to the altered surface tension forces.

Some lung function studies following CdCl_2 inhalation have been reported by Bus et al. (1978) and they found decreased compliance in the treated animals on days 1, 5, and 11. Among their suggestions for the decreased compliance are the presence of pulmonary edema, altered alveolar surfactant activity, altered connective tissue fibers, alveolar cells, and other tissue elements that affect the mechanical properties of the lungs.

Our earliest time point for measuring compliance was at three weeks at which time there is no pulmonary edema evident. It is, possible therefore that there was decreased compliance but that compliance had returned to normal values by 3 weeks and the ensuing time points.

Snider and Karlinsky (1977) induced a fibrotic lesion in hamsters by a single endotracheal dose of 0.025% CdCl_2 . They found that at 21 days the V-P curves were shifted downward to the right of normal and lung volumes were diminished, at 44 days the V-P curve and lung volume had returned to normal and at 110 days they were greater than normal. Our measurements at none of the time points showed any dramatic variations from the control ($\text{H}_2\text{O} + \text{air}$) except that at 6 months there was a slight tendency of the V-P curve for $\text{Cd} + \text{air}$ to shift to the right denoting a decrease in lung volume.

In the animals that received H_2O aerosols followed by 80% O_2 , the V-P curves at 3 weeks, 3 months, and 1 year showed a slight shift to the left, signifying an increase in volume but these results were not markedly different from those of the other groups.

The lowest values noted for chord compliance in our experiments were at 3 months in the two treated groups and at this time point there is residual

fibrosis as seen by light microscopy and also a high hydroxyproline content, attesting to the presence of collagen.

In experiments with Cd-induced injury there is difficulty correlating the reductions in lung volume and the physiologic parameters such as compliance with the increased collagen content of the lungs. There are several factors to account for this. In the early stages after Cd treatment there is cellular infiltration and edema which cuts down on the air space of the alveoli and causes a decreased lung volume, resulting in decreased compliance before the connective tissue elements are increased. Later the connective elements are increased and the focal areas of fibrosis are so incompressible that they decrease the lung volume, but now there is an increase in lung collagen due to the increased connective tissue elements. Finally, late in the course of interstitial fibrosis, emphysema develops which is typified by excessive compliance, but this added to the stiff fibrotic areas of the lung may cause compliance to return to normal, in spite of the high level of connective tissue elements (and hydroxyproline content) (Karlinsky and Goldstein, 1980). Other factors to be considered are the possibility of increased stiffness of surviving alveoli, the change in surfactant and lung shrinkage. Thus great caution should be exercised in interpreting the results of lung function studies.

3. Histopathology

One distinguishing feature of the toxic effects of Cd on the lungs as seen by light microscopy is the development of fibrosis, and this tends to be resolved as time goes by and the lung returns to an almost normal state. Cd-induced fibrosis was described by Dervan and Hayes (1979) who noted that the

fibrosis had a peribronchiolar localization with distortion of the bronchioli and adjacent alveoli. They suggested that the peribronchiolar localization of the fibrosis was the probable cause of centriacinar emphysema caused by acute Cd fume poisoning in man. Devan and Hayes (1979) also noted that at 6 months after Cd exposure there was only fine scarring about the bronchioles, and their findings are in agreement with our observations that the acute injury does not become progressive but there were only traces of residual fibrosis at one year.

A study of Cd chloride administered as a single endotracheal dose to hamsters by Snider and Karlinsky (1977) showed persistent irregular fine scars at 44 days along with distribution and dilation of alveoli giving the appearance of emphysema. Their last end point was 110 days at which time there was still remnants of the insult.

While performing light microscopy examinations, one is struck by the variability in response of different animals within a group, where one animal may show very slight injury and another may have very severe damage. The suggestions offered have been the possibility of differing exposure levels of CdCl_2 , for example, due to a position close to or away from a linear flow of the aerosol, or some mice may have increased or decreased susceptibility to CdCl_2 which may be associated with intrinsic or extrinsic causes.

One explanation could be if there were diseased animals in a group, the disease would affect the mechanism of clearance and deposition of the Cd particles. For instance if there is mucus blocking the airways, the decreased cross section of the airway would cause an increase in linear velocity and turbulence, leading to increased inertial impaction and central deposition of the particles. Disease also alters the breathing pattern which would affect the deposition of particles.

Whatever the individual variations, it was seen at one year that the damage in the Cd and oxygen group remained a little more severe than the Cd only group, due no doubt to the greater initial damage and therefore requiring more time for resolution. The high values of the hydroxyproline determination at one year suggests that the fibrotic changes have persisted, but as mentioned before, the morphologic examination show that the interstitial fibrosis has subsided considerably. What is more apparent at one year are the irregular airspace enlargements, suggestive of emphysematous change and these are interspersed between the few areas of residual fibrosis.

The observation of tumors in the year old animals was considered incidental since tumors were found in both treated and control groups. Kauffman et al. (1979) present a good report on similar lung adenomas which were induced in mice by transplacental exposure to nitrosourea on the 16th day of gestation. They classified the tumors on the basis of predominant histologic pattern as either alveolar or bronchiolar, and they identified by electron microscopy, the cell type comprising the tumors. The electron microscopic examination showed that the bronchiolar papillary tumors consisted of Clara cells, while the alveolar tumors consisted of Type II alveolar cells. It was suggested that the Clara cell tumors may become malignant while Type II tumors would not, providing added importance to the ability to identify lung adenomas histologically. In our study both types of tumors were present, and occasionally mixed types were found.

F. Cell Kinetics

1. Overall repair after Cd and Cd + O₂ ¹⁴C-thymidine incorporation

Cell renewal is necessary to maintain the integrity of the tissue, and in the case where cells are damaged by injurious agents, cells are renewed and proliferate to repair the injury. The proliferation response of lung tissue damaged by Cd and hyperoxia was studied and provided valuable information on such points as the changes in pulmonary cell populations during the early phases of fibrosis, the magnitude of the lesion, and how well repair takes place when another injurious agent is applied.

In the study with exposure of mice to Cd only, it is noted that there is a marked increase in DNA synthesis on day 5, while in the group that was exposed to Cd followed by 70% O₂, the peak is shifted to the right and occurs on day 7. The results of the experiment dealing with exposure to Cd only can be compared with those of Palmer et al. (1975) where rats were exposed for 2 hr to a 0.1% aerosol of CdCl₂ and the lung content of DNA was found to reach a peak on day 4.

There are no reports in the literature of exposure to a second agent following Cd (one of the major contributions of this study). In our study of exposure to O₂ following Cd, it is interesting to note the increase in injury as evidenced by the greater synthesis of DNA in the Cd + O₂ group compared with the Cd only group. Another striking fact is the delay in cell proliferation in the O₂ treated group, but once the animals are out of O₂, cell proliferation markedly increases and results in greater synthesis, at a later time than that which occurs in the Cd only group. One should also note the persistence of the injury, and the fact that the DNA content was always at a

higher level in the Cd + O₂ group than in the Cd alone group once the animals were taken out of the O₂. This emphasizes the synergistic effect of O₂ in promoting lung injury and the fact that it takes a longer time for the lesion to be resolved, for example in day 11 the DPM/mg DNA for Cd + O₂ is 7332 ± 4450 compared with 1079 ± 739 for Cd alone which is closer to the control values of 298 ± 71 for H₂O + O₂ and 475 ± 137 for H₂O + air.

It has been shown by Witschi and Côté (1977a) in an experiment where mice were injected with BHT and then exposed to O₂ at various times thereafter, that the O₂ inhibited DNA synthesis and this effect was most pronounced at 2 days after BHT. The effects were progressively less until on day 6 and 7 after BHT it was no longer possible to depress DNA synthesis with 100% O₂. In our Cd experiments the animals were kept in 70 or 80% from day 0 to day 6 and here the inhibitory effect of O₂ on DNA synthesis was also shown. However once the animals were removed from O₂ the DPM/mg DNA shot way up (Figure 25) (page 96) and surpassed all values for Cd alone animals.

The cell types that are dividing and contributing to the curve in Figure 25 (page 96) will be discussed later under cell differentiation.

2. Parenchymal labelling index

In this experiment animals subjected to Cd aerosols or Cd + O₂ were sacrificed at selected time intervals and lung sections were prepared for autoradiography. The labelling index (LI) was determined and was defined as the percentage of ³H-thymidine labelled pulmonary cells per total number of

cells counted. It is remarkable how similar the LI curves (Figure 26) (page 98) are to those of Figure 25 (page 98) for the overall repair study by ^{14}C -thymidine incorporation.

Again the peak of cell division as shown by the LI was seen on day 5 for the animals exposed to Cd alone while for the ones treated with both Cd + 80% O_2 the labelling index was not as high until the animals were taken out of O_2 at day 6 and then the values rose, attaining peak values at day 9. The labelling index for the Cd + O_2 group maintained this high level and in comparison again at day 11 it can be seen that the LI for Cd + O_2 was 5.2 ± 1.9 while for Cd + air it was 0.8 ± 0.1 , which is close to the control values of 0.9 ± 0.2 for H_2O + air and 1.5 ± 0.5 for H_2 + O_2 .

In the experiment of Haschek et al. (1983) where animals were injected with 400 mg BHT/kg and placed immediately in 70% O_2 for 6 days similar results were seen to those described for Cd + O_2 . Labeling indices on days 4 and 6 for BHT and O_2 were lower than in BHT alone. When the animals were removed from O_2 , a sharp increase in the labelling index occurred, and it remained significantly elevated from day 8 to day 14 compared with the values in animals receiving BHT alone. Thus O_2 exposure after the BHT insult in this experiment also caused a delay in cell proliferation and the results are in agreement with the Cd ones except for the different times that the peaks are reached. It is expected that there would be some slight differences between the overall repair curves (Figure 25) (page 96) and the ones for parenchymal labelling index (Figure 26) (page 98), since in the former any cell that incorporates ^{14}C thymidine is counted. Thus all the

dividing cells of the bronchioles and airways are included in that count, but not in the latter.

It was interesting however to check the correlation between these two curves and the results can be seen in Figure 27 (page 99) where DPM per mg DNA on the abscissa is plotted against LI on the ordinate for both the Cd only group and the Cd + O₂ group. In the Cd + O₂ group the correlation coefficient, *r*, was found to be 0.8738 and for the Cd only group it was 0.9877 which shows remarkably good agreement.

3. Differential cell counts

The cell kinetics after administration of Cd have not been described previously but there are some light and electron microscope studies by Dervan and Hayes (1979) which describe the lung lesion in rats after the inhalation of 0.1% CdCl₂. Their results are similar to ours in that they found the maximum cell proliferation to be on the 4th and 5th days.

They also found that on days 7–10 the majority of interstitial cells were fibroblasts and they were associated with an increase in interstitial collagen. Special attention should be paid to these fibroblasts because they play an important role in fibrosis. Haschek et al. (1983) showed that in the differential cell count for mice treated with BHT + O₂, at two weeks 82% of all labelled cells were interstitial (mainly fibroblasts) as compared to 27% in animals treated with BHT alone. The result with Cd is different in that at day 15 the Cd + O₂ group had 68% interstitial cells and the Cd alone group has 62%, which shows that in this system the interstitial component is elevated for a long time in both groups. This corresponds well with the high

hydroxyproline values found at this time (though they are always higher in the Cd + O₂ group).

The origin of the increased numbers of interstitial fibroblasts has led to much speculation. Dervan and Hayes (1978) suggest that they may arise from division and multiplication of fibroblasts already present, or it is possible they may arise from primitive uncommitted interstitial mesenchymal cells. In examining the labelling index graphs (Figure 26) (page 98), one realizes that it is made up of various populations of alveolar cells, and it can be broken down into various components after studying the graphs for differentiation (Figure 29) (page 104) and the graphs of labelled cells/10⁻⁴ lung cells (Figure 30) (page 106).

The labelling index graph of animals exposed to Cd only has its peak at day 5 and the labelled cell populations contributing to this peak according to the graph for labelled cells/10⁻⁴ lung cells are not only the Type II cells, but also the interstitial and fibroblast populations and some macrophages. Thus it seems that all these cells are actively dividing at this time (day 5) though in different numbers, and only the endothelial population has a later peak at day 9.

From the study of the differential percentages (Figure 29) (page 104) one observes that the Type II cells gradually taper off in number from 62% on day 3 down to 13% on day 11 for Cd + air while the interstitial cells still constitute a high percentage (56%) of the cells that are dividing at day 11. If we examine the graph for labelled cells/10⁻⁴ (Figure 30) (page 106) however it can also be seen that at day 5 there are 511 ± 44 labelled Type II cells compared with 12 ± 2 at day 11 for Cd + air

and with the interstitial cells at day 5 there are 452 ± 41 compared with 52 ± 14 at day 11. This difference in the two sets of graphs arises because the labelling index at day 11 is very low and since it is used in the calculation for number of cells it shows a reduced number of dividing cells (even if the differentiation percentage is high). The graphs of Cd + O₂ are similar to the Cd + air ones except that all peaks are shifted to the right for Cd + O₂ showing that cell proliferation takes place later, and in the case of labelled cells per 10⁻⁴ lung cells the peaks for Cd + O₂ are higher, since the labelling index is higher comparatively.

In the cytodynamic study of Adamson et al. (1977) of lung injury in BHT treated mice they showed that the epithelial cells peaked at 2 to 3 days, the interstitial cells plateaued at day 5 to 9 and the endothelial cells showed a late peak at 6 days. Our peak for Type II cells in the cell differentiation study is similar, occurring at day 3 and the interstitial cells have a plateau at days 5 to 9 but continue to increase to day 13. The endothelial population is also similar in that there is a late peak, at day 9 in our study.

One cell kinetic study of treating animals with a second agent, namely O₂, after lung damage induced by BHT is the one noted earlier by Haschek et al. (1983). The BHT is a blood-borne agent compared to Cd which is administered by inhalation so it was interesting to note the similarity in results in that there was an initial delay in cell proliferation followed by greater proliferation once the animals were taken out of O₂, and also the differential cell counts were not remarkably different in the group exposed to O₂.

From an examination of the graphs of the cell populations represented as labelled cells/10,000 lung cells the main conclusion reached is that the

major difference in the Cd treated group and the Cd + O₂ group is the time of onset for the various changes and also the final extent and persistence of the changes.

In regard to the hypothesis of Haschek and Witschi (1979) that the O₂ inhibits the division of the epithelial cell population, thus allowing the interstitial cells to grow undisturbed, our results do not show conclusively that the suppression of the Type II cells is the direct cause of the resulting fibrosis. There is the slowing down of cell division of the Type II cells while the animals are still in the O₂ enriched atmosphere but as soon as they are taken out, the Type II cells proliferate extensively and reach a peak at day 7 with still a high number at day 9. The interstitial cell population behaves similarly, that is they are repressed while still in O₂ but show great proliferation when taken out of O₂, reaching a peak at day 9. It would therefore be difficult to conclude from this observation that it is repression of the Type II cell population that causes the interstitial population to increase. What is remarkable however is that the Type II cell population returns to a low level on day 15 in the Cd + O₂ group while the interstitial population remains elevated even at day 15. This could mean that enough Type II cells have then divided and differentiated into Type I to repair the epithelial lining. Knowing the reason for the prolonged elevated interstitial cell population would be one clue in solving the fibrosis puzzle. It does seem that whatever the stimulus is that caused these cells to increase in number must still be present [whether the stimulus be from macrophages, polymorphonuclear leukocyte (PMN), or platelets as has been discussed]. This data does suggest however that it is greater proliferation and persistence of this interstitial (fibroblast) population

which is the dominant cause for the longer persistence of fibrosis in the lungs of Cd + O₂ treated mice compared to that of Cd only treated mice.

One other population of cells that bears discussion is the alveolar macrophage (AM). In cases of acute inflammation, the typical response is an initial phase of PMN exudation, which is superceded by a population of AM. Indeed Asvadi and Hayes (1978) after treating rats to a single 2 hr exposure of 0.1% CdCl₂ found a peak of PMN at 3 days and one of AM at 4 days after exposure but the increase in AM persisted during the repair phase, being elevated at 10 days after injury. They suggested that the PMN wave was a response to the death of a large number of cells in the lung (mainly the Type I alveolar cells).

In our differentiation studies there did not appear to be any dramatic increases in AM during the period of injury, but in the graph representing the number of labelled cells/10,000 lung cells there was a peak of AM on day 5 in the Cd only group. The percentage of macrophages in the Cd + O₂ treated animals was usually higher than in the Cd only treated animals, perhaps related to the greater damage requiring more phagocytosis.

Our microscopic observations of mouse lung at early time periods after Cd exposure have shown an increase in PMN, and this has also been observed in lungs of rats exposed to hyperoxia (Crapo et al., 1980). The PMN may release some factors that would attract the AM to the site of injury to clear up the damage, and this would account for the small peak of AM at day 5 mentioned earlier.

There is however a resident population of AM in the lung and one theory is that the AM may be the reason that the PMN are attracted to the

lung during injury (Hunninghake et al., 1978). Bell et al. (1979), in studying the ultrastructure of macrophages exposed to CdCl_2 , suggested that CdCl_2 may react directly with AM, causing them to release a chemotactic factor which attracts PMN to the air spaces of the lung. These PMN in order to reach the alveoli need to migrate across the pulmonary endothelium. It is postulated that stimulants from AM may cause O_2 radical release by PMN which may damage endothelial cells. This is substantiated by cases of pulmonary edema found in O_2 and Cd toxicity which is probably due to loss of endothelial integrity. There is strong belief that PMN may contribute to O_2 toxicity and there are reports on the release by PMN of O_2 intermediates, such as superoxide anion (Babior et al., 1973), hydrogen peroxide (Iyer et al., 1961), and hydroxyl radical (Repine et al., 1979), which can all cause tissue injury.

The PMN are also thought to release proteolytic enzymes notably elastase, which could degrade elastin in the alveolar wall. Other PMN products such as collagenase and cathepsin G could cause alterations in pulmonary architecture.

Yamada et al. (1982) confirmed the release of elastase from neutrophils migrating into the lung when they compared the elastase content of neutrophils isolated from peripheral blood with that of neutrophils from airways during acute pulmonary inflammation induced by CdCl_2 . They showed that the airway neutrophils contained 40–60% less elastase than peripheral blood neutrophils, so the airway neutrophils must have released their elastase to cause the observed inflammation.

In a study of pulmonary inflammation due to O₂ toxicity, Fox et al. (1981) showed that there was an increase in chemoattractant activity which was closely associated with the increases in the number of PMN recovered in lavages, and the sharp onset of mortality in rats. The source of the chemoattractant has attracted some attention, and some evidence has been found that exposure of AM to hyperoxia *in vitro* stimulated a release of chemoattractant (Harada et al., 1980).

These studies all point to the important role of AM and PMN in lung damage and a better understanding of this function would possibly shed some light on the intricacies of the Cd-induced lung injury.

Another cell population of interest in the cell kinetics study is the endothelial cell population. In the differentiation study it is seen that this cell population does not reach its peak of production until day 9 which is much later than the day 3 seen in Type II cells. An explanation could be that since the exposure is by inhalation, the Type II cells which line the alveoli will be first exposed, while the Cd aerosols have to penetrate the epithelium and interstitium before they can damage the endothelial cells. Thus, since they are damaged later, they will start their proliferation at a later time.

This theory however does not explain why BHT which is bloodborne shows the same pattern as Cd with the peak of Type II regeneration at days 2 to 3 and that of endothelial cells at day 6 while in O₂ toxicity (a gaseous toxicant) the endothelial regeneration precedes the Type II. One other explanation is offered by Kistler et al. (1967) who postulate that in the case of O₂ toxicity to endothelial cells "additional factors" must be present before the O₂ can exert its damage. If these factors are endogenous and conveyed by the

blood, then the endothelial cells of alveolar capillaries would be the site of most "intense interaction" between O_2 from the alveolar air and these factors supplied by the blood. Thus these cells would be injured first and therefore regenerate first.

One conclusion seems to be that different toxicants attack different cell populations, and at various times, and the regenerative patterns also differ. Adamson et al. (1977) in explaining the differences in regenerative pattern following BHT damage and O_2 damage suggest that the result could be related to the severity of the initial damage. In the case of O_2 poisoning, there is early endothelial necrosis while after BHT, the early changes are caused more by increased permeability rather than frank necrosis.

In Strauss et al.'s (1976) study of lung injury by $CdCl_2$ they found there was death of Type I cells on the first day after exposure which triggered Type II cell division which reached a maximum on days 2-4 after the injury. It is not surprising that the Type I cells should be damaged first because its large surface area makes it vulnerable to rapid build up of intracytoplasmic concentrations of the Cd. The Type II cell has a smaller absorbing surface area, and thus may account for the reason there is no damage there. Another suggestion by Strauss for the lack of injury to Type II cells is that these cells are rich in glucose-6-phosphate dehydrogenase which shows that there is a well-developed hexose-monophosphate shunt in the cytoplasm. This pathway is one of the suppliers of the cofactor NADPH, an important contributor to maintaining the tissue concentration of glutathione peroxidase, which provides one of the major means of protection against lipid peroxidation. Thus Type II cells may have some built-in protection against toxicants that produce free

radicals. In summary, some cells are more vulnerable than others because of their shape and size, location in the tissue, or presence or absence of certain protective chemicals in their cytoplasm.

4. Bronchiolar labelling index

No literature reports were found on labelling indexes in the terminal bronchiolar epithelium of mice following Cd injury but some studies have been done on renewal of terminal bronchiolar epithelium following exposure to NO₂ and O₃ (Evans et al., 1976) and to urethane (Kauffman, 1971). Some of these studies were designed to show which are the progenitor cells for the terminal bronchiolar epithelium, whose chief cell type are the nonciliated (Clara cells) and the ciliated cells. The Clara cells as mentioned in Chapter II are the columnar ones with rough endoplasmic reticulum and a large Golgi apparatus and they are concerned with secretory processes, while the ciliated cells are cuboidal with numerous cilia projecting into the lumen of the terminal bronchioles, and their function is to move the mucous layer that covers the surface of the terminal bronchiole toward the trachea and out of the lung.

The results of Evans et al. (1976) suggest that the nonciliated (Clara cells) are the progenitor cells for the terminal bronchiolar epithelium. They found that NO₂ or O₃ damaged the ciliated cells, and the Clara cells then divided, migrated, and matured to form new ciliated and nonciliated cells.

Our study did not differentiate between ciliated and nonciliated cells but rather the LI was determined for all labelled cells of the terminal bronchiolar epithelium. With Cd + air the highest LI was seen on day 1, showing that following damage by the Cd the repair process began very soon

thereafter. The LI's fell on subsequent days until at day 9 they were similar to the control values.

The difference between individual animals was variable and a similar result was seen in the work of Evans et al. (1976) with rats where they found that the average variability in LI between animals at each time interval was approximately 35%.

The peak of the bronchiolar LI with Cd + O₂ at no time attained the peak height of Cd + air, but after day 3 the values were always higher for Cd + O₂ and this persisted until the end of the experiment at 15 days. The pattern for the bronchiolar LI is quite different from that of the parenchymal cells. With the Cd + air animals the peak of cell division in the bronchiolar epithelium is earlier, perhaps because these cells are damaged earlier than alveolar cells (the aerosols would reach the bronchioles before they would arrive at the alveoli), or they may be more sensitive to Cd than are alveolar cells.

With the Cd + O₂ there is an initial delay in damage but there is no dramatic high peak after the animals are taken out of the O₂ at day 6. There is a slight peak at day 7 but only as high as it was on day 3. However, the damage to the bronchiolar epithelium is definitely more severe with Cd + O₂ than with Cd + air as can be seen by weighing the areas under the curve. This was done by tracing from the graph onto standard paper the area under both curves, cutting them out and weighing them on a sensitive balance (Mettler A30). For Cd + air the weight was 0.3132 g versus 0.4888 g for Cd + O₂, showing more severe damage in the latter.

G. Mechanism of Action of Cd

The question of how Cd exerts its toxic action has intrigued researchers for a long time and several theories have been advanced. One theory is that Cd uncouples phosphorylation associated with the oxidation of succinate and of citrate in mitochondria. Jacobs et al. (1956) suggest two schemes to account for this uncoupling. One is that Cd^{++} might displace another cation essential for phosphorylation from its active site, and the other is that Cd^{++} might block a free active site, but more data were required before any conclusions could be drawn.

Cadmium has also been shown to cause significant alterations in the adenosine triphosphatase system, which is stimulated at low Cd concentrations and depressed at high concentrations (Cross et al., 1971).

The effects of Cd on serum α_1 antitrypsin levels have been suggested as one explanation for the Cd injury in lungs. Chowdhury et al. (1982) have shown that there is a link between Cd exposure, serum α_1 antitrypsin level, and the pathologic changes which occur in the lung. Indeed Chowdhury et al. (1983) in a recent paper showed that by suppressing the rat's α_1 -AT system with galactosamine and then treating them with CdCl_2 (i.p.) extensive lung lesions were produced including PMN infiltration, distortion, and distention of alveolar spaces and massive hemorrhages. This suggests that though the α_1 -AT deficiency itself may not be pathogenic, it does increase the lung's susceptibility to tissue damage. It is interesting to note that Cd itself possesses α_1 -AT suppressive ability and is unique in that other metals such as Pb, Hg, Ni, Zn, and Fe do not have this property (Chowdhury and Louria, 1976).

The electron transport system may also be disrupted by the action of Cd. Cd is known to be a dithiol-binding agent and could inhibit alpha-ketoglutarate dehydrogenase activity and the activity of the flavoproteins of the electron transport system by specifically binding to functional thiol groups (Fluharty and Sanadi, 1960). These reactions would cause a major change in cellular metabolism and that might cause cell death or act as a stimulus for cell proliferation.

One other explanation of the toxic effects of Cd is its interaction with nucleic acids. Shin (1973) showed that Cd^{2+} because of its strong affinity for the nucleotide bases of DNA, would bind these bases and mediate the unstacking of the bases and produce the random coil form of the ordered structure of the polynucleotides. Cd^{2+} ions also prevented poly(U) and poly(I) from forming double and triple-stranded helices respectively. The unstacking process if carried too far could possibly lead to cell death.

Much has been said on the subject of cell death, so the question can be asked, what is the actual mechanism of cell death following Cd treatment? Morselt et al. (1983) have shown that chromatin condensation is the first event demonstrable by light microscopy. It precedes membrane leakage and leads to cell death. Chromatin condensation occurred within 15 min to 1 hr after adding 1 $\mu\text{g/ml}$ CdCl_2 to a liver cell culture and was irreversible to the case of CdCl_2 , but not in some other treatments. It is interesting to note in the study of Morselt et al. (1983a) that a small percentage of cells survive the toxic effects of Cd. The existence of Cd resistant cells may be related to an increased content of metallothionein and this could explain the many variations in animal response in that some animals will have more Cd resistant cells than others (or more metallothionein induced when stress is applied).

The role of metallothionein in the development of resistance to Cd has been discussed in Chapter II.

The effects of Cd on the cytoplasm and plasma membrane occur after the ones seen in the nucleus and this is thought to be due to the interval needed for some nuclear components like ribosomal RNA and messenger RNA, after their inhibition, to extend their effects on the cytoplasm (Morselt et al., 1983a). After studying the changes that occur in the cytoplasm of endothelial cells of small uterine vessels and cultured liver parenchymal cells following Cd administration, Morselt et al. (1983b) suggest that a disturbance in ribosomal RNA synthesis is the main factor leading to ultimate death.

Whatever the mechanism, some cells die and some may survive after the Cd insult and one is inspired to seek the answers for survival. Schlaepfer (1971) pointed out that some endothelial cells of the rat spinal sensory ganglia showed great sensitivity to Cd by undergoing severe degenerative changes, while other endothelial cells were more resistant even to high Cd doses. There could be various reasons for this, such as inequalities of the exposed surface membrane area, variations in specific metabolic activities, or qualitative or quantitative differences among constituent elements, for example membrane porosity or intrinsic chelation mechanisms. One sidelight of this is that selective survival and perpetuation of Cd-resistant endothelial cells could lead to the development of tolerance toward a second exposure to Cd.

We still do not have all the answers for the mechanism of Cd damage; nor do we know why one cell population is targeted for injury instead of another, or even why one population, for example the fibroblasts, will proliferate beyond bounds to provide excess collagen (and hence fibrosis), but

we have provided an experimental model for the interactions of two harmful inhalants, namely Cd and O₂, elucidated the behavior of different cell populations following the injury and discussed modifications in response imposed by one agent on another.

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VITA

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