To the Graduate Council:

I am submitting herewith a dissertation written by Louis E. Sendelbach, Jr. entitled "Lung Injury in Mice and Rats Acutely Exposed to Beryllium." 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 Biomedical Science.

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We have read this dissertation
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LUNG INJURY IN MICE AND RATS ACUTELY EXPOSED TO BERYLLIUM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Louis E. Sendelbach, Jr.

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ABSTRACT

The effect of lung injury, in rats and mice, exposed to an aerosol of beryllium sulfate (BE) for one hour, through nose-only inhalation, was evaluated by the methods of bronchoalveolar lavage (BAL) and lung cell kinetics. The BAL in rats, sacrificed over a 21 day period following exposure, showed lactate dehydrogenase (LDH) and alkaline phosphatase (Alk Pase) activities as the most sensitive indicators of lung damage. LDH activity peaked at day 8 while Alk Pase activity peaked at day 5, both being 30 times greater than comparable control values. Acid phosphatase activity and albumin levels were also increased, but not to the same extent as LDH and Alk Pase. The BAL of mice showed LDH activity as the most sensitive indicator of lung damage, with a maximum response 3 times greater than controls at day 5.

Lung cell kinetics in rats showed a maximum value at day 8. Cell labeling was predominated by interstitial cell replication. Cell turnover in mice, reduced in comparison to rats, peaked at day 5 with interstitial cell replication predominating. A second rise in labeling was seen at 21 days, consisting of endothelial cell turnover. Differences in response of the two species to Be toxicity was best explained by the differences in initial dose received to the lung. Comparison of lung lavage parameters and cell kinetics showed a high correlation between the two methods.

In another series of experiments, animals were treated with a known immunosuppressant, Cyclosporin A (Cy A), to deplete T cell dependent responses during the development of lung fibrosis. Rats and mice were treated with three agents capable of inducing fibrosis: beryllium sulfate, bleomycin,

and butylated hydroxytoluene (BHT). Cy A completely inhibited the fibrogenic effects of BHT in mice, as measured through total lung hydroxyproline content. Bleomycin-induced fibrosis was significantly reduced by Cy A treatment in rats, but showed no effect in mice.

Additionally, the effect of iron salt administration to rats decreased the intravenous LD_{50} dose, and significantly reduced the inhalation toxicity, of beryllium sulfate. The protective mechanism of iron salt administration, through the induction of ferritin synthesis, is postulated.

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INTRODUCTION

To charge such an admirable metal with having poisonous properties is about as distasteful as accusing a trusted butler of stealing the family plate. Editorial, The Lancet, 1951.

A. Historical Context

The first reported discovery of beryllium (Be), by Vanquelin in 1797, claimed that the substance was biologically inert and of little medical significance (Berman, 1980). By 1882, Blake (1881, 1882) described this element to have the general physiological properties of aluminum and iron. Brenton and Cash (1884), two years later, reported the toxicity of Be in frogs to be no greater than that of potassium chloride or calcium chloride. Subsequent work, as to the toxic nature of Be, throughout the late 1800's and early 1900's was generally inconclusive. Richter (1930) showed that the subcutaneous injection of beryllium nitrate was lethal in mice while Steidle (1937) concluded that the pharmacological activity of Be was quite unique unto itself.

European literature reports of the late 1930's continued documenting experimental evidence as to the toxic nature of Be. Weber and Englehardt (1933) described patients from a German beryllium extraction plant showing clinical signs of bronchitis, bronchiolitis and pneumonitis with cyanosis. In Russia, workers from extraction plants were found to have similar lesions (Zamakhovskaia, et al., 1934). Morradi-Fabrini (1935) reported the occurrence of similar cases in Italy and attributed the disease to Be, terming it

"berylliosis". During these years, and into the early 1940's, continued descriptions of the diseases associated with Be production were reported in the European literature. Unbelieveably, these reports of Be toxicity in Europe gained very little attention within the scientific and medical community of the United States. Regrettably, it was only until production and usage of Be reached large scale development within the U.S., causing increased exposure and subsequent harm to workers, that the toxic nature of Be was finally recognized.

The outbreak of World War II increased demand for Be. The Manhattan project alone required more Be than total world consumption prior to 1940 (Reeves, 1979). When Be atoms are bombarded with alpha particles, their nuclei disintegrate and yield a profusion of neutrons. Chadwick, in 1933, utilized this reaction to discover the neutron (Reeves, 1979). It was a radium-beryllium source that provided the neutrons which led Fermi to the discovery of uranium fission. Beryllium proved most valuable for slowing down the speed of neutrons and as an excellent neutron reflector. From the time of the early 1940's, it took more than a decade to firmly establish that berylliosis was a compensable occupational disease. It was only when Be production was begun in large scale use in the fluorescent lamp industry in the U.S., that berylliosis was first noted as a disease. The confusion apparently stemmed from the variability in clinical descriptions, now recognized to be the acute and chronic forms of the same disease.

In the early 1940's, zinc beryllium silicate began to be used as an ingredient in the making of phosphors, which give fluorescent tubes their glow. The first reported cases of symptoms experienced with the fluorescent tube

industry were in Ohio in 1943 (Van Ordstrand, et al., 1943). These "Cleveland Cases", as they have now become known as, were essentially chemical pneumonias, due to the inhalation of high doses of soluble beryllium compounds with acidic pH. By 1949, DeNardi (DeNardi, et al., 1949) had reviewed over 400 clinical cases in an eight year period. Over this time, a total of ten deaths had occurred. The fulminating type of pneumonitis observed was the most common form described, developing over a period of many weeks.

At the same time of these cases being reported from plants in Ohio, two patients from a fluorescent lamp plant in Massachusetts were reported with pulmonary problems. Within weeks, one patient had died with postmortem findings suggesting the possible cause of death as Boeck's sarcoid (a disease having no known etiology). Other patients were being observed from the same working environment with the diagnosis of pulmonary sarcoidosis (Shipman, 1950). These "Massachusetts Cases", unlike those reported cases in Ohio, were of a chronic rather than acute form. This form apparently resulted from much smaller exposures to Be than those of the acute onset. In almost every instance, the use of one Be compound, beryllium oxide, was prominent in the manufacturing process. Beryllium oxide is now generally regarded as the sole compound responsible for the chronic disease (Breslin, 1966).

The differences in clinical description reported from the plants in Ohio and Massachusettes led to considerable confusion as to the epidemiology of Be disease. Compounding this confusion were patients reported from so called "Neighborhood Cases", who although were not working in an environment exposed to Be, developed berylliosis. These patients had only the common factor of living near a manufacturing plant or being in close contact with

workers from such a plant. Not all residents living near Be plants developed the disease. In some cases, the number of neighborhood cases even exceeded that reported within the plant. In one case, a woman who had come in contact with contaminated clothing of her husband developed the disease while her husband did not. It seemed that the incidence of disease occurring outside the plant was a function of the beryllium air concentration or contact of Be from the clothes of workers at the plant.

The evidence for the cause and effect relationship of Be developed slowly. Medical diagnosis of the disease was difficult due to the difficult clinical pathology between the two diseases reported. Proper methods for the quantitative determination of Be were not available, thereby preventing the accurate determination of exposure levels. Certain factories, including those whose quality of cleanliness were poor, reported no fatalities while factories with some of the cleanest reported environments had the most cases. In addition to the pulmonary problems, it was soon realized that Be caused granulomatous ulcerations of the skin in some individuals, suggesting an allergic etiology. In 1951, it was postulated that berylliosis was an autoimmune disorder with certain individuals capable of extraodinary sensitivity (Sterner and Eisenbad, 1951).

With the occurrence of the disease becoming more evident, in 1947 the staff of the Saranac Laboratory in New York called for a symposium to discuss all the available information on Be. From this meeting, it was recognized that Be was the cause of a disease that occurred in the lungs in both acute and chronic forms. Later, from the suggestion of those present at

this conference, the Beryllium Case Registry was established in 1952 to review all relevant data and material concerning this disease.

In retrospect, it is difficult to believe that with the preponderence of available evidence as to the toxic nature of Be in the working environment, such a long time would elapse until the recognition by the scientific community of its adverse effects. One must try to understand the problems and confounding information afforded the scientist during these times, and the high regard industry had placed on the use of this "admirable metal". Shipman and Vorwald (1966) have accurately summarized this situation:

> Beryllium disease was born in the early 1940's of the union of industry and beryllium. It was a lusty and very obnoxious baby, hated by all its neighbors. It isn't dead, but it has been forced into an institution where, if the rules are obeyed, it will stay. Some day we shall understand the genetic accident that produced it.

B. Chemical and Physical Properties

Beryllium is one of the lightest elements of the periodic table, being the smallest of the Group II metals. The s-orbital of its outer shell is occupied by two valence electrons with the 3 p-orbitals being vacant. The small radius of the beryllium ion permits unusually close approach of its ion centers. The compounds of Be with the smallest anions; F^{-2}(r=1.36 Angstroms) and O^{-2} (r=1.40 Angstroms) have great stability since the bond strength increases directly as the product of the charges but inversely as the square of the distance between charges (Krejci and Scheel, 1966).

The size constraints which favor the smaller ions of Be limit its ability to form stable compounds with larger anions. The oxide ion, with its high ratio

of charge to radius, forms the most stable bond, with fluorine and oxygen forming the next most stable. Ultimately. the reaction product of any beryllium compound is beryllium oxide, or compounds of beryllium oxide.

Beryllium has such a strong tendency to coordinate with oxygen that beryllium compounds are generally hydrolyzed in solution, with liberation of hydrogen ions. Thus, aqueous solutions of beryllium salts are strongly acidic. Furthermore, coordination of Be with the hydroxyl ion converts the highly insoluble beryllium hydroxide to a soluble anion. In this regard, Be resembles the Group III element, aluminum, with the same high ratio of charge to radius. Beryllium resembles trivalent aluminum so closely that separation of the two elements is often difficult. Some of the physical properties of Be are summarized in Table 1. Beryllium occurs naturally as the ^{9}Be isotope with commercially prepared isotopes of ^{6}Be, ^{7}Be, ^{8}Be, and ^{10}Be available.

The small atomic volume of Be, and the high charge to volume ratio of the atom, are characteristics that affect the behavior of the element to form alloys. Initially, difficulties were encountered in extracting the pure element. The solubility of Be in copper was used in an attempt to extract the metal. Alloys thus formed from beryllium were found to be increased in hardness, tensile strength and resistance to fatigue and corrosion. Subsequent studies found Be to inhibit surface oxidation by diffusing to the surface of the alloy, forming a protective oxide coat on the surface.

Beryllium has the capacity to form complexes with many elements and compounds. Table 2 shows a partial listing of these along with some of their chemical and physical properties. Since this study involved one of the salts of

Table 1

Physical Properties of Beryllium[a]

Property	Value
Atomic Number	4
Atomic Weight	9.013
Atomic Radius	1.123
Atomic Volume(cm^3/mole)25°C	4.877
Electron Configuration	$1s^2 2s^2$
First Ionization Potential (ev)	9.320
Second Ionization Potential (ev)	18.206
Ionic Radius(Be^{+2})in Angstroms	0.31
Electronegativity(Pauling's)	1.5
Density(g/cm^3)25°C	1.8477 ± 0.0007
Melting Point	1283°C
Boiling Point	2970°C

[a]Adapted from Krejci and Scheel (1966).

Be, beryllium sulfate tetrahydrate (hereafter referred to as $BeSO_4$), discussion will be limited to this compound.

Beryllium salts dissociate readily in aqueous solution. At pH values less than 5, Be acts as a cation. With pH values ranging from 5 to 8, Be tends to form insoluble hydroxides, while at values above a pH of 8, beryllium complexes form. In general, at pH 7, the strong acid salts of Be hydrolyze to

Table 2

Chemical Properties of Some Beryllium Compounds

Chemical Name	M.W.	Solubility[a] Cold H_2O	Hot H_2O	Other
Beryllium Acetate	127.1	i		i al,eth
Beryllium Acetate (basic)	406.32	sl d	d	s chl,ac a sl s al,eth
Beryllium Carbonate	112.05	i	d	s a,alk
Beryllium Chloride	79.92	vs	vs,d	vs alc,eth sl s bz,chl
Beryllium Fluoride	47.01	inf	inf	sl s alc
Beryllium Hydroxide		i	i	
Beryllium Oxide	25.01	.002		s conc. acid
Beryllium Silicate	110.11			i a
Beryllium Sulfate	105.07	i		
Beryllium Sulfate Tetrahydrate	177.14	42.5	100	sl s conc a i al

Adapted from Weast (1972).

[a]i - insoluble, al - alcohol, eth - ether, sl - slightly, chl - chloroform, ac a - acetic acid, alk - alkaline, vs - very soluble, sl s - slightly soluble, bz - benzene, con a - concentrated acid, inf- infinite, d - decomposes. Numbers indicate solubility in g/ml.

the same extent at any particular concentraton (Krejci and Schell, 1966).

In the dissociation of water, the equilibrium obtained is expressed in the following reaction:

$$HOH \rightarrow H^+ + OH^-$$

With beryllium salts, the accumulation of H^+ in solution in large quantities can occur only if the OH^- ion concentration is reduced below its normal equilibrium in water.

For a biunivalent salt (e.g., $Be(NO_3)_2$), hydrolysis may proceed by:

$$Be(NO_3)_2 + HOH \rightarrow Be(OH)NO_3 + H^+ + NO_3^-$$

In the case of a bi-bivalent salt (e.g., $BeSO_4$), complete hydrolysis of a large portion of the salt may occurr:

$$BeSO_4 + 2HOH \rightarrow Be(OH)_2 + 2H^+ + SO_4^{-2}$$

Because of the amphoteric character of the Be atom, in aqueous solution its common salts are not neutral. Hydrolysis of Be^{+2} solutions are strongly acidic. Vorwarld, et al., (1966) states that these circumstances have a number of important implications with respect to biological systems, and are briefly summarized as follows:

1. Solutions of beryllium sulfate, chloride, or nitrate have a strongly acidic character capable of causing tissue injury by virtue of the pH effect alone. Such is the effect on mammalian erythrocytes and pulmonary tissue causing the chemical pneumonitis which may lead to the rapid death of the experimental animal.

2. Continuing hydrolysis of dissolved beryllium salts causes these solutions to have a high buffer reserve. Hydrogen ions consumed in the course of neutralization are constantly replenished by a shifting ion equilibrium, making the acidity of Be^{+2} solutions more persistent and damaging to tissues than a solution of hydrochloric acid of equal pH.

3. Due to its acidity, Be^{+2} administered to an animal will either overwhelm the buffer systems of the organism or be overwhelmed by them. Overwhelming the buffering capacity will inevitable kill the animal. Overwhelming the Be administered by the buffering systems of the organism will cause *in situ* precipitation of Be, allowing development of the chronic effects of the challenge.

Krejci and Scheel (1966) emphasize the importance of the relationship between the hydrogen ion, beryllium ion and water molecules as essential for an understanding of the biological interactions that may take place within body tissues. The removal of hydrogen ions in the living cell is provided for by the buffering system of proteins, rapid combination with bicarbonate and excretion with organic acids and phosphate salts. The inhibition of hydrolysis of such salts as those of beryllium by hydrogen ions can not occur, therefore, the complete hydrolysis of Be salts must occur. Be must exert its effects in the body at some stage as a hydrolytic product or as part of a hydrolytic complex. Beryllium salts hydrolyze at body pH to first form basic salt ions or complexes, and then insoluble $Be(OH)_2$.

C. Analysis

Three general procedures are used for the detection of trace quantities of Be; colorimetric, fluorometric and spectrographic.

Beryllium forms color reactions with dyes, but in most cases the reactions are not specific (Reeves, 1979). Reagents such as alkanin, naphthazarin, naphthachrome Green G and chrome azurol S were widely used

at one time. These methods allow the determinations of Be with a limit of detection from 0.05 to 0.2 ug Be/ml (Stokinger, 1958). Fluorescent methods utilizing the dye morin, yield levels of detection ranging from 0.001 to 0.025 mg/l of pure solutions.

Spectrographic methods are often the method of choice. Trace quantities of Be can be determined with little interference in biological specimens (Reeves, 1979). As little as 0.0005 ug of Be may be detected. More recently, gas chromotography coupled with a mass spectrometer allows the analysis of quantities in the range of 0.04 to 10 pg Be/sample (Reeves, 1979).

D. Source, Production and Use

Beryllium, discovered in 1798 by Vanquelin as a constituent of the mineral beryl, was isolated in 1828 by Wohler and by Bussy through the reduction of beryllium chloride with potassium (IARC Monographs, 1972). Most Be is present in localized deposits of beryl (Reeves, 1979). The gemstones emerald and aquamarine are colored variants of beryl.

Fluoride and sulfate processes are used to convert beryl to BeO. The fluoride process involves the formation of a berylsodium ferric fluoride mixture, followed by precipitation of $Be(OH)_2$ and igniting the precipitate to form BeO (Stokinger, 1958). The sulfate process involves melting the beryl at 1625°C, quenching in cold water, leaching with strong sulfuric acid, extracting $BeSO_4$, followed by precipitating beryllium as the hydroxide and igniting to form BeO (Stokinger, 1958).

Beryllium sulfate tetrahydrate has been produced commercially in the U.S. since the 1920's. Beryllium sulfate tetrahydrate was first produced by Vanquelin in 1798 (Mellor, 1946). It is produced by fractional distillation from a beryllium sulfate solution prepared by the reaction of beryllium hydroxide with sulfuric acid. Beryllium sulfate tetrahydrate is used as a chemical intermediate in the processing of beryl (Ballance, 1978).

Beryllium has been in significant industrial use since 1920 with U.S. production begun commercially since the 1940's (IARC Monographs, 1972). U.S. consumption of Be began to decline by 1974, attributed to reductions in defense and aerospace programs. Total world production in 1979 was 10,000 tons (Reeves, 1979).

Most Be produced is used as a hardening agent in alloys. Be-Cu alloys are used in parts subject to abnormal wear such as non-sparking tools, welding components, and bearing sleeves. Beryllium, added to zinc with other metals, confers on these alloys a higher resistance to corrosion and a higher tensile strength. Beryllium nickel alloys have high tensile strength and greater hardness than Be-Cu alloys. Be-Ni alloys are used in diamond drill bit matrixes, watch wheel balances and aircraft and spacecraft parts. Applications of the free metal include; missile and nuclear reactor components, rocket nozzles, aircraft brakes, electrical relays, space vehicle re-entry cones and x-ray windows. An early major use of Be was as beryllium phosphors in the production of fluorescent tubes. This use was discontinued in the U.S. and other countries in the early 1950's.

E. Toxicity

Perhaps the earliest reported study of Be toxicity *in vivo* was that of Brenton and Cash in 1884 (Brenton and Cash, 1884). The authors reported that beryllium chloride was no more toxic to frogs than potassium chloride or calcium chloride. Two years later, Siem reported the first evidence of Be toxicity, showing adverse affects in dogs, cats and rabbits following intravenous and subcutaneous injections of beryllium lactate and tartate (Siem, 1886). By 1930, Richter reported that the subcutaneous injection of 50 mg/kg of beryllium nitrate was lethal in mice (Richter, 1930). Inhaled beryllium oxide exposure to guinea pigs resulted in no observeable pathology while inhaled beryllium fluoride caused acute pneumonitis (Weber and Englehardt, 1933). The conclusion at this time was that the toxicity was due to the fluorine component, rather than to Be. Marradi-Fabroni (1935) later disputed this view, showing pneumonitis in guinea pigs following administration of beryllium carbonate free of any fluorides, naming the response "berylliosis".

Numerous accounts of the harmful effects of Be became available in the 1940's with the increased concern of Be toxicity in the working environment. Much of the early experimental work regarded the inhalation toxicity of Be compounds. The acute form of berylliosis was readily reproduced. Sprague, in 1947, exposed dogs, rabbits, rats, guinea pigs, mice and hamsters to $BeSO_4$ dusts at a concentration of 83.3 mg $BeSO_4/m^3$ (Sprague, 1947). Rats were also exposed to the metal fume yielding a concentration of 800 mg Be/m^3. The results showed a variation in toxicity among the different species with mice

being the most sensitive. Acute damage was observed in the lungs, liver and kidneys.

Stokinger (1958) described the acute inhalation effects of beryllium sulfate mist in rats, mice, monkeys, dogs, cats, goats and chickens. Four separate concentrations of the mist were used: 1, 10, 50 and 100 mg $BeSO_4/m^3$. Control animals were exposed to the inhalation of a sodium sulfate mist. Again, as in the study of Sprague, the response among species varied with rats being the most susceptible, showing an LC_{50} at 10 mg $BeSO_4/m^3$. The authors concluded that an acute pulmonary pneumonitis was produced which closely resembled that seen in man. Yet, even after 3 months of exposure, there was no evidence of the chronic granulomatous pneumonitis seen in humans.

Hall, in 1950, produced acute pneumonitis in animals by exposure to insoluble Be compounds (Hall, et al., 1950). Six species of animals were exposed to the inhalation of beryllium oxide dusts at a mean concentration of 85 mg BeO/m^3. Histological lesions were produced solely in the lungs and attributed to the toxic agent.

In 1953, Vorwald, et al., exposed albino rats to the inhalation of beryllium sulfate aerosols at 194, 42, 21 and 2.8 ug Be/m^3 (Vorwald, et al., 1953). Exposures were for 7 days a week, up to 560 days. At 42 ug Be/m^3, a chemical pneumonitis was produced. The higher dose caused a high acute toxicity while lower doses produced little significant inflammation. After 3 months of exposure at 42 ug Be/m^3, animals sacrificed showed subpleural foci consisting of macrophages grouped in 4-6 alveoli with thickened walls. By 6 months of exposure, these focal lesions had progressed into a granulomatous

response. The pneumonitis had worsened, being predominantly interstitial with proliferation of histiocytic elements and lymphocyte infiltration. Multi-nucleated giant cells were seen with occasional crystal-like structures within the alveolar walls. Gradually, rats exposed to $BeSO_4$ showed an epithelial proliferation ultimately resulting in malignant pulmonary cancer. This was the first reported instance of malignancy in test animals exposed to any beryllium compound.

The chronic form of the disease, i.e., the granulomatous reaction, has been reproduced with only partial succes in experimental animals. As previously mentioned, Vorwald (1953) reported the occurence of granulomata in lungs of rats continually exposed to an aerosol of $BeSO_4$. Policard (1950) reported the formation of granulomas in the lungs of guinea pigs following inhalation and intratracheal exposures to BeO. Elias, et al., (1981) produced histological findings, similar to the human respiratory disease, in outbred guinea pigs following intratracheal instillation of 0.05 M $BeSO_4$, buffered to a pH of 5. More recently, Barna, et al., (1984) observed the granulomatous disease in guinea pigs by the intratracheal injection of 5 mg BeO.

The toxic effects of beryllium and its compunds have been widely studied. Table 3 presents a short summary of some of these results. The toxicity of Be compounds appears to be related to their solubility, with the more soluble forms being the more toxic.

F. Metabolism

The distribution pattern of Be within the body is dependent on the solubility of the compound and the route of administration. Dogs administered

Table 3

Toxicity of Beryllium Salts[a]

Compound	Species	Route[b]	LD_{50}	Reference
Beryllium fluoride	Mice	oral	100	c
	Rats	oral	100	c
	Mice	s.c.	20	c
	Mice	i.v.	1.8	c
	Hamsters	i.p.	20	d
Beryllium hydroxide	Rats	i.v.	3.8	c
Beryllium chloride	Mice	i.m.	12	c
	Rats	oral	86	c
	Rats	i.p.	4.4	c
	Guinea Pig	i.p.	50	c
Beryllium sulfate	Rats	oral	80	c
	Mice	oral	80	c
	Rats	s.c.	1.5	c
	Mice	s.c.	1.5	c
	Rats	i.p.	18	c
	Rats	i.v.	7.2	c
	Monkeys	i.v.	0.6	c
	Rabbits	s.c.	1.5	c
Beryllium phosphate	Rats	oral	82	c
	Rats	i.v.	4.2	c
	Mice	i.v.	1.4	d
Beryllium carbonate	Guinea Pig	i.p.	50	c
Beryllium sulfate tetrahydrate	Mice	i.v.	.265	d
		i.p.	10	e
	Rats	i.v.	0.4	e
		i.p.	5	e
		i.t.	1	e
	Rabbits	i.p.	.1	e

[a]Values given are mg/kg body weight.

[b]s.c.-subcutaneous, i.v.-intravascular, i.p.-intraperitoneal, i.m.-intramuscular, i.t-intratracheal

[c]Luchey and Venugopal (1977).

[d]Vacher, et al., (1973).

[e]Hodge, et al., (1947).

an aerosol of $BeSO_4$ for 100 days at 40 ug Be/m^3 accumulate 200 times more Be in the lung than in the kidney, and 20 times that found within bone (Stokinger, et al., 1950a). Administration of BeF_2 to dogs showed similar distribution after 87 days at 100 ug Be/m^3 (Stokinger, et al., 1953). The lungs contained 40 times the amount of Be found in the kidney and 4 times that in bone. In rats exposed to $BeSO_4$, pulmonary Be concentrations reached a plateau after 36 weeks of continual exposure (Reeves, 1979). Reeves and Vorwald (1967) demonstrated the clearance of Be from the lungs of rats exposed to a $BeSO_4$ aerosol of 34 ug Be/m^3. The authors reported that Be was cleared from the lungs in a multiphasic response. After discontinuation of exposure, half the dose remained in the lungs after two weeks with a smaller percentage remaining for many months.

Intratracheal injection of ^7Be- beryllium chloride, administered in a non specified carrier, showed a pulmonary half-life of 20 days (Kuznetsov, et al., 1974). Administration without carrier showed a half-life of 24 hours. Fifteen minutes following i.v., i.m., and i.p. injection of carrier free ^7Be-beryllium chloride, Bugryshev, et al., (1976) demonstrated the highest concentrations of Be to be found within the skeleton (50%) with liver (10%) and spleen (10%) being the next highest. Fifteen minutes following intratracheal administration, 72% of the total amount of Be injected was found in the lungs and 36% within the skeleton. Twenty percent of the radioactivity remained within the lungs after 94 days.

Van Cleave and Taylor (1955), administering $BeSO_4$ to rats, showed 70% of the initial dose, delivered by intratracheal administration, to remain within the lungs at 16 days. Half the dose, after 16 days was eliminated

through the feces. Clearance of Be from the lung was presumably due to migration, and diffusion, of phagocytes through the lymphatics to the peritracheal nodes.

The intravenous injection of Be, and Be compounds, cause liver toxicity. Scott (1948) reported mid-zonal focal necrosis of the liver following an LD_{50} dose of 0.36 mg Be/kg. Beryllium metal, when injected i.v. into rabbits at 40 mg Be/animal, resulted in 19 deaths out of 24 animals within one month due to acute liver necrosis (Barnes, 1950). Vacher,et al., (1973) showed that beryllium phosphate blocks the functioning of the reticuloendothelial system of mice following i.v. injection of 12 ug Be/kg. Beryllium sulfate exerted the same effect at 75 ug Be/kg. Skilleter and Price injected rats with beryllium phosphate, at 12.5 - 520 umole Be/kg, or beryllium sulfate at 12.5 - 85 umole Be/kg (Skilleter and Price, 1978). Beryllium phosphate was removed from the blood predominantly by the non-parenchymal sinusoidal cells and later distributed to the parenchymal cells.

The distribution of Be following intravenous administration appears to be dose dependent (Reeves, 1979). Two and one-half hours following i.v. injection to rats of 50 ug Be/kg, Be accumulation occurred within the skeleton, while at dosages of 500 ug Be/kg, Be preferentially located to the liver. Stokinger notes that the distribution pattern of intravenously injected ^{7}Be differed depending upon whether a carrier was present or not (Stokinger, 1953).

Oral administration of Be will induce hemorrhagic necrosis of the gastric mucosa, but the metal is poorly absorbed. Repeated administration of Be in the diet will ultimately result in a rachitic condition in rats (Stokinger,

1958). Absorption of Be by the gastrointestinal tract is minimal. Following administration of $BeSO_4$ at 0.16 and 1.66 mg Be/ml in the drinking water, rats absorbed only 20% with 60- 80% passing unabsorbed through the feces (Reeves, 1965). Following absorption, transport of Be is thought to be mainly in the form of a colloidal phosphate, absorbed on plasma alpha- globulin (Reeves and Vorwald, 1961; Vacher and Stoner, 1968a).

Because of the colloidal binding of Be within the plasma, Be is only partially excreted in the urine. Reeves (1965) and Van Cleave and Taylor (1955) showed 20-60% of intratracheally injected Be to be excreted in the urine. Since Be is bound to colloids within the plasma, Be does not cross the glomerular membrane and is excreted via the tubules.

G. Mechanism of Action

The biochemical basis for Be toxicity has been studied for many enzyme systems. In 1949, it was discovered that Be is one of the strongest inhibitors of alkaline phosphatase (DuBois, et al., 1949; Grier, et al., 1949). Noncompetitive inhibition of alkaline phosphatase was measured at 10^{-7} M, being maximal at 10^{-5} M. Beryllium also inhibits a number of other Mg^{+2} or K^{+} activated phosphatases besides alkaline phosphatase; phosphatidic acid phosphatase (Hakin, et al., 1963), adenosine triphosphatase (Cochran, et al., 1951) and beta- glycerophosphatase (Naido and Pratt, 1953). Beryllium was also found to inhibit other enzyme systems; phosphoglucomutase (Cochran, et al., 1951), phosphoglyceromutase (Thomas and Aldridge, 1966), hexokinase and deoxythimidine kinase (Mainigi and Busnick, 1969), lactate dehydrogenase

(Schormuller and Stan, 1965), malic, succinic and alpha ketoglutaric dehydrogenases (Mukhira, 1967), amylase from pancreatic tissue, saliva, serum and urine (McGeachin, et al., 1962), nonspecific esterases (Mietkienski and Malendowicz, 1967) and enolphosphopyruvate hydrolase (Malmstrom, 1955). Tepper (1972) concludes that the mechanism of inhibition of enzyme systems by Be remains unclear, and that although Be may exert an effect on a number of enzyme systems, only alkaline phosphatase and phosphoglucomutase appear to be directly acted upon by Be.

Labeled Be in rat liver cells following intravenous injection was deposited with high specific activity within lysosomes (Witschi and Aldridge, 1968). Purification confirmed the deposition of Be within lysosomes. This uptake of Be by lysosomes is most likely nonspecific, since circulating Be is in the form of phosphate associated with serum globulin. The mechanism of lysosomal uptake is most probably the same as that of many colloidal compounds (Tepper, 1972). It is not known if the toxic action of Be reflects a release of lysosomal enzymes secondary to damage of the lysosomal membrane. Beryllium possibly exerts its cytotoxic effects at sites outside of the lysosome. Dinsdale speculates that Be may be largely nontoxic until degraded within the environment of secondary lysosomes (Dinsdale, 1982). Death of the cell may then result from liberation of Be from the distended membranes of these organelles.

Beryllium accumulated in the nuclear fraction of cells following administration (Witschi and Aldridge, 1968). While the precise nuclear structure to which Be is attached is not yet identified, it has been shown that Be inhibits DNA synthesis (Witschi, 1970) and the synthesis of nucleolar

proteins (Bassleer, 1965). RNA and protein synthesis are not adversely affected (Witschi and Aldridge, 1967; Witschi, 1968). Beryllium diminishes the increased response of thymidine kinase and DNA polymerase in regenerating rat liver (Witschi, 1970). Here, the author concludes that since DNA polymerase and thymidine kinase have different locations within the cell (the former in the nucleus with the later in the cytoplasm) and since the nucleus had ten times as much Be as the cytoplasm, direct enzyme inhibition would not explain the cytoplasmic enzyme being more inhibited relative to controls. Witschi (1970) concludes that Be probably influences events leading to DNA synthesis. Perry, et al., (1980) provided more direct evidence that Be may exert its toxic effects by interfering with regulatory mechanisms controlling transcriptional events in gene regulation.

Skilleter and Price (1980) speculate that the affinity of Be for cell nuclei may reflect a redistribution during homogenation. Dinsdale concludes ". . . . the localization of this element within the intact cell remains a major prerequisite for an understanding of the mechanism of its toxicity." (Dinsdale, 1982).

H. Scope of the Study

It is the purpose of this study to examine, in further detail, the toxic effects of Be within the lung following acute exposure. Although Be remains one of the most toxic substances known, the mechanism of its action remains unclear.

In Chapter 1, the fibrogenic effects of $BeSO_4$ within the lung are examined. Beryllium compounds have been reported to cause increase collagen deposition within the lung following administration. These reports are largely histological, thereby being qualitative rather than quantitative in description. The fibrogenic response can be quantitatively measured by the assay of hydroxyproline, an amino acid present virtually only in the collagen molecule. Chapter 1 reports the induction of fibrosis within the lungs of experimental animals through inhalation exposure of beryllium sulfate.

Many causes for the induction of fibrosis have been postulated. The histological sequelae seen throughout fibrosis shows an early influx of inflammatory cells to precede the increased accumulation of collagen. Many of the cells responsible for this early inflammation have been implicated in the fibrotic response which follows. One of these cells, the T lymphocyte, is examined in Chapter 1. Through the use of a known immunosuppressant, Cyclosporin A, the response of the T cell is blocked. The extent of the fibrotic reaction is then measured following administration of $BeSO_4$, bleomycin and butylated hydroxytoluene, all of which are known agents capable of inducing fibrosis in experimental animals. Chapter 1 reports measurements of the fibrotic response of these agents following Cyclosporin A treatment and speculates as to the involvement of the T cell in the process.

Chapters 2 and 3 deal with quantitative measurements of lung damage and repair following $BeSO_4$ insult. Chapter 2 reports the measurement of lung repair in rats and mice by the measurement of lung cell kinetics. In Chapter 3, the biochemical response of the lung lavage fluid is measured for these two species. The technique of bronchoalveolar lavage has been recently used to

evaluate lung damage following toxic injury. Both the techniques of bronchoalveolar lavage and cell kinetics have been shown to be valuable tools in understanding lung repair following pneumotoxicant injury.

Chapter 4 describes the protective effect of iron salt administration upon the toxicity of $BeSO_4$ in rats. Ferritin, generally recognized as an iron storage protein within the body, has been shown to strongly bind Be *in vitro*. Administration of iron induces *in vivo* ferritin synthesis and may then protect animals from the toxic effects of $BeSO_4$ when given i.v. or through nose-only inhalation.

CHAPTER 1

AFFECT OF CYCLOSPORIN A ON BERYLLIUM SULFATE, BUTYLATED HYDROXYTOLUENE AND BLEOMYCIN - INDUCED LUNG FIBROSIS

A. Introduction

Interstitial Pulmonary Fibrosis (IPF) is characterized by an increase, and rearrangement, of interstitial collagen and by changes in lung parenchymal cell architecture. The normal pulmonary epithelium is replaced by an abnormal and distorted accumulation of fibroblasts and the collagenous extracellular matrix produced by these cells (Snider, 1983).

The process of IPF often begins with edema, hemorrhage and a cellular infiltration of neutrophils. This neutrophilic response diminishes with time and is replaced by increased numbers of alveolar macrophages and lymphocytes. Injury of Type I epithelial cells and capillary endothelial cells is also observed. Type II pneumocytes differentiate and divide to repair the epithelial lining. Alveolar walls become thickened and the granulation tissue formed encroaches into adjacent airspaces. Ultimately, connective tissue begins to appear in the alveolar walls with many alveoli becoming partially or totally obstructed.

Many agents have been shown to cause IPF: inhaled inorganic dusts such as silica (Heppleston, 1969); inhaled gases such as ozone (Last, et al., 1979) and oxygen in high concentrations (Chvapil and Peng, 1975; Katzenstein, et al., 1976; Zapol, et al., 1979); ionizing radiation (Gross, 1977; Hakkinen, et al., 1982); and cytotoxic drugs (Hakkinen, et al., 1983; Schoenberger, et al., 1984).

In the fibrotic process, much attention has been paid to those cells which constitute the initial alveolitis reaction: namely macrophages, neutrophils and lymphocytes.

Macrophages are pluripotent cells with phagocytosis as only one of their many functions. Macrophages, when activated, have been shown to produce a number of chemicals which influence fibroblast activity. Tsukamoto, et al., (1981) have shown that activated macrophages produce fibronectin, a glycoprotein that can stimulate the migration of fibroblasts. Activated alveolar macrophages, *in vitro*, have been shown to produce growth factors for fibroblasts (Bitterman, et al., 1982). Interleukin 1, a nondialyzable factor derived from human mononuclear cells with thymocyte proliferation activity, has also been shown to stimulate fibroblast activity in culture (Schmidt, et al., 1982). Alveolar macrophages from humans with idiopathic pulmonary fibrosis and sarcoidosis have been shown to secrete a growth factor which stimulates human lung fibroblasts to replicate (Bitterman, et al., 1981). Alveolar macrophages not only secrete factors that influence fibroblast regulation, but also release chemoattractants for polymorphonuclear leukocytes (Hunninghake, et al., 1978; Valone, et al., 1980).

The second major cell type involved in the initial alveolitis, the polymorphonuclear leukocyte (PMN) has been studied in the involvement of the induction of fibrosis as well. Rats were depleted of PMN's by treatment with antineutrophil serum for one week (Thrall, et al., 1981). This was followed by the induction of fibrosis by the intratracheal administration of bleomycin. Neutrophil depletion resulted in an increase in total lung collagen content at one week. The authors speculated that neutrophils may play a role

in regulating collagen levels either at the synthesis or degradation stage. Since these cells have been shown to contain high levels of collagenase, the breakdown of collagen may be impeded in the absence of neutrophils.

Phan, et al., (1983) further explored the possible role of neutrophils in the fibrotic response of bleomycin in the beige mutation mouse. This mutation is known to impair certain neutrophil functions. After a single intratracheal instillation of bleomycin, beige mice showed a higher rate of lung collagen synthesis and a higher total lung collagen content than the controls. Beige mice were found to be defective in the release of hydrolytic enzymes from neutrophils while the ability to produce oxygen free radicals remained unimpaired. The inability to degranulate hydrolytic enzymes did not reduce the fibrotic response but did instead enhance the repsonse. The authors suggested a protective role for these hydrolytic enzymes of neutrophils and furhter suggested an important role of oxygen free radical production in the regulation of the fibrotic response. The toxic oxidant molecules generated by these cells seemed to be more important in fibrogenesis than the proteolytic enzymes.

The third major cell type seen in the initial alveolitis in fibrosis, the lymphocyte, is the topic of this chapter. Human IPF is often associated with autoimmune disorders (Snider, 1983). This correlation of IPF and immune function has been demonstrated in experimental animals following bleomycin administration. Treatment of animals with the corticosteroid, methylprednisolone, decreased lung collagen synthetic rates in bleomycin treated rats as opposed to bleomycin treated controls (Phan, et al., 1981). Methylprednisolone also reduced lung collagen synthetic rates in rats exposed to ozone (Hesterburg and Last, 1981). Methylprednisolone, like all

corticosteroids, is known for its antiinflammatory and immunosuppressive properties. Quantitative information of the effects on lung macrophages or lymphocytes is not reported in these studies and the immunosuppressive effects are conjectoral. Prednisolone, given in large doses, prevented to some degree, the development of fibrosis in mouse lungs (Hakkinen, et al., 1983). The authors postulated that prednisolone was acting in some manner to inhibit the proliferation of fibroblasts and the synthesis of collagen from these cells. The possibility of immunosuppressive effects following corticosteroid treatment is not presented.

Thrall, et al., (1979) provided more direct evidence for the involvement of lymphocytes in bleomycin-induced fibrosis. Bleomycin treated rats receiving antilymphocyte globulin, showed a 50% decrease in lung collagen compared to untreated controls. Thrall, and associates, (1980) further studied the affect of the T-lymphocyte in bleomycin-induced fibrosis in thymectomized rats. In this study, lung collagen was decreased in thymectomized animals treated with bleomycin as compared to normal animals. Recently, Schrier, et al, (1983) treating athymic nude mice with bleomycin, reduced lung collagen versus normal (heterozygous) controls (Schrier, et al., 1983). In all of these studies, the histological appearance between bleomycin treated and bleomycin control animals was similar. Only the quantitative determination of total lung collagen showed significant changes.

Since many studies have investigated the involvement of the T-lymphocyte in IPF, this chapter investigated the role of lymphocytes in lung fibrosis. It has been established that Cyclosporin A (Cy A) suppresses T helper lymphocytes, blocking the release of interleukin-2, thereby preventing primary

and terminating secondary T-dependent responses (Borel and Lafferty, 1983). Animals were depleted of T-cell dependent responses through the use of Cy A, to investigate the importance of T-cells in the development of IPF by three agents known to induce fibrosis; bleomycin, butylated hydroxytoluene (BHT) and beryllium (Be).

B. Materials

1. Animals

Specific pathogen-free male Fischer 344 rats, 6 weeks of age, were obtained from Charles River Laboratory (Kingston, N.Y.). Male Balb/C mice, 20-30 g, were from the breeding colony maintained in the Biology Division of the Oak Ridge National Laboratory. All animals were group housed in plastic shoebox cages with hardwood chip bedding (four rats or ten mice to a cage) and were placed on study at 10 weeks of age. Fluorescent lighting was maintained on a 12 hour on/off cycle. Food and water were provided, *ad libitum*, in pellet form (Purina Rodent Laboratory Chow #5001, Ralston Purina Company, St. Louis, MO) with the water chlorinated at a concentration of 16-18 ppm. Temperature was maintained at 72 ± 2° F and humidity monitored at 55 ± 5%.

2. Chemicals

Beryllium sulfate tetrahydrate was purchased from Fisher Scientific Co. (Fair Lawn, NJ), butylated hydroxytoluene (BHT), chrome azurol S, and para-dimethylaminobenzaldehyde from Sigma Chemical Co. (St. Louis, MO),

Chloramine T from Kodak (Rochester, NY), Metofane from Pitman-Moore, Inc. (Washington Crossing, NJ), bleomycin sulfate was a gift from Bristol Laboratories (Syracuse, NY) and Cyclosporin A was generously donated by Sandoz, Ltd. (Basel, Switzerland). All other chemicals were reagent grade.

C. Methods

1. Nose - Only Inhalation Exposure to Beryllium

The inhalation chamber used for the nose only exposure of animals was that described by Hakkinen, et al., (1983a). The chamber apparatus was rectangular in shape with external dimensions of 50 cm in width, 10 cm in depth and 40 cm in height. On each side of the chamber were 20 exposure ports into which the animal holding tubes were placed. Incoming air flowed from the upper cone (at a 60° angle) into the rectangular exposure area making the aerosol mixture accessible to each animal as it breathed. From here, the aerosol mixture was forced through a baffle plate at the bottom of the exposure area and passed through two separate filters before entering an exhaust port.

Rats were placed into plexiglass tubes 21 cm long, 6.5 cm in outer diamater, 5.9 cm in inner diameter with a wall thickness of 0.3 cm. These tubes were opened at one end to insert the animal while the opposite end contained a small opening through which the animal's nose protruded. A paper clip was positioned under the upper incisors of the rat and bent to hold the animal in place during the exposure period.

The entire exposure apparatus was contained within a fume hood to prevent any accidental exposure of aerosol to personnel. The $BeSO_4$ aerosol was generated using a Collison Nebulizer (BGI, Inc., Waltham, MA) at a concentration of 30% (w/v) $BeSO_4$. The airflow generated by the Collison Nebulizer was maintained at 46 l/m. This was then diluted with air at a flowrate of 14 l/m before being passed to a charge neutralizer of ^{85}Kr to prevent electrostatic precipitation of aerosol particles. At this point, the aerosol entered the inhalation chamber for exposure to the animals. Air was forced through the system by the combined effects of air entering through the Collison Nebulizer and dilution flask at a total flowrate of 60 l/m.

A vacuum pump at the end of the line pulled the aerosol mixture from the chamber through a series of two separate filters. The first filter the aerosol encountered was a Grade DX Balston Type 92 Microfiber Coalescing Filter (Balston Filter Products, Lexington, MA) which stopped greater than 95% of all particles. After passing through this first roughing filter, the chamber air then entered a second final filter (Heppa) which effectively trapped all remaining particles before being passed to an exhaust port.

After the animals were placed within individual holders, they were secured into the exposure ports of the chamber. When all animals had been loaded into the chamber, the vacuum pump was turned on. The valves for the air inlets to the Collison Nebulizer and the Dilution Flask were opened. The fume hood was closed and the chamber was run for approximately 20 minutes. This allowed ample time for the chamber to reach maximum (99%) concentration according to the equation:

$$t(99)=4.6\ V/L$$

where V is the volume of the chamber (liters), L is the airflow through the chamber (liters/minute) and t(99) is the time (minutes) for the chamber to reach 99% total concentration (MacFarland, 1976).

For sampling the chamber atmosphere, after reaching 99% maximum concentration, a cascade impactor (Type D7, Aries Inc., Davis, CA) was placed into one of the exposure ports kept vacant for this purpose. A vacuum line was connected to the impactor with airflow at a constant rate of 0.675 l/m over the entire sampling period. All animals were exposed for one hour following the time required to reach 99% maximum concentration. Negative pressure was maintained within the chamber at 0.5 psi during the entire procedure to prevent any unwarranted leakage of aerosol.

Following the one hour exposure, airflow to the Collison Nebulizer was discontinued. Negative pressure was maintained and airflow through the chamber was continued for an additional twenty minutes to flush out remaining test material.

2. Treatment of Animals with Cyclosporin A

Cy A dissolved in olive oil, or olive oil alone, was administered orally by gavage for three consecutive days prior to bleomycin, BHT, or beryllium sulfate administration, and each day thereafter until animals were sacrificed. The dosages of Cy A were based on previously described effective levels, which differed according to species and duration of study (Thomson, et al., 1981; Whiting, et al., 1983).

3. Beryllium Sulfate Administration

Beryllium sulfate was administered to rats for one hour via nose-only administration, as previously described. A 30% (w/v) aqueous solution of $BeSO_4$, with a final pH of approximately 2, was used to generate the beryllium sulfate aerosol. As controls, animals were exposed to an aerosol of H_2SO_4 with identical pH. The beryllium concentration from each stage of the cascade impactor was assayed by the methods of Wood (1950). The Be chamber concentration was determined as 3.3 ug Be/L with a particle mass median aerodynamic diameter of 1.95 with a geometric standard deviation (σg) of 1.38. All animals were sacrificed 21 days following exposure. Cy A was administered at 10 mg/kg.

4. Intratracheal Administration of Bleomycin

Rats, under light Metofane anesthesia, were placed on a stainless steel holder, tilted at an angle of approximately 60 degrees, and secured to prevent movement. One-half an ml of air was drawn through a 21 gauge blunted stainless steel gavage needle into a 1 ml plastic syringe. The remaining 0.5 ml was filled from a solution of bleomycin. This 0.5 ml aliquot was injected into the trachea, through the oral cavity, followed by the 0.5 ml of air to ensure complete administration. Animals were revived following instillation in an upright position, placed back into cages and sacrificed later for total lung hydroxyproline analysis.

Mice received the same treatment, with the exception that an incision was made through the ventral surface of the neck to expose the trachea.

Bleomycin was administered directly into the trachea from a 1 ml plastic syringe by perforating the trachea with a 25 gauge needle. The incision was closed by a stainless steel surgical clip and the animals were placed back into their cages until the day of sacrifice.

5. Bleomycin Administration

Bleomycin-treated rats received an intratracheal dose at 1.5 units/animal. Controls received isotonic saline. Cy A treatment consisted of a dosage of 10 mg/kg. Mice received bleomycin at 4 units/kg with Cy A treatment at 60 mg/kg. Both rats and mice were sacrificed 21 days following bleomycin administration.

6. BHT Administration

BHT, dissolved in corn oil, was injected intraperitoneally into mice at a dosage of 400 mg/kg. Control animals received corn oil alone. Cy A treatment was administered at 125 mg/kg until sacrifice at 14 days post-treatment.

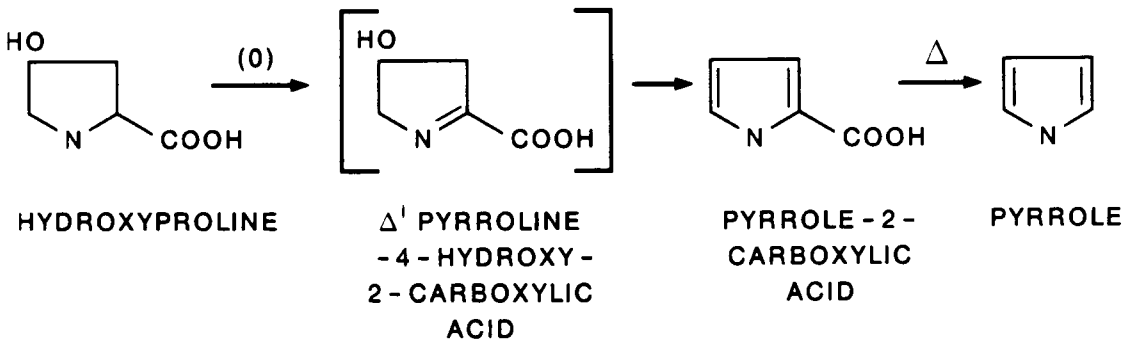
7. Total Lung Hydroxyproline Analysis

On the day of sacrifice, animals were anesthetized with Metofane and sacrificed by exsanguination via the abdominal aorta. The thorax was opened, the lungs exposed and perfused *in situ* with isotonic saline through the right ventricle of the heart. Following excision, the lungs were lyophilized for 48 hours and assayed for total lung hydroxyproline according to Lindenschmidt and Witschi (1985). This method is a modification of previously described procedures of Prockop and Udenfriend (1960), Kivirikho, et al., (1967), and

Blumenkrantz and Asboe-Hansen (1975). Following lyophilization, the lungs were hydrolyzed with 6 N HCl in capped tubes for 2 days at approximately 110°C. This hydrolysate was then neutralized with 10 N NaOH, diluted with distilled water and allowed to cool. Each sample was then vortexed, and centrifuged at 2000xg for 5 minutes at room temperature. An aliquot of the neutralized hydrolysate was pipetted into screw capped tubes followed by the addition of a borate-alanine buffer (0.112 M alanine at pH 8.7, 1 M borate; with 100 ml of a 1:9 dilution of the borate buffer added to 50 ml of 1:9 dilution of alanine). Samples were saturated with KCl, and vortexed, followed by addition of Chloramine T. All samples were allowed to stand at room temperature for 30-40 minutes to allow complete oxidation. After adding 3 ml of toluene to all samples, each was capped tightly and placed in boiling water for 30 minutes. After cooling, the tubes were shaken vigorously and centrifuged on an International Equipment model PR-2 centrifuge (International Equipment Co., Needham Heights, MA) at 1000xg for 5 minutes. Following centrifugation, 1.5 ml of the top toluene phase was pipetted into separate tubes with Ehrlich's reagent (Prockop and Udenfriend, 1960) and vortexed. Thirty minutes later, the solutions were read at 560 nm, against a reagent blank, on a Gilford Stasar II spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). Standard curves were prepared of 0 to 16 ug hydroxyproline and carried through all steps of the procedure. Results were calculated, using the appropriate dilution factor and from the standard curve generated for each sample run, as micrograms hydroxyproline per total lung.

Hydroxyproline is found almost exclusively in animals within the collagen molecule and has been used extensively to study collagen metabolism

and formation (Prockop and Udenfriend, 1960; Jackson and Cleary, 1967). The reaction for most methods employed to assay hydroxyproline involves the oxidation of this imino acid to pyrrole-2-carboxylic acid (or pyrrole) and the subsequent formation of a chromophore with paradimethylaminobenzaldehyde. This reaction is summarized as follows (Prockop and Udenfriend, 1960):



In the methods described above, hydroxyproline is oxidized in the presence of excess alanine and chloramine T. Since the first oxidation products formed (pyrrole-4-hydroxy-2-carboxylic acid and pyrrole-2-carboxylic acid) are not soluble in toluene, materials which may interfere with the color reaction are removed prior to the formation of the pyrrole.

8. Statistical Analysis

All statistics were performed by the one way analysis of variance and Tukey's Honestly Significant Difference Test (Daniel, 1977). Significant differences were determined at the 0.05 level.

D. Results

Figure 1 shows the results of all four treatment regimes. Rats exposed to $BeSO_4$, when given Cy A, showed no difference in total lung hydroxyproline values when compared to rats administered $BeSO_4$ only. Yet, when these Cy A-treated animals were compared to both control groups, no significant alteration in lung collagen was noted. In all treatment regimes, Cy A-treated control animals showed no significant difference to untreated controls, thereby demonstrating no effect in total lung hydroxyproline through Cy A administration.

BHT-induced fibrosis in mice was significantly ($p<0.05$) reduced by Cy A-treatment compared to Cy A-untreated controls. BHT given alone significantly increased total lung hydroxyproline over BHT+CyA-untreated controls. Cy A, given with BHT showed no significant differences when compared to both control groups.

Mice given bleomycin showed no effect with CyA treatment. Mice given bleomycin and treated with Cy A showed no difference in hydroxyproline content versus mice treated with bleomycin alone. However, rats administered bleomycin showed a significant ($p<0.05$) effect due to Cy A. Rats given bleomycin and treated with Cy A, showed significantly reduced levels of lung hydroxyproline against bleomycin+Cy A-untreated animals. These Cy A treated animals also showed a significant elevation compared to both Cy A groups. Although Cy A treatment did not completely abolish the fibrotic response, it did result in significant reduction.

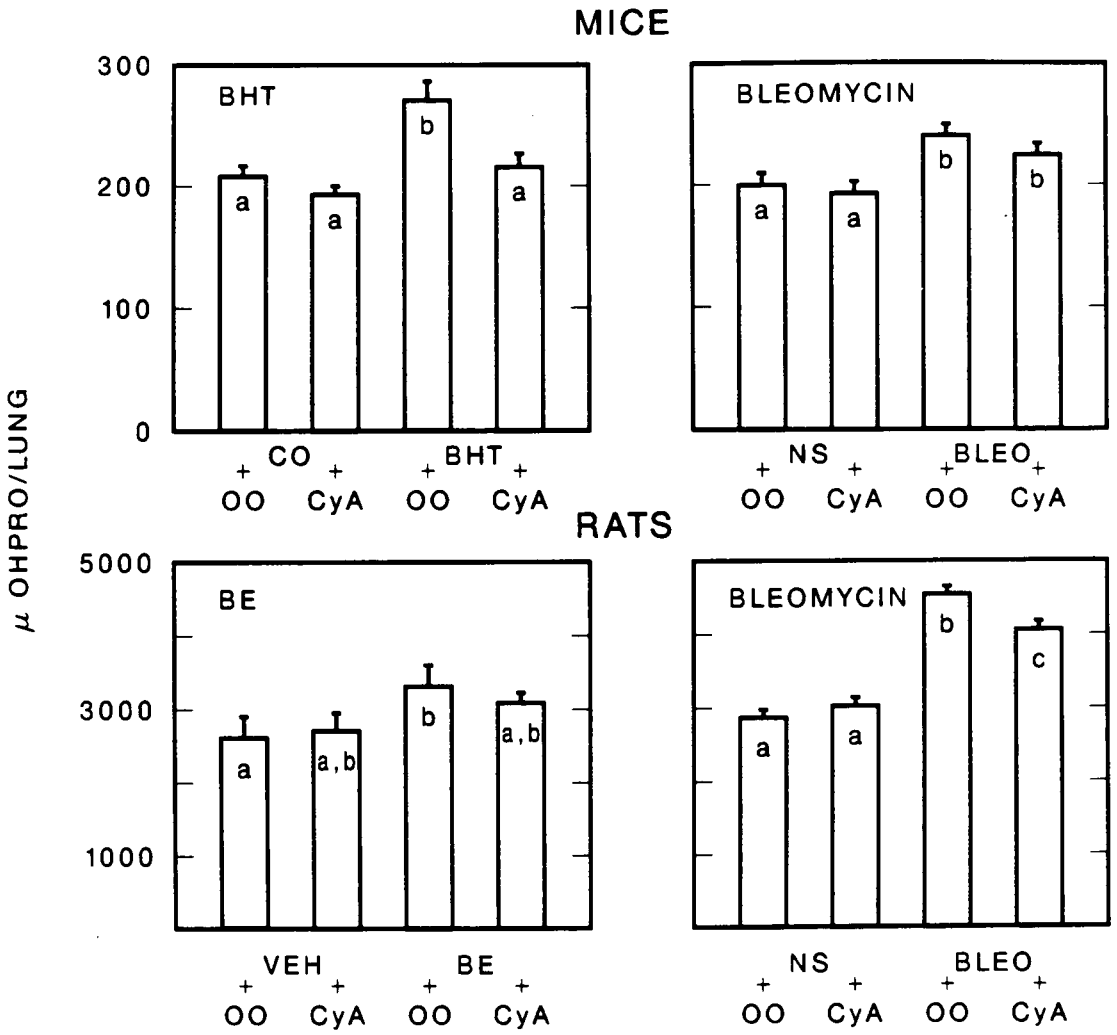


Figure 1. Total lung hydroxyproline following administration of beryllium sulfate, butylated hydroxytoluene, and bleomycin. Lung damage was induced by either BHT (400 mg/kg) in mice; bleomycin (bleo) in mice (4 units/kg) and rats (1.5 units/animal); or beryllium (BE) inhalation in rats. Control animals were given either corn oil (CO), normal saline (NS) or H_2SO_4 vehicle (Veh) or olive oil (OO). Animals were sacrificed either day 14 after BHT treatment or day 21 after bleomycin or beryllium treatment. Results are expressed as ug hydroxyproline per total lung (mean ± SE). Bars with different letters differ significantly ($p<0.05$).

E. Discussion

Animals treated with Cy A were analyzed for total lung hydroxyproline content following administration of fibrogenic agents; $BeSO_4$, BHT, and bleomycin. Total lung hydroxyproline was analyzed as an index of the extent of the fibrotic process. The assay of hydroxyproline is a quantitative method widely used to detect changes in lung collagen following exposure to pneumotoxicants. With the single exception of $BeSO_4$, the three agents used in this study ($BeSO_4$, BHT, and bleomycin) were selected based on their use in previous studies as models of IPF.

Beryllium sulfate was the principal subject of study in the experiments described in this dissertation. Initially, this study was designed solely to assess the effects of Cy A on $BeSO_4$-induced fibrosis and was later redefined to include its effects on other established fibrotic agents.

This study is the first reported instance of the quantitative measurement of fibrosis due to the administration of $BeSO_4$. Most research has previously assessed the fibrotic content of lungs following administration of Be compounds through a histological description (Sprague, et al., 1947; Ploicard, 1950; Stokinger, 1966). The assay of total lung hydroxyproline does indeed quantitate significant fibrosis due to $BeSO_4$. Results showed significantly increased total lung hydroxyproline values following inhalation exposure of $BeSO_4$ versus controls.

Rats treated with Cy A, and exposed to a $BeSO_4$ aerosol, showed no significant reduction in total lung collagen when compared to $BeSO_4$+Cy A-untreated rats. Conversely, these animals showed no significant elevation of

lung collagen when compared to both control groups. The extent of the fibrotic process due to $BeSO_4$, at 21 days, did not allow for a sufficiently great enough increase in total lung collagen. The $BeSO_4$+Cy A-treated group had a decreased lung collagen content compared to the $BeSO_4$+Cy A-untreated group, and an identical lung collagen content compared to controls. Perhaps through methods that would increase lung collagen, either through higher chamber concentrations or prolonged exposure times, these results would be better defined.

Unlike the response of $BeSO_4$ in rats, mice administered BHT showed a much clearer picture. Here, the treatment of animals with Cy A showed no change in lung collagen between BHT+Cy A-treated animals and controls. Comparing values of BHT+Cy A-untreated mice and BHT+Cy A-treated mice, Cy A completely inhibited the fibrogenic response. In this model of IPF, Cy A appeared to completely abolish the BHT-induced fibrosis.

This is not the case for the bleomycin-induced model of fibrosis. Mice, administered bleomycin showed no effect of Cy A treatment. Bleomycin+Cy A-treated mice showed a significant elevation of lung collagen versus Cy A-treated and Cy A-untreated controls and no difference when compared to Bleomycin+Cy A-untreated animals. One possible explanation for the differences observed in BHT and bleomycin treatments may be that the dosage of Cy A given to the two groups differed. Mice dosed with BHT received 125 mg/kg body weight of Cy A while animals given bleomycin received 60 mg/kg. Both groups received an optimum dose of Cy A dependent on their treatment. Animals receiving BHT were allowed a greater dosage of Cy A due to decreased observance of acute toxicity with these two agents when

combined. Bleomycin and Cy A treatment did not allow for such a high dosage level of Cy A. Animals showed an acute toxicity of Cy A at 125 mg/kg with bleomycin and needed the dosage decreased to a level of 60 mg/kg. Therefore, it is difficult to directly compare the results of these two studies since the dosage levels of Cy A treatment differed.

Rats, under Cy A treatment, did show positive effects with bleomycin administration. Cy A administered to bleomycin treated rats decreased lung collagen in comparison to Bleomycin+Cy A- untreated animals. The effect of Cy A did not completely abolish the fibrogenic response. The Cy A treated group given bleomycin still showed elevated total lung hydroxyproline when compared to each control group. In general, these results of bleomycin treated rats agree with previously reported results of immunosuppressed rats and bleomycin-induced fibrosis (Thrall, et al., 1980; Thrall, et al., 1979) showing a reduction in total lung hydroxyproline, but not a complete inhibtion of the fibrotic response.

In these reported cases of decrease lung collagen content following immunosuppression (Thrall, et al., 1979; Thrall, et al., 1980; Schrier, et al., 1983) the histological findings showed no difference between T-cell suppressed and non-suppressed treated animals. Conclusions drawn from these studies are based solely on the biochemical description of total lung collagen content. A histological description often results in a subjective determination while a biochemical determination provides a more objective quantitative approach. In contrast, although biochemical determinations may reveal subtle differences in lung collagen content, these measurements do not provide all the information required in an understanding of the disease process. Witschi, et al., have shown

the differences obtained between a pathological description and biochemical determination of lung damage following insult from a variety of pneumotoxicants (Witschi, et al., 1985). Animals exposed to either oleic acid, or bleomycin and oxygen, showed a different histological location of their lesions. The oleic acid lesion was a subpleural infarct with the remaining lung being normal. Bleomycin and oxygen treatment showed the lesion to be uniformly distributed throughout the centriacinar regions of the lung. Biochemical determinations of total lung hydroxyproline, for both oleic acid and bleomycin treatments were, 140% of their respective controls. The authors concluded that the combined use of biochemical measurements and histopathological methods will further the exploration of collagen metabolism in models of pulmonary fibrosis.

Human T lymphocytes, when activated by mitogens or specific antigens *in vitro*, produce a nondialyzable factor which stimulates fibroblast proliferation and synthesis of collagen (Snider, 1983). Lymphocytes can also release factors that are chemotactic for fibroblasts. T lymphocytes may then be directly, or indirectly, acting upon lung fibroblasts to increase their growth or secretion of collagen, thereby developing a fibrotic lung. Immunosuppression of T cells, by treatment with Cy A, may mitigate the development of lung fibrosis by blocking these responses. The immunosuppressive effects of Cy A were not investigated in these studies. Therefore, the selective inhibitory effect of Cy A on T cell activation is speculative. Further studies are required to test whether Cy A administration depletes circulating blood lymphocytes or lymphoid tissue before a more exact conclusion may be drawn.

This is the first reported study implying the involvement of T cells in BHT-induced lung fibrosis. Previous research of BHT acute lung damage has investigated the use of corticosteroids in the fibrotic response caused by this drug. Kehrer and Witschi (1981) demonstrated that prednisolone given to mice following BHT and O_2 treatment significantly elevated pulmonary fibrosis. Hakkinen, et al., (1983b) further studied the effects of prednisolone in BHT plus O_2 induced lung fibrosis. The authors concluded that prednisolone, given early in the acute phase of lung injury enhanced the development of fibrosis, while administration late after acute lung injury mitigated development. In these two studies, prednisolone was postulated to affect collagen synthesis rates of lung fibroblasts by an unexplained mechanism. The immunosuppressive effects of corticosteroid treatment was not implied. It is possible that Cy A in BHT, bleomycin and $BeSO_4$ induced lung fibrosis may have an effect on collagen synthesis rates of lung fibroblasts similar to the case for corticosteroids.

In conclusion, the administration of Cy A to animals dosed with beryllium, butylated hydroxytoluene or bleomycin, showed a variable response dependent on the pneumotoxicant administered and the species tested. BHT - induced fibrosis in mice was completely inhibited with CY A treatment. Bleomycin administered to mice showed no effect under CY A treatment while rats demonstrated a significant lowering of total lung hydroxyproline. Rats exposed to beryllium, when treated with CY A, showed no significant elevation in total lung hydroxyproline. Since a consistent effect was not demonstrated for all models of induced fibrosis, the T-lymphocyte was not the sole causative agent of the fibrotic response but did contribute to the overall process.

CHAPTER 2

ACUTE PULMONARY TOXICITY OF BERYLLIUM SULFATE INHALATION IN RATS AND MICE AS MEASURED BY CELL KINETICS AND HISTOPATHOLOGY

A. Introduction

The cellular proliferative response of the lung following toxic insult has been measured for a variety of pneumotoxicants (Kaufman, 1980; Adamson, 1985) and has proved a valuable description of the repair process of the lung following damage. Cell turnover is a normal process where dying cells may be desquamated and replaced by newly dividing cells. This process may be rapid, as in the epithelium of the gut, or very slow, as for hepatocytes. In the adult lung, cell turnover is low with a mitotic rate of about 3 cells per thousand (Adamson, 1985). Beserga (1981) has grouped the cells types of the lung (estimated by Sorokin (1970) to be aroung 40) into 3 populations based on their proliferative capacity:

1. Cells that continually divide and pass through all phases of the cell cycle.

2. Cells that leave the cycle after a number of divisions and then differentiate. These cells are no longer capable of division and ultimately die.

3. Cells which leave the cycle temporarily and remain dormant. A change in the local environment can induce these cells to re-enter the cycle and proliferate.

Exposure of the lung to a pneumotoxin usually results in enhanced cell division, most often in the form of proliferation of an unharmed stem cell

followed by differentiation to repair the cellular damage. Normal structure is usually restored if repair occurs rapidly.

The process of autoradiography is made by exposing a photographic emulsion to a radiographic specimen. Selective molecules, labeled by an isotope, can be localized to specific cells by light microscopy, and to specific organelles by electron microscopy. A section of tissue containing the radioactive substance is covered by a photographic emulsion. Following exposure, the section is developed photographically and the location of the radiographic molecule is marked by silver grains in the exposed emulsion.

Low beta emitters such as tritiated compounds are widely used due to their low energy. This allows for dissapation of collisions close to the source, resulting in a good correlation between the location of the silver grains and site of isotope incorporation. By administering a radioactive precursor of DNA, nuclei of cells about to divide become labeled and can be detected by autoradiography. Tritium labeled DNA precursors, such as tritiated thymidine, are commonly used since their low energy will not cause harm to any cells.

Thymidine is incorporated into DNA during the S phase of the cell cycle. It is generally accepted that almost all labeling will be in cells of renewing populations. Circulating tritiated thymidine is incorporated into nuclei within one hour following injection (Hughes, et al., 1958: Rogers, 1973). Once labeled, these cells retain the isotope which can only be diluted by subsequent cell division.

Precise cellular identification can be made on autoradiographs prepared for electron microscopy. Unfortuneately, these ultrathin sections represent only a small sample of the whole lung. Since the normal cell turnover is so low, very

few labeled nuclei can be seen. This makes ultrastructural autoradiography of the lung an expensive and time consuming process. Instead, the use of plastic sections for light microscopy autoradiography is frequently used. Plastics of the methacrylate group allow for sections to be cut of less than 1 um in thickness, This technique can then be used to prepare an entire lobe of a mouse lung such that morphologic and autoradiographic differences throughout the entire lung may be examined, and enough cells can be counted for statistical analysis.

This chapter utilized autoradiographic methods in pulse labeling experiments to evaluate changes in lung cell kinetics following Be insult. At selected intervals following $BeSO_4$ administration through nose-only inhalation, animals were injected with tritiated thymidine. Ninety minutes later the animals were sacrificed and lungs excised for autoradiography. After about 30 minutes, all the thymidine will be incorporated by cells in the S phase (Adamson and Bowden, 1974; Adamson, et al., 1977). By killing animals at 90 minutes, it is unlikely that these cells will have progressed much further in the cell cycle. Sections were cut from each lung embedded in glycol methacrylate and prepared for autoradiograpghic analysis. In random microscopic fields, the number of labeled nuclei in a total of 2000 cells were determined and a labeling percentage, termed the Labeling Index (LI), was calculated for all lung cells at each time point. Once the LI was determined, analysis of labeled cells was performed. A differential count was made where each labeled nucleus was categorized as being; endothelial, type II epithelial, interstitial or alveolar macrophage. Using a sample of 100 labeled cells per lung, differential counts were calculated for each cell type.

B. Materials

1. Animals

Specific pathogen-free male Fischer 344 rats, 6 weeks of age, were obtained from Charles River Laboratories (Kingston, NY). Male, Balb/C mice, were from the breeding colony maintained in the Biology Division of the Oak Ridge National Laboratory. Animal maintenance and housing were as previously described (Chapter 1).

2. Chemicals

Beryllium sulfate tetrahydrate was purchased from Fisher Scientific Co. (Fair Lawn, NJ), and methyl-^{3}H thymidine (sp. act. of 1.9 Ci/mmole) was obtained from Schwartz-Mann, a division of Becton, Dickinson and Co. (Orangeburg, NY), chrome azurol S was from Sigma Chemical Co. (St. Louis, MO) and Metofane from Pitman-Moore, Inc. (Washington Crossing, NJ). All other chemicals were reagent grade.

C. Methods

1. Determination of Intratracheal LD_{50} Values for Beryllium Sulfate

The intratracheal procedures used to administer solutions of $BeSO_4$ were previously described (Chapter 2). Thirty mice were randomly divided into 5 equal groups with each group receiving a single intratracheal dose of $BeSO_4$ ranging from 2.5 mg/kg to 40 mg/kg. Sixteen rats were randomly

divided into 4 equal groups with each group receiving a single intratracheal dose of $BeSO_4$ ranging from 5 mg/kg to 40 mg/kg. The LD_{50} values and 95% confidence intervals for the two species were determined.

2. Inhalation Exposure to Beryllium Sulfate

Animals were exposed to an aerosol of $BeSO_4$, or H_2SO_4, as controls. The inhalation procedures, chamber design and operation, and measurement of Be chamber concentration were previously described (Chapter 1).

a. Short Term Study

Rats and mice were exposed to the $BeSO_4$ aerosol for one hour. The average Be content of the chamber was 13 ug Be/L with a particle mass median aerodynamic diameter of 1.9 um ($\sigma g = 1.8$). Immediately following exposure, animals were returned to their cages. Four mice from each treatment group were sacrificed 1,3,5,7,9,11,13,15,17 and 21 days following exposure (day 0 designated as time of exposure). Four rats from each group were sacrificed 1,2,5,6,8,10,14,17 and 21 days after exposure.

b. Long Term Study

Rats were exposed to an aerosol of $BeSO_4$ for one hour, to a chamber concentration of 4.05 ug Be/L, with a particle mass median aerodynamic diameter of 1.9 um ($\sigma g = 1.89$). Four rats from each treatment group were sacrificed at 3 weeks, and 3, 6, and 12 months following exposure.

3. Beryllium Lung Burdens

In a separate study to assess the initial lung deposition of Be, 15 male mice and 15 male rats were simultaneously exposed to an aerosol of $BeSO_4$ for one hour, as previously described. The chamber concentration was measured as 3.3 ug Be/L with a particle mass median aerodynamic diameter of 1.7 um ($\sigma g = 1.5$). Animals were then sacrificed at 0, 3, 6, 9 and 24 hours following exposure. Lungs were excised and lyopholized to constant dry weight and assayed for total Be analysis by atomic absorption spectroscopy (Revis, 1985).

4. Autoradiographic and Histopathologic Analysis

On each day of sacrifice, animals were injected i.p. with methyl-^{3}H thymidine (400 uCi/rat; 50 uCi/mouse). Ninety minutes later, animals were anesthetized with Metofane, the abdomen was opened by midline incision, and animals sacrificed by exsanguination via the abdominal aorta. Lungs were fixed by intratracheal instillation of 10% neutral buffered formalin. Sections from three lung lobes of each mouse and two lung lobes of each rat were dehydrated and embedded in glycol methacrylate. Sections were cut at 1 um on a Sorvall JB4 microtome and mounted on glass slides. Slides were then covered with type NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and stored in light-tight boxes at 0-4°C. After 10 days, the slides were developed, rinsed, fixed and stained with Lee's methylene blue-basic fuschin.

5. Labeling Index

The Labeling Index (LI) was determined by counting the number of cells in the alveolar wall with 5 or more grains per nucleus per 2,000 to 3,000 cells in each lung. The LI was defined as the number of labeled cells per total number of cells counted. Only alveolar parenchymal cells were counted, bronchiolar cells were excluded from the count. Differential counts were performed by counting and identifying 100 labeled cells per lung, as previously described (Witschi and Morse, 1983). Alveolar cells were identified as to four main categories: capillary endothelial, interstitial, alveolar macrophage and type II epithelial. Capillary endothelial cells are elongated occasionally cuboidal cells, lining blood vessels where the nucleus protrudes to some extent into the blood vessel or is continuous with the lumen. Alveolar macrophages are present in the alveolar space or may sit on the alveolar wall, having a large nucleus with a vacuolated or granulated cytoplasm whose cytoplasmic edges may be irregular or feathered. Type II epithelium are located in the corner of the alveolus, sitting on the basal lamina, protruding into the alveolus. Cytoplasmic vacuolation is fairly uniform and eosinophilic. Type I epithelial cells were excluded from the count due to their extremely low labeling index. Due to the low LI of control animals, it was impossible to count 100 labeled cells in the lungs of all animals. Individual counts, in each treatment group, were then summed. The percentage of each individual cell type labeled was then calculated based on the number of total cells labeled for all animals. Data from the LI (number of cells labeled/total cells counted) and the percentage of each individual cell type resulted in an estimate of the total number of

individual cells labeled/10,000 parenchymal cells. Therefore, numbers expressed in this manner do not represent individual observations but are group estimates and statistical analysis could not be performed. Data obtained from differential cell counts is expressed as number of cells labeled/10,000 parenchymal cells.

6. Statistical Analysis

Statistical analysis was performed by the Student's t test. Data is expressed as mean ± SE for each treatment group, where appropriate. LD_{50} values and 95% confidence intervals were determined by the methods of Weil (1952).

D. Results

The intratracheal LD_{50} values and 95% confidence intervals for $BeSO_4$ in mice and rats were 7.07 mg/kg (6.07 to 8.07) and 11.89 mg/kg (10.47 to 13.30), respectively.

Results of the 24 hour Be retention study of mice and rats simultaneously exposed to a chamber concentration of 3.3 ug Be/L, showed differences in the Be deposition between the two species. Rats, immediately taken from the chamber, and lungs excised for Be content (0 hour), showed an initial deposition of Be as 127 (±7.9) ug Be/g dry lung weight (Table 4). Three hours later, the retention of Be was 61% of the initial dose. An unexplainable increase in Be lung content was seen at 9 hours. By 24 hours, Be retention decreased to 55% of the initial dose. Essentially, half the initially received Be was cleared from the lung 1 day following exposure. In mice, at 0

Table 4

Be Lung Burdens[a]
(ug/g dry lung weight)

	Control	0 Hrs	3 Hrs	6 Hrs	9 Hrs	24 Hrs
Rat	.06	127	76.9	79.8	112	69.8
	(0.0)	(7.9)	(5.31)	(16.2)	(4.73)	(17.2)
Mouse	.35	18.7	15.5	14.2	13.0	12.8
	(0.04)	(3.34)	(2.98)	(0.78)	(4.03)	(2.97)

[a]Values are mean (SE), n=3/group.

hours, 18.7 (±3.34) ug Be/g dry weight was deposited. By three hours, 83% of the initial dose remained. At 24 hours, clearance of inhaled Be increased to allow 68% of the inhaled dose to remain.

In comparison to rats, the initial Be deposition in mice was 15% the value at this time point (0 hours). At 24 hours, rats cleared 45% of the inhaled dose, mice cleared 32%.

The overall LI for rats is seen in Figure 2. As early as 2 days post exposure, a significant increase was seen in the LI of lung cells of $BeSO_4$ treated animals versus controls. This increase continued to rise which peaked at a value of 3.41% by day 8. After 8 days post exposure, the LI diminished over time until by 17 days, the effect was no longer significant.

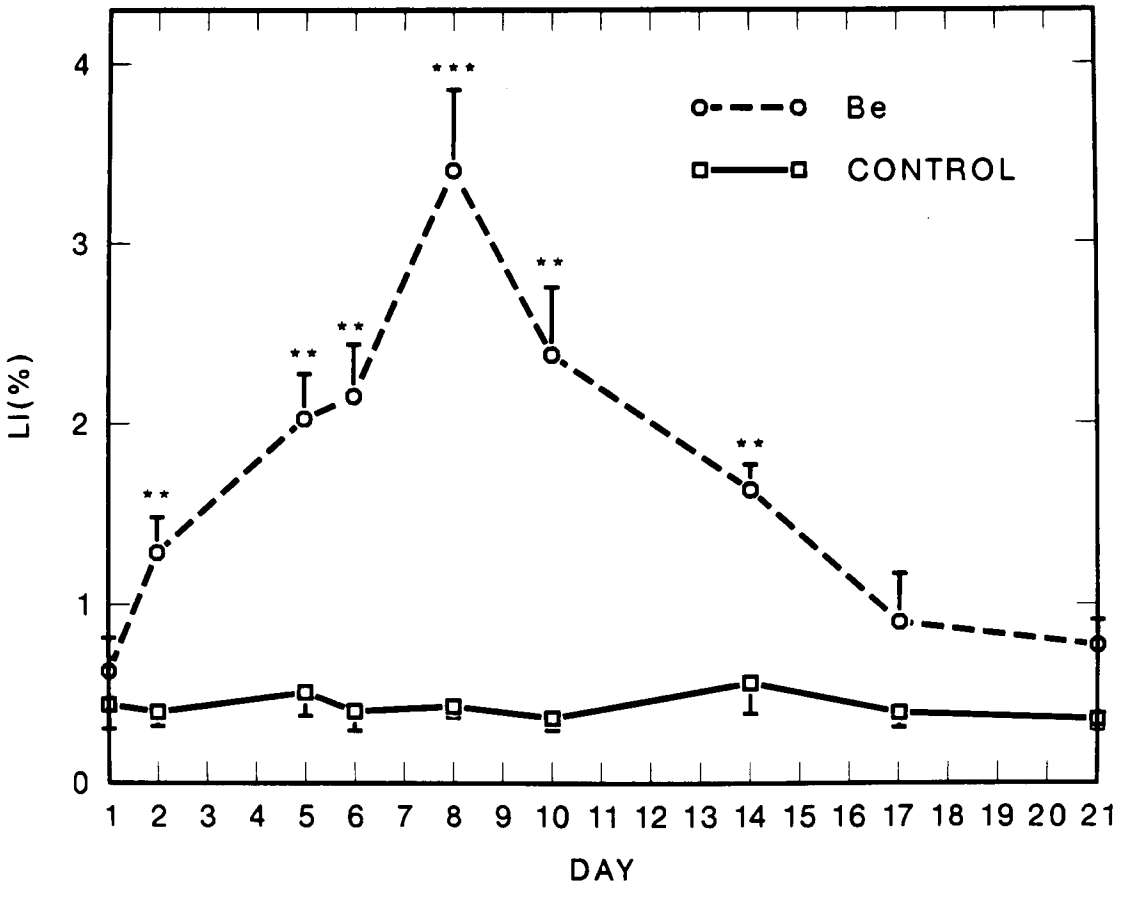


Figure 2. Labeling index of pulmonary parenchymal cells in rats following $BeSO_4$ inhalation. Animals were exposed to a $BeSO_4$ aerosol of 13 ug Be/L or H_2SO_4 as controls, and sacrificed over a 21 day period. Each point represents mean ± SE (n=3-4). *,**,*** Denotes $p<0.05$, 0.01. and 0.005, respectively.

Analysis of the differential cell counts in rats (Figure 3) showed that all cell populations roughly paralleled the response as shown in the LI. The interstitial cell population showed the greatest response. This cell population included fibroblasts, some endothelial cells, monocytes and macrophages. Interstitial cell labeling dramatically increased by the second day of exposure (73 labeled cells/10^4). By 8 days, this response reached a maximum (128 labeled cells/10^4), which decreased at 21 days to 39 labeled cells/10^4. Endothelial cell proliferation followed the pattern as that of the interstitial cells, being somewhat reduced in magnitude, with a maximum response seen at day 8 (100 labeled cells/10^4). Likewise, kinetics of the type II pneumocyte replication was roughly parallel to the endothelial response. A slight decrease by type II epithelial cells was seen at 2 days following exposure with the response at a maximum by day 8 (92 labeled cells/10^4). The alveolar macrophage turnover, unlike all other cell populations studied, remained relatively constant over the entire 21 days.

The LI in rats over the one year measurement period is shown in Figure 4 with differential counts in Figure 5. At no time point did the LI of $BeSO_4$ treated rats show any significant changes when compared to control groups.

In mice, exposed to the same Be chamber concentration as rats, the LI showed a different pattern (Figure 6). A biphasic nature to the response was seen over 21 days. Significant differences between Be treated (.72%) and control groups (.25%) were obtained by 3 days following exposure. This response continued to rise until 5 days (1.16% vs .25%), decreasing in magnitude which by day 7 (.74% vs .37%) was no longer significantly elevated. This first phase of the curve continued to decrease, reaching a minium value

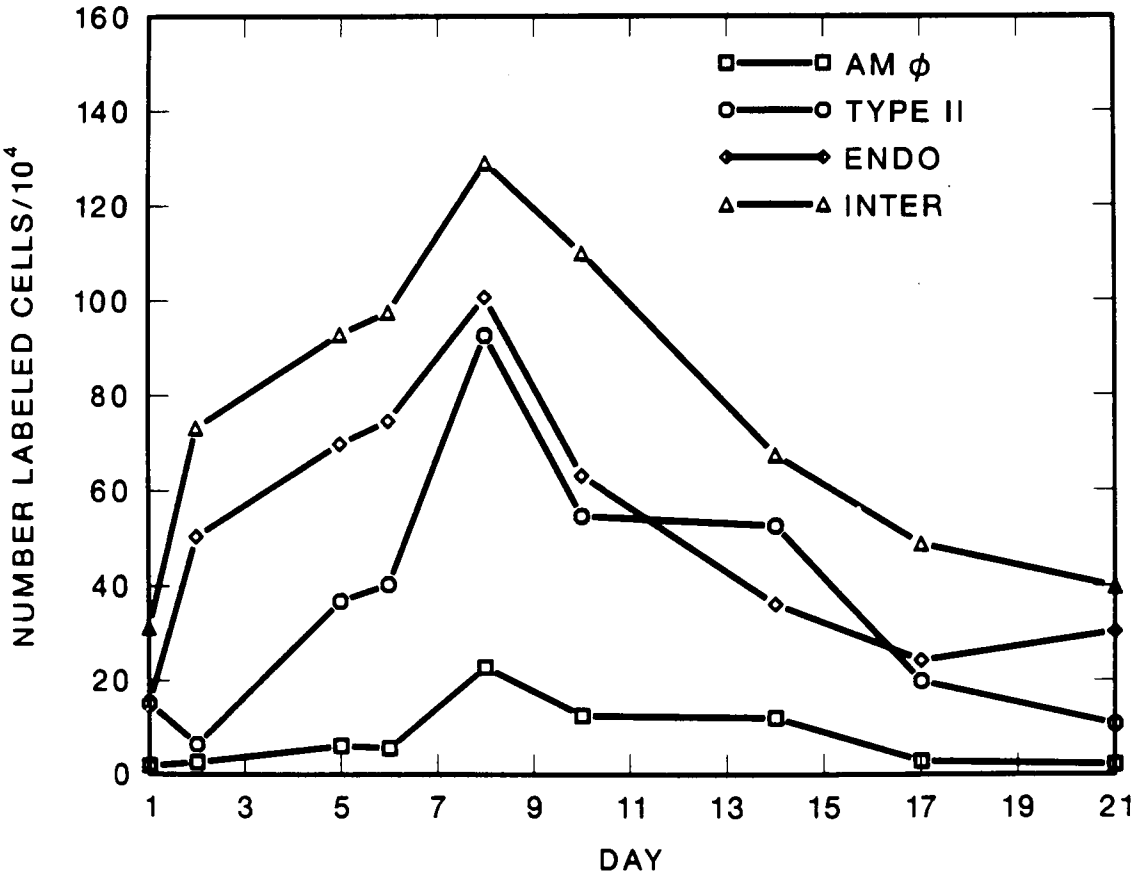


Figure 3. Number of labeled cells per 10,000 in rats on days 1-21 after $BeSO_4$ inhalation. Animals were exposed to an aerosol of $BeSO_4$ at 13 ug Be/L and sacrificed over a 21 day period. Data represents alveolar macrophage (AM), type II epithelial (type II), endothelial (endo) and interstitial (inter) cells.

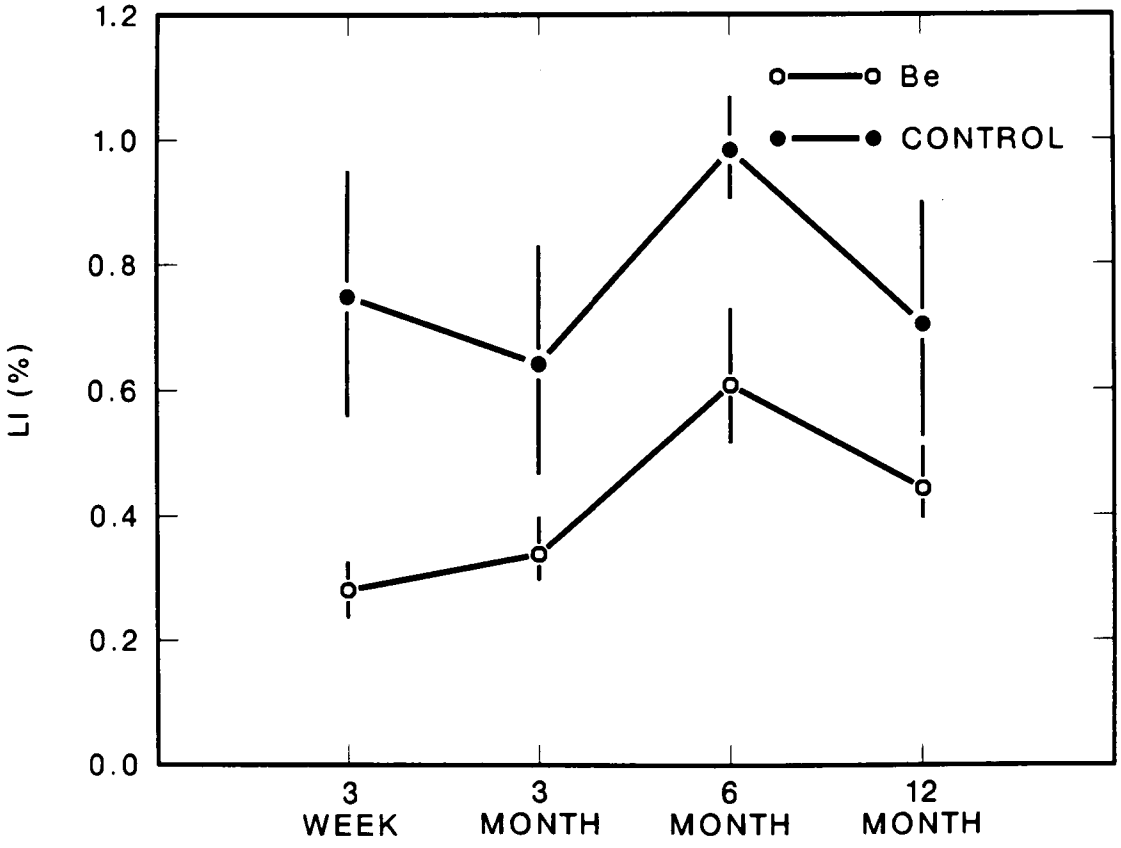


Figure 4. Labeling index of pulmonary parenchymal cells in rats over a one year period following $BeSO_4$ inhalation. Animals were exposed to a $BeSO_4$ aerosol of 4.05 ug Be/L or H_2SO_4 as controls. Each point represents the mean ± SE (n=4).

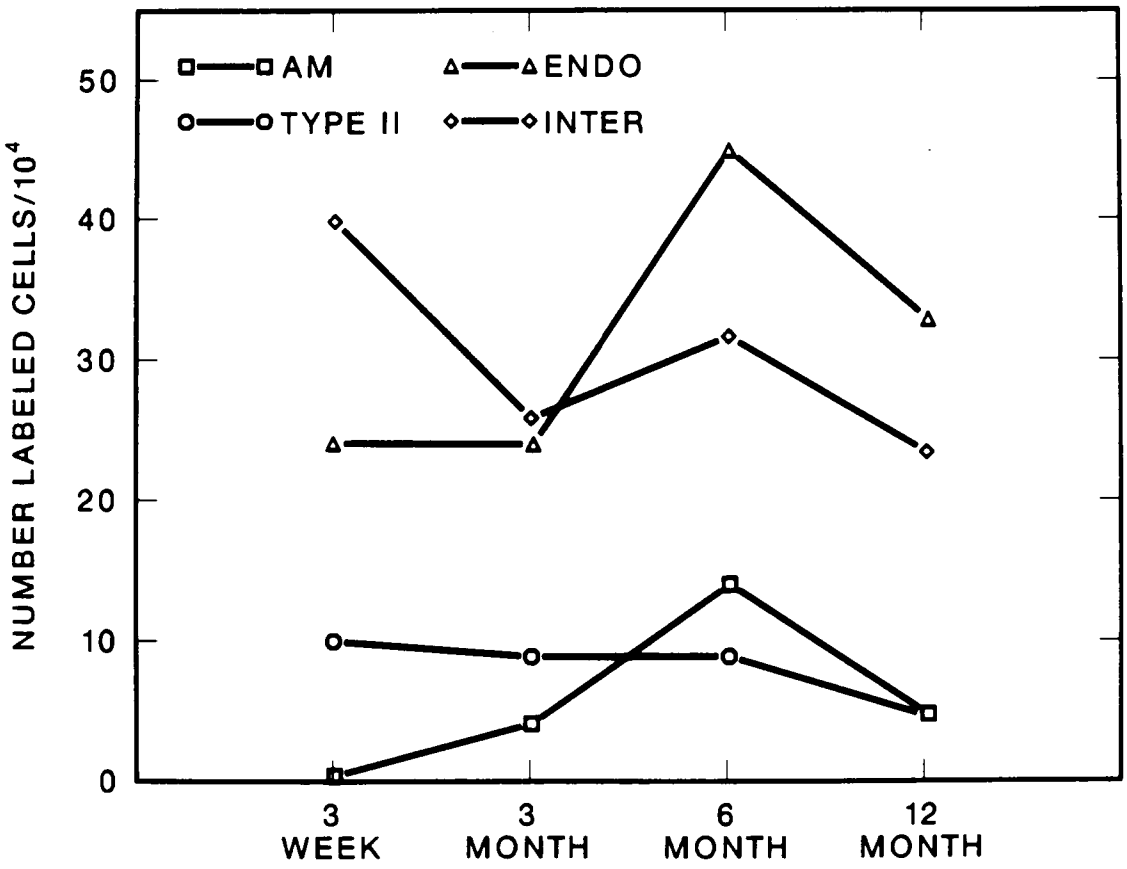


Figure 5. Number of labeled cells per 10,000 in rats over a one year period following $BeSO_4$ inhalation. Animals exposed to an aerosol of 4.05 ug Be/L and sacrificed over one year. Data represents alveolar macrophages (AM), type II epithelial (type II), endothelial (endo) and interstitial (inter) cells.

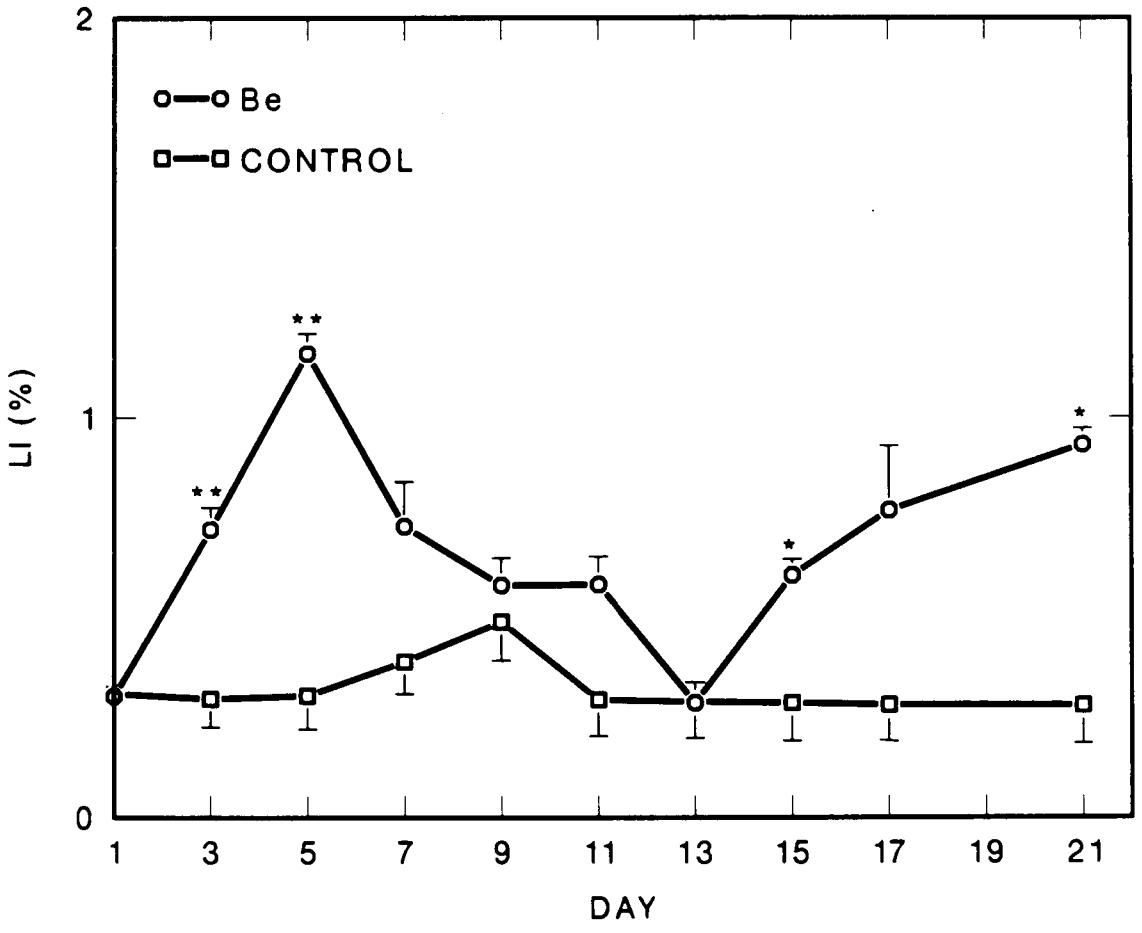


Figure 6. Labeling index of pulmonary parenchymal cells in mice following $BeSO_4$ inhalation. Animals were exposed to a $BeSO_4$ aerosol of 13 ug Be/L or H_2SO_4 as controls and sacrificed over a 21 day period. Each point represents mean ± SE (n=3-4). *,**, Denotes $p<0.05$ or 0.01 respectively.

by 13 days (.34% vs .32%). At this time, the LI began to rise, becoming significantly elevated at 15 days (.63% vs .19%). By 21 days, the LI remained greater than control (.96% vs .18%), reaching a value comparable to that seen at day 5.

Differential cell counts in mice (Figure 7) showed the predominant cell population responsible for the peak response at day 5 as the interstitial cells. The other cell populations responding at this time were endothelial and alveolar macrophages. The response of all cells diminished at day 13. At this time, the endothelial cells again began to proliferate. By day 21, this was the predominant cell population accounting for the second phase of the curve, closely followed by interstitial cell replication. The type II pneumocyte, unlike the response seen in rats, remained relatively stable over the 21 day measurement period.

The histological description of lung damage in rats showed that by one day after exposure to beryllium there was a mild increase of interstitial macrophages at the bifurcations of the alveolar ducts (Figure 8). Segmented leukocytes were observed in the respiratory epithelium, and amorphous material was present in these respiratory epithelial cells. Similar changes were seen two days after exposure, with a greater increase in the interstitial accumulation of macrophages at the alveolar duct bifurcations, accompanied by interstitial segmented leukocytes.

Five days after exposure, the increase in interstitial macrophages and segmented leukocytes continued to be observed predominantly in the centriacinar regions of the lung. In addition, throughout the lung, type II pneumocytes became prominent with large distinct vacuoles in their cytoplasm.

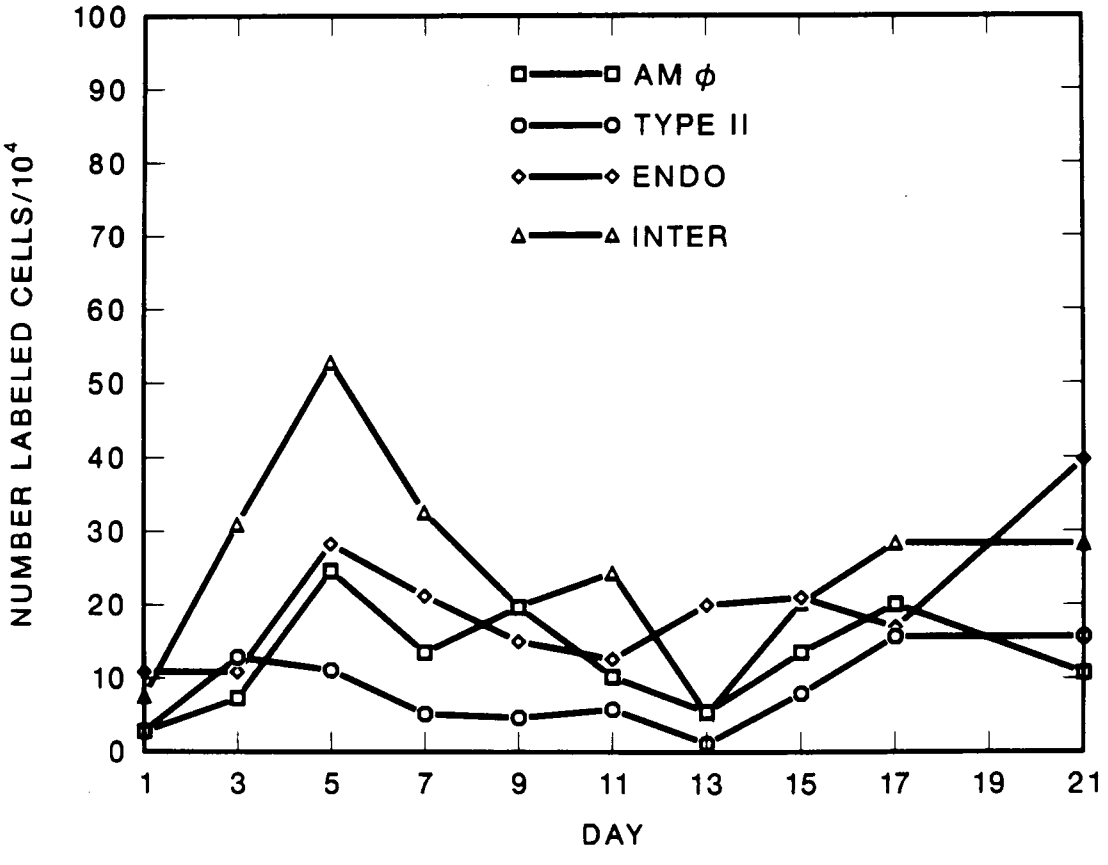


Figure 7. Number of labeled cells per 10,000 in mice on days 1-21 after $BeSO_4$ inhalation. Animals exposed to an aerosol of 13 ug Be/L and sacrificed over a 21 day period. Data represents alveolar macrophage (AM), type II epithelial (type II), endothelial (endo) and interstitial (inter) cells.

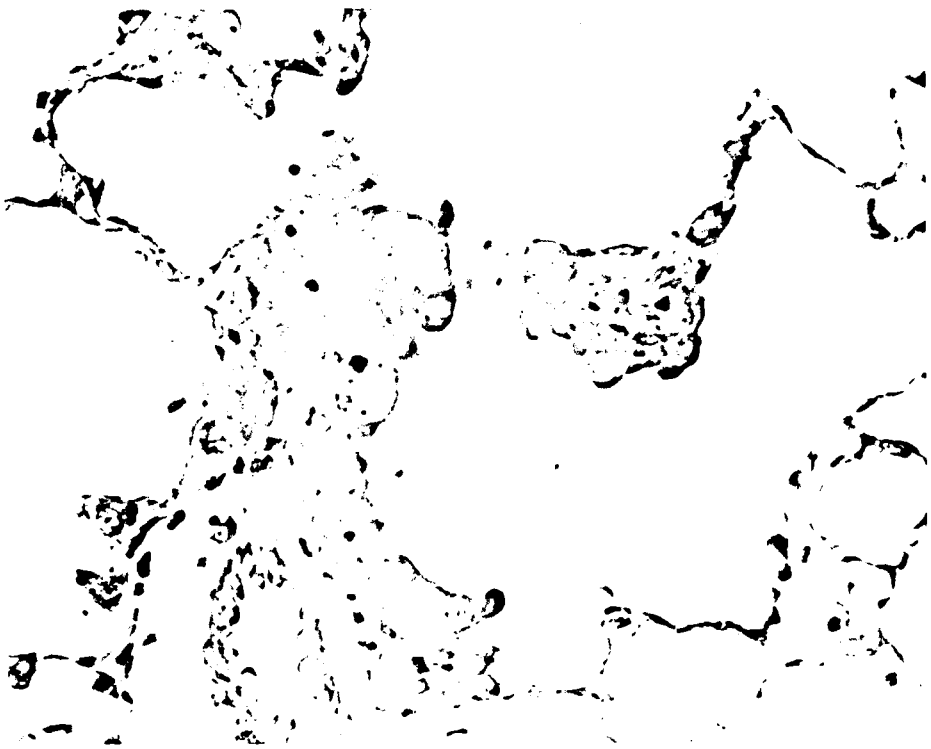


Figure 8. Lung of rat after 1 day post exposure. Mild increase in interstitial macrophages at alveolar duct bifurcations (1150x).

Fibrin, and occasionally segmented leukocytes, were observed in the centriacinar regions. By six days, segmented and degenerating leukocytes were observed in the alveoli of all animals. Small alveolar macrophages and macrophages with ragged cell membranes were present. Large macrophages with degenerated leukocytes within their cytoplasm were also seen (Figure 9). Eight days after exposure, all regions of the parenchyma were abnormal. The interstitium was thickened with an infiltrate of interstitial macrophages and segmented leukocytes. Type II pneumocytes were increased with prominent cytoplasmic vacuoles. Alveolar fibrin, hemorrhage, and degenerating segmented leukocytes were frequently observed. The increase in alveolar macrophages included cells with ragged membranes and cells with phagocytized leukocytes and erythrocytes in their cytoplasm. Changes similar to those seen on day 8 were seen on days 10 and 14. By this time, the tissue response was most prominent (Figure 10). Also noted was the appearance of alveolar macrophages with fine multivesciculated cytoplasms.

By 17 and 21 days after exposure, the prominent interstitial response seen at 14 days had resolved leaving residual focal interstitial macrophages with few segmented leukocytes. Alveolar edema and hemorrhage was infrequent. Focally, alveolar macrophages continued to be increased, some with erythrocytes or degenerated leukocytes in their cytoplasm. Macrophages with fine multivesciculated cytoplasms were observed. Occasionally, these cells also contained erythrocyte fragments.

A few control animals had an occaisional foci consisting of an increase in alveolar macrophages. Otherwise, no pathologic changes were observed.

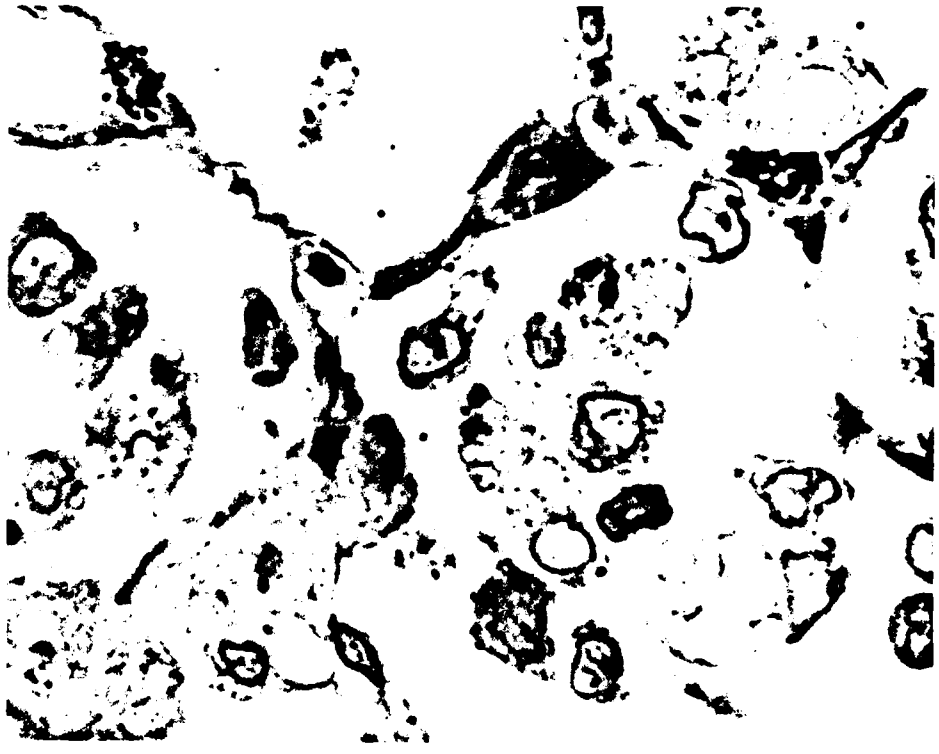


Figure 9. Lung of rat after 6 days post exposure. Macrophages with ragged cell membranes and cytoplasms containing degenerative leokocytes (2900x).

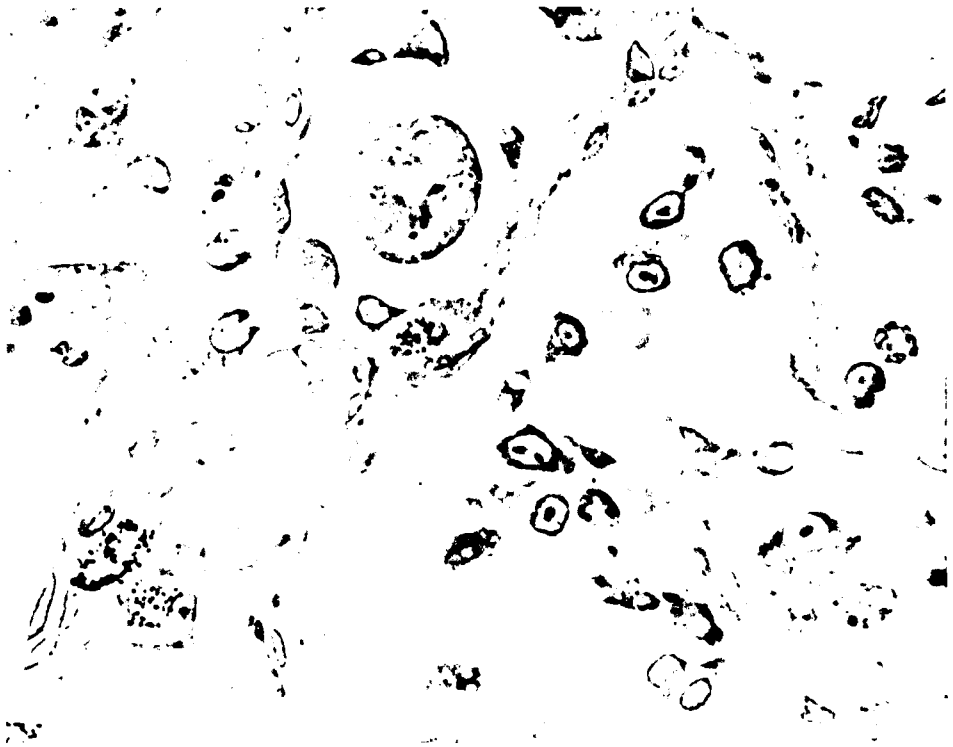


Figure 10. Lung of rat after 14 days post exposure. Time point of most prominent tissue response (1150x).

Histopathologically, the response in the mouse to beryllium sulfate inhalation was less dramatic than that seen in the rat. One and 3 days after exposure, there was a mild increase of interstitial macrophages and leukocytes at the bifurcations of the alveolar ducts. By 5 and 7 days, this increase in interstitial macrophages and segmented leukocytes was observed in the adjoining parenchyma. Also at this time, large alveolar macrophages were seen. There was little change in the distribution or morphology of the pneumonitis at 9 days, except that this time marked the appearance of vacuolated alveolar macrophages and macrophages with erythrocytes in their cytoplasm. These characteristics seen at 9 days persisted in the remaining time points studied.

E. Discussion

Rats and mice exposed to a $BeSO_4$ aerosol of 13 ug Be/L for one hour through nose-only inhalation showed differences in lung damage measured by the analysis of lung cell kinetics. Rats showed a much greater degree of damage, with the LI approximately 3 times that seen in mice. This difference in lung toxicity of $BeSO_4$ can be partially explained by differences in respiratory volume, and thus, differences in amount of inhaled dose delivered to the lungs. Lesions seen in the lungs of rats in the first 6 days after exposure were localized to the bifurcations of the alveolar ducts and alveoli of these centriacinar regions of the lung. The distribution of lesions correlates with the deposition of particles after exposures of silica (Brody, et al, 1982) and asbestos (Washeit, et al, 1984). By eight days after exposure, the pulmonary

parenchyma was involved diffusely with an interstitial increase in macrophages and segmented leukocytes, with an increase in these cell spaces as well. These lesions were most severe 10 and 14 days after exposure. Why does a lung reaction that was initially focal later become diffuse? One possibility is ongoing lung injury. There were several indications of continuing lung injury. These included pycnotic leukocytes in alveoli, leukocytes engulfed by macrophages, alveolar erythrocytes, and erythrophagocytosis by macrophages. Seventeen days after exposure, the diffuse pulmonary reaction had subsided, although focal regions remained. Al veolar segmented leukocytes became less frequent at these later time points. By 17 days and later, most alveolar macrophages had acquired the microvescicular appearance to their cytoplasm.

Cell kinetics for both species roughly paralleled the response seen histologically. The greatest LI response in rats, at day 8, occured slightly before the maximum tissue response noted at days 10 and 14. In mice, both the peak LI, and tissue response, occured simultaneously at day 5.

Little information is available on the response in mice to Be exposure. Stokinger, et al, (1950b) in studying the acute toxicity of 11 species of animals, reported the toxicity of mice and rats continually exposed to an aerosol of $BeSO_4$ at 4.3 mg Be/m^3. At this concentration (Concentration x Time=6,600 ug hr/L), rats showed 100% mortality, while mice had no mortality.

What might explain these differences in $BeSO_4$ toxicity between mice and rats? When both rats and mice were exposed to an aerosol of $BeSO_4$, with a chamber concentration of 3.3 ug Be/L, a dramatic difference was seen in the initial Be lung burdens. Mice retained 20% less of the inhaled Be (ug Be/g dry

lung weight) compared to rats over the 24 hour period. Is the difference explained by the ability of these two species to differ in capability to clear Be from the lungs? If the pattern of clearance over 24 hours is evaluated, the retension of Be is similar, with rats retaining 55% of the inhaled dose, mice 68%. It appeared that the two species have the same capacity for Be clearance with the response difference explained by the difference in the initial dose received to the lung.

In analyzing the respiratory rate and tidal volumes of these two species, the discrepencies in initial dose received to the lungs becomes apparent. Guyton (1947) in studying the minute volumes of 7 different species of animals, reported the following equation for the Respiratory Volume:

$$\text{Respiratory Volume/min. (ml)} = 2.10 \times (\text{Wt. in grams})^{3/4}$$

Using this equation, and the approximate average body weights for 10 week old Fischer 344 rats (220g) and 10 week old Balb/C mice (20g), the respiratory volumes for the animals used in this study were calculated (Table 5). Rats have approximately 6.04 times a greater respiratory volume than mice. The data presented in Table 4 shows rat lungs to contain 6.79 times greater a concentration of Be (ug/g dry lung weight) than mice at 0 hours. Since these animals were simultaneously exposed to the same chamber concentration of Be, and assuming that these two species are able to identically retain the same amount of inhaled Be, then the differences in Be lung content may be explained by the difference in respiratory volumes of the two species studied.

Table 5

Calculated Respiratory Volumes

	Rats	Mice
Age(weeks)	10	10
Weight(grams)	220	20
Respiratory Volume/Min(ml)	119.96	19.86

These results are the first reported instance of the cellular proliferative process in the lung following insult from any compound of Be. Data from the differential cell counts showed the predominant cell population responsible for the peak LI, at day 8, as being interstitial. This population of cells comprises both fibroblasts and mononuclaer phagocytic cells undergoing division within the interstitium. Alveolar macrophages may be derived from this mononuclear cell population (Adamson and Bowden, 1980, 1981). The increase in this cell population turnover apparently resulted from an increased demand for phagocytic ingestion, and clearance, of the inhaled Be particles, or from destruction and replacement of existing cells.

Injury to the lung results in increased cell division and repair by a surviving stem cell. Exposure of animals to high concentrations of oxygen produced endothelial injury with subsequent endothelial proliferation to return normal lung structure (Kistler, et al, 1967; Bowden, et al, 1968; Evans and Bils, 1969; Bowden and Adamson, 1971; Adamson and Bowden, 1974; Bowden and Adamson, 1974). The histologic appearance of alveolar fibrin and

hemorrhage at day 8 was indicative of capillary damage. Endothelial cell labeling was increased by this timepoint, indicating an attempt to replace damaged cells.

Damage to type I epithelial cells is followed by increased type II epithelial cell proliferation and differentiation to replace the damaged epithelial lining. This process has been described following lung injury from a variety of pneumotoxicants (Adamson and Bowden, 1979; Kaufman, 1980; Hakkinen, et al, 1982; Haschek, et al, 1983; Adamson, 1985). The pattern reported here following $BeSO_4$ inhalation showed this population replicating to repair the damaged epithelial lining from destruction of type I epithelium.

The cellular replication in the lungs of mice following $BeSO_4$ exposure is much reduced in magnitude compared to that of rats. A maximum response in the LI was reached at day 5, a time point earlier than that of rats, at day 8. This pattern also revealed a biphasic nature, with a distinct rise at day 21. By 21 days, the LI was significantly elevated compared to controls. Although time points beyond this were not included in the study protocol design, it would be of importance to determine when, if at all, values would approach control levels. This biphasic response is, as yet, unexplained.

The maximum LI response in mice was approximately one-third the value of that determined for rats. Tissue damage was much reduced in the lungs of mice and even though a statistical difference can be measured between control and treated groups through the LI, the histopathological description was minimal.

Since the degree of lung damage in mice is marginal, an adequate evaluation of the cellular proliferative response following $BeSO_4$ administration

is difficult. Differential cell counts showed that the cell populations replicating were similar to those in the rat lung, except that type II epithelial cells remained at a fairly stable value throughout the measurement period. The response of rat lungs showed type II cells to respond greater to $BeSO_4$ insult while the alveolar macrophage population remained at a fairly uniform value.

For rats given a single one hour exposure of $BeSO_4$ at a concentration of 4.05 ug Be/L, the cellular kinetics over a one year period showed no increase in cell proliferation of the lung. This is in agreement with the replication process of the lung following injury. Adamson (1985) illustrates that repair from exposure to a potential toxin will be characterized by a proliferative burst of a particular stem cell. Extended mitotic activity might be indicative of a neoplastic process, whereas predominant interstitial proliferation usually suggests abnormal repair and the development of fibrosis, neither of which are indicated in this study.

The difference in response between rats and mice would lead to the question of which species is more sensitive to Be toxicity? Lung burden studies showed that rats retained 6.79 times a greater concentration of Be than mice. Cell kinetic measurements demonstrated that the maximum LI response obtained in rats was 3 times the maximum response of mice, although rats retained nearly 7 times the amount of inhaled Be. Intratracheal LD_{50} determinations showed mice to be more sensitive than rats to $BeSO_4$. Mice had a LD_{50} value nearly twice that of rats. Based on these results, it appeared that mice were more sensitive to $BeSO_4$ lung toxicity than rats.

CHAPTER 3

ACUTE TOXICITY OF BERYLLIUM SULFATE INHALATION IN RATS AND MICE AS MEASURED THROUGH BRONCHOALVEOLAR LAVAGE

A. Introduction

The technique of bronchoalveolar lavage (BAL) has proven to be a valuable tool in assaying lung damage from a variety of toxicants. Analysis of the BAL is used both in the experimental and clinical settings as an aid in understanding the repair mechanisms of the lung following insult or during the disease process. Bronchoalveolar lavage allows safe and repetitive sampling in patients, gaining insight into how the the pulmonary system functions in both health and disease (Hunninghake, et al., 1979). The lavage fluid has been studied for many clinical disease states such as tuberculosis, unilateral pneumonitis, alveolar proteinosis, neoplastic lung diseases, interstitial lung diseases, sarcoidosis, hypersensitivity pneumonitis and patients with lung disorders from chronic smoking (Hunninghake, et al., 1979).

Biochemical and cellular parameters of the BAL have been used as an experimental tool in animals following lung damage from a variety of toxicants, whether inhaled (Henderson, et al., 1979; DeNicola, et al., 1981; Hu, et al., 1982), administered intratracheally (Sykes, et al., 1983), intravascularly (Eierman, et al., 1983; Chang and Lesser, 1984), intranasally (Terai, et al., 1979) or intraperitoneally (Roth, 1980). Cellular profiles of the lung lavage fluid have been used extensively to quantitate and study the early inflammatory changes seen following toxic lung injury. It is the purpose of this

study to assess only the biochemical changes in the BAL following exposure of animals to a beryllium sulfate aerosol through nose - only inhalation.

Biochemical paramaters of the lung lavage fluid are chosen based on their ability to detect pulmonary injury or repair processes (Henderson, et al., 1979). The enzymes chosen for this study were lactate dehydrogenase (LDH), alkaline phosphatase (Alk Pase) and acid phosphatase (Ac Pase). Lactate dehydrogenase is a cytosolic enzyme found within all tissues of the body. Damaged, or lysed cells, will release this enzyme into the BAL. LDH is then regarded as a general measure of cellular damage but the specific cell of the lung which releases this enzyme can not be determined by routine spectrophotographic methods. Alkaline phosphatase is a plasma membrane enzyme which has been detected histochemically and biochemically in the alveolar type II pneumocyte (Meban, 1975; Fisher and Furia, 1977) and in isolated lamellar bodies of the type II cell (DiAugustine, 1974). It is therefore thought that accumulation of Alk Pase in the BAL is a measure of type II cell destruction or a general indicator of broken cell membranes (Henderson, et al., 1979). Acid phosphatase is a lysosomal enzyme which, if found in the BAL, would indicate phagocytic activity or lysed phagocytic cells (Henderson, et al., 1979). As a further biochemical marker, the amount of albumin was measured in the lavage fluid. Albumin is a serum protein and would serve as an indicator of capillary damage or leakage (Beck, et al., 1982).

Quantities of cellular enzymes released into the BAL are not an exact index of lung damage. The specific cell types which release these enzymes are difficult to determine. Analysis of isoenzyme patterns following lung damage has been used in an attempt to differentiate types of pulmonary injury. The

isoenzyme profile of LDH has been studied in lung homogenates and lung lavage fluid following exposure of pneumotoxicants.

LDH exists as five different isoenzymes with differing electrophoretic mobilities. Cahn, et al., (1962) and Markert (1963) determined that LDH formation is controlled by two genes, each responsible for synthesis of a unique peptide. The two peptides combine in various combinations to form a tetramer. The tetramers formed are the electrophoretic variants. LDH - 1(H_4) and LDH - 5(M_4) represent the most anodic and cathodic variants, respectively. H represents the peptide associated with the main cardiac enzyme while M represents that peptide from skeletal muscle. Intermediate isoenzymes are hybrids formed by random association of H an M subunits into tetramers; LDH - 2(H_3M), LDH - 3(H_2M_2) and LDH - 4(HM_3). LDH - 1 and LDH - 2 are sometimes refered to as fast isoenzymes while LDH - 4 and LDH - 5 are the slow.

LDH - 1, - 2, and - 3 are prominent in tissues such as heart, kidney, brain, pancreas, and diaphragm (Starkweather, et al., 1966). LDH - 4, and LDH - 5 are major fractions of liver, and skeletal muscle. Prostate, thyroid, lung, adrenal, gastric mucosa, and ovary are characterized by fractions intermediate to the other groups. Isoenzyme patterns are also dependent upon species and age of the animal chosen from which the tissue is derived (Wilkinson, 1965).

Increases in lung LDH, or changes of lung LDH isoenzyme patterns, have been described in animals following exposure to oxidant gases (Buckley and Balchum, 1967; Chvapil, 1975), mineral dusts (Lindy, et al., 1970; Moores, et al., 1981; Beck, et al., 1981), metal salts (Henderson, et al., 1979),

chemical poisons (Roth, 1981; Forkert, et al., 1982), and during the development of silicosis (Bansal and Kaw, 1981). This sudy also analyzes the lavage fluid for the LDH isoenzyme profile as an assessment of lung damage more exact than changes in LDH measured through spectrophotometry.

Rats and mice were exposed to an aerosol of $BeSO_4$, or H_2SO_4 as controls, in a nose-only inhalation chamber for one hour. Animals were sacrificed thereafter over a time period of 21 days, and the lungs lavaged *in situ* for biochemical analysis. The BAL of these two species showed differences in degree and onset of lung injury.

B. Materials

1. Animals

Specific pathogen-free male Fischer 344 rats were obtained from Charles River Laboratories (Kingston, NY). Male, Balb/C mice, were from the breeding colony maintained in the Biology Division of the Oak Ridge National Laboratory. Animal maintenance and housing were as previously described (Chapter 1).

2. Chemicals

Beryllium sulfate tetrahydrate was supplied from Fischer Scientific Co. (Fair Lawn, NJ). Reduced nicotinamide adenine dinucleotide (NADH), sodium pyruvate, p-nitrophenyl phosphate, alkaline buffer, citrate buffer, bromocresol green, protein standard, LDH Agaro-Stain, and HEPES (N - 2 -

hydroxyethylpiperazine - N' - 2 - ethanesulfonic acid) were all supplied from Sigma Chemical Co. (St. Louis, MO). Metofane was supplied from Pitman-Moore, Inc. (Washington Crossing, NJ). Anode and cathode buffers were from Isolab, Inc. (Akron, OH). All other chemicals were reagent grade.

C. Methods

1. Inhalation Exposure

Animals were exposed to an aerosol of $BeSO_4$, or H_2SO_4 as controls. Inhalation procedures, chamber design and operation, and measurement of Be chamber concentration were previously described (Chapter 1).

a. Short Term Study

Rats were exposed to a chamber concentration of 3.3 ug Be/L with a particle mass median aerodynamic diameter of 1.95 um (σg = 1.38) or 7.0 ug Be/L with a particle mass median aerodynamic diameter of 2.0 um (σg = 1.85). Mice were exposed to a Be chamber concentration of 7.2 ug Be/L with a particle mass median aerodynamic diameter of 1.85 um (σg = 1.59).

Immediately following exposure, all animals were returned to their cages. On days 1, 3, 5, 7, 9, 11, 13, 15, 17, and 21 following exposure (day 0 designated as day of exposure) four mice from each treatment group were sacrificed for bronchoalveolar lavage analysis. On days 1, 2, 3, 5, 8, 10, 14, 17, and 21, 3 rats exposed to the 3.3 ug Be/L chamber concentration were sacrificed. Four rats exposed to the 7.0 ug Be/L chamber concentration were sacrificed on days 1, 5, 8, 14, and 21 post exposure.

b. Long Term Study

Rats were exposed to a chamber concentration of 4.05 ug Be/L with a particle mass median aerodynamic diameter of 1.9 um (σg = 1.89). Four rats from each treatment group were lavaged at 3 weeks, 3, 6, and 12 months following exposure.

2. Bronchoalveolar Lavage

On each day of sacrifice, animals were anesthetized under Metofane, the abdomen opened by midline incision and animals exsanguinated via the abdominal aorta. The diaphragm was opened, creating a pneumothorax, and the trachea exposed. For rats, the trachea was cannulated with polyethylene tubing (P.E. 205, Intrademic, Clay Adams, Becton, Dickinson and Co., Parsippany, NJ) and ligated. Lungs were then lavaged, *in situ*, with 4 ml of room temperature isotonic saline introduced gently through a plastic syringe. After 2 minutes, this first lavage was withdrawn and collected in clean plastic centrifuge tubes. Following recovery of the first lavage, each lung was washed 5 subsequent times with 4 ml of isotonic saline. All 6 lavages were pooled and the volume of each recorded as separate. Mice were lavaged according to the same procedure omitting use of the polyethylene tubing. Each mouse lung was lavaged with two separate washings of 0.5 ml isotonic saline at room temperature.

Collected lavage fluids were then centrifuged at 600xg for 10 minutes. The cell pellet was separated and enzyme assays were immediately performed on the supernatant.

Rats exposed to a chamber concentration of 7.0 ug Be/L were additionally assayed for LDH isoenzyme analysis. Procedures were identical to those previously described with the exception that the first two lavages collected from each animal were pooled and kept separate from the subsequent four lavages. Assaying for LDH activity of each six separate lavages showed that greater than 65% of the total LDH activity was recovered in the first two lavages (unpublished data). Subsequent lavages yielded LDH activity diluted beyond the sensitivity of the isoenzyme assay. All lavage fluids were then centrifuged at 600xg and the supernatant removed. The supernatants of the first two combined lavages were assayed for LDH isoenzyme analysis. Following isoenzyme separation, all six lavages were combined for biochemical analysis, for consistency with previous methods of lavage collection.

3. Biochemical Analysis

Cell free portions of each lavage were assayed spectrophotometrically for enzyme activity. The procedure for LDH determination was based upon the spectrophotometric method of Wroblewski and LaDue (1955). The activity of LDH was measured by monitoring the rate at which the substrate, pyruvate, was reduced to lactate. This reaction is coupled with the oxidation of nicotinamide adenine dinucleotide (NADH) which is followed spectrophotometrically in terms of reduced absorbance at 340 nm (A_{340}):

$$\text{pyruvate} + \text{NADH(high } A_{340}) \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD(low } A_{340})$$

For the assay of LDH activity in lavage fluid, 2.8 ml of potassium phosphate buffer (0.1 M, pH 7.5 at 25°C) and 0.1 ml of lavage sample were added to 0.2 mg of NADH and left at 25°C for 20 minutes. A 0.1 ml aliquot of sodium pyruvate (0.02 M in 0.1 M phosphate buffer, pH 7.5 at 25°C) was added to the mixture,. The decrease in absorbance at 340nm was read on a Gilford Stasar II spectrophotometer over 30 second intervals for 3 minutes versus water as a reference. Assaying different amounts of lavage sample showed the decrease in absorbance at 340 nm to be linear (unpublished data).

Values obtained by this procedure were in LDH units, as described by Wroblewski and LaDue (1955), based on the extinction of NADH at 340nm. LDH activity was then expressed as units/ml/minute where one unit of LDH will cause a decrease in A_{340} of 0.001 per minute at 25°C in a 3 ml reaction mixture in a cuvet of 1 cm lightpath. One International Unit (IU) of an enzyme is defined as that amount which will convert 1 umole of substrate per minute under the specified conditions of the procedure. The units of LDH (units/ml/minute) were then converted to IU by multiplying by 0.48 (Sigma Technical Bulletin No. 340-UV, 1980). Data is expressed as IU/lung by multiplying the LDH activity (IU/ml) by the total amount of lavage recovered for each animal (ml/lung).

Calculations of LDH activity in the BAL were performed using the following formula:

$$\text{LDH (units/ml)} = \text{A/min} \times \text{TCF}/(0.001 \times 0.1 \times \text{lightpath (cm)})$$

Where: ΔA = Δ Absorbance decrease at 340nm

0.001 = Δ A equivalent to 1 unit of LDH activity in a 3 ml volume with 1 cm lightpath at 25°C

0.1 = sample volume

TCF = Temperature Correction Factor (1.0 at 25°C)

For determining phosphatase activity in the BAL, p-nitrophenyl phosphate was used as the substrate. Upon hydrolysis of p-nitrophenyl phosphate, p-nitrophenol and inorganic phosphate are formed (Walter and Schutt, 1974):

$$\text{p-nitrophenyl phosphate} + H_2O \rightarrow \text{p-nitrophenol} + H_3PO_4$$

After a 30 minute incubation period, the phosphatases are completely inhibited by NaOH and the p-nitrophenol is converted to a yellow complex, readily measured at 400nm. The intensity of the color formed is directly proportional to the phosphatase activity.

For the determination of alkaline phosphatase in lung lavage, the following procedure was used (Sigma Technical Bulletin No. 104, 1982). One - half an ml of alkaline buffer (glycine at 0.1 M and magnesium chloride at 0.001 M, pH 10.5 at 25°C) and 0.5 ml of substrate (p-nitrophenyl phosphate, 4mg/ml) were pipetted into two separate tubes. One - tenth an ml of distilled water was pipetted into one tube labeled blank and 0.1 ml of lung lavage was pipetted into a second tube labeled test. Tubes were placed in a water bath at 37°C for 30 minutes. After exactly 30 minutes, 10 ml of 0.02 N NaOH was

pipetted into each tube and mixed. The absorbance of the sample was read, using the blank as reference, at 400nm using a Gilford Stasar II spectrophotometer. Two drops (0.1 ml) of concentrated HCl was added to each tube and mixed. Addition of acid removed the color due to p-nitrophenol leaving the absorbance of the lavage fluid. The absorbance of the test was again read using the blank as reference. This reading of the test sample was subtracted from its previous absorbance to yield the corrected alkaline phosphatase value. To calculate alkaline phosphatase units from the measured absorbance, a standard curve was made by diluting p-nitrophenol with alkali, and measuring its absorbance at 400nm. From the linear regression analysis of these results, the best fit line was determined with a correlation coefficient of 0.9999 as:

$$y = mx + b$$

with

y = absorbance at 400nm

m = slope

x = alakline phosphatse units (mIU/ml)

b = intercept (absorbance at 400nm)

Data is expressed as mIU/lung by multiplying the Alk Pase activity (mIU/ml) by the total amount of lavage recovered (ml/lung). To determine the alkaline phosphatase units:

$$\text{alkaline phosphatase units} = \frac{\text{sample absorbance} - \text{intercept}}{\text{slope}}$$

To determine acid phosphatase activity of the lung lavage, the following procedure was used (Sigma Technical Bulletin No. 104, 1982). One - half an ml of substrate and 0.5 ml of citrate buffer (citric acid at 0.09 M and chloride at 0.01 M, pH 4.8 at 25°C) were pipetted into two separate tubes. Two - tenths of an ml of distilled water was pipetted into one tube labeled reagent blank and 0.2 ml of lavage was pipetted into a second tube labeled test. Each were placed into a water bath at 37°C for 30 minutes. After 30 minutes, 5 ml of 0.1 N NaOH was added to each tube and mixed. A second blank was prepared by mixing 6 ml of 0.1 N NaOH and 0.2 ml of lavage fluid. The absorbance of the blank was read versus water as reference at 400nm. Next, the absorbance of the test sample was read versus the reagent blank as reference at 400nm. The blank reading was subtracted from the absorbance of the test sample to yield the corrected acid phosphatase units.

To calculate acid phosphatase units from the measured absorbance, a standard curve was made as was done for the alkaline phosphatase determinations. From the linear regression analysis of these results, a best fit line was determined, with a correleation coefficient of 0.9999 as:

$$y = mx + b$$

with:

y = absorbance at 400nm

m = slope

x = acid phosphatase units (mIU/ml)

b = intercept (absorbance at 400nm)

Data is expressed as mIU/lung by multiplying the Ac Pase activity (mIU/ml) by the total amount of lavage recovered (ml/lung). To determine the acid phosphatase units:

$$\text{acid phosphatase units} = \frac{\text{sample absorbance} - \text{intercept}}{\text{slope}}$$

The determination of albumin in lung lavage was based on the methods of Doumas (1972) utilizing the direct binding of the anionic dye, bromocresol green (BCG), to albumin. Under ideal conditions, the dye binds to albumin but not to other serum proteins. At pH 4.0, bromocresol green displaces most substances bound to albumin, and at a wavelength of 625nm, most other serum constituents show negligible absorption (Sigma Technical Bulletin No. 630, 1982).

When albumin binds bromocresol green an intense green colored albumin - bromocresol green complex is formed:

albumin + BCG → albumin - BCG

(yellow) (green)

The intensity of the green color formed is measured at 625nm and is directly proportional to the amount of albumin present. For the assay of albumin in lung lavage, the following procedures were used (Sigma Technical Bulletin No. 630, 1982). The following reagents were added to tubes containing 5 ml of bromocresol green (0.01%, w/v), as indicated; blank, 0.02 ml of 0.9% sodium chloride; standard, 0.02 ml of protein standard (albumin (human), 5 g/dl and gamma-globulin (human), 3 g/dl); test, 0.02 ml of lavage

sample. Each was mixed and allowed to stand for 10 minutes. The absorbance of the standard and test sample was read versus the blank as reference at 625nm.

To calcuate the amount of albumin present:

$$\text{albumin (g/dl)} = \frac{\text{absorbance of test sample}}{\text{absorbance of standard}} \times 5$$

Data is expressed as mg/lung by multiplying the amount of albumin (mg/ml) by the total amount of lavage recovered (ml/lung).

4. LDH Isoenzyme Analysis

Rats exposed to a chamber air concentration of 7.0 ug Be/L were subjected to BAL, and the lavage fluid analyzed for isoenzymes of LDH. On each day of sacrifice, animals were anesthetized under Metofane and the abdomen opened by midline incision. The diaphragm was cut and the thorax opened to expose the lungs. Blood (from one control animal) was collected by cardiac puncture and stored on ice for one hour for collection of serum. All other animals were exsanguinated via the abdominal aorta. Lungs were lavaged (as previously described) and perfused through the right ventricle of the heart with isotonic saline until uniformly white in appearance. From one control animal, the heart and liver were excised, trimmed of excess tissue and stored on ice to serve as control markers for LDH isoenzyme separation. All tissues (heart, liver, and lung) were homogenized in 5 volumes (5 ml buffer to 1 ml of homogenate) of cold Hepes KCl buffer (Hepes, 0.02 M and KCl, 1.15%, pH

7.4 at 4°C) in a Polytron homogenizer (Beckman Instruments, Westbury, NY) and centrifuged at 20,000xg for 30 minutes on a Sorvall RC2-B centrifuge (DuPont Instruments, Wilmington, DE). The homogenized supernatant was removed for separation of tissue LDH isoenzymes. Blood was centrifuged at 600xg on a International Equipment model PR-2 centrifuge for 30 minutes and the serum was collected.

For the separation of LDH isoenzymes, sample aliquots were applied to precast agarose gels containing isolytes of the Isolab Resolve Electrophoresis System (Isolab, Inc., Akron, OH). Eight ul aliquots from each lavage and serum sample and 2 ul aliquots from each tissue homogenate were applied to the precast gels. These sample amounts allowed optimum visualization of the electrophoretic bands. Samples were allowed to diffuse into the gel until all sample wells appeared dry. Gels were then placed on a Resolve Electrophoresis Unit (Resolve Electrophoresis System, Isolab, Inc., Akron, OH). Precut wicks, containg cathode (0.5 M NaOH) and anode (0.5 M acetic acid) buffers were aligned at their respective electrodes. The leads from the Resolve Unit were connected to a Bio - Rad model 3000/300 constant power supply (Bio - Rad Laboratories, Richmond, CA) with a constant power setting of 15 Watts. Electrophoresis was allowed to progress for 30 minutes for isoenzyme separation. The gel was removed from the unit and an even layer of LDH Agaro-Stain (10 parts NAD, 85 parts Lithium L(+) lactate, 5 parts tetranitrobluetetrazolium (TNBT), and 0.1 part phenazine methosulfate (PMS)) was applied over the surface of the gel. Following incubation at 37°C for 15 minutes, excess LDH Agaro - Stain was removed and a fresh solution

reapplied for an additional 15 minute incubation period. After a total 30 minute incubation, the staining solution was discarded and gels were fixed for one hour in methanol and acetic acid (5 parts glacial acetic acid and 20 parts distilled water to 75 parts methanol). This methanol acetic acid solution was then discarded and the gels soaked for one hour in distilled water to remove ampholytes and destaining solution. Gels were then dried overnight at 90°C and stored in the dark to prevent fading.

The areas of enzyme activity were visualized by histochemical procedures according to the following equations (Sigma Technical Bulletin No. 705 - EP, 1979):

1. L(+) lactate + NAD $\xrightarrow{LDH}$ pyruvate + NADH
2. NADH + PMS $\rightarrow$ NAD + PMS - H
3. PMS - H + TNBT $\rightarrow$ PMS + TNBT - formazan

The TNBT - formazan complex is highly colored and insoluble, thereby localizing the electrophoretic zones of LDH activity in the gel.

5. Statistical Analysis

All statistical analyses were performed by the Student's t test. Data is expressed as mean ± S.E.

D. Results

The amount of the lavage analyzed was slightly lower for mice than rats, averaging 75-76% (Table 6). Rats returned a higher per cent of the instilled lavage. Animals exposed to: 3.3 ug Be/L averaged 95%; 4.05 ug Be/L averaged 87%; and 7.0 ug Be/L averaged 87-89%. In mice, the lower total lung volume required a smaller amount of lavage fluid instilled into the lungs and hence, a greater difficulty in manipulation.

Table 6

Per Cent Lavage Recovered[a]

Concentration (ug Be/L)	Species	Treatment	Per Cent Recovery
3.3	Rat	Be	95
		Control	95
4.05	Rat	Be	87
		Control	87
7.0	Rat	Be	87
		Control	89
7.2	Mice	Be	75
		Control	76

[a]Values are the means of combined average values from each day of sacrifice.

The LDH activity of the BAL in rats is shown in Figure 11. Animals exposed to a chamber concentration of 3.3 ug Be/L showed significantly different values from that of control groups at the earliest measured time point of day 1 (0.97 vs 0.26). These levels increased to a maximum at day 8 (6.68 vs 0.22), returning to control levels by day 21 (0.42 vs 0.26). Alkaline phosphatase (Figure 12) was also significantly increased by day 1 (310 vs 52.3). This rose to a maximum at day 5 (1289 vs 56.9) and decreased by day 21 (246 vs 89.0), remaining significantly elevated. Acid phosphatase lung lavage levels (Figure 13) were significantly elevated at day 1 (22.2 vs 9.27). The maximum response plateaued on day 2 (47.8 vs 19.5) and day 3 (47.8 vs 13.9), followed by a decrease to control levels at day 21 (17.3 vs 17.0).

Rats exposed to a chamber concentration of 7.0 ug Be/L showed a similar pattern in enzyme activity as that of animals exposed to 3.3 ug Be/L. Lactate dehydrogenase activity in the lung lavage showed a slightly greater response at this level of exposure (Figure 11). Values were significantly greater at day 1 (1.15 vs 0.10), maximum at day 8 (7.59 vs 0.26) and decreased to day 21 (0.23 vs 0.12). Alkaline phosphatase showed the same response pattern at this higher dose level (Figure 12). Day 1 values (523 vs 216) were significantly elevated compared to controls and rose to a peak response at day 5 (2316 vs 161), nearly twice the value as the lower dose at the same time point. Alkaline phosphatase activity decreased to day 21 (269 vs 119) remaining significantly higher than controls. Acid phosphatase activity (Figure 13) was significantly elevated by day 1 post exposure (44.4 vs 12.2), approximately twice the value as that obtained from the lower exposure concentration. By day 5 (43.8 vs 22.4) the response remained similar as at day

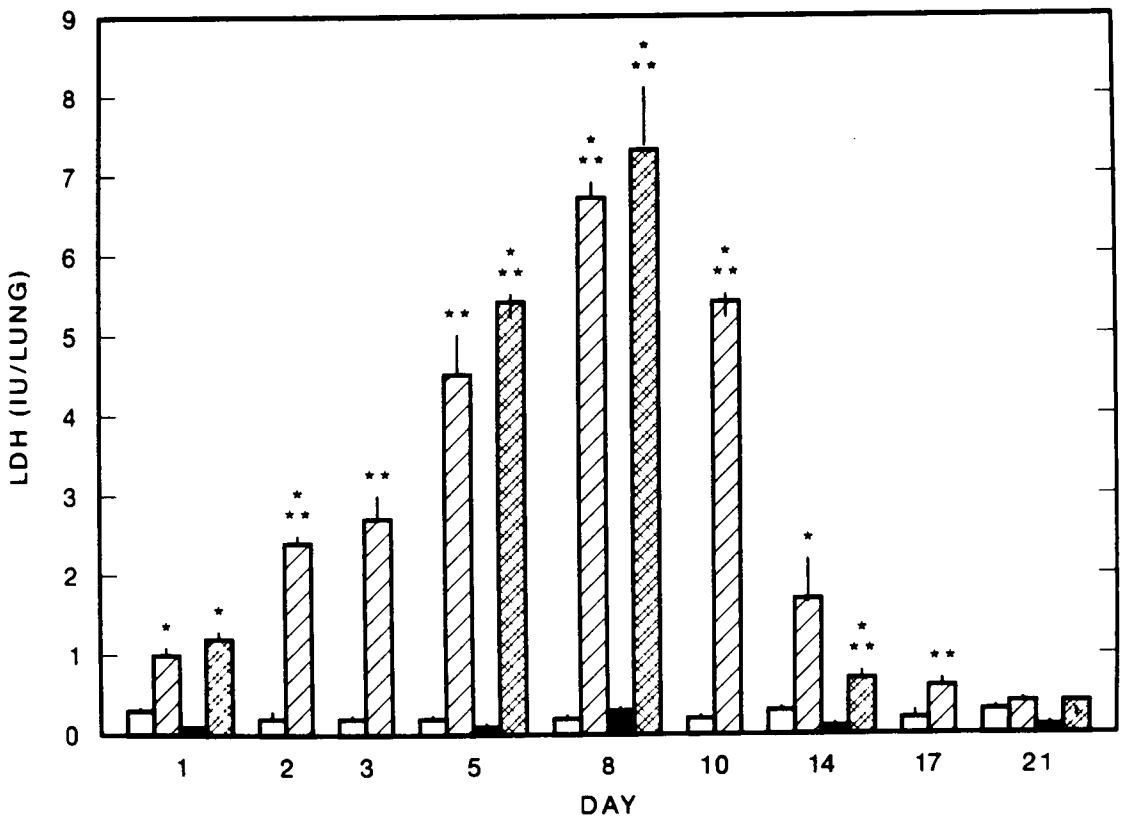


Figure 11. Lactate dehydrogenase activity in lung lavage of rats on days 1-21. Animals exposed to an aerosol of 3.3 ug Be/L (▨) or H_2SO_4 as controls (□) or 7.0 ug Be/L (▨) and H_2SO_4 as controls (■). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

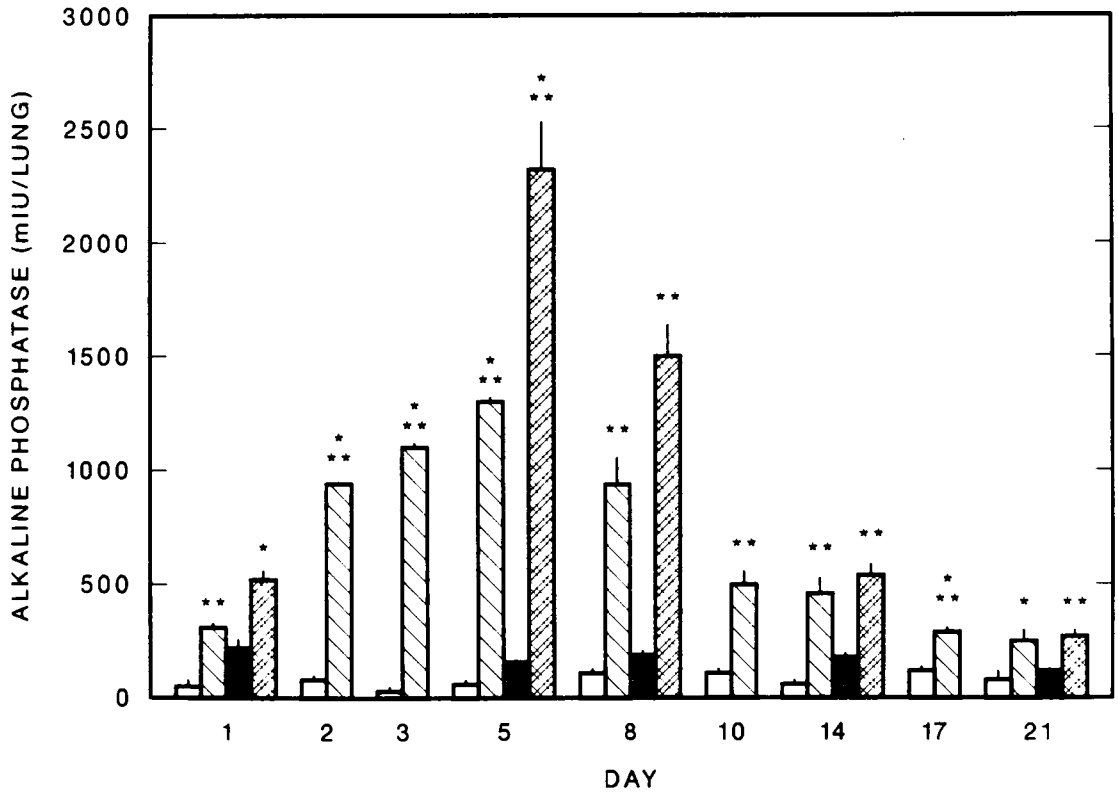


Figure 12. Alkaline phosphatase activity in lung lavage of rats on days 1-21. Animals exposed to an aerosol of 3.3 ug Be/l (▨) and H_2SO_4 as controls (□) or 7.0 ug Be/L (▨) and H_2SO_4 as controls (■). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

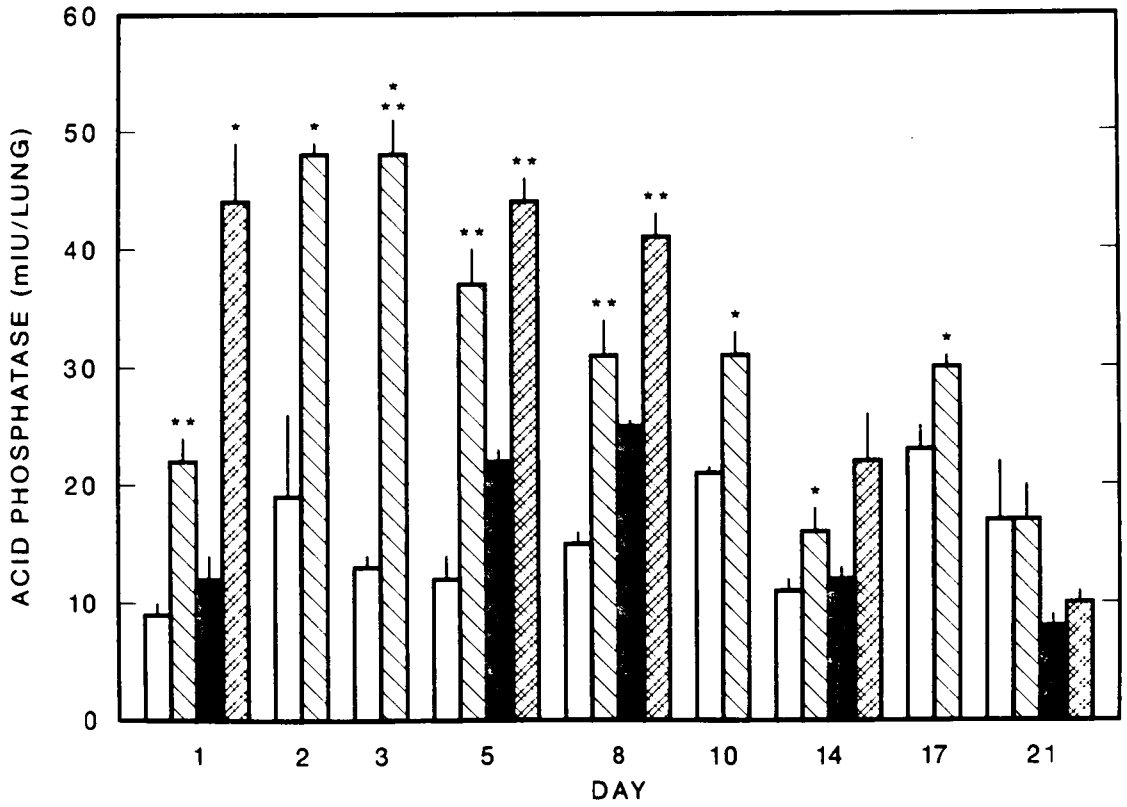


Figure 13. Acid phosphatase activity in lung lavage of rats on days 1-21. Animals exposed to an aerosol of 3.3 ug Be/L (▨) and H_2SO_4 as controls (▭) or 7.0 ug Be/L (▩) and H_2SO_4 as controls (■). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

1, slightly greater than the lower dose. At day 14 (22.2 vs 11.6) the response was no longer significant against control values.

Albumin (Figure 14), measured in the BAL of rats exposed to the higher chamber concentration (7ug Be/L), was significantly elevated by day 5 (6.97 vs 1.72). Although albumin could be detected in the BAL of rats exposed to $BeSO_4$ at day 1, none was measured in the control animals at this time point. Quantities of albumin in the BAL of $BeSO_4$ treated rats continued to rise by day 8. However, since control values also rose, there was no significant difference between groups. By day 14 (4.00 vs 8.42), BAL albumin of $BeSO_4$ treated rats showed an unexplained depression versus control values. At day 21 (2.61 vs 2.07) both groups were comparable.

With the extremely large increase in LDH and Alk Pase seen in the lung lavage of rats, the analysis was centered on these two enzymes in the BAL of mice. Additionally, as an index of capillary damage, albumin was also measured.

Lactate dehydrogenase activity in the BAL of mice was significantly elevated by day 3 (147 vs 33.9) post exposure (Figure 15) and remained so by day 5 (149 vs 39.1). The activity of LDH in the BAL decreased by day 11 (67.5 vs 43.4). Day 13 values (67.6 vs 40.7) were significantly elevated, returning to control values by 21 days (44.4 vs 54.7). Alkaline phosphatase activity showed a varied response in the BAL of mice over the 21 day period (Figure 16). Values were significantly elevated by day 3 (2.32 vs 0.72). Alk Pase activity began to decrease over the remaining time points tested. The response was never consistent after day 3, being unmeasureable in control groups at 7, 11, 13, 15, and 21 days following exposure. Treated groups

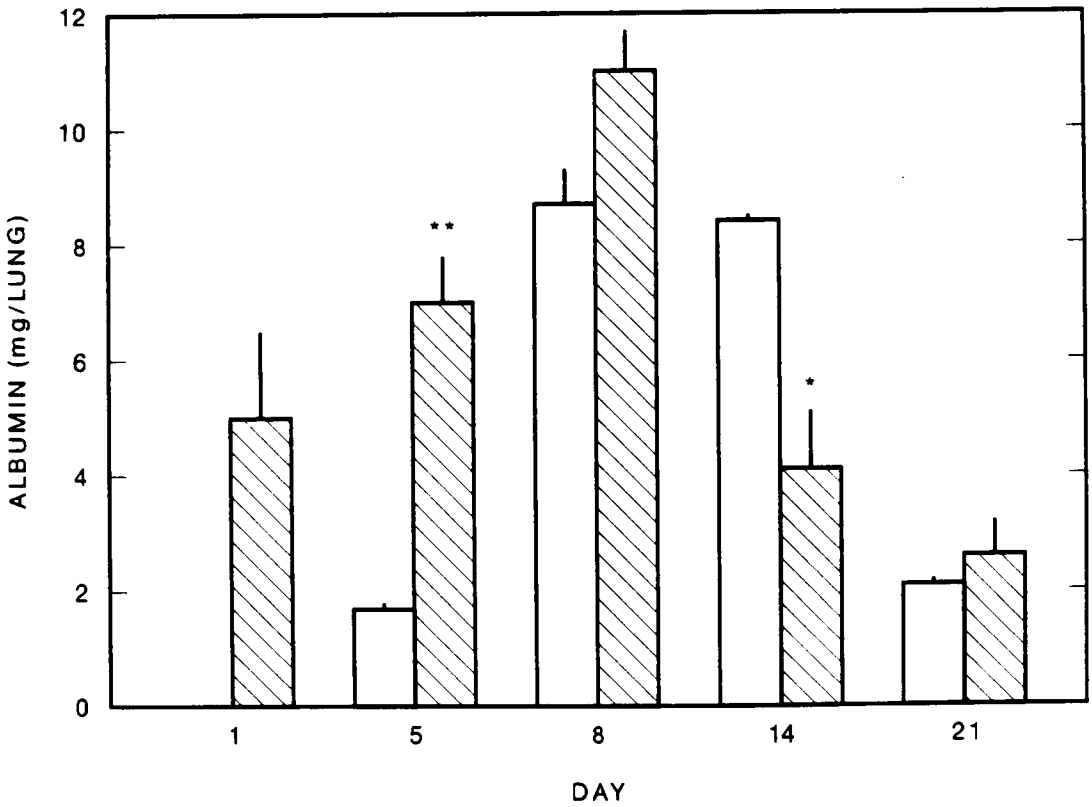


Figure 14. Albumin in lung lavage of rats on days 1-21. Animals exposed to an aerosol of 7.0 ug Be/L (▨) and H_2SO_4 as controls (▭). Values are mean (SE). *,**, Denotes $p<0.05$, or 0.01, respectively.

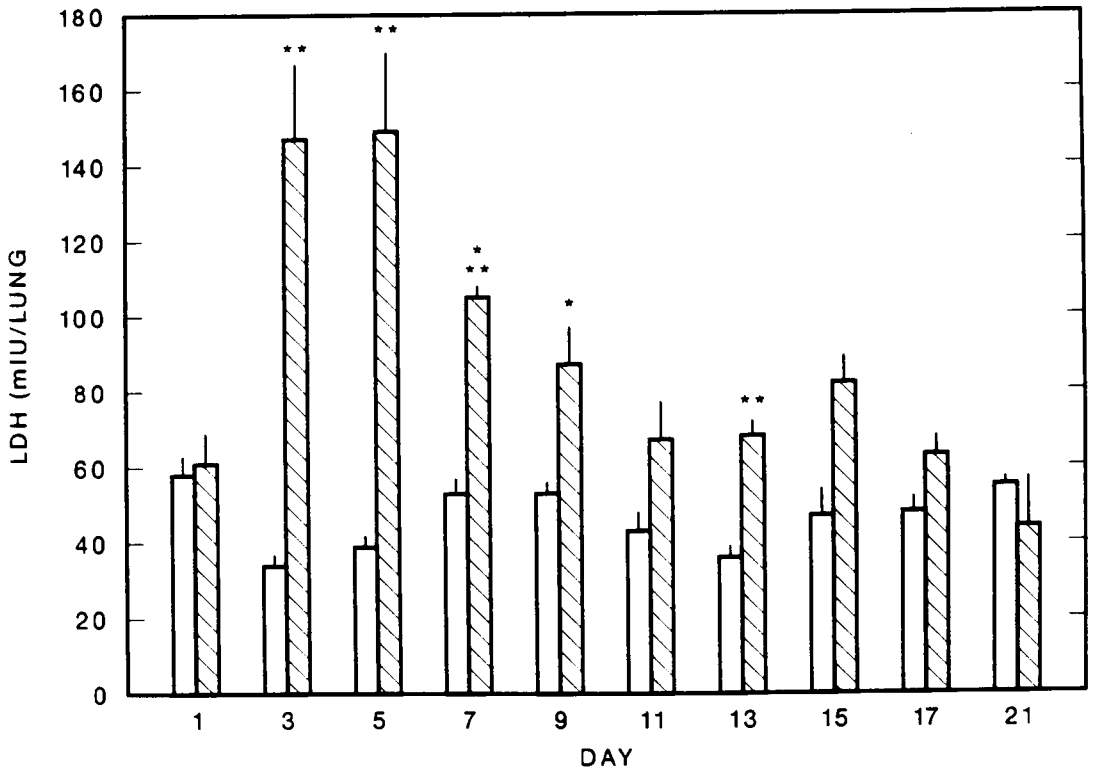


Figure 15. Lactate dehygrogenase activity in lung lavage of mice on days 1-21. Animals exposed to an aerosol of 7.2 ug Be/L (▨) and H_2SO_4 as controls (▭). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

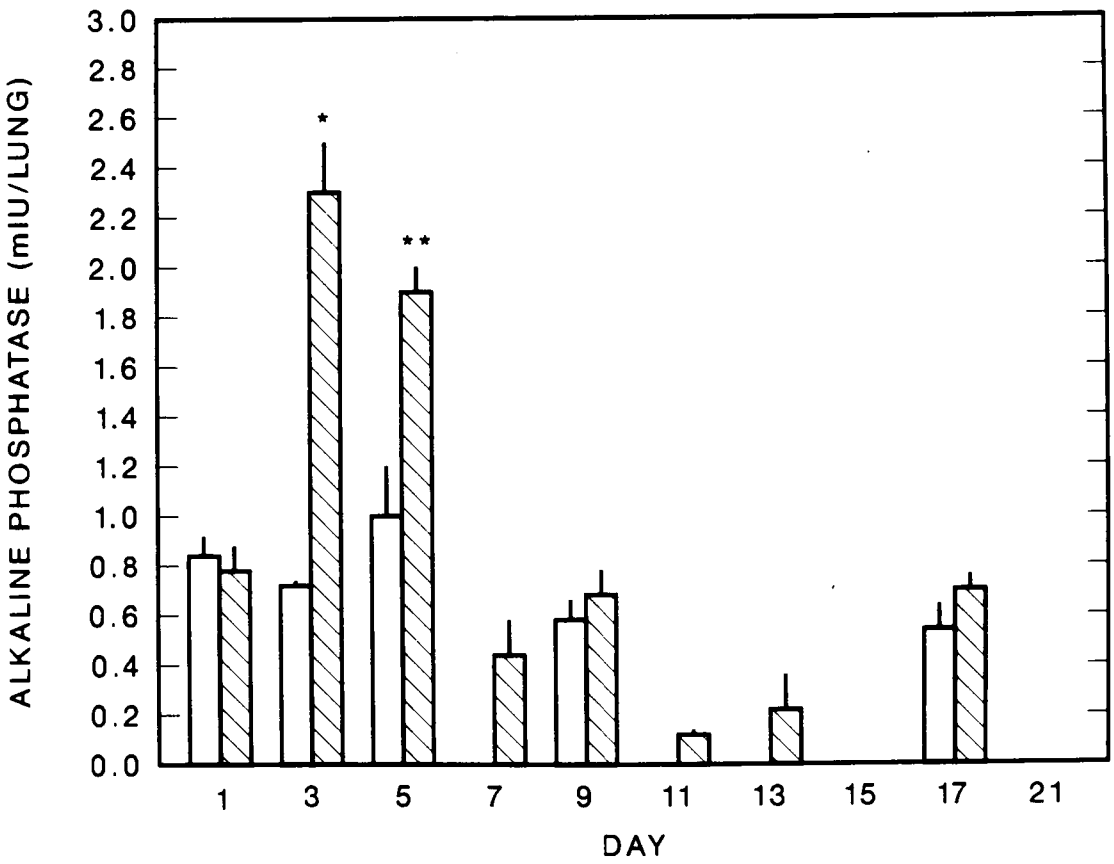


Figure 16. Alkaline phosphatase activity in lung lavage of mice on days 1-21. Animals exposed to an aerosol of 7.2 ug Be/L (▨) and H_2SO_4 as controls (▭). Values are mean (SE). *,**, Denotes $p<0.05$, or 0.01, respectively.

showed no measureable quantities on days 15 and 21 post exposure. Albumin measured in the BAL of mice was significantly elevated only at day 5 (0.30 vs 0.19) although the response of both control and treated groups showed a variation over the entire 21 day period (Figure 17).

As a determination of the origin of LDH activity in the BAL of rats exposed to 7.0 ug Be/L, the isoenzyme profile of treated and control animals was determined. With the increase in LDH activity seen at day 1, a concommittant increase in the intensity of band staining in the lavage profile was seen in $BeSO_4$ treated animals (Figure 18). The pattern of staining, at this time, was more characteristic of the serum than the lung homogenate profile. By day 5 post exposure (Figure 19), staining intensity increased, taking on the profile more similar to that of the lung homogenate. This staining intensity continued to increase to day 8 (Figure 20), the maximum response of LDH activity seen within the lavage fluid of treated rats. The isoenzyme profile of the lung lavage at day 8 was very similar to the lung homogenate profile of treated animals. Day 14 isoenzyme patterns (Figure 21) showed a decreased staining intensity with little similarity to lung homogenate profiles, and by day 21 (Figure 22), no difference between control and Be treated rats was apparent.

Long term results of LDH activity in the BAL of rats is graphically illustrated in Figure 23. Rats were exposed to different Be chamber concentrations than animals of the short term study since a consistent Be chamber concentration could not be reproduced. Three week LDH values, as in the acute study, were not different from control. By 3 months, LDH activity reached a maximum value, significantly greater than controls. The amount of

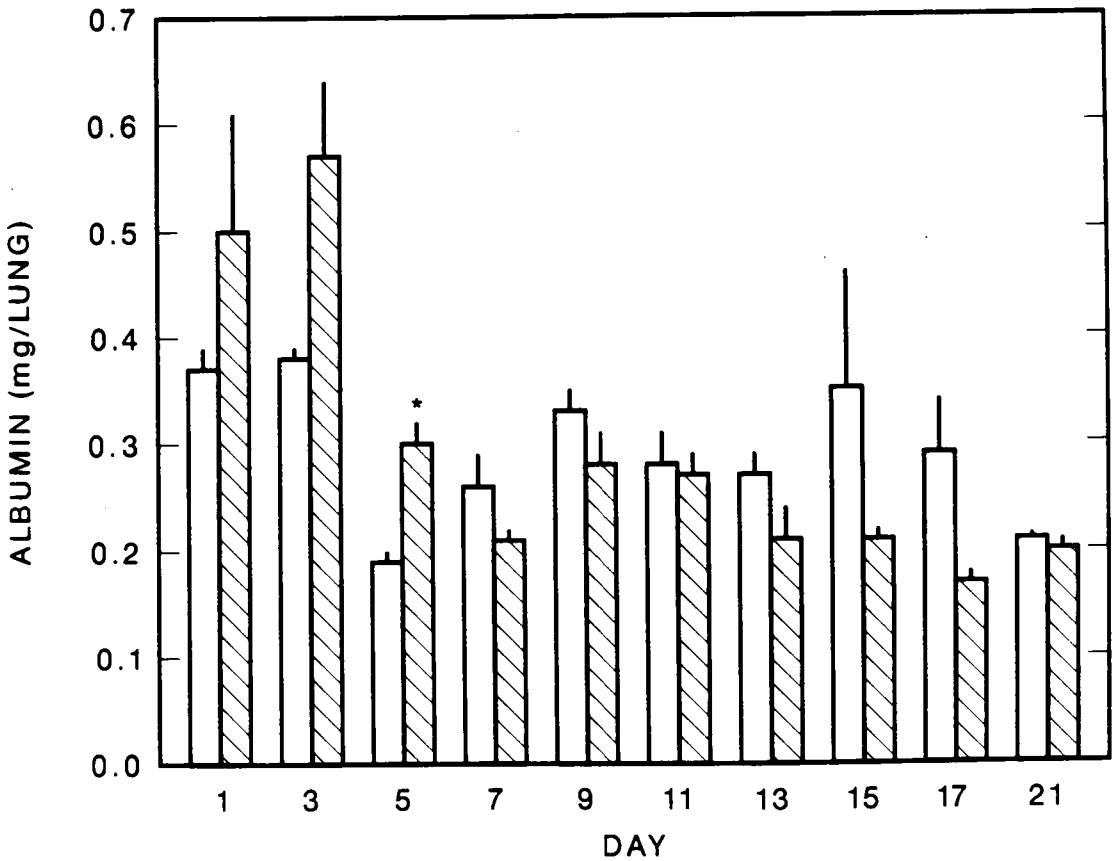


Figure 17. Albumin in lung lavage of mice on days 1-21. Animals exposed to an aerosol of 7.2 ug Be/L (▨) and H_2SO_4 as controls (▭). Values are mean (SE). * Denotes $p<0.05$.

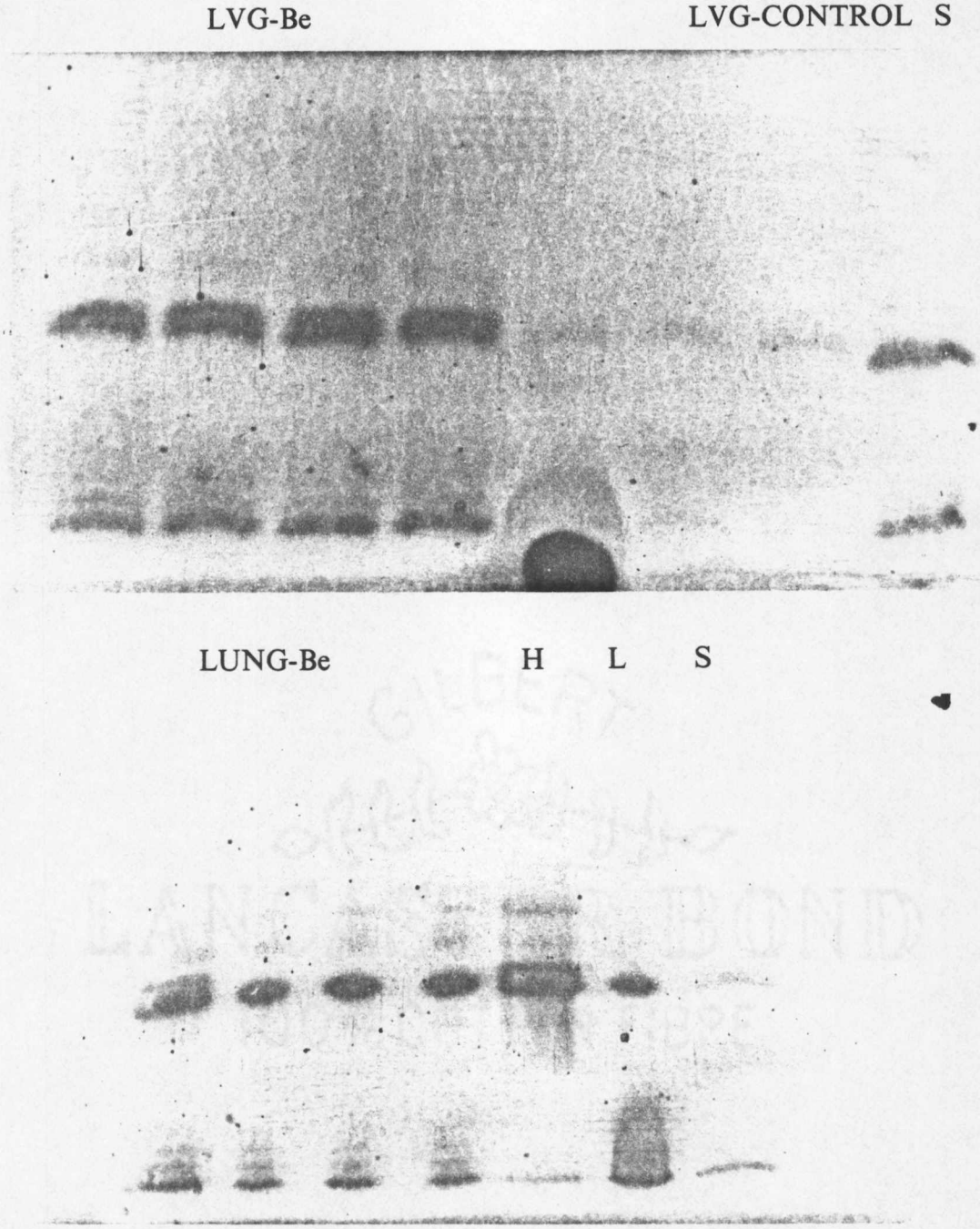


Figure 18. Lactate dehydrogenase isoenzyme profile 1 day post exposure. Animals exposed to an aerosol of 7.0 ug Be/L. LVG-Be, lavage from $BeSO_4$ treated rats; LVG-Control, lavage from H_2SO_4 exposed rats; H, heart; L, liver; S, serum: LUNG-Be, lung homogenate from $BeSO_4$ treated rats.

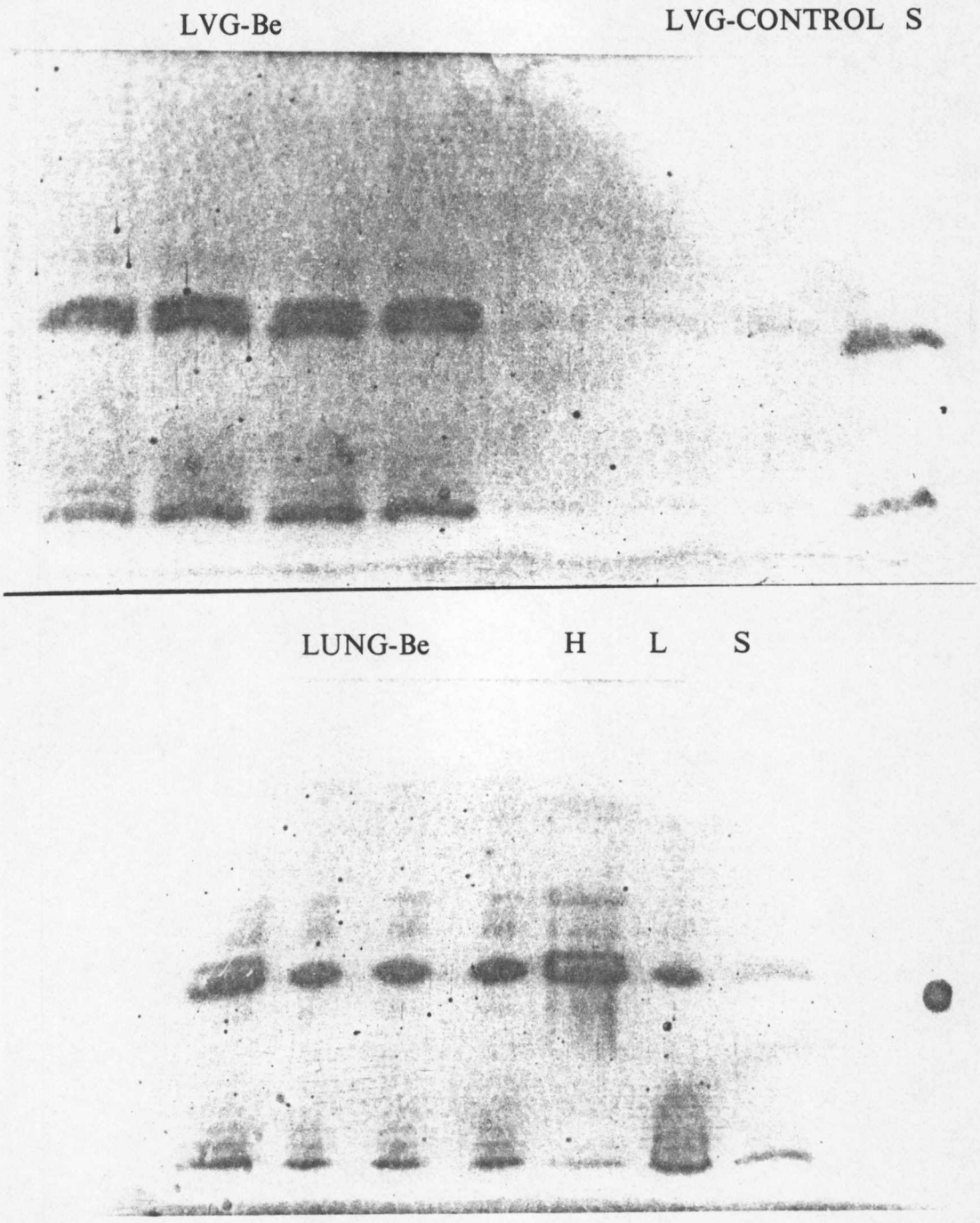


Figure 19. Lactate dehydrogenase isoenzyme profile 5 days post exposure. Animals exposed to an aerosol of 7.0 ug Be/L. LVG-Be, lavage from $BeSO_4$ treated rats; LVG-Control, lavage from H_2SO_4 exposed rats; H, heart; L, liver; S, serum: LUNG-Be, lung homogenate from $BeSO_4$ treated rats.

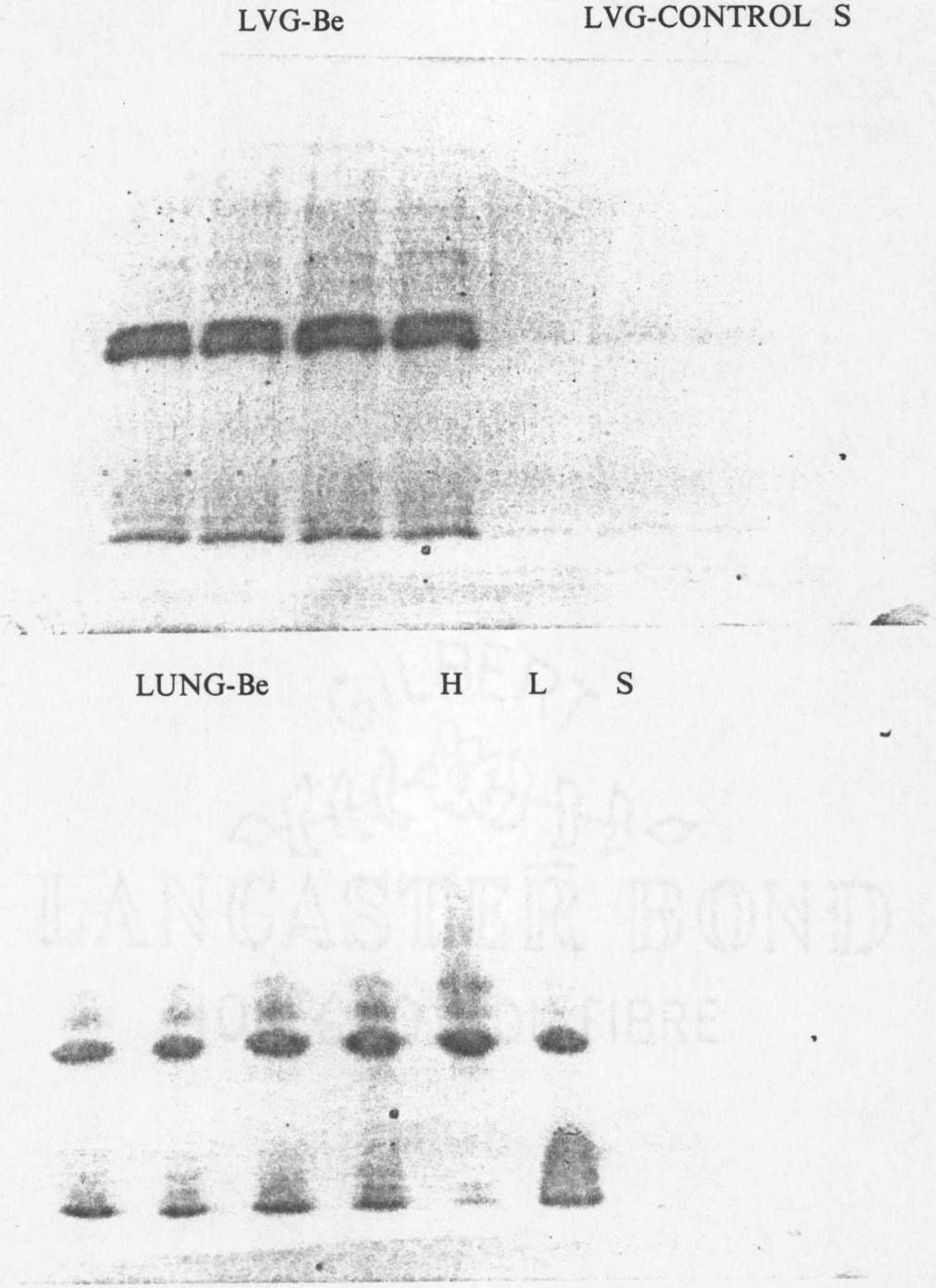


Figure 20. Lactate dehydrogenase isoenzyme profile 8 days post exposure. Animals exposed to an aerosol of 7.0 ug Be/L. LVG-Be, lavage from $BeSO_4$ treated rats; LVG-Control, lavage from H_2SO_4 exposed rats; H, heart; L, liver; S, serum: LUNG-Be, lung homogenate from $BeSO_4$ treated rats.

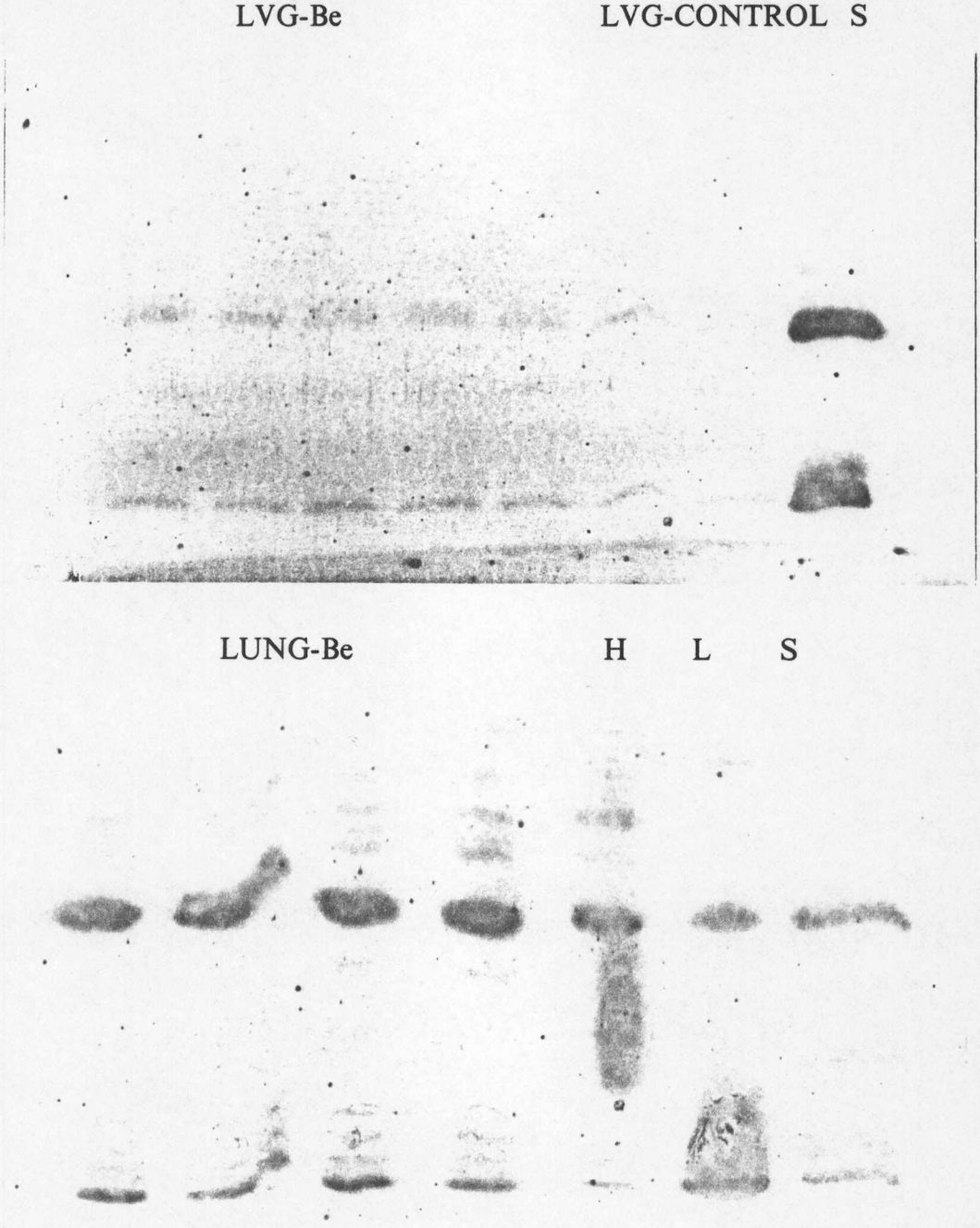


Figure 21. Lactate dehydrogenase isoenzyme profile 14 days post exposure. Animals exposed to an aerosol of 7.0 ug Be/L. LVG-Be, lavage from $BeSO_4$ treated rats; LVG-Control, lavage from H_2SO_4 exposed rats; H, heart; L, liver; S, serum: LUNG-Be, lung homogenate from $BeSO_4$ treated rats.

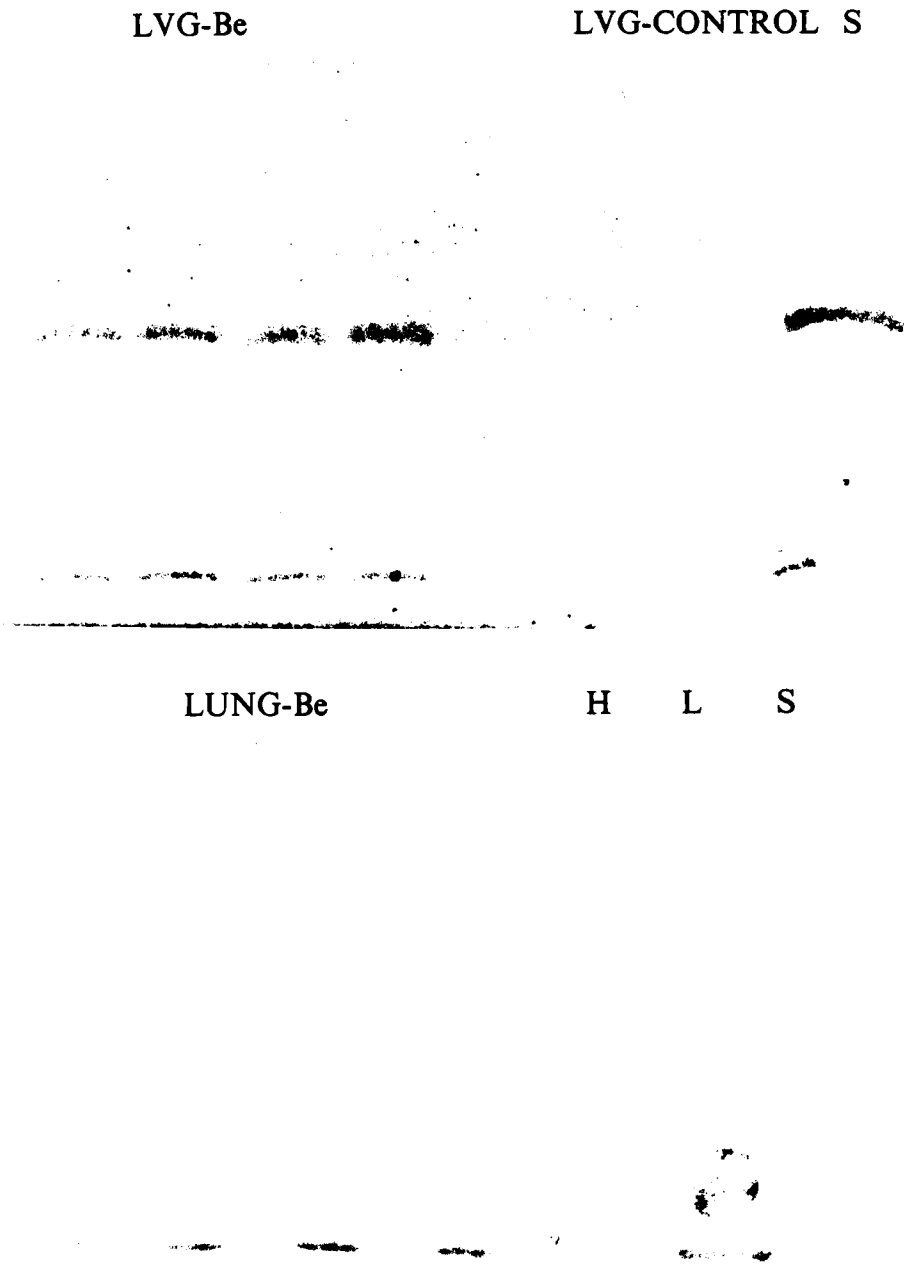


Figure 22. Lactate dehydrogenase isoenzyme profile 21 days post exposure. Animals exposed to an aerosol of 7.0 ug Be/L. LVG-Be, lavage from $BeSO_4$ treated rats; LVG-Control, lavage from H_2SO_4 exposed rats; H, heart; L, liver; S, serum: LUNG-Be, lung homogenate from $BeSO_4$ treated rats.

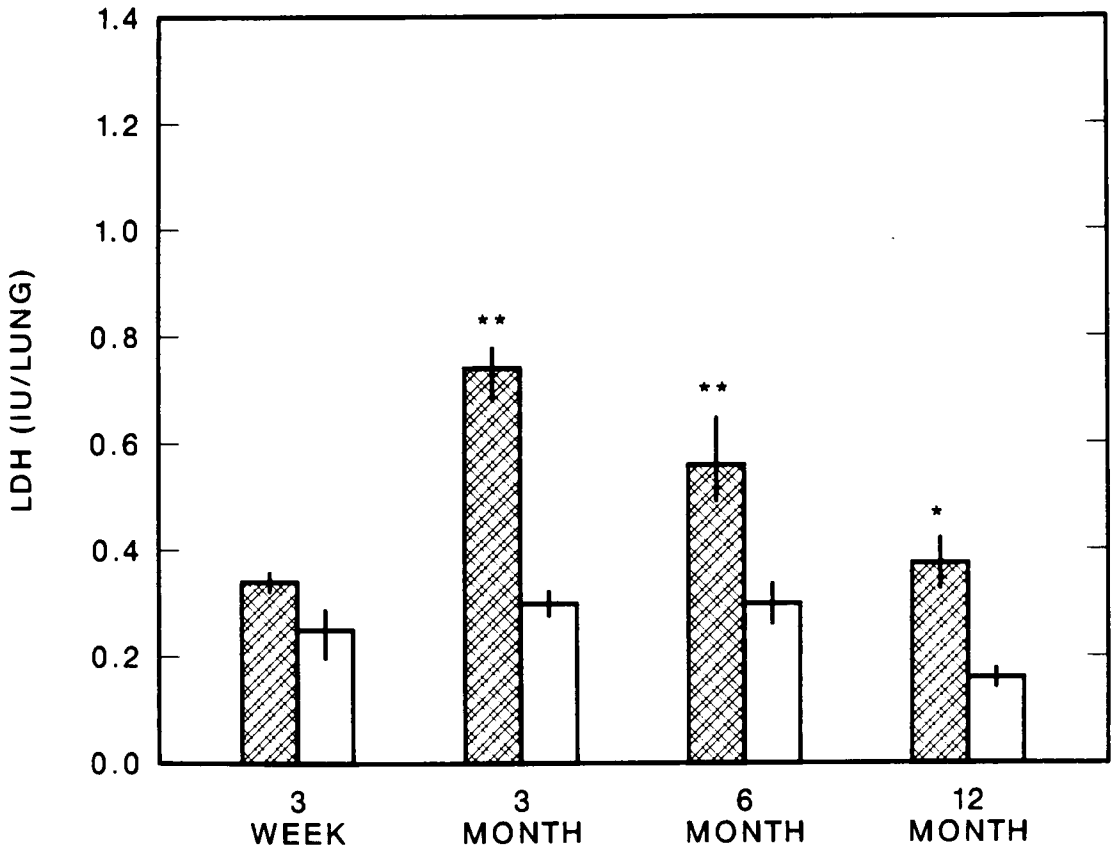


Figure 23. Lactate dehydrogenase activity in lung lavage of rats over a one year period. Animals exposed to an aerosol of 4.05 ug Be/L (▨) or H_2SO_4 as controls (□). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

LDH gradually declined over the remaining one year. Alkaline phosphatase showed the same general pattern (Figure 24). Values at 3 weeks were significantly different from controls, similar to the acute study. As was the case for LDH, Alk Pase reached a maximum over the 12 month period at 3 months post exposure. This activity then declined until by 12 months, no significant difference was observed between groups. Acid phosphatase activity, unlike that of the acute study, was significantly elevated at 3 weeks (Figure 25). Total activity declined by 3 months, remaining significantly greater than comparable controls. Both control and treated groups showed a relatively constant level following this time with no differences noted between groups.

In a separate experiment to better determine the origin of Alk Pase in the BAL, one male Fischer 344 rat was dosed intratracheally with 10 mg/kg of $BeSO_4$. The first two lavages (4 ml) were collected as previously described (Chapter 3). The pooled lavages were centrifuged at 600xg, to remove cells, and the supernatant collected. This supernatant was then centrifuged at 100,000xg, for 90 minutes, to pellet the phospholipid fraction. Following centrifugation, the supernatant was withdrawn and the phospholipid pellet resuspended in 1 ml of isotonic saline. Both the resuspended pellet and supernatant were analyzed for Alk Pase activity (Chapter 3). Results (Table 7) show that 98% of the total Alk Pase activity of the lung lavage was recovered in the phospholipid (surfactant) portion.

E. Discussion

The technique of bronchoalveolar lavage is based upon the presumption that biochemical parameters of lung washings will be indicative of the degree

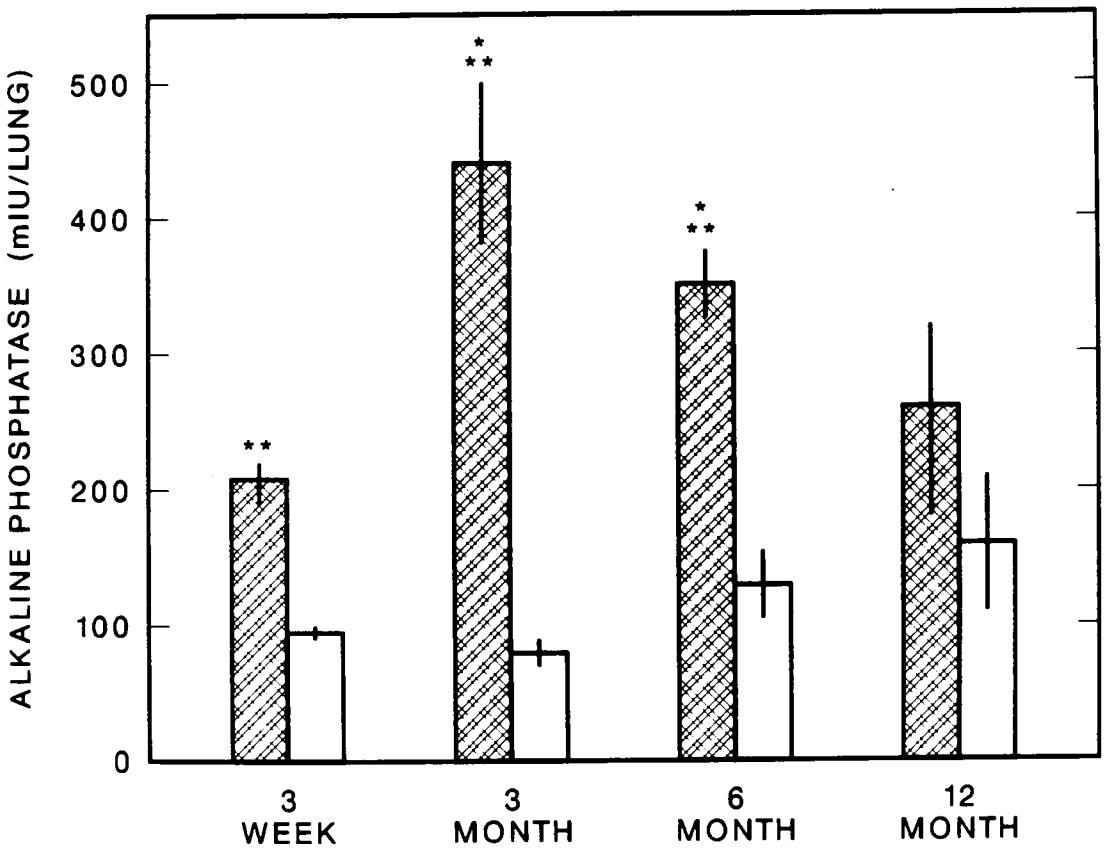


Figure 24. Alkaline phosphatase activity in lung lavage of rats over a one year period. Animals exposed to an aerosol of 4.05 ug Be/L (▨) or H_2SO_4 as controls (□). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

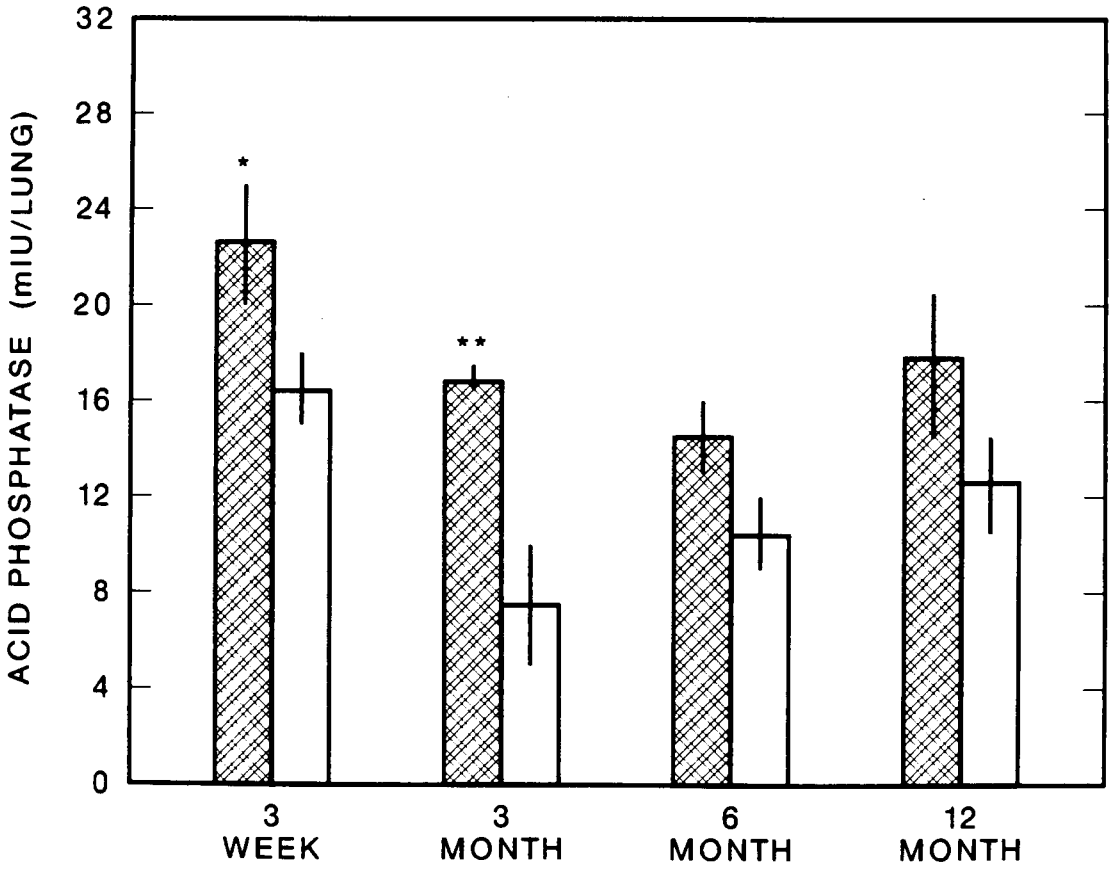


Figure 25. Acid phosphatase activity in lung lavage of rats over a one year period. Animals exposed to an aerosol of 4.05 ug Be/L (▨) or H_2SO_4 as controls (□). Values are mean (SE). *,**,***, Denotes $p<0.05$, 0.01, or 0.005, respectively.

Table 7

Alkaline Phosphatase Activity (mIU/L)[a]

Sample	mIU/L	% of Total
Pellet	169	97.9
Supernatant	3.6	2.1

[a]Activity of alkaline phosphatase in surfactant (pellet) vs supernatant of lung lavage.

and type of lung injury induced by toxicants. Henderson, et al., (1981), reported differing lung lavage profiles depending on the type of lung injury induced, which included both biochemical measurements as well as the cytological response found within the lavage.

When rats were exposed to an aerosol of $BeSO_4$, at chamber concentrations of 3.3 and 7.0 ug Be/L, LDH and Alk Pase activity measured in the BAL were the most sensitive indicators of lung damage. The specific cell types from which these two enzymes originate can not be discerned by the spectrophotometric methods commonly employed. Beck, et al, (1983) reported the variation of LDH isoenzymes following lung injury from pneumotoxic agents. The study by Beck demonstrated that the LDH isoenzyme profile can be used to differentiate among types of pulmonary injury which may be more useful than estimates of total LDH levels. In this regard, the results of the work presented in this chapter did not determine the specific cell origin of the LDH released into the BAL. The purpose of studying the isoenzyme profile

was to assess contamination of the lavage fluid from enzymes of serum source. Results then suggested that the LDH isoenzyme profiles of treated rats showed a similarity to lung tissue LDH.

Likewise, as in the measurement of LDH, spectrophotometric results of Alk Pase levels can not discriminate its source. Reasor, et al., (1978) has characterized pulmonary Alk Pase from rabbits and reports three separate isoenzymes within the BAL. Two isoenzymes are located extracellularly within the airways with the third located entirely within the lung tissue. The authors speculated that the two extracellular isoenzymes did not arise from the free cells of the airways and that the enzyme may be associated with the surfactant fraction of the acellular lining. Measurements of Alk Pase in the BAL are based upon the determination of this enzyme as an index of type II pneumocyte destruction (Henderson, et al., 1979). Therefore, the dramatic increase of Alk Pase in the BAL of rats would indicate a massive destruction of type II epithelial cells. Histopathological analysis of rats exposed to 13 ug Be/L (Chapter 2) indicates little type II epithelial destruction. Therefore, the Alk Pase is not appearing as a consequence of damage. From the results of the Alk Pase activity associated with the surfactant, rather than freely soluble, portion of the BAL (Table 7), and the lack of histologic damage to type II cells, it then seems possible that the Alk Pase recovered may be that type described by Reasor, et al., (1978) associated with the surfactant of the acellular lining of the lung and not that associated with lung tissue or serum.

Quantities of albumin in the BAL is an indication of serum leakage. Although albumin was detected at day 1 in the lavage of treated rats, none could be found within the BAL of controls. Albumin levels in the BAL of

control animals following day 1 were inconsistent and gradually rose to a peak at day 8 post exposure. This might have suggested that the lungs of control animals, exposed to the H_2SO_4 aerosol, were damaged and released higher quantities of albumin into the lung lavage. This event is unlikely since histologic findings (Chapter 2) and cell kinetic data (Chapter 2) revealed no lung damage from this agent. Further, Henderson, et al., (1978) exposed rats to an aerosol of H_2SO_4 at 100 mg/m^3, and found that alterations in the BAL were limited to increases in sialic acid content and acid phosphatase activity in some, but not all, animals. Henderson, et al., (1985) later concluded that the BAL is ineffective in detecting lung injury from H_2SO_4. The variability in albumin measurements of the BAL of control animals can not be fully explained by H_2SO_4 exposure, but perhaps to an unexplained variation in daily sampling methods.

Results showed albumin within the BAL of rats to be significantly elevated from controls only at day 5 post exposure. At the time point of maximum LDH response, at day 8, no significant differences in albumin were noted between treated and control groups. The maximum response of albumin in the BAL of rats was 4 times the value of controls, while the maximim response of LDH and Alk Pase was 30 times greater than respective controls. This suggests that only a very small portion of the proteins within the BAL were of serum source.

Acid phosphatase activity of the BAL in rats showed a 2 to 4 fold increase following $BeSO_4$ exposure. Significant differences between groups were obtained early in the time periods studied. If Ac Pase is a measure of increased phagocytic activity, or lysed phagocytic cells, this then coincided with

results of histopathological analysis showing an increased number of recruited macrophages with phagocytyzed particles and degenerating leukocytes (Chapter 2) at the same time points as increased Ac Pase activity.

The increase in LDH and Alk Pase was not evident in the BAL of mice exposed to a $BeSO_4$ aerosol of 7.2 ug Be/L. Lactate dehydrogenase values were 2% of the maximum values obtained with rats exposed to approximately the same chamber concentration. The Alk Pase response was inconsistent and unmeasureable at certain time points. Using albumin as an indicator of serum transudation, the maximum values obtained for Alk Pase and albumin coincided. It is likely that the increase of Alk Pase in mice was in part due to capillary leakage, or damage, resulting in an increase in serum enzymes into the lung washings. This, no doubt, also contributed to the rise in LDH levels in the BAL.

In Chapter 3, it was postulated that the differences in lung cell kinetics between mice and rats was due to the difference in initial dose received to the lungs. The large dissimilarities in the BAL response of the two species may also be attributed to this inequal deposition of inhaled beryllium.

In a separate study, rats were assessed for the effects of long term toxicity, following $BeSO_4$ exposure, over a period of one year. Animals exposed to an aerosol of 4.05 ug Be/L, for one hour, showed LDH activity in the BAL to be similar to control values by three weeks, confirming the previously reported data of acute toxicity. A maximum value was seen at 3 months, progressively decreasing to one year. As was seen for LDH activity, Alk Pase in the BAL was consistent with previous results, being significantly elevated at 3 weeks. A peak value was obtained at 3 months, which declined until one year

where no differences were noted between groups. Acid phosphatase, unlike previous results, was significantly elevated at 3 weeks. Three month Ac Pase values, although comparable to time points at 6 and 12 months, were significantly higher than concurrent controls.

Figure 26 shows the lungs of animals sacrificed at 12 months of exposure. Circular foci, opaque in appearance, were noted on the surface of the lungs of treated animals. The microscopic findings showed large aggregates of foamy or activated macrophages (Figure 27). This effect was first seen at 3 months post exposure. The appearance of these aggregates of activated macrophages coincided with the maximum response in the BAL of LDH and Alk Pase. Further study between 3 weeks and 3 months would be necessary to understand if any morphologic measurements correlate with the appearance of biochemical parameters in the BAL.

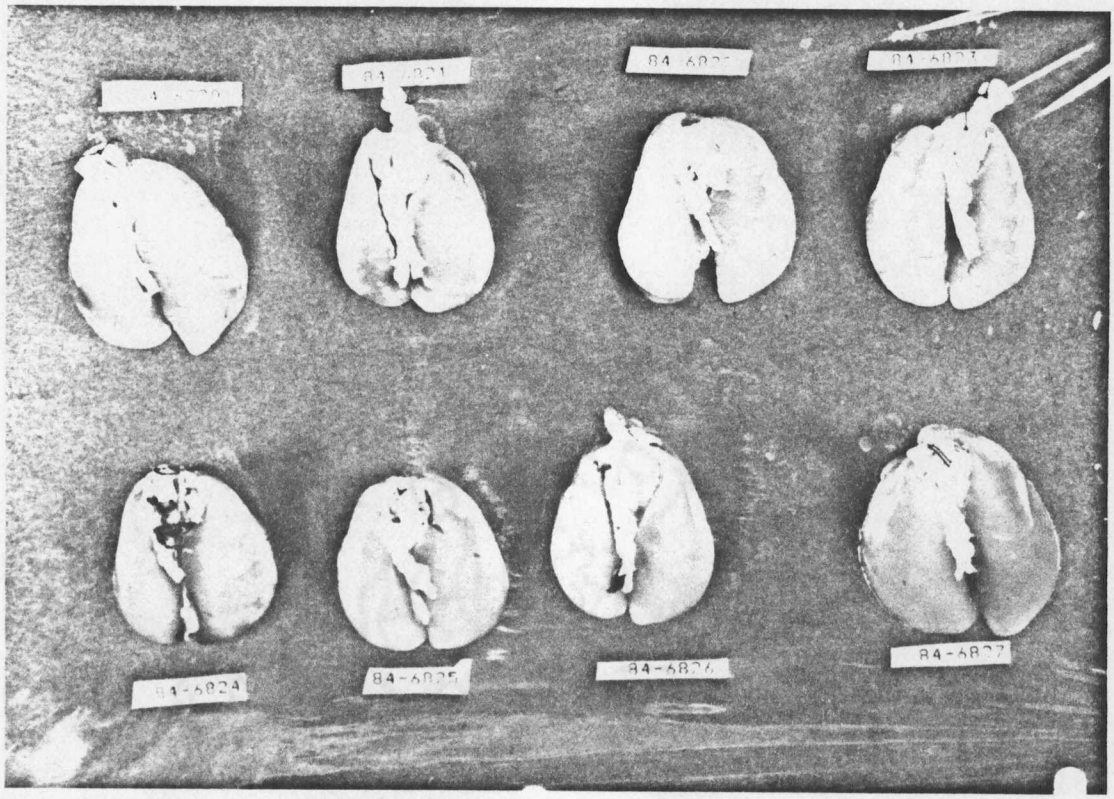


Figure 26. Lungs excised from rats sacrificed one year post exposure. Lungs in the top row were from animals exposed to an aerosol of $BeSO_4$. Lesions seen were circular, opaque foci, on dorsal surface of lungs.

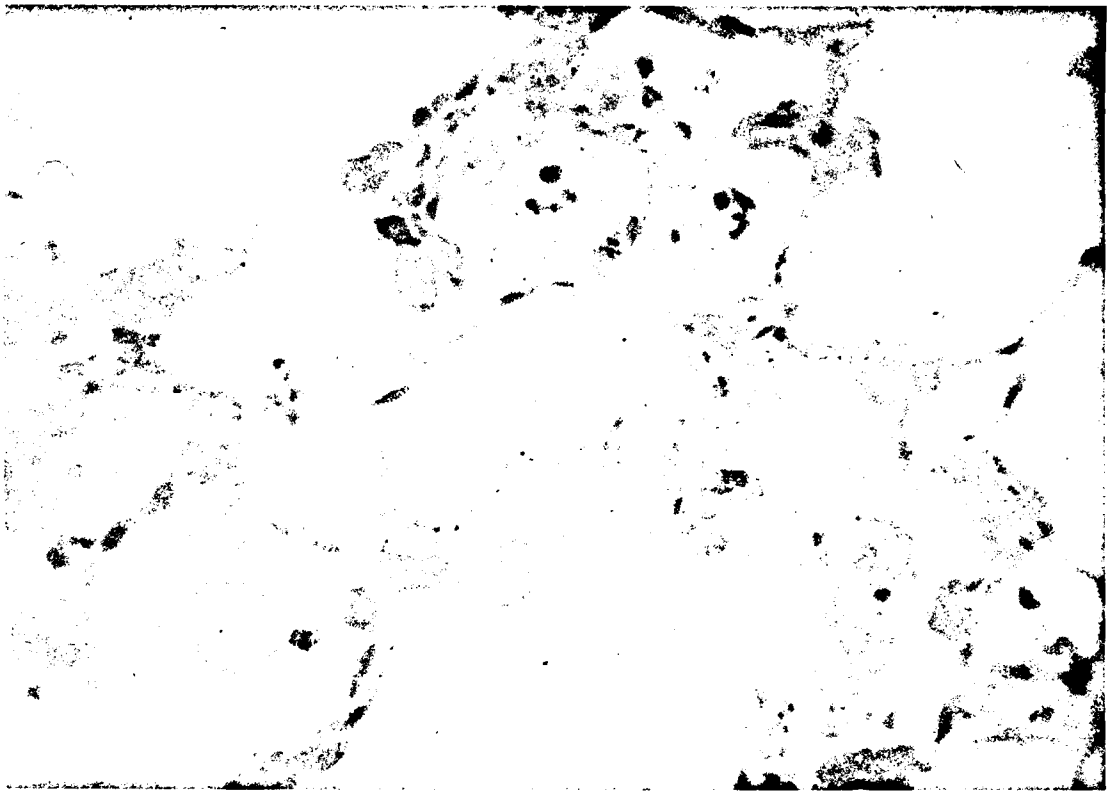


Figure 27. Lungs from rats one year post exposure. Animals exposed to an aerosol of 4.05 ug Be/L. Accumulation of activated macrophages. Lee's methylene blue basic fuschin (440X).

CHAPTER 4

PROTECTION BY IRON SALT AGAINST BERYLLIUM SULFATE TOXICITY IN VIVO

A. Introduction

While the molecular mechanism of beryllium toxicity is yet unclear, it is, however, well established that *in vivo* beryllium has a marked affinity for cell nuclei (Witschi and Aldridge, 1968) and specifically inhibits certain enzymes: alkaline phosphatase, Na-K ATPase and phosphoglucomutase (Aldridge and Thomas, 1966; Thomas and Aldridge, 1966; Toda et al., 1967). Extension of previous studies on the inhibition of phosphoglucomutase by beryllium (Hashimoto et al., 1967) revealed that purified preparations of this enzyme from rat liver were completely inhibited at micromolar concentrations of beryllium ion but the enzyme in crude extract was inhibited partially (Fleming, 1971). This suggested the presence of a protecting factor in the crude extract, and led to the isolation from liver homogenates of a high molecular-weight protein capable of binding large quantities of beryllium. The protein was later identified as ferritin (Price and Joshi, 1983) and protected alkaline phosphatase, Na-K ATPase and phosphoglucomutase from inhibition by Be, and reversed the inhibition *in vitro* (Price and Joshi, 1981, 1984).

Ferritin, with a molecular weight of about 480,000, is composed of 24 subunits of molecular weights 19,000 and 22,000. It can store up to 4,500 g atoms of Fe (III) and release it as Fe (II) whenever needed. Thus, its function as a major protein of iron storage and transport appear well established (Aisen

and Litowsky, 1980). Indeed, Granick who discovered ferritin also observed that the synthesis of this protein increases in response to iron intake (Granick, 1946). Ferritin not only binds iron, but can bind beryllium in significantly greater amounts than other divalent metals (Price and Joshi, 1983).

It appeared likely that ferritin would also would bind beryllium *in vivo*. The purpose of this study was to determine whether iron salt administration would offer protection against beryllium toxicity *in vivo*, by the induction of ferritin synthesis.

B. Materials

1. Animals

Specific pathogen free male Fischer 344 rats, weighing between 200-250 g were purchased from Taconic Farms (Germantown, NY) and Charles River (Portage, MI). Animal maintenance and housing were as previously described (Chapter 1).

2. Chemicals

Beryllium sulfate tetrahydrate and ferric ammonium citrate (FAC) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). ^{7}Beryllium chloride was purchased from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade.

C. Methods

1. Effect of Iron Salt on Intravenous Beryllium Sulfate Toxicity

Sixty animals were equally divided into two groups. Animals in one group received daily intraperitoneal injections of FAC (40 mg/kg body weight) for 3 days while the other group received daily injections of normal saline (controls). On the fourth day, both groups were subdivided into 5 groups of 6 rats each. Each of the five groups received a single intravenous dose of $BeSO_4$ ranging from 2.2 mg/kg to 8.4 mg/kg body weight. Animals remained on their treatment regimens (FAC or normal saline) throughout the rest of the study (14 days). The LD_{50} values and 95% confidence intervals for the two groups were determined.

2. Effect of Iron Salt on Inhalation Toxicity of Beryllium Sulfate

Forty animals were divided equally into two groups. Animals in one group received daily intraperitoneal injections of FAC (40 mg/kg body weight) while animals in the second group received an equal volume of normal saline. On the fourth consecutive day, all animals were simultaneously exposed to an aerosol of $BeSO_4$ for two hours through nose-only inhalation. The inhalation procedures, chamber design and operation, and measurement of Be chamber concentration were previously described (Chapter 1). Animals were exposed for 14 days to the $BeSO_4$ aerosol. By day 15, less than one half the animals in the $BeSO_4$+Saline group had survived, at which time, exposure was discontinued. Animals were continued on FAC treatment, or normal saline, and observed

daily for mortality. Figure 28 shows the results of chamber concentration analysis over the 14 day exposure period. The average daily Be concentration was 2.59 ± 0.32 ug Be/L with a particle mass median aerodynamic diameter of 2.05 ± 0.123 um (σg=2.08 ± 0.107).

3. Distribution and Excretion of Beryllium Over a 48 Hour Period Following Intravenous Injection

Ten animals were divided equally into two groups and placed in metabolism cages (Techniplast, Italy) for a five day adaptation period. Food and water were provided *ad libitum.* On the sixth day, one group began receiving daily intraperitoneal injections of FAC (40 mg/kg) for three consecutive days. The second group received normal salne. On the fourth day, all animals received 6.0 mg/kg of $BeSO_4$ plus 20 uCi/200 g of $^7BeCl_2$. This dose was based on the predetermined LD_{50} values so that deaths were not expected over the 48 hour period. Urine and feces were collected every 24 hours. After 48 hours, animals were sacrificed and blood, liver, kidney, spleen, lung, femur, heart, brain, large and small intestines were collected and analyzed for Be content. Radioactivity of tissue aliquots was measured on an Intertechnique gamma counter (Fairfield, NJ). Results are expressed as per cent of total dose injected.

4. Distribution and Excretion of Beryllium Over a 12 Day Period Following Intravenous Injection

Ten animals were divided into 2 groups of 5 and placed in metabolic cages where they had food and water, *ad libitum.* After a five day adaptation

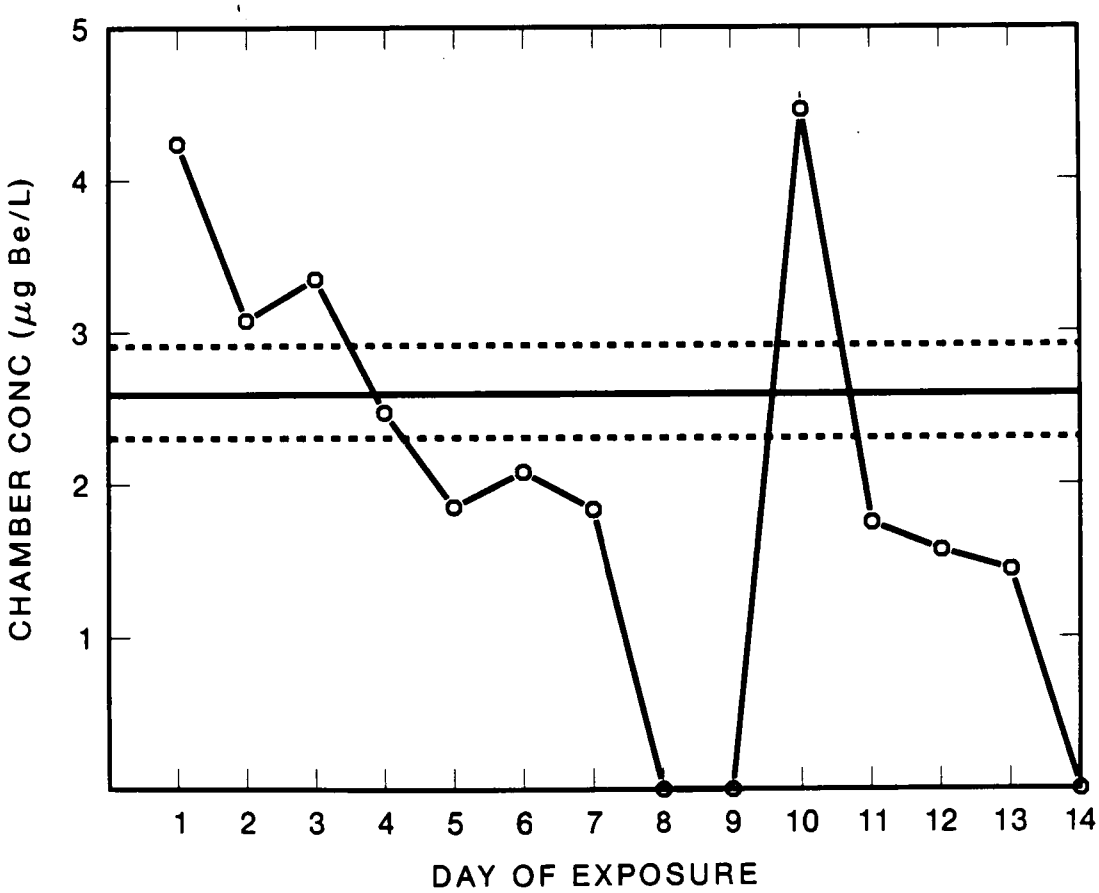


Figure 28. Be chamber concentration during continual daily exposure. Rats exposed each day for two hours to a mean daily concentration of 2.59 ug Be/L.

period, one group received 3 daily injections of FAC (40 mg/kg body wt) intraperitoneally while the other group received normal saline. On the fourth day, all animals received 3.1 mg/kg of $BeSO_4$ plus 20 uCi/200 g body weight of ^7Be. Urine and feces were collected every 24 hours for 12 days. After 12 days, animals were sacrificed and the following tissues removed: blood, liver, spleen, kidney, lung, femur, heart, brain, large and small intestines. Radioactivity of tissue aliquots was measured with results calculated as nmoles of beryllium per gram of tissue.

5. Statistical Analysis

LD_{50} values and 95% confidence intervals were determined by the method of Weil (1952). Comparisons between control and FAC treated animals of beryllium content in tissues, and urine and fecal output, were made by Student's t test. Analysis of survival data following inhalation exposure to Be was made by the Mann-Whitney two sample rank test (Daniel, 1977). Significant differences were determined at the 0.05 level, unless otherwise indicated.

D. Results

Following the pretreatment protocol of 3 daily injection of FAC or normal saline, the LD_{50} values following intravenous injection of $BeSO_4$ were determined. The LD_{50} values, and the 95% confidence intervals, following the intravenous administration of $BeSO_4$ with normal saline or FAC pretreatment were 3.85 (3.45-4.31) mg $BeSO_4$/kg and 8.43 (7.25-9.80) mg $BeSO_4$/kg body

wt, respectively. The data indicate that pretreatment with FAC, at a dose regimen shown to increase liver ferritin values (Joshi, 1985), was able to increase the LD_{50} value two fold for intravenous $BeSO_4$.

The dose of beryllium sulfate used in the distribution and excretion studies did not result in deaths in either group (normal saline or FAC) over the 2 or 12 day study period. Results of the 48 hour Be tissue distribution are shown in Table 8. Be concentrations (expressed as per cent of injected dose) were significantly lower ($p<0.05$) in the liver of FAC treated animals versus controls. Spleen and lung of the FAC treated group also showed a significant reduction ($p<0.05$) of Be, while the kidneys and large intestines contained elevated levels. Total Be eliminated in the urine was significantly reduced in FAC treated animals. Be, eliminated through the feces, showed a significant reduction at day 1 and a significant elevation at day 2 in FAC treated animals. Total fecal output of Be over the two day measurement were not significantly different between groups.

The tissue distribution of beryllium after 12 days is shown in Table 9. Twelve days after administration, the concentration of beryllium was significantly lower in the liver of FAC treated animals, while values for the large intestine were significantly elevated in this group compared to saline controls. Total urinary and fecal output of beryllium over 12 days is shown in Table 10. Compared to controls, the total output of beryllium in urine was significantly less in the FAC treated animals while the fecal output was significantly elevated. However, combined fecal and urine output of beryllium over 12 days was not significantly different between the two groups.

Table 8

Tissue Distribution of Be 2 Days After Intravenous Injection[a]

Tissue	Control	FAC
Blood/0.1 ml	.01 ± 0.0	.01 ± 0.0
Liver	37.30 ± 1.01	28.86 ± 1.95[b]
Spleen	2.25 ± 0.11	1.56 ± 0.04[b]
Kidneys	1.08 ± 0.02	1.43 ± 0.02[c]
Lung	.28 ± 0.02	.21 ± 0.01[b]
Bone (femur)	.97 ± 0.03	.96 ± 0.05
Adrenals	.07 ± 0.01	.09 ± 0.01
Heart	.05 ± 0.00	.03 ± 0.00
Brain	.01 ± 0.03	.01 ± 0.02
Large Intestine	.16 ± 0.03	.34 ± 0.02[c]
Small Intestine	1.81 ± 0.14	1.46 ± 0.23
Urine		
Day 1	9.1 ± 0.66	4.3 ± 0.87[b]
Day 2	1.7 ± 0.16	0.9 ± 0.17
Total	10.8 ± 0.76	5.2 ± 0.97[b]
Feces		
Day 1	1.4 ± 0.19	0.6 ± 0.19[b]
Day 2	1.7 ± 0.09	3.0 ± 0.41[c]
Total	3.1 ± .018	3.6 ± 0.38
Total Urine + Feces	13.9	8.8
Total Recovery	57.9	43.7

[a]Values are expressed as the average per cent (mean ± SE, n=5/group) of the injected dose of ^{7}Be per tissue (Normalized to the amount of ^{7}Be received).

[b]Values were significantly lower than controls ($p<0.05$).

[c]Values were significantly higher than controls ($p<0.05$).

Table 9

Tissue Distribution of Beryllium
12 Days After Intravenous Administration[a]

Tissue	Control	FAC Treatment[b]
Blood[c]	.24 ± 0.02	.21 ± 0.03
Liver	45.06 ± 4.41	30.67 ± 2.02[d]
Spleen	132.65 ± 5.18	119.13 ± 12.64
Kidneys	12.66 ± 2.28	11.07 ± 0.86
Lung	3.46 ± 0.33	3.85 ± 0.29
Bone[e]	46.27 ± 2.47	44.34 ± 1.98
Heart	1.75 ± 0.22	1.40 ± 0.11
Brain	0.15 ± 0.05	0.08 ± 0.02
Large Intestine	1.29 ± 0.10	4.31 ± 0.58[f]
Small Intestine	8.54 ± 1.08	8.81 ± 1.14

[a] Values are expressed as nmoles beryllium per g wet weight of tissue (mean ± SE, n=5/group) following intravenous injection with 3.1 mg/kg $BeSO_4$ and 20 uCi/200 g body weight of ^7Be.

[b]Ferric ammonium citrate (40 mg/kg body wt).

[c]Beryllium values in blood are nmoles per 0.1 ml whole blood.

[d]Values were significantly lower than controls ($p<0.05$).

[e]Values are nmoles beryllium per femur.

[f]Values were significantly higher than controls ($p<0.01$).

Table 10

Urine and Fecal Output of
Intravenous Administered Beryllium[a]

	Urine	Feces	Total
Normal saline	541.3 ± 43.3	743.5 ± 63.6	1284.8
FAC[b]	406.2 ± 35.3[c]	904.0 ± 29.4[c]	1310.2

[a]Values are expressed as nmoles beryllium excreted over the 12 day study (mean ± SE, n=5/treatment) following intravenous injection with 3.1 mg/kg $BeSO_4$ and 20 uCi/200 g body weight of ^7Be

[b]Ferric ammonium citrate (40 mg/kg body wt)

[c]Significantly different from normal saline group, $p<0.05$

Tissue distribution of Be after 2 days (Table 8) and 12 days (Table 9) were calculated in terms of the total amount of Be injected. The 2 day values expressed the percentage of total Be recovered, being approximately 58% in the control group, and 44% in the FAC group. Both distribution studies had amounts of Be unaccounted for, presumably in the carcass of the animals, which was not measured. This indicates that the FAC treated animals had accumulated Be at some site, or sites, not directly measured in this experiment. Values, when expressed in total amounts of Be injected, did not account for the distribution of Be in the carcass.

Figure 29 shows the cumulative mortality of animals treated with FAC or normal saline given continuous inhalation exposure to $BeSO_4$. By day 15, an 80% mortality in the $BeSO_4$+Saline group had been achieved compared to a 33% mortality in the $BeSO_4$+FAC animals. Exposure was discontinued at this

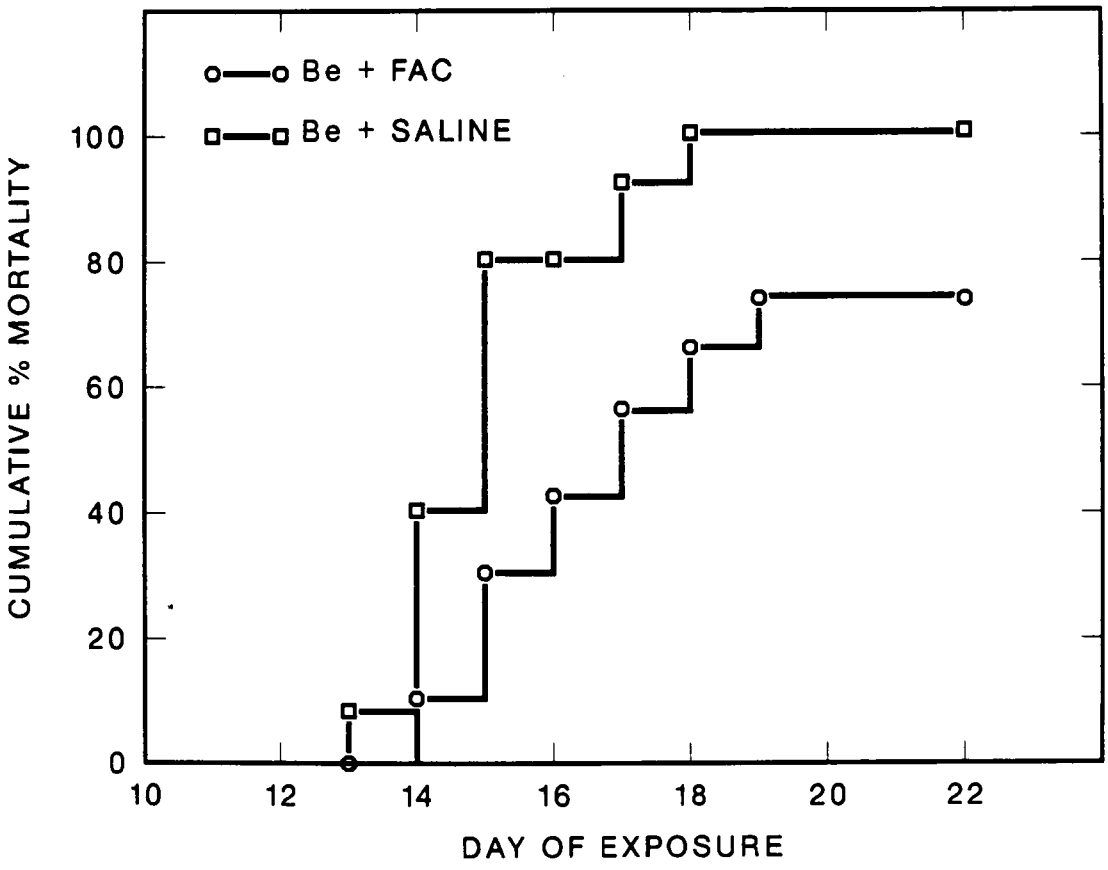


Figure 29. Cumulative per cent mortality of rats during continual daily exposure. Rats exposed for 14 days to mean daily chamber concentration of 2.59 ug Be/L and given daily injections of normal saline (Saline) or iron salt (FAC).

time since the effect had reached a value greater than a dose lethal to half the animals. By day 18, all animals in the $BeSO_4$+Saline group had died while the cumulative mortality for the $BeSO_4$+FAC group was 66%. At day 19, mortality in this group increased to a value of 76% and remained at this point until all remaining animals were sacrificed at day 22.

E. Discussion

Most forms of beryllium adversely affect living systems (Tepper, 1972). The main lesion usually prominent following intravenous injection of soluble beryllium compounds is necrosis of the liver (Scott, 1948; Aldridge et al., 1949; Aldridge, 1966). Beryllium accumulates primarily in the liver, presumably due to uptake by the Kupffer cells (Cheng, 1956). This seems to be the result of the conversion of soluble beryllium sulfate to beryllium phosphate aggregates which are then phagocytized by the reticuloendothelial system of the liver (Vacher and Stoner, 1968b). However, it has also been shown that depression of the reticuloendothelial system does not alter the acute tissue toxicity induced by beryllium (Vacher, et al., 1974). Although the effect on the reticuloendothelial system appears to reside with the particulate moiety of beryllium, the toxicity appears more related to its soluble form (Hard et al., 1977). Whether in the soluble or insoluble form, the resulting toxicity may be due to the high affinity that Be^{+2} has for the nuclei of liver cells (Witschi and Aldridge, 1968), although whether this plays a direct role in the acute cell necrosis has been disputed (Skilleter and Price, 1980). The inhibition of certain liver enzymes may also play a role (Aldridge and Thomas, 1966;

Thomas and Aldridge, 1966, Hashimoto et al., 1967: Toda et al., 1967). The actual mechanism behind the toxicity is yet undetermined. Complexing of Be, by ferritin, would lower its toxicity by any of these proposed mechanisms.

In response to toxic levels of metals such as Cd^{+2}, Cu^{+2}, or Zn^{+2}, the living system can synthesize metallothionein to sequester the toxicant (Mogilnicka et al., 1975; Kojima and Kagi, 1978). Unlike the other divalent metal ions tested, beryllium injection did not induce the synthesis of metallothionein (Joshi, 1985). This agrees with previous *in vitro* studies (Piotrowski and Szymanska, 1976; Price and Joshi, 1983) which indicate that metallothionein does not play an important role in beryllium binding. The different metal ions tested were all able to bind with ferritin to some degree. However, the affinity for beryllium by ferritin (15.33 g atoms/mole), *in vivo*, was much greater than Cu (1.65 g atoms/mole), Cd (1.6 g atoms/mole), or Pb(3.1 g atoms/mole), which correlates with the high affinity seen *in vitro* (Price and Joshi, 1983). Previous work has shown that ferritin was able to protect susceptible enzymes against inhibition by beryllium *in vitro* (Price and Joshi, 1984). This seems to be the result of beryllium binding to the iron core of ferritin. Because the iron core is located within the ferritin shell, the bound Be^{+2} may become less accessible to other proteins, such as enzymes, which are susceptible to inhibition.

The present study indicates that iron salt administration, through the induction of ferritin synthesis, may play a major role in attenuating beryllium sulfate toxicity. By increasing ferritin levels in the liver with iron salt administration, the LD_{50} dose of beryllium sulfate was elevated by a factor of 2. The distribution and excretion study was performed to determine a possible

explanation for the reduction in the toxicity of beryllium sulfate. The results of both the 2 and 12 day studies indicated that the liver and spleen accumulated more beryllium than the other organs, supporting an earlier study (Witschi and Aldridge, 1968). As shown, the liver concentration of beryllium in the iron salt treated group was reduced compared to the control group after 2 and 12 days, inspite of elevated levels of the beryllium binding protein, ferritin. This may suggest that the initially increased ferritin levels in the liver bind beryllium and transport it elsewhere, possibly via the bile. It appears that ferritin-induced animals excreted more beryllium via the gastrointestinal tract since the concentration of Be in the large intestine (after 2 and 12 days) and fecal output (after 12 days) was significantly elevated. However, increased excretion alone cannot explain the decreased liver values of beryllium. Total fecal output at 2 and 12 days were similar for the two groups. Urine output for both time points showed significant reduction. After 48 hours, control animals excrete more total Be than FAC treated rats while both groups excrete a similar amount over a 12 day period.

Animals continually exposed to an aerosol of $BeSO_4$ showed a significant ($p<0.05$) decrease in mortality when given intraperitoneal injections of iron salt. This study was performed to determine if the protective effects of iron salt administration could be obtained by routes of $BeSO_4$ administration other than intravenous. Ferritin levels within the lung were not measured following FAC treatment. Thus, the protective mechanism of the effects of FAC, after inhalation of $BeSO_4$, is not known.

The tissue distribution of radiolabeled Be following iron salt administration showed a very similar correlation to the pattern observed

following treatment of animals with aurintricarboxylic acid (ATA). Schubert, and associates, studied the mechanism of protection by ATA in rats and mice given intravenous $BeSO_4$ (Schubert, et al., 1952). Each of the female CF - 1 mice, received an LD_{95} dose (0.7 mg Be/kg) of non-radioactive $BeSO_4$ and about 0.1 mCi of ^{7}Be. Treated animals received an intravenous injection of 4 mg of the ammonium salt of ATA either 1 hour before or 1 or 7 hours after Be injection. ATA treatment at either of these times had been reported previously to increase survival of Be poisoned mice (White, et al., 1951). Of the tissues analyzed (spleen, femurs, kidney, liver, lungs, blood and carcass) the authors concluded that only the kidneys showed an appreciable effect of ATA on the distribution of Be, increasing at least 2 fold in all ATA-treatment groups.

The data obtained (in per cent of injected dose) between ATA and FAC treatment shows a similarity, with the exception of liver values (Table 11). Shubert, et al., (1952) concluded that ATA had no effect on Be distribution or excretion in mice, except to increase the amount of Be in the kidney. White and Schubert (1954) further investigated the role of ATA in Be poisoning and stated that increased excretion of Be is not neccessarily a concomitant of protection from acute Be poisoning and may even be associated with a greater toxicity of the retained Be. In addition, citric acid has been demonstrated to increase Be excretion while it may actually hasten death (Schubert and White, 1950). Mice receiving sulfosalicylic acid excrete 40% of injected Be but are not prevented from dying from acute Be poisoning (White and Schubert, 1954). White and Schubert further conclude:

....lowering the Be content in tissue is not necessarily sufficient to prevent death of the animal....Then there is little doubt that the protection of Be poisoned animals by chelating agents [ATA and sulfosalicylic acid] involves the inactivation of deposited Be.

Table 11

Comparative Distribution of Be Between FAC and ATA Treatment[a]

Tissue	FAC (Rats) Control	FAC (Rats) Treated	ATA (Mice) Control	ATA (Mice) Treated
Spleen	2.3	1.6	2.6	1.9
Liver	37.3	28.9	25.0	30.0
Kidneys	1.1	1.4	1.2	2.5
Lung	0.281	0.211	1.0	1.2
Femurs	.974	.965	2.1	1.7
Carcass	—	—	41.0	44.0

[a]FAC treatment days -3 to 2. ATA treatment 1 hour before Be injection. Values represent per cent of intravenous dose from animals sacrificed 48 hours following injection.

The results presented in this study, on the protective effects of iron salt administration on $BeSO_4$ toxicity, are in general agreement with the conclusions of these authors. Since ATA is a chelator for Be, and the tissue distribution and excretion of labeled Be following FAC treatment is similar to these previous results of ATA treatment, it may be that iron salt administration is exerting its protective effects by acting through ferritin

induction. However, the tempting conclusion that the protective effect of iron salt was solely due to increased synthesis of ferritin, and subsequent inactivation of bound Be, can be accepted only with caution. This is because iron injections reduced liver beryllium levels inspite of increased liver ferritin. Most probably, elevated ferritin levels reduce Be toxicity by an inactivation of deposited Be, combined with a mobilization of Be from the liver.

CHAPTER 5

SUMMARY

After more than 40 years of intense research, the mechanism of action of beryllium still remains poorly understood. It was the intent of these studies to add to the existing knowledge of this unique element, using methods not previously employeed. In this chapter, these studies will be summarized.

The involvement of Cy A in lung fibrosis caused from $BeSO_4$ exposure was the subject of study presented in Chapter 1. The chronic form of Be disease appears to have many characteristics of hypersensitivity disease. Patients with chronic berylliosis show cutaneous delayed hypersensitivity reactions to Be compounds (Curtis, 1951). Peripheral blood lymphocytes are able to undergo blast transformation (Deodhar, et al., 1973) and release macrophage inhibition factor (Marx and Barrell, 1973). Epstein, et al., report increased numbers of activated T cells in the bronchoalveolar lavage of patients with berylliosis, with both blood and BAL lymphocytes proliferating in response to $BeSO_4$ and BeF_2 (Epstein, et al., 1982). The authors concluded that results from this study were consistent with the concept that chronic berylliosis is a form of hypersensitivity pneumonitis. With this concept of T cell involvement in beryllium disease, animals were blocked of their T cell dependent responses with the use of Cyclosporin A. The question asked then was: Could immunosuppression, by the use of Cy A, block the response of the lung in experimental animals following $BeSO_4$ exposure? Lung response in this study was the induction of fibrosis, measure by the amount of total lung hydroxyproline.

Previous research investigating experimentally induced lung fibrosis had implied a possible role of T lymphocytes in the mechanism. The study was then designed to answer three possible questions:

1. Does $BeSO_4$ induce fibrosis?

2. Will inhibiting treatment with Cy A inhibit the fibrotic response caused by $BeSO_4$?

3. If Cy A treatment will reduce the fibrotic response due to $BeSO_4$, will it also inhibit the fibrotic response in other animal models and in more than one species?

Results presented in Chapter 1 indicated that $BeSO_4$ did cause a significant increase in total lung hydroxyproline measured at 21 days. Treatment of animals with Cy A did not completely abolish the fibrotic response in rats due to $BeSO_4$ exposure. Animals did not show a significant increase in total lung hydroxyproline when given Cy A and exposed to $BeSO_4$. Yet, these animals also did not show any significant reduction in hydroxyproline content when compared to Cy A - untreated animals exposed to $BeSO_4$. Although $BeSO_4$ inhalation, with the methods employed, was able to cause a fibrotic response, the degree of this reaction may not have be great enough to support the conclusions that Cy A had an influence on the initial lung response.

Mice exposed to BHT, when given Cy A, showed complete inhibition of the fibrotic response. Rats, administered bleomycin and treated with Cy A, showed a significant reduction in total lung hydroxyproline, while mice showed no effect. It is difficult to draw generalized conclusions based on these results

for there are many agents used to induce fibrosis. However, the results with BHT in mice, and bleomycin in rats, appear promising and could lead to further research to investigate the possible involvement of T cells in these models. Cy A has other effects and the immunusuppressive effects are only conjectoral. Additionally, work on the use of Cy A on lung fibrosis has even concluded that T lymphocytes are not necessary for the development of acute or chronic interstitial fibrosis in the phorbol myristate acetate model of lung injury (Hendricks, et al., 1984). The use of Cy A should not be overlooked based on this conflicting evidence. Cy A, given to patients diagnosed with sarcoidosis, resulted in significant stable lung improvement in all three patients studied (Rebuck, et al., 1984). With the close clinical association of sarcoidosis and berylliosis, and the results demonstrated herein, the involvement of Cy A in the production of granulomata due to Be would be an interesting case for further experimental study.

Chapter 2 presented the cellular proliferation pattern in the lung following acute $BeSO_4$ inhalation. Rats and mice were shown to have large differences in their respective patterns of cell renewal. The discrepencies in response were explained by the differences in the respiratory volumes of the two species, resulting in differences in the amount of inhaled dose to the lungs. In Chapter 3, the biochemical parameters of the BAL in rats and mice again showed large differences, accounted for by the difference in dose received. The cellular kinetics and biochemical assessment of lung injury caused by $BeSO_4$ showed a remarkable correlation. Adamson (1985) has stated that studies are needed to combine structure - function parameters with kinetic determinations for more complete information on toxicological assessment in the lung. These

two methods of BAL and cell kinetics were correlated to define the toxicological assessment of $BeSO_4$ damage, as recommended by Adamson (1985).

Table 12 shows the correlation coefficients of the cellular kinetic and biochemical parameters following $BeSO_4$ exposure in rats. Animals exposed to either 3.3 or 7.0 ug Be/L showed a similarity in the calculated correlation coefficients of lung lavage parameters vs. the cell kinetics of rats exposed to 13 ug Be/L. The biochemical parameters of LDH activity and albumin showed the highest correlation against all cell kinetic measurements, with the exception of alveolar macrophage differential cell counts. Alkaline and acid phosphatase activities showed less correlation between the cell kinetic data.

Table 12

Correlation Coefficient (r) of Lung Lavage vs. Cell Kinetics

BAL Parameters	Chamber Conc.[a]	Type II	Inter	AM	Endo	LI
LDH	3.3	.864	.975	.333	.963	.962
	7.0	.776	.926	-.09	.966	.907
Alk Pase	3.3	.547	.698	-.13	.786	.686
	7.0	.427	.721	-.26	.738	.633
Ac Pase	3.3	.336	.616	-.14	.613	.530
	7.0	.333	.419	-.19	.409	.385
Albumin	7.0	.841	.893	.049	.900	.903

[a]Valeus are ug Be/L. Animals exposed to 3.3 or 7.0 ug Be/L (BAL parameters) or 13 ug Be/L (cell kinetic parameters)

Type II - Type II epithelial, Inter - Interstitial, Am - Alveolar Macrophage, Endo - Endothelial, LI - Labeling Index

Measurements of BAL parameters are based on the presumption that the epithelial lining of the respiratory tract is the site of initial contact of inhaled materials. If the inhaled material is toxic, indicators of that toxicity should be present in the fluid lining the tract, able to be sampled by lavage (Henderson, et al., 1985). BAL is then an analysis of lung injury. Conversely, cell kinetic measurements detect cell proliferation.

The Labeling Index is a general measure of cell proliferation within the lung. This measurement consisted of cells categorized as: type II epithelial, endothelial, interstitial and alveolar macrophages. In rats, the predominat cell types constituting the increase in the LI following $BeSO_4$ exposure were endothelial and interstitial. It is possible then, that the endothelial response is measuring capillary damage, which caused leakage of serum proteins into the BAL (LDH, Alk Pase and albumin). Correlation of cell kinetics and biochemical parameters of the BAL may then be nonspecific to lung damage.

Analysis of LDH isoenzyme patterns was performed in an attempt to answer this problem of serum transudation. Could the LDH isoenzyme profile distinguish the source of LDH as being from the serum or lung tissue? Results suggested that the LDH within the BAL of rats was of lung tissue source. Henderson, et al., (1985) stated that some attempts have been made to correlate LDH isoenzyme patterns with cell type (Beck, et al., 1983), but in general the BAL analysis does not indicate the site of injury. In the event of this realization, conclusion as to LDH source, based on isoenzyme profiles, are difficult to determine. Measurements of albumin in the BAL is an indicator of serum transudation of proteins. Initial measurements showed increased BAL albumin, but, on the day of maximum response (day 8) there was no difference

in amounts of albumin in the BAL of control or treated groups. It can be presumed that the greatest amount of LDH in the BAL of rats was from lung tissue and not from serum transudation. The correlation of BAL parameters, and cell kinetic measurements, may then be determining an identical response. Bronchoalveolar lavage, instead of simply indicating lung damage, may also be measuring lung repair. Correlating the two measurements over time should be approached with caution. The kinetics for the appearance, and clearance, of proteins within the BAL is not known. The exact time of cell death, measured by BAL, can not then be determined.

This study is not the first reported instance of BAL analysis following Be inhalation. Hart, et al., exposed male rats to an aerosol of BeO (.447 ug Be/L) for one hour through nose-only inhalation (Hart, et al., 1984). Animals were killed at 2.5 hours, and 2, 12 and 21 days following exposure. Increases in all biochemical parameters were seen to peak on day 5. LDH activity was five times higher and Alk Pase activity was six times greater than comparable controls. Acid phosphatase activity peaked at day 2, being three times higher than controls. Initial lung burdens of Be for this study were determined as 447 ng Be per whole lung. Results of the initial lung burdens in Chapter 2 showed levels of 25 ug Be/whole lung (unpublished data), nearly 50 times greater than animals of the Hart study. BAL analysis of enzyme activity also showed marked differences in comparison to the Hart study. LDH and Alk Pase values reported in Chapter 3 were 30 times control values. Although different results were obtained in both the studies, and exposures were with different compounds of Be, similarities do exist. Hart's peak BAL response was on day 5 post exposure. Time points between day 5 and 12 in this study were not

reported. It would be of interest to note if more time points had been sampled, if a close correlation of results in both studies would have been obtained.

In the last chapter, the protective effects of iron salt against $BeSO_4$ toxicity were studied. Aurintricarboxylic acid had been previously determined to be an effective chelator against $BeSO_4$ toxicity in experimental animals (White, et al., 1951; Schubert, et al., 1952; White and Schubert, 1954). ATA chelates Be ions resulting in reduced toxicity. The chelate tended to accumulate in the kidneys and spleen, but did not enhance urinary excretion of Be. Price and Joshi (1981) have shown ferritin to be a chelator for Be, *in vitro.* Ferritin has been demonstrated to protect the enzymatic activity of alkaline phosphatase, Na-K ATPase, and phosphoglucomutase against the inhibtion caused by Be (Price and Joshi, 1984). Could the protection of ferritin against Be, *in vitro*, be applied to *in vivo* use? Chapter 4 demonstrated that this is the case. Induction of ferritin synthesis by the administration of an iron salt protected animals against the toxicity of Be whether administered intravenously or inhaled. These results showed good agreement with earlier studies using ATA, supporting the conclusion that the protective effects are due in part to the inactivation of deposited Be.

Iron salt administration may exert its effects by other mechanisms than through the elevation of liver ferritin. All cells contain enzymes which depend on the presence of iron for activation; ribonucleotide reductase, aconitate hydratase and cytochrome P_{450} are some examples (Jacobs, 1977). The possibility of iron administration potentiating enzyme activation, thereby exerting a beneficial effect on beryllium toxicity, can not be overlooked. Additional studies are warranted to investigate this possibility.

Beryllium disease, is indeed, ". . . . a very obnoxious baby . . . born of the union of industry and beryllium. . . ." (Shipman and Vorwald, 1966). These studies have given some additional insight to the understanding of this very complex element. Yet, questions still remain unanswered.

LIST OF REFERENCES

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Adamson, I.Y.R. (1985). Cellular Kinetics of the Lung. *Handbook of Experimental Pharmacology,* **75**,289-317.

Adamson, I.Y.R., Bowden, D.H., Cote, M.G., and Witschi, H.P. (1977). Lung Injury Induced by Butylated Hydroxytoluene. Cytodynamic and Biochemical Studies in Mice. *Lab Invest.,* **36**,28-32.

Adamson, I.Y.R., and Bowden, D.H. (1974). The Type 2 Cell as the Progenitor of Alveolar Regeneration. *Lab Invest.,* **30**(1),35-42.

Adamson, I.Y.R., and Bowden, D.H. (1979). Bleomycin - Induced Injury and Metaplasia of Alveolar Type 2 Cells. *Am. J. Pathol.,* **96**(2),531-538.

Adamson, I.Y.R., and Bowden, D.H. (1980). Role of Monocytes and Interstitial Cells in the Generation of Alveolar Macrophages. II. Kinetic Studies After Carbon Loading. *Lab Invest.,* **42**,518-524.

Adamson, I.Y.R., and Bowden, D.H. (1981). Dose Response of Pulmonary Macrophage System to Various Particles and its Relationship to Transepithelial Passage of Free Particles. *Exp. Lung Res.,* **2**,165-175.

Aisen, P., and Litowsky, I. (1984). Iron Transport and Storage Proteins. *Ann. Rev. Biochem.,* **49**,357-393.

Aldridge, W.N. (1966). The Toxicity of Beryllium. *Lab Invest.,* **15**,176-180.

Aldridge, W.N., Barnes, J.M., and Denz, F.A. (1949). Experimental Beryllium Poisoning. *Brit. J. Exp. Path.,* **30**,375-389.

Aldridge, W.N., and Thomas, M. (1966). The Inhibition of Phosphoglucomutase by Beryllium. *Biochem. J.,* **98**,100-104.

Bansal, S.K., and Kaw, J.L. (1981). Lactate Dehydrogenase Isoenzymes in Macrophages and Serum During the Development of Pulmonary Silicosis in the Rat. *Tox. Letters,* **7**,279-283.

Barna, B.P., Deodhar, S.D., Chiang, T., Gautam,S. and Edinger, M. (1984). Experimental Beryllium-Induced Lung Disease I. Differences in Immunological Responses to Beryllium Compounds in Strain 2 and 13 Guinea Pigs. *Int. Arch. Allergy Appl. Immun.*, **73**, 42-48.

Barnes, J.M. (1950). Experimental Production of Malignant Tumors by Beryllium. *Lancet,* **i**,463.

Bassler, R. (1965). Effects of Beryllium on Synthesis of Nucleoproteins and DNA in Fibroblasts Cultivated In Vitro. *C.R. Soc. Biol.*, **159**,1620.

Beck, B.D., Brain, J.D., and Bohannon, D.E. (1981). The Pulmonary Toxicity of an Ash Sample from Mt. St. Helens Volcano. *Exp. Lung Res.*, **2**, 289-301.

Beck, B.D., Brain, J.D., and Bohannon, D.E. (1982). An In Vivo Hamster Bioassay to Assess the Toxicity of Particulates for the Lungs. *Toxicol. Appl. Pharmacol.*, **66**,9-29.

Beck, B.D., Genson. B., Feldman, H.A., and Brain, J.D. (1983). Lactate Dehydrogenase Isoenzymes in Hamster Lung Lavage Fluid After Lung Injury. *Toxicol. Appl. Pharmacol., 71*,59-71.

Berman, E. (1980). *In Toxic Metals and Their Analysis* (Berman, E., ed.), Hayden and Son, Ltd.

Beserga, R. (1981). The Cell Cycle. *N. Eng. J. Med.*, **304**, 453-459.

Bitterman, P.B., and Crystal, R.G. (1981). Alveolar Macrophage (AM) in Idiopathic Pulmonary Fibrosis (IPF) and Sarcoidosis are Spontaneously Secreting a Growth Factor Causing Human Lung Fibroblasts to Replicate. *Clin. Res.*, 49-53A.

Bitterman, P.B., Rennard, S.I., Hunninghake, G.W., and Crystal, R.G. (1982). Human Alveolar Macrophage Growth Factor for Fibroblasts. Regulation and Partial Characterization. *J. Clin. Invest.*, **70**,806-822.

Blake, J. (1881). On the Relationship of the Molecular Properties of Inorganic Compounds and Their Effect Upon the Living Animal Organism. *Ber. Devt. Chem. Ges.*, **14**,394.

Blake, J. (1882). Atomic Weights of Beryllium as Determined by Physiological Action. *Chem. News,* **65**,111.

Blumenkrantz, N., and Asboe-Hansen, G. (1975). An Assay for Hydroxyproline and Proline on One Sample and a Simplified Method for Hydroxyproline. *Anal. Biochem.*, **63**,331-340.

Borel, J.F., and Lafferty, K.J. (1983). Cyclosporin: Speculation About its Mechanism of Action. *Transplant. Proc.*, **25**,1881-1885.

Bowden, D.H., and Adamson, I.Y.R. (1968). Reaction of Lung Cells to a High Concentration of Oxygen. *Arch. Pathol.*, **86**,671-675.

Bowden, D.H., and Adamson, I.Y.R. (1971). Reparative Changes Following Pulmonary Cell Injury. Ultrastructural, Cytodynamic and Surfactant Studies in Mice After Oxygen Exposure. *Arch. Pathol.*, **92**,279-283.

Bowden, D.H., and Adamson, I.Y.R. (1974). Endothelial Regeneration as a Marker of the Differential Vascular Response in Oxygen - Induced Pulmonary Edema. *Lab Invest.*, **30**(3),350-357.

Breslin, A.J. (1966). Exposures and Pattern of Disease in the Beryllium Industry. In *Beryllium: Its Industrial Hygiene Aspects* (Stokinger, H.E., ed.) Academic Press, NY.

Brody, A.R., Roe, M.W., Evans, J.N., and Davis, G.S. (1982). Deposition and Translocation of Inhaled Silica in Rats. *Lab Invest.*, **47**,533-542.

Brunton, T.L., and Cash, J.T. (1884). Comparative Toxicity of the Alkali Earths. *Phil. Trans. Roy. Soc. London,* **175**,231.

Buckley, R.D., and Balchum, O.J. (1967). Effects of Nitrogen Dioxide on Lactate Dehydrogenase Isozymes. *Arch. Environ. Health,* **14**,424-428.

Bugryshev, P.F., Moskalev, Y.I., Kuznetsov, A.A., and Nazarova, V.A. (1976). Beryllium Distribution in Rats (Russ.). *Formakol. Toksikol.*, **39**,615-618.

Cahn, R.D., Kaplan, N.O., Levine, L., and Zwilling, L.E. (1962). Nature and Development of Lactate Dehydrogenase. *Science,* **136**,962.

Chang, J.G., and Lesser, M. (1984). Quantitation of Leukocytes in Bronchoalveolar Lavage Samples from Rats After Intravascular Injection of Endotoxin. *Am. Rev. Resp. Dis.*, **129**,72-75.

Chvapil, M. (1975). Lactate Dehydrogenase Isoenzyme Pattern and Total LDH Activity in the Lung and Liver of Rats Chronically Exposed to Low and High Oxygen Concentrations. *Life Sci.*, **17**,761-766.

Chvapil, M., and Peng, Y.M. (1975). Oxygen and Lung Fibrosis. *Arch. Environ. Health,* **30**,528-532.

Cheng, K.K. (1956). Experimental Studies on the Mechanism of the Zonal Distribution of Beryllium Liver Necrosis. *J. Path. Bact.*, **71**,265.

Cochran, K.W., Zerwic, M.M., and DuBois, K.P. (1951). Studies on the Mechanism of Acute Beryllium Poisoning. *J. Pharmacol. Exp. Ther.*, **102**,165-178.

Conover, W.J. (1980). *Practical Nonparametric Statistics.* 2nd. ed., John Wiley and Sons, NY.

Crystal, R.G., Roberts, W.C., Moss, M.L., Line. B.R., and Reynolds, H.Y. (1976). Idiopathic Pulmonary Fibrosis. Clinical, Histologic, Radiographic, Physiologic, Scintographic, Cytologic and Biochemical Aspects. *Ann. Int. Med.*, **85**,769-788.

Curtis, G.H. (1951). Cutaneous Hypersensitivity due to Beryllium. *Arch. Derm. Syph.*, **64**,470-482.

Daniel, W.W. (1977). *Introductory Statistics with Applications.* Houghton Mifflin Company, Boston.

Deadhar, S.V., Barna, B., and Van Ordstrand, H.S. (1973). A Study of the Immunological Aspects of Chronic Berylliosis. *Chest,* **63**,309-313.

DeNardi, J.M., Van Ordstrand, H.S., and Carmody, M.G. (1949). Acute Dermititis and Pneumonitis in Beryllium Workers: Review of 406 Cases in 8 Year Period with Follow-up on Recoveries. *Ohio State Med. J.*, **45**,467.

DeNicola, D.B., Rebar, A.H., and Henderson, R.H. (1981). Early Damage Indicators in the Lung. V. Biochemical and Cytological Response to NO_2 Inhalation. *Toxicol. Appl. Pharmacol.*, **60**,301-312.

DiAugustine, R.P. (1974). Lung Concentric Laminar Organelle. Hydrolase Activity and Compositional Analysis. *J. Biol. Chem.*, **249**,584-593.

Dinsdale, D. (1982). Lysosomal Involvement in Beryllium Phosphate Toxicity. *Br. J. exp. Path.*, **63**,103-108.

Doumas, B.T., and Biggs, H.G. (1972). Determination of Serum Albumin. In *Standard Methods of Clinical Chemistry* (Cooper, G.R., ed.), vol. 7, Academic Press, New York, NY.

DuBois, K.P., Cochran, K.W., and Mazur, M. (1949). Inhibition of Phosphatase by Beryllium and Antagonism of the Inhibition by Manganese. *Science,* **110**,420.

Editorial (1951). The Toxicity of Beryllium. *The Lancet,* **260**,357.

Eierman, G.J., Dickey, B.F., and Thrall, R.S. (1983). Polymorphonuclear Leokocyte Participation in Acute Oleic Acid - Induced Lung Injury. *Am. Rev. Resp. Dis.*, **128**,845-850.

Elias, J.A., Rossman, M.D., Atkinson, B.J., Phillips, S.M., and Danielle, R.P. (1981). Experimental Berylliosis and Beryllium-Induced Interstitial Fibrosis. *Am. Rev. Resp. Dis.*, **123**(4),141.

Evans, M.J., and Bils, R.F. (1969). Identification of Cells Labeled with Tritiated Thymidine in the Pulmonary Alveolar Walls of the Mouse. *Am. Rev. Resp. Dis.*, **100**,372-378.

Fisher, A.B., and Furia, L. (1977). Isolation and Metabolism of Granular Pneumocytes from Rat lungs. *Lung,* **154**,155-165.

Fleming, K.A. (1971). M.S. Thesis. University of Tennessee, Knoxville,TN.

Forkert, P.G., Custer, E.M., Alpert, A.J., Ansari, G.A.S., and Reynolds, E.S. (1982). Lactate Dehydrogenase Activity in Mouse Lung Following 1,1 - dichloroethylene: Index of Airway Injury. *Exp. Lung Res.*, **4**,67-77.

Granik, S. (1946). Ferritin. IX. Increase of the Protien Apoferritin in the Gastrointestinal Mucosa as a Direct Response to Iron Loading. The Function of Ferritin in the Regulation of Absorption. *J. Biol. Chem.*, **164**,737-746.

Grier, R.S., Hood, D.B., and Hoagland, M.B. (1949). Observations on the Effects of Beryllium on Alkaline Phosphatase. *J. Biol. Chem.*, **180**,289-298.

Gross, H. (1977). Pulmonary Effects of Radiation Therapy. *Ann. Int. Med.*, **86**,81-92.

Guyton, A.C. (1947). Measurement of Respiratory Volumes in Laboratory Animals. *Am. J. Physiol.*, **150**,70-77.

Hakkinen, P.J., Whiteley, J.W., and Witschi, H.P. (1982a). Hyperoxia, but not Thoraxic X-Irradiation, Potentiates Bleomycin and Cyclophosphamide - Induced Lung Damage in Mice. *Am. Rev. Resp. Dis.*, **126**,281-285.

Hakkinen, P.J., and Haschek, W.M. (1982b). Pulmonary Toxicity of Methylcyclopentadienyl Manganese Tricarbonyl: Nonciliated Bronchiolar Epithelial (Clara) Cell Necrosis and Alveolar Damage in the Mouse, Rat and Hamster. *Toxicol. Appl. Pharmacol.*, **65**,11-22.

Hakkinen, P.J., Morse, C.C., Martin, F.M., Dalbey, W.E., Haschek, W.M., and Witschi, H.P. (1983a). Potentiating Effects of Oxygen in Lungs Damaged by Methylcyclopentadienyl Manganese Tricarbonyl, Cadmium Chloride, Oleic Acid, and Antitumor Drugs. *Toxicol. Appl. Pharmacol.*, **67**,55-69.

Hakkinen, P.J., Schmoyer, R.L., and Witschi, H.P. (1983b). Potentiation of Butylated Hydroxytoluene-Induced Acute Lung Damage by Oxygen. Effects of Prednisolone and Indomethacin. *Am. Rev. Resp. Dis.*, **128**,648-651.

Hall, R.H., Scott, J.K., Laskin, S., Stroud, C.A., and Stokinger, H.E. (1950). Acute Toxicity of Inhaled Beryllium, III. Observations Relating Toxicity with the Physiochemical Properties of Beryllium Oxide Dust, *A.M.A. Arch. Ind. Hyg. Occupatioal Med.*, **2**,25.

Hamman, L., and Richy, A.R. (1935). Fulminating Diffuse Interstitial Fibrosis of the Lungs. *Trans. Am. Clin. Climatol. Assoc.*, **51**,154-163.

Hard, G.C., Skilleter, D.N., and Reiner, E. (1977). Correlation of Pathology with Distribution of Be Following Administration of Beryllium Sulfate and Beryllium Sulfosalicylate Complexes to the Rat. *Exp. Mol. Path.*, **27**,197-212.

Hart, B.A., Harmsen, A.G., Low, R.B., and Emerson, R. (1984). Biochemical, Cytological, and Histological Alterations in Rat Lung Following Acute Beryllium Aerosol. *Toxicol. Appl. Pharmacol.*, **75**,454-465.

Haschek, W.M., Reiser, K.M., Klein - Szanto, A.J.P., Kehrer, J.P., Smith, L.H., Last, J.A., and Witschi, H.P. (1983). Potentiation of Butylated Hydroxytoluene - Induced Lung Damage by Oxygen. Cell Kinetic and Collagen Metabolism. *Toxicol. Appl. Pharmacol.*, **75**,454-465.

Hashimoto, T., Joshi, J.G., del Rio, C., and Handler, P. (1967). Phosphoglucomutase: IV. Inactivation by Beryllium Ions. *J. Biol. Chem.*, **242**,1671-1679.

Henderson, R.F., Wolff, R.K., Rebar, A.H., DeNicola, D.B., and Beethe, R.L. (1978). Early Indicators of Lung Damage from Inhaled Sulfuric Acid Mist. *Lovelace Inhalation Toxicology Research Institute Annual Report,* LF-60, pp.352-355, National Technical Information Service, United States Department of Commerce, Springfield, VA.

Henderson, R.F., Rebar, A.H., Pickrell, L.A., and Newton, G.J. (1979). Early Damage Indicators in the Lung. III. Biochemical and Cytological Response of the Lung to Inhaled Metal Salts. *Toxicol. Appl. Pharmacol.*, **50**,123-136.

Henderson, R.F., Rebar, A.H., DeNicola, D.B., Henderson, T.R., and Damon, E.G. (1981). The Use of Pulmonary Washings as a Probe to Detect Lung Injury. *Chest,* **80**(1),125-155.

Henderson, R.F., Benson, J.M., Hahn, F.F., Hobbs, C.H., Jones, R.K., Mauderly, J.L., McClellan, R.O., and Pickrell, J.A. (1985). New Approaches for the Evaluation of Pulmonary Toxicity: Bronchoalveolar Lavage Analysis. *Fund. Appl. Toxicol.*, **5**,451-458.

Hendricks, C., Taylor, R., and McCall, C. (1984). The Effect of Cyclosporin A (CyA) on Lung Fibrogenesis. *Am. Rev. Resp. Dis.*, **129**(4),12.

Heppleston, A.G. (1969). The Fibrogenic Action of Silica. *Brit. Med. Bull.*, **25**,282.

Hodge, H.C., LaBelle, C.W., Cucci, M.R., Sprague, G.F., Pettingill, A.G., Stokinger, H.E., Laskin, S., Turner, R.A.N., Neuman, W.F., Scott, J.K., Maynard, E.A., and Downe, W.L. (1947). *Progress Report of Preliminary Studies of Beryllium Toxicity.* University of Rochester, Atomic Energy Project, No. M-1997.

Hokin, L.E., Hokin, M.R., and Mathison, D. (1963). Phosphatidic Acid Phosphatase in the Erythrocyte Membrane. *Biochem. Biophys. Acta,* **67**,485-497.

Hu, P.C., Miller, F.J., Daniels, M.J., Hatch, G.E., Graham, J.A., Gardner, D.E., and Selgrade, M.K. (1982). Protein Accumulation in Lavage Fluid Following Ozone Exposure. *Environ. Res.,* **29**,377-388.

Hughes, W.L., Bond, V.P., Brechner, G., Cronkite, E.P., Painter, R.B., Quastler, H., and Sherman, F. (1958). Cellular Proliferation in the Mouse as Revealed by Autoradiography with Tritiated Thymidine. *Proc. Nat. Acad. Sci.,* **44**,476-483.

Hunninghake, G.W., Gadek, J.E., Kawanami, O., Ferrans, V.J., and Crystal, R.G. (1979). Inflammatory and Immune Processes in the Human Lung in Health and Disease: Evaluation by Bronchoalveolar Lavage. *Am. J. Pathol.,* **97**(1),149-205.

Hunninghake, G.W., Garrett, K.C., Richerson, H.B., Fantone, J.C., Ward, P.A., Rennard, S.I., Bitterman, P.B., and Crystal, R.G. (1984). Pathogenesis of the Granulomatous Lung Disease. *Am. Rev. Resp. Dis.,* **130**,476-496.

Hunninghake, G.W., Gallin, J.I., and Fauci, A.S. (1978). Immunologic Reactivity of the Lung: The In Vivo Generation of a Neutrophil Chemotactic Factor by Alveolar Macrophages. *Am. Rev. Resp. Dis.,* **117**,15-23.

IARC Monographs (1972). IARC Monographs on the Evaluation of Carcinogenic Risk of Chemiclas to Man. Vol. 1, IARC, Lyon, France.

Jackson, D.S., and Cleary, E.G. (1967). The Determination of Collagen and Elastin. *Methods Biochem. Anal.,* **15**,25-76.

Jacobs, A. (1977). An Intracellular Transit Iron Pool. In *Iron Metabolism,* Ciba Foundation Symposium 51, pp. 91-106, Elsevier Scientific Publishing Co., Amsterdam.

Joshi, J. (1985). Personal communication. Iron salt administration significantly elevates liver ferritin. Be administration following iron salt treatment does not induce metalothionein synthesis and binds with a greater affinity than Pb, Cd, Cu, and Zn.

Katzenstein, A.L.A., Bloor, C.M., and Leibow, A.A. (1976). Diffuse Alveolar Damage - The Role of Oxygen, Shock, and Related Factors. *Amer. J. Pathol.*, **85**,210-228.

Kaufman, S.L. (1980). Cell Proliferation in the Mammalian Lung. *In International Review of Experimental Pathology* (Richter, G.W. and Epstein, M.A., eds.), vol. 22.

Kehrer, J.P., and Witschi, H.P. (1981). The Effect of Indomethacin, Prednisolone and Cis-4-Hydroxyproline in Pulmonary Fibrosis Produced by Butylated Hydroxytoluene and Oxygen. *Toxicology,* **20**,281-288.

Kistler, G.S., Caldwell, P.R.B., and Wiebel, E.R. (1967). Development of Fine Structural Damage to Alveolar and Capillary Lining Cells in Oxygen - Poisoned Rat Lungs. *J. Cell Biol.,* **32**,605-628.

Kivirikko, K.I., Laitinen, O., and Prockop, D.J. (1967). Modification of a Specific Assay for Hydroxyproline in Urine, *Anal. Biochem.,* **19**,249-255.

Kojima, Y., and Kagi, J.H.R. (1978). Metallothionein. *Trends Biochem. Sci.,* **3**,90-93.

Krejci, L.E., and Scheel, L.D. (1966). The Chemistry of Beryllium. In *Beryllium: Its Industrial Hygiene Aspects.* (Stokinger, H.E., ed.) Academic Press, New York, NY.

Last, J.A., Greenberg, D.B., and Castleman, W.L. (1979). Ozone-Induced Alteration in Collagen Metabolism in Rat Lungs. *Tox. Appl. Pharmacol.,* **51**,247-258.

Lindenschmidt, R.C., and Witschi, H.P. (1985). Propranolol-Induced Elevation of Pulmonary Collagen. *J. Pharmacol. Exp. Ther.,* **232**,346-350.

Lindy, S., Kahanpaa, K., Karhunen, P., Halme, J., and Uitto, J. (1970). Lactate Dehydrogenase Isoenzymes During the Development of Experimental Fibrosis. *J. Lab Clin. Med.,* **76**(5),756-760.

Linhardt, K., and Walter, K. (1974). Phosphatases (Phosphomonoesterases). In *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.). Academic Press, New York, NY.

Luckey, T.D., and Venugopal, B. (1977). *Metal Toxicity in Mammals,* vol. 2, Plenum Press, NY.

MacFarland, H.N. (1976). Respiratory Toxicology. In *Essays in Toxicology,* (Hayes, W.L.,ed.), vol. 7, Academic Press, NY.

Mainigi, K.P. and Brusnick, E. (1969). Inhibition of Deoxythymidine Kinase by Beryllium. *Biochem. Pharmacol.,* **18,**2003-2007.

Malmstrom, B.G. (1955). Metal-Ion Specificity on the Action of Enolase. *Arch. Biochem. Biophys.,* **58,**381-397.

Market, C.L. (1963). Lactate Dehydrogenase Isozymes: Dissociation and Recombination of Subunits. *Science,* **140,**1329.

Marradi-Fabroni, S. (1935). Pulmonary Disease Due to Beryllium. *Med. Lavoro,* **26,**297.

Marx, J.J., and Burrell, R. (1984). Delayed Hypersensitivity to Beryllium Compounds. *J. Immunol.,* **111,**590-598.

McGeachin, R.L., Pavord, W.M., and Pavord, W.L. (1982). Inhibition of Various Mammalian Amylases by Beryllium and Aluminum. *Biochem. Pharmacol.,* **11,**493-498.

Meban, C. (1975). The Structural Localization of Alkaline Phosphatase in the Granular Pneumocytes of Hamster Lung. *Histochemistry,* **43,**367-372.

Mellor, J.W. (1946). *A Comprehensive Treatise on Inorganic and Theoretical Chemistry,* Vol. 4, Longmans, Green and Co., London.

Mietkiewski, K., and Malendowicz, L. (1966). *Acta Histochem.,* 23,259-275. Cited In *Handbook on the Toxicology of Metals* (Friberg, L., Nordberg, G.F., and Vouk, V.B., eds.), Elsevier Biomedical Press, NY, (1979).

Mogilnicka, E.M., Piotrowski, J.K., and Trojanowska, B. (1975). The Influence of Certain Metals (Cd, Hg, Zn, Cu, Pb, Mn, V) on the Level of Metallothionein in the Liver and Kidneys of Rats. *Med. Pracy.,* **26,**147-155.

Moores, S.R., Black, A., Evans, J.C., Evans, N. Holmes, A., and Morgan, A. (1981). The Effect of Quartz, Administered by Intratracheal Instillation on the Rat Lung. II. The Short Term Biochemical Response. *Environ. Res.,* **24,**275-285.

Mukhina, S.T. (1967). *Gig. Tr. Prof. Zabol.,* 11,43-46. Cited In *Handbook on the Toxicology of Metals* (Friberg, L., Nordberg, G.F., and Vouk, V.B., eds.,), Elsevier Biomedical Press, NY, (1979).

Naido, D., and Pratt, O.E. (1953). *Enzymolgia,* 16,91-98. Cited In *Handbook on the Toxicology of Metals* (Friberg, L., Nordberg, G.F., and Vouk, V.B., eds.,) Elsevier Biomedical Press, NY, (1979).

Phan, S.H., Thrall, R.S., and Williams, C. (1981). Bleomycin-Induced Pulmonary Fibrosis. Effects of Steroid on Lung Collagen Metabolism. *Am. Rev. Resp. Dis.,* **124**(4),428-434.

Piotrowski, J.K., and Szymanska, J.A. (1976). Influence of Certain Metals on the Level of Metallothionein - Like Proteins in the Liver and Kidneys of Rats. *J. Toxicol. Environ. Health,* **1**,991-1002.

Policard, A. (1950). Histological Studies of the Effects of Beryllium Oxide (Glucine) on Animal Tissues. *Br. J. Ind. Med.,* **7**,117-121.

Price, D.J., and Joshi, J.G. (1981). Ferritin, a Natural Chelator for Beryllium. *Fed. Proc.,* **40**,1665.

Price, D.J., and Joshi, J.G. (1983). Ferritin: Binding of Beryllium and Other Divalent Metal Ions. *J. Biol. Chem.,* **258**,10873-10880.

Price, D.J., and Joshi, J.G. (1984). Ferritin: Protection of Enzymatic Activity Against the Inhibition by Divalent Metal Ions. *Toxicology,* **31**,151-163

Prockop, D.J., and Udenfriend, S. (1960). A Specific Method for the Analysis of Hydroxyproline in Tissue and Urine. *Anal. Biochem.,* **1**,228-239.

Raghu, G., Striker, L.J., Hudson. L.D., and Striker, G.E. (1985). Extracellular Matrix in Normal and Fibrotic Human Lungs. *Am. Rev. Resp. Dis.,* **131**,281-289.

Reasor, M.J., Nadeau, D., and Hook, G.E.R. (1978). Extracellular Alkaline Phosphatase in the Airways of the Rabbit Lung. *Lung,* **155**,321-335.

Rebuck, A.S., Stiller, C.R., Braude, A.C., Laupacis, A., Cohen, R.D., and Chapman, K.R. (1984). Cyclosporin for Pulmonary Sarcoidosis. *The Lancet,* **1**(8387),1174.

Reeves, A.L. and Vorwald, A.J. (1961). The Humoral Transport of Beryllium. *J. Occup. Med.*, **3**,567-574.

Reeves, A.L. (1965). The Absorption of Beryllium from the Gastrointestinal Tract, *Arch. Environ. Hlth.*, **11**,209.

Reeves, A.L., and Vorwald, A.J. (1967). Beryllium Carcinogenesis. II. Pulmonary Deposition and Clearance of Inhaled Beryllium Sulfate in the Rat. *Cancer Res.*, **27**,446-451.

Reeves, A.C. (1979). Beryllium. In *Handbook of the Toxicology of Metals* (Friberg, L., Nordberg, G.F., and Vouk, V.B., eds.) Elsevier Biomedical Press, NY.

Revis, N. (1985). Acknowledgement is made to Dr. N. Revis of Oak Ridge Research Institute for providing the atomic absorption analysis for beryllium.

Richter, V. (1930). Contribution to the Pharmacology of Beryllium. Inaugural Dissertation, Wurzburg.

Rogers, A.W. (1973). *Techniques of Autoradiography,* second ed., Elsevier, Amsterdam.

Roth, R.A. (1981). Effect of Pneumotoxicants on Lactate Dehydrogenase Activity in Airways of Rats. *Toxicol. Appl. Pharmacol.*, **57**,69-78.

Schepers, G.W.H., Durkan, T.M., Dilahunt, A.B., and Creedan, F.T. (1957). The Biological Action of Inhaled Beryllium Sulfate. *A.M.A. Arch. Ind. Health,* **15**,32-58.

Schmidt, J.A., Mizel, S.B., Cohen, D., and Green, I. (1982). Interleukin I, a Potential Regulator of Fibroblast Proliferation. *J. Immunol.*, **128**,2177-2182.

Schoenberger, C.I., Rennard, S.I., Bitterman, P.B., Fukuda, Y., Ferrans, V.J., and Crystal, R.G. (1984). Paraquat-Induced Pulmonary Fibrosis. Role of the Alveolitis in Modulating the Development of Fibrosis. *Am. Rev. Resp. Dis.*, **129**,168-173.

Schormuller, J., and Stan, H.J. (1965). *Nahrung,* 9,435-444. Cited In *Handbook of the Toxicology of Metals* (Friberg, L., Nordberg, G.F., and Vouk, V.B., eds.) Elsevier Biomedical Press, NY, (1979).

Schrier, D.J., Phan, S.H., and McGarry, B.M. (1983). The Effect of the Nude (nu/nu) Mutation on Bleomycin Induced Pulmonary Fibrosis. *Am. Rev. Resp. Dis.*, **127**,614-617.

Schubert, J., White, M.R., and Lindenbaum, A. (1952). Studies on the Mechanism of Protection by Aurintricarboxylic Acid on Beryllium Poisoning. I. *J. Biol. Chem.*, **196**,279-288.

Schubert, J., and White, M.R. (1950). Effect of Citrate Salts and Other Chemical Factors on the Distribution and Excretion of Beryllium. *J. Lab. Clin. Med.*, **35**,854-864.

Scott, J.K. (1948). Pathologic Anatomy of Acute Experimental Beryllium Poisoning. *Arch. Pathol.*, **45**,354.

Shipman, T.L. (1950). History of the Beryllium Problem. In *Sixth Saranac Symposium (1947), Pneumoconiosis: Beryllium, Bauxite Fumes, Compensation.* (Vorwald, A.J., ed.), Paul B. Hoeber, Inc., NY.

Shipman, T.L., and Vorwald, A.J. (1966). History of Beryllium Disease. In *Beryllium: Its Industrial Hygiene Aspects* (Stokinger, H.E., ed.) Academic Press, NY.

Siem, P. (1886). The Influence of Aluminum and Beryllium on Animals, Dissertation Report. Cited In *Beryllium: Its Industrial Hygiene Aspects* (Stokinger, H.E., ed.) Academic Press, NY.

Sigma Technical Bulletin No. 340-UV (1980). The Quantitative Ultraviolet Determination of Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) in Serum at 340 nm. Sigma Chemical Co., St. Louis, MO.

Sigma Technical Bulletin No. 705-EP (1982). The Electrophoretic Fractionation and Determination of Lactate Dehydrogenase Isoenzymes in Serum. Sigma Chemical Co., St. Louis, MO.

Sigma Technical Bulletin No. 104 (1982). The Colorimetric Determination of Phosphatase. Acid, Alkaline and Prostatic in Serum and Alkaline in Urine at 400-420nm. Sigma Chemical Co., St. Louis, MO.

Skilleter, D.N., and Price, R.J. (1978). The Uptake and Subsequent Loss of Beryllium by Rat Liver Parenchymal and Non-Parenchymal Cells After the Intravenous Administration of Particulate and Soluble Forms; *Chem. Biol. Interactions,* **20**,383-396.

Skilleter, D.N., and Price, R.J. (1980). Apparent Two Phase Beryllium Labeling of Hepatic Cell Nuclei Isolated from Intravenous Administration of Beryllium Compounds to Rats. A Possible Explanation. *Arch. Toxicol.*, **45**,75.

Snider, G.L. (1983). Interstitial Pulmonary Fibrosis-Which Cell is the Culprit? *Am. Rev. Resp. Dis.*, **127**,535-539.

Sorokin, S.P. (1970). The Cells of the Lung. In *Morphology of Experimental Respiratory Carcinogenesis.* (Nettesheim, P., Hanna, M.G., and Deatherage, J.W., eds.), U.S. Atomic Energy Commision.

Sprague, G.F., LaBelle, C.W., Pettingill, A.G., and Stokinger, H.E. (1947). Initial Studies of the Toxicity of Inhaled Beryllium Sulfate Dusts and Beryllium Fume. In *Sixth Saranac Symposium (1947), Pneumoconiosis: Beryllium, Bauxite Fumes, Compensation* (Vorwald, A.J., ed.) Paul B. Hoeber, Inc., NY.

Starkweather, W.H., Green, R.A., Spencer, H.H., and Schoch, H.K. (1966). Alterations of Serum Lactate Dehydrogenase Isoenzymes During Therapy Directed at Lung Cancer. *J. Lab. Clin. Med.*, **68**,314.

Steidle, H. (1937). Pharmacology of Beryllium. *Arch. Exptl. Pathol. Pharmacol.*, **187**,533.

Stokinger, H.E. (1953). *Nucleonics,* 11,24. Cited In *Industrial Hygiene and Toxicology* (Patty, F., ed.), vol. 2, second ed., Interscience, NY.

Stokinger, H.E. (1958). The Metals (Excluding Lead). In *Industrial Hygiene and Toxicology* (Patty, F., ed.). Vol. 2, second ed., Interscience, NY.

Stokinger, H.E., Ashenburg, N.J., DeVoldre, J., Scott, J.K., and Smith, F.A. (1950a). Acute Inhalation Toxicity of Beryllium. II. The Enhancing Effect of the Inhalation of Hydrogen Flouride Vapor in Beryllium Sulfate Poisoning in Animals. *Arch. Ind. Hyg. Occupational Med.*, **1**,398.

Stokinger, H.E., Sprague, G.F., Hall, R.H., Ashenburg, N.J., Scott, J.K., and Steadman, L.T. (1950b). Acute Inhalation Toxicity of Beryllium. I. Four Definitive Studies of Beryllium Sulfate at Exposure Concentrations of 100, 50, and 1 mg per Cubic Meter. *Arch. Ind. Hyg. Occup. Med.*, **1**(4),379-397.

Stokinger, H.E., Spiegel, C.F., Root, R.E., Hall, R.H., Steadman, L.T., Stroud, C.A., Scott, J.K., Smith, F.A., and Gardner, D.F. (1953). Acute Inhalation Toxicity of Beryllium. IV. Beryllium Flouride at Exposure Concentrations of One and Ten Milligrams per Cubic Meter. *Arch. Ind. Hyg. Occupational Med.,* **8**,493.

Sykes, S.E., Morgan, S.R., Holmes, A., and Davison, W. (1983). Dose Dependent Effects on the Subacute Response of the Rat Lung to Quartz. I. The Cellular Response and the Activity of Lactate Dehydrogenase in the Airways. *Exp. Lung Res.,* **5**,229-243.

Tepper, L.B. (1972). Beryllium. *CRC Crit. Rev. Toxicol.,* **1,** 235-259.

Terai, T., Gangrely, R., and Waldman, R.H. (1979). Guinea Pig Lung Lavage Cells After Intranasal BCG Sensitization. *Infection and Immunity,* **25**(3),844-848.

Thomas, M., and Aldridge, W.N. (1966). The Inhibition of Enzymes by Beryllium. *Biochem. J.,* **98**,94-99.

Thomson, A.W., Whiting, P.H., Blair, J.T., Davidson, R.J.L., and Simpson, J.G. (1981). Pathological Changes Developing in the Rat Lung During a 3-Week Course of High Dosage Cyclosporin A and Their Reversal Following Drug Withdrawal. *Transplantation,* **32**,271-277.

Thrall, R.S., McCormick, J.R., Phan. S.H., Jack, R.H., and Ward, P.A. (1979). Effect of Antilymphocyte Globulin on the Development of Bleomycin Induced Pulmonary Fibrosis in the Rat. *Am. Rev. Resp. Dis.,* **119**(Suppl),83.

Thrall, R.S., Lovett, R.J., Barton, R.W., McCormick, J.R., Phan, S.H., and Ward, P.A. (1980). The Effect of T-Cell Depletion on the Development of Bleomycin Induced Fibrosis in the Rat. *Am. Rev. Resp. Dis.,* **119**(Suppl),99.

Thrall, R.S., Phan, S.M., McCormick, J.R., and Ward, P.A. (1981). The Development of Bleomycin-Induced Fibrosis in Neutrophil-Depleted and Complement-Depleted Rats. *Am. J. Pathol.,* **105**,76-81.

Toda, G., Hashimoto, J., Asakura, T., and Minakami, S. (1967). Inhibition of Na-K Activated ATPase by Beryllium. *Biochem. Biophys. Acta.,* **135**,570-572.

Tsukamoto, Y., Helsel, W.E., and Wahl, S.M. (1981). Macrophage Production of Fibronectin, a Chemoattractant for Fibroblasts. *J. Immunol.*, **127**,673-678.

Vacher, J. and Stoner, H.B. (1968a). The Transport of Beryllium in Rat Blood. *Biochem. Pharmacol.*, **17**,93-107.

Vacher, J., and Stoner, H.P. (1968b). The Removal of Injected Beryllium from the Blood of the Rat: The Role of the Reticuloendothelial System. *Br. J. Exp. Path.*, **49**,315-323.

Vacher, J., Deraedt, R., and Berzoni, J. (1973). Compared Effects of Two Beryllium Salts (soluble and insoluble): Toxicity and Blockage of the Reticuloendothelial System. *Toxicol. Appl. Pharmacol.*, **24**,497-506.

Vacher, J., Deraedt, R., and Berzoni, J. (1974). Role of the Reticuloendothelial System in the Production of Alpha-Macrofetoprotein in the Rat Following Intravenous Injection of Beryllium and Other Particles. *Toxicol. Appl. Pharmacol.*, **28**,28-37.

Valone, F.H., Franklin, M., Sun, F.F., and Goetzl, E.J. (1980). Alveolar Macrophage Products of Arachidonic Acid: Isolation and Recognition as the Predominant Constituents of the Neutrophil Chemotactic Activity Elaborated by Alveolar Macrophages. *Cell Immunol.*, **54**,390-401.

Van Cleave, C.D. and Taylor, C.T. (1955). Distribution, Retention and Elimination of Be in the Rat After Intratracheal Injection. *Arch. Ind. Health,* **11**,375-392.

Van Ordstrand, H.S., Hughes, R., and Carmody, M.G. (1943). Chemical Pneumonia in Workers Extracting Beryllium Oxide. *Clev. Clinic. Quart.*, **10**,10.

Vorwald, A.J. (1953). Adenocarcinoma in the Lung of Albino Rats Exposed to Compounds of Beryllium: Cancer of the Lung; an Evaluation of the Problem. *Proc. Sci. Session, Am. Cancer Soc. Ann. Meeting,* Detroit, Nov. 3-4.

Vorwald, A.J., Reeves, A.L., and Urban, E.C.J. (1966). Experimental Beryllium Toxicity. In *Beryllium: Its Industrial Hygiene Aspects* (Stokinger, H.E., ed.). Academic Press, NY.

Walter, K., and Schutt, C. (1974). Acid and Alkaline Phosphatase in Serum (Two Point Method). In *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.), Vol. 2, second ed., Academic Press, NY.

Washeit, D.B., Chang, L.Y., Hiu, L.H., Hook, G.E.R., Crapo, J.D., and Brody, A.R. (1984). Pulmonary Macrophage Accumulation and Asbestos - Induced Lesions at Sites of Fiber Deposition. *Am. Rev. Resp. Dis.*, **129**,301-310.

Weast, R.C. (1972). *Handbook of Chemistry and Physics,* 53rd ed., p.B72, The Chemical Rubber Co., Cleveland, OH.

Weber, H.H., and Englehardt, W.E. (1933). Investigation of Dusts Arising Out of Beryllium Extraction. *Zentr. Gewerbehyg. Unfallverhiit.*, **10**,41

Weil, C.S. (1952). Tables for Convenient Calculation of Median Effective Dose (LD_{50} or ED_{50}) and Instructions in their Use. *Biometrics,* **8**,249-263.

White, M.R., Finkel, A.J., and Schubert, J. (1951). Protection Against Experimental Beryllium Poisoning by Aurintricarboxylic Acid. *J. Pharmacol. and Exp. Therap.*, **102**,88-93.

White, M.R., and Schubert, J. (1954). Studies on the Mechanism of Protection by Aurintricarboxylic Acid on Beryllium Poisoning. V. Comparative Effects of Related Compounds on Survival of Be Poisoned Animals and on Distribution of Be. *Arch. Biochem. Biophys.*, **52**,133-142.

Whiting, P.H., Simpson, J.G., Davidson, R.J.L., and Thomson, A.W. (1983). Pathological Changes in Rats Receiving Cyclosporin A at Immunotherapeutic Doses for 7 Weeks. *Br. J. Exp. Path.*, **64**,437-444.

Wilkinson, J.H. (1965). *Isoenzymes.* Spon, London.

Witschi, H.P. (1970). Effects of Beryllium on Deoxyribonucleic Acid - Synthesizing Enzymes in Regenerating Rat Liver. *Biochem. J.*, **120**,623.

Witschi, H.P., and Aldridge, W.N. (1967). Biochemical Changes in Rat Liver After Acute Beryllium Poisoning. *Biochem. Pharmacol.*, **16**,263

Witschi, H.P., and Aldridge, W.N. (1968). Uptake Distribution and Binding of Beryllium to Organelles of Rat Liver Cell. *Biochem. J.*, **106**,811-820.

Witschi, H.P., and Morse, C.C. (1983). Enhancement of Lung Tumor Formation in Mice by Dietary Butylated Hydroxytoluene: Dose - Time Relationships and Cell Kinetics. *J. Natl. Cancer Inst.*, **71**(4),359-366.

Witschi. H.P. (1968). Inhibition of Deoxyribonucleic Acid Synthesis in Regenerating Rat Liver by Beryllium. *Lab Invest.*, **19**,67-70.

Wood, J.H. (1950). A New Organic Reagent for the Determination of Microgram Quantities of Beryllium by a Spectrophotometric Method. *Mikrochim. Acta.*, **1**,11-24.

Wroblewski, F., and LaDue, J.S. (1955). Lactic Dehydrogenase Activity in Blood. *Proc. Soc. Exp. Biol. Med.*, **90**,210.

Wyler, D.J., Wahl, S.M., Cheever, A.W., and Wahl, L.M. (1981). Fibroblast Stimulation in Schistosomiasis. I. Stimulation In Vitro of Fibroblasts by Soluble Products of Egg Granulomas. *J. Infect. Dis.*, **144**,254-262.

Zamakhovskaia, E.M., Markinkovskii, B.I., and Syroechkovskii, E.E. (1934). On the Question of the Effect of Beryllium Fluoride on an Organism. *Gigiena Truda:Tekh. Bezopasnosti,* **2**,23.

Zapol, W.M., Coffey, J.W., Tsai, I., and Salvador, K.A. (1979). Pulmonary Fibrosis in Severe Acute Respiratory Failure. *Am. Rev. Resp. Dis.*, **119**,547-554.

APPENDIX

LIST OF ABBREVIATIONS

Ac Pase	acid phosphatase
Alk Pase	alkaline phosphatase
ATA	aurintricarboxylic acid
BAL	bronchoalveolar lavage
Be	beryllium
BHT	butylated hydroxytoluene
C	centigrade
cm	centimeter
Cu	copper
Cy A	cyclosporin A
F	fahrenheit
FAC	ferric ammonium citrate
g	gram
IPF	interstitial pulmonary fibrosis
kg	kilogram
Kr	krypton
L	liter
LC_{50}	lethal concentration to 50% of population
LD_{50}	lethal dose to 50% of population
LD_{95}	lethal dose to 95% of population
LDH	lactate dehydrogenase
l/m	liter per minute
M	molar
m^3	cubic meter
mCi	milliCurie
mg	milligram
ml	milliliter
N	normal
Ni	nickel
nmoles	nanomoles
OHPro	hydroxyproline
pH	-log hydrogen ion concentration
pg	picogram
ppm	part per million
psi	pounds per square inch
RNA	ribonucleic acid
SE	standard error
wt	weight
w/v	weight per volume
Zn	zinc
uCi	microCurie
ug	microgram
um	micron
σg	geometric standard deviation

VITA

Louis E. Sendelbach, Jr. obtained his Bachelor of Science degree, cum laude, from Western Michigan University in April 1976. The following two years were spent as a graduate assistant, enrolled in the Master of Science program at Western Michigan University, teaching undergraduate and graduate level classes in anatomy, histology and physiology. In August 1978, he accepted the position of Assistant Department Manager in a private contract toxicology testing laboratory, International Research and Development Corporation, in Mattawan, Michigan.

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