THE EFFECTS OF PYROLYSIS PRODUCTS OF POLYTETRAFLUOROETHYLENE (PTFE) ON PULMONARY ALVEOLAR MACROPHAGES (PAM)

by

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ABSTRACT

Rabbits were exposed for one hour to fumes of Polytetrafluoroethylene (PTFE) heated between the range of 350 and 450°C. Following the exposure, animals were sacrificed and lungs lavaged to remove pulmonary alveolar macrophages. An in vitro assay followed which determined the phagocytic activity of the macrophages incubated with ^{85}Strontium labeled carbon particles.

The macrophage assay demonstrated that rabbits exposed to concentrations of PTFE fumes of 1.0 mg/l and above (and/or exposure to fumes produced when PTFE was heated to 450°C) exhibited an initial inhibition of macrophage phagocytosis. At lower concentrations and temperatures, no effect was observed. White rats and rabbits exposed to lethal concentrations of PTFE fumes demonstrated similar lung pathology of emphysema and hemorrhage. Rats showed no sublethal effects on the kidney (pathologic or enzymatic), however, their lungs showed mild edema, congestion and inflammation.

Thermal Gravimetric Analysis (TGA) of PTFE samples showed a single degradation peak at temperatures below 600°C.

It was concluded that a particulate produced when PTFE was heated to 450°C caused the macrophage phagocytic inhibition in rabbits.

CHAPTER 1

INTRODUCTION

The production of plastics, including man-made fibers and synthetic rubbers, went into a period of rapid growth in the 1930's and has continued to expand until today (Vouk 1976). The plastic industry is considered one of the most important of all the chemical industries. Worldwide production (Youle and Stammers 1976) of polymer materials is estimated annually to be 80 million tons. Of the 80 million tons; 40 million are products such as polystyrene, polyvinylchloride and polyalkanes, 30 million tons of synthetic fibers such as polyester, nylon and acrylics, and 10 million tons of rubber products such as styrenebutadiene and polybutadiene. The end product use of these plastics include construction, packaging, consumer "throw away" products, home furnishings, clothing, transportation, and miscellaneous products. It was also estimated by Youle and Stammers (1976) that the United States and Japan are the biggest users of these polymer materials with an annual use of 44 kg per person. Europe is thought to be in second place with 35 kg per person per year, whereas the rest of the world consumes only 2 kg per person per year.

The popularity of plastics is due mainly to their basic properties. Generally, the polymer plastics are chemically inert and mechanically either exhibit plastic properties (i.e., ability of a solid to suffer permanent deformation), elastic properties (i.e., ability to suffer reversible deformation), or resistance to stretching (Lefaux 1968).

The widespread use of these polymers has raised a question of their toxicity and their potential hazard to human health and the environment. Generally, the principle hazards with plastics are associated with the inhalation of their monomers and the plasticizers, stabilizers, activators, fillers, and pigments used in their formation (Eckardt and Hindin 1973). These are, of course, evolved during the manufacturing process from the combination of raw ingredients, but also during the pyrolysis and combustion of the final product in the industrial fabrication, salvage, or consumer use (Cornish, Hahn and Barth 1975).

Besides deaths associated with polymeric smoke inhalation during fires (Dyer and Esch 1976, Einhorn 1975), examples of occupational related injuries include; "Meat Wrappers Asthma" (Jones and Weill 1977, Vandervort and Brooks 1977, Johnson and Anderson 1976) and cancer associated with polyvinyl chloride production (Casterline, Casterline and Jaques 1977, Lilis et al. 1976, Eckardt 1976), asthma-like symptoms associated with the manufacture of polyurethanes

(Eckardt and Hindin 1973), and "Polymer Fume Fever" associated with the combustion of polytetrafluoroethylene (Eckardt 1976, Lee, Zapp and Sarver 1976, Waritz 1975, Eckardt and Hindin 1973, Lefaux 1968, Clayton 1967).

Polytetrafluoroethylene, the latter of these polymers associated with occupational related injuries, is commonly known as PTFE. Polytetrafluoroethylene is manufactured under the trade name of Teflon^{R} by E. I. du Pont de Nemours and Company. PTFE has a widespread use due to its basic characteristics of plasticity, lubricity, chemical inertness, low toxicity, and thermal stability (Waritz and Kwon 1968). Because of these factors PTFE is used in wire insulation and coating, non-stick cookware, mold lining, and numerous industrial linings for the handling of caustic materials (Waritz 1975, Lefaux 1968).

Because of the widespread use of PTFE and its thermal stability, the opportunity for thermal abuse or misuse is increased and makes this an ideal plastic polymer to conduct toxicological investigation of its pyrolysis and combustion products. Waritz (1975) has suggested many types of thermal abuse including reclaiming clad metal, wire coating and stripping, coated cookware, fires, incineration, and machining.

Polytetrafluoroethylene (PTFE) is made by direct polymerization of tetrafluoroethylene ($CF_2 = CF_2$), which is obtained by pyrolysis of monochlorodifluoromethane (Freon 22)

in the presence of platinum at red heat.

$$2CHF_2Cl \longrightarrow CF_2 = CF_2 + 2HCl$$

Tetrafluoroethylene readily polymerizes under pressure in the presence of oxygen or peroxides (Lefaux 1968).

The monomer of PTFE is relatively toxic in comparison to the polymer. Clayton (1967) demonstrated that the acute inhalation approximate lethal concentration (ALC) of tetrafluoroethylene (TFE) was 40,000 PPM. At sub-lethal concentrations TFE caused respiratory tract irritation and kidney injury. With chlorotrifluoroethylene (CTFE), a similar type fluoroalkene, Zapp (1959) reported that urine solute concentration dropped as CTFE exposure concentration increased. This correlated with urine solute concentration seen in animals injected subcutaneously with increasing doses of the known nephrotoxic agent, mercuric chloride.

In addition, the association between the inability of the kidney to concentrate urine and actual renal damage was substantiated by Zapp, who microscopically noted kidney injury. With this type of kidney damage (i.e., lack of concentrating ability), one would not only expect to see decreased urine solute concentration, but also increased water intake and urine output. Potter, Gandolfi and Clayton (1979) observed, as expected, that after exposure to CTFE, rats took in more water and excreted more urine in dilute concentration. On microscopic examination of the kidney,

they noted necrosis in the proximal renal tubules and in some cases total degeneration of the epithelial cells leaving only a basement membrane. Similarly, Clayton (1977) has reported that hexafluoropropylene (HFP) impairs renal function in the same way as CTFE. In contrast, the threshold concentration for HFP is 300 PPM, while CTFE is 100 PPM.

Clayton (1967), in a summary of Russian literature found that researchers Danishevskii and Kochanov observed degenerative changes in the kidney of rats not only exposed to CTFE, but also TFE.

It must be remembered that although the monomer fluoralkenes are relatively toxic, the polymer species are extremely inert and have low toxicity. PTFE is a white powder, insoluble in organic solvents and extremely inert. The Toxic Substances List (1974) indicates that the lowest toxic dose for a rat implanted subcutaneously with PTFE Film is 80 mg/kg, and at this concentration caused a neoplastic effect. This information was derived from cancer research (Oppenheimer et al.) in 1955 and listed in Suspected Carcinogens: A Subfile of the N.I.O.S.H. Toxic Substances List, 1975. However only film (0.02 mm thick), not powdered PTFE was proven to be neoplastic in the rat and the purity of samples used at that time (1955) is questionable.

The oral toxicity of PTFE is very low (Lefaux 1968). With a diet of 25% finely ground Teflon^{R}, no symptoms of poisoning or adverse histopathological results were observed

(Clayton 1967). In this 90-day feeding study conducted by Clayton, there were no detrimental effects on growth rate or behavior of the rats. The only effects seen were a shift in the distribution and number of white blood cells and in one case an increase in the liver/body weight ratio. As stated above, there was no evidence of microscopic tissue change.

As mentioned earlier, fluoropolymer toxicity especially in the case of polytetrafluoroethylene results from the heated polymer. With the high thermal stability of PTFE, this polymer is subjected to heating as part of its normal use pattern.

The main toxic materials of polytetrafluoroethylene are evolved at temperatures above 350°C, and could result in an influenza-like illness in humans known as "Polymer Fume Fever" (Waritz and Kwon 1968).

The flu-like illness was first noted by Harris in 1951. He reported at that time that workers demonstrated influenza-like symptoms for several hours after being exposed to vapors from polytetrafluoroethylene heated above 300°C. These symptoms included chest discomfort, fever, sweating, cough, chills, and slight tachycardia. Harris also showed that these same workers were asymptomatic after one or two days, with little or no sign of physical damage.

Since this initial investigation, numerous researchers have reported on the effects of breathing fumes of polytetrafluoroethylene and the polymer fume fever syndrome

(Kuntz and McCord 1974, Okawa and Polakoff 1974, Williams, Atkinson and Patchefski 1974, Evans 1973, Clayton 1967, Lewis and Kerby 1965, Robbins and Ware 1964).

In addition to the general symptoms of malaise and fatigue associated with polymer fume fever, other investigators have seen pulmonary edema (Evans 1973, Robbins and Ware 1964). Like the other symptoms of polymer fume fever reported, pulmonary edema also disappeared within a few days with no apparent permanent effects.

The prevalence of these symptoms among cigarette smokers has also been documented (Evans 1973, Milby, Welti and Hipp 1968, Lewis and Kerby 1965). Whether these symptoms resulted from the accidental contamination of the cigarette or the increased susceptibility among cigarette smokers, the exact etiological agent produced from heated polymer is not known. It is known that the PTFE material must be heated above 300°C to produce polymer fume fever (Clayton 1962). Some PTFE resins however, can be heated to 350°C without producing toxic materials (Clayton, Hood and Raynsford 1959). Possible agents which might cause this fever are numerous and experts are undecided regarding the specific agent (Coleman et al. 1968, Scheel, Lane and Coleman 1968, Waritz and Kwon 1968, Clayton 1962).

Coleman et al. (1968) and Scheel, Lane and Coleman (1968) indentified the predominant and toxic product of PTFE

heated above 500°C to be carbonyl fluoride (COF_2). At this temperature other gaseous products were toxicologically insignificant.

To further substantiate that COF_2 was the toxic component of the pyrolysis gases, comparison exposures were made using pure COF_2 and heated PTFE. The toxicity of the pure COF_2 correlated with the pyrolyzed PTFE to confirm their theory of COF_2 as the etiological agent at temperatures above 500°C.

However, this theory is not necessarily correct for two reasons. First, although the experimental animals exhibited signs of respiratory difficulty after breathing fumes of PTFE, they did not demonstrate the classical symptoms of polymer fume fever. In fact, while high fatal concentrations produced pulmonary edema and petechial hemorrhage in animals, only the typical short-term polymer fume fever has been demonstrated in humans, with no deaths reported (Evans 1973, Milby et al. 1968). Second, the products evolved from heated PTFE change as the temperature changes (Waritz and Kwon 1968). At the Approximate Lethal Temperature (ATL) identified by Waritz and Kwon to be 450°C, COF_2 was of toxicological insignificance.

Nonclinical research on pyrolysis products of polytetrafluoroethylene have been ongoing for nearly three decades. Zapp, Limperos and Brinker (1955) reported that when PTFE was heated at 300°C in an air atmosphere, products

lethal to rats were evolved. Animals subjected to pyrolysis products of PTFE showed rapid shallow respiration and pallor. On necropsy, rats showed severe respiratory tract injury, pulmonary congestion and edema. They identified the 300°C pyrolysis products by infrared absorption spectophotometry to include tetrafluoroethylene (TFE), hexafluoroethane (HFE), hexafluoropropylene (HFP), octafluorocyclobutane (OFCB), and octafluoroisobutylene (PFIB). Zapp et al. also reported that PFIB was the most toxic of the chemical species with 0.5 PPM PFIB being lethal to rats exposed for six hours. Although there are numerous effluents given off by PTFE at different temperatures, it was thought that a particulate was an important toxic agent (Zapp et al. 1955). Clayton et al. (1959) confirmed this by showing a positive correlation between the amount of particulate from heated PTFE and mortality.

Clayton et al. (1959) also reported that when the particlate effluent was filtered out, toxicity of the pyrolysis products was drastically reduced. When a 0.45μ pore size millipore filter was used to filter the pyrolysate from PTFE heated to the Approximate Lethal Temperature (450°C), the result was an absence of mortality. This experiment clearly demonstrated that toxicity of the PTFE pyrolysis products was associated with a particulate.

Since these initial investigations, Waritz and Kwon (1968) have also conducted toxicological studies on the

pyrolysis products of PTFE. Gas chromatography was utilized in the identification and quantitation of the gaseous pyrolysis products. Waritz and Kwon showed a .04% weight loss of sample during the exposure of PTFE heated to 400°C with no TFE, HFP, PFIB, or OFCB detected. 17.5 mg of hydrolyzable fluoride was evolved. There were no deaths with a 1 l/min diluting stream (i.e., 3 l/min total volume passing through the exposure chamber). Waritz and Kwon also showed that at 450°C there was 100% mortality with 22 l/min diluting air, and less than 4% of the sample weight was lost. Amounts of hydrolyzable fluoride, TFE and HFP were detected however, at levels that were of toxicological insignificance. These products were also not reduced upon filtration, however the toxicity of the pyrolysate was. This experimentation clearly confirmed the fact that a particulate was the toxic agent, however at the same time denied the particulate to be PFIB, TFE or HFP.

A summary of the significant products evolved at different temperatures is tabulated in Table 1.

Thermogravimetric analysis (TGA) is a method by which the thermal characteristics of a material are measured. The plot of weight loss versus constant temperature increase shown by TGA can often give an insight into a material's thermal stability. PTFE was analyzed by TGA and exhibited a single weight loss (Alarie and Barrow 1977).

Table 1. Pyrolysis Products of PTFE

350°C & Below	350 - 425°C	450°C	470 - 480°C	500 - 650°C	650°C & ABOVE
INSIGNIFICANT	HYDROLYZABLE FLUORIDE	ACIDIC PARTICULATE* (0.02 to 0.04 micron in diameter)	PFIB*	COF_2*	COF_2
			HFP		CF_4
			TFE		CO_2
		TFE#			
		HFP#			

TOXICOLOGICALLY INSIGNIFICANT

* PRINCIPLE TOXIC COMPONENT

The number of weight losses observed during TGA is indicative of the number of chemical reactions which take place at different temperatures (Daniels 1973). Therefore, the single peak observed with PTFE corroborates its thermal stability and lack of side reactions. Paciorek, Kratzer and Kaufman (1973) determined the autoignition temperature of PTFE in an air atmosphere to be 575°C. The main products evolved at this temperature were saturated fluorocarbons, COF_2, CO_2 and CO. Similarly, Alarie and Barrow (1977) reported that the entire sample was consumed by 625°C. Therefore complete combustion takes place between 575°C and 625°C. The differences seen between investigators could be from differences in TGA equipment, PTFE samples or operational procedures.

Symptoms of polymer fume fever seen in humans has never been reproducibly demonstrated in animal models (Lee et al. 1976, Waritz 1975). Lee et al. (1976) have demonstrated ultrastructural changes in rat lung exposed to Teflon^R fume generated at 450°C. They consistently showed degeneration of the membranous pneumocytes of the alveolar tissue. By use of electron microscopy, Lee et al. noted three major changes in the lungs exposed to pyrolysis products of PTFE at 450°C. First was the damage to the alveolar lining epithelial cells which was mainly represented in the membranous pneumocytes by blebbing and sloughing. Second was the damage of the endothelial cells of the alveolar

capillaries characterized by swelling, blebbing and denudation. The third major change consisted of septal edema formation. Similarly, light and electron microscopy disclosed extensive perivascular and peribronchial interstitial edema which is thought to result from epithelial damage.

Of those animals exposed to heated Teflon^{R} fumes of 425°C and below, Lee et al. noted no significant morphological changes of the lungs. At the ALT (i.e., 450°C) they showed severe respiratory difficulty, pulmonary edema, hemorrhage, and necrosis of the tracheobronchial epithelium. Similar to the work of Clayton (1962), Zapp (1962) and Waritz and Kwon (1968), Lee et al. also filtered the pyrolysate stream of PTFE heated to ALT to find a dramatic reduction in mortality. By electron microscopy they sized the particulate to be in the range of 0.02 to 0.04μ in diameter. Furthermore, they observed this particulate in the alveolar tissue and postulated it to be the primary toxic component and the agent responsible for the pulmonary edema reported.

Besides the respiratory damage, other organs have been affected by inhalation of pyrolysis products of PTFE (Scheel, Lane and Coleman 1968, Scheel, McMillan and Phipps 1968, Lucia et al. 1978). It was shown by Scheel, Lane and Coleman that TFE polymer heated above 550°C produced fatty liver degeneration. This was explained by the investigators to be caused by the principle gas evolved at that temperature, carbonyl fluoride (COF_2).

In another paper by Scheel, McMillan and Phipps (1968), they presented data that indicated biochemical changes in the kidney of animals exposed to PTFE fumes. In an attempt to show that PTFE pyrolysis products were toxic because of fluoride generation (i.e., carbonyl fluoride), they measured urinary fluoride concentration and activity of succinic dehydrogenase. Scheel, McMillan and Phipps (1968) observed an inverse correlation between high levels of urinary fluoride and reduced succinic dehydrogenase activity of the kidney, however, no pathological changes in the kidney were observed.

Most recently Lucia et al. (1978) reported renal damage in mice following exposure to pyrolysis products of PTFE. The histological observation of proximal tubular necrosis by Lucia et al. was similar to those seen after exposing rats to gaseous PFIB, HFP and TFE (Clayton 1967). Additionally, Lucia et al. observed a reduction in the kidney/body ratio at all levels of exposure after 24 hours. This significant weight change disappeared at all exposure levels after 48 hours and returned to control limits, while the pathological states at 24 hours mentioned above were only seen at the two higher concentrations (i.e., 0.5 and 1.0 mg/l), and not the lower ones (i.e., 0.25 and 0.4 mg/l). The urine volume was also measured by Lucia et al. as a parameter of toxicity. These data showed that with a 1 mg/l exposure concentration, mice had an increase in urine output

only after one week post exposure. These data seem to be inconsistent with the kidney pathology seen at 24 hours, since proximal tubular necrosis would immediately inhibit water reabsorption by the kidney. Instead of a one week delay between renal damage and increased urine output as was actually observed, one would have anticipated increased urine output with corresponding kidney necrosis at 24 hours (Potter et al. 1979, Clayton 1977).

As mentioned earlier, it was shown that CTFE, HFP and TFE have similar effects on the kidney as mercuric chloride ($HgCl_2$), a potent nephrotoxic agent (Clayton 1967). These effects were seen as a decline in urine osmolality which exemplified the functional impairment of the renal concentrating mechanism. Figure 1 compares the effect of $HgCl_2$ and CTFE on rat kidneys (Clayton 1967). Besides the functional impairment of the kidney, there is an associated physical damage seen in the proximal renal tubules (Clayton 1967).

Potter et al. (1979) have also seen the effect of CTFE on the kidney. In addition to the increase in water intake and large volumes of diluted urine excreted, there was also a proportional increase in urinary lactic dehydrogenase (LDH). All of these parameters of toxicity directly correlated with proximal renal tubule necrosis observed after exposure to CTFE.

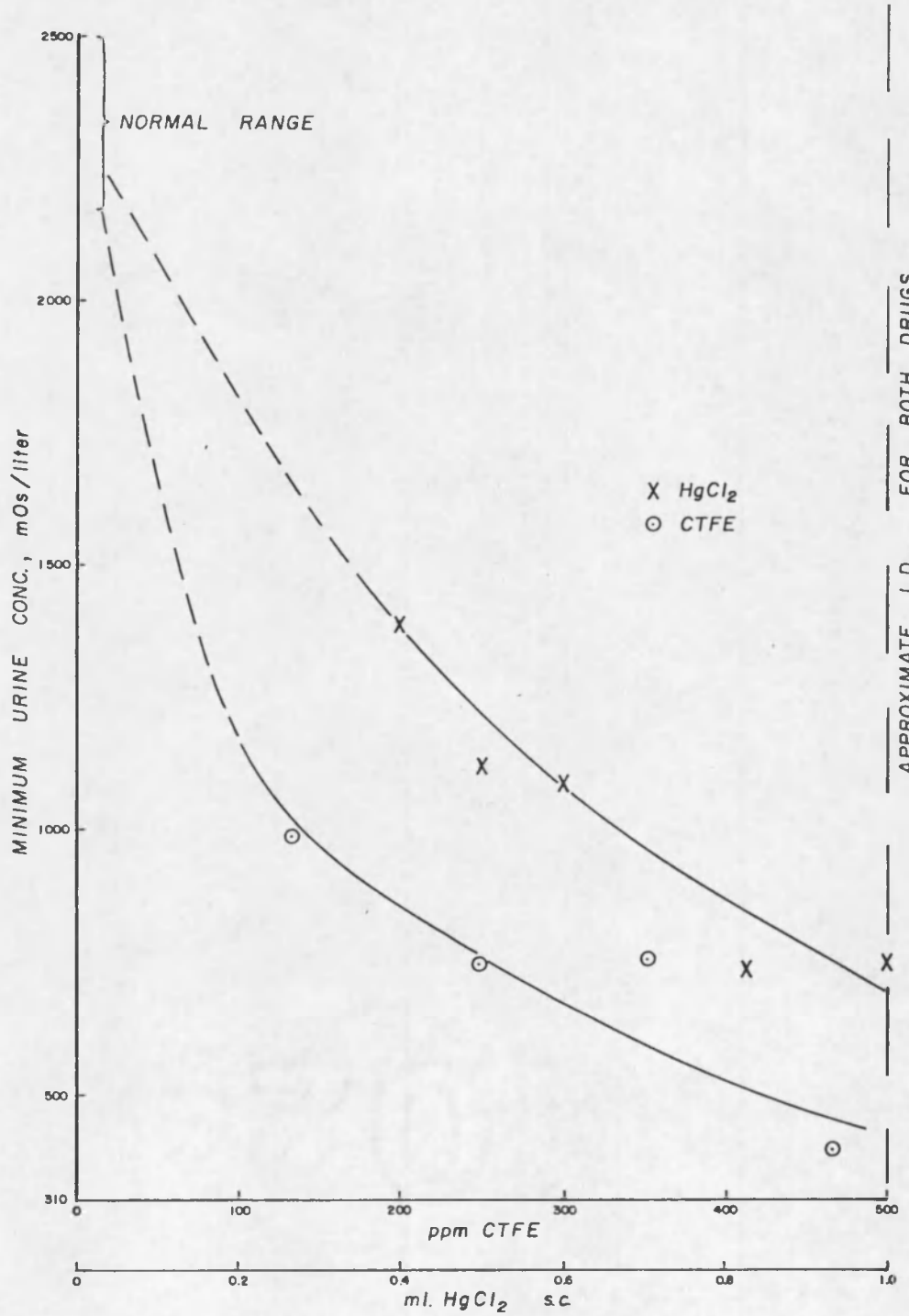

Figure 1. The Effect of $HgCl_2$ and CTFE on Rat Kidneys (Clayton 1967)

Most recently, Bhargava, Khater and Günzell (1978) also showed a correlation between the urinary LDH activity and kidney damage in the rat. After intravenous treatment with mercuric chloride there was a definite increase in urinary LDH activity. High LDH activity in the urine resulting from kidney tubular damage has been proven and is attributed to cellular breakdown of the tubular epithelium (Crockson 1961, Rosalki and Wilkinson 1959). Moreover, Bhargava et al. (1978) explained that the increase in LDH activity in the urine occurred in proportion to and subsequent to the severity of renal damage. In addition, only actual necrosis of the renal tubule resulted in increased LDH activity in the urine. Therefore, measurement of this enzyme would be an excellent indication of kidney damage. However, this renal damage is not seen in every animal model. There even seems to be an intraspecies variation. Mazze, Cousins and Kosek (1973) reported differences between similarly treated white rats of Fischer 344 and Buffalo strains. After treatment with methoxyflurane or inorganic fluoride, a metabolite of the former, proximal tubular necrosis and swelling of the mitochondria in the epithelial cells were observed only in the Fischer 344 rats. Likewise, only the Fischer 344 rats were polyuric after 24 hours with a decrease in urine osmolality. Therefore, even differences in animal strain have a variable effect on metabolism and susceptibility.

Mazze et al. (1973) explained that proximal tubular damage caused the increase in urine output. They stated that this injury interfered with reabsorption of the tubular fluid causing an excessive load on the more distal nephron (i.e., the ascending loop of Henle). This resulted in a decrease in renal medullary hyperosmolality with reduced reabsorption of water from the collecting ducts. Therefore despite hormonal controls, abnormally large volumes of diluted urine were formed.

Wisemann (1970) explained the increase in urine output by means of the inhibitory effect of inorganic fluoride on the energy transfer mechanism. He suggested that formation of adenosine triphosphate (ATP) used for the sodium pump in the ascending loop of Henle could be inhibited by inorganic fluoride. This could result in reduced renal medullary hyperosmolality and polyuric renal insufficiency.

Whatever the cause of increased urinary output, it is clear that in certain strains of white rats (i.e., Fischer 344) inorganic fluoride treatment or materials that breakdown through metabolism into inorganic fluoride can cause proximal renal tubule damage.

The toxicity of combusted PTFE is not attributable to inorganic fluoride, but rather the particulate evolved at 450°C (Clayton 1967, Waritz and Kwon 1968, Lee et al. 1976).

As mentioned earlier, Zapp (1962), Clayton (1962), and most recently Lee et al. (1976), demonstrated that the

toxic component of PTFE at the ALT (i.e., 450°C) was a particulate. It has been shown that the toxicity of airborne material is related to the particulate size (Pavia and Thomson 1976), as well as to its atmospheric concentration (Guyton 1976). If a particulate is smaller than 5μ, there are good grounds for assuming penetration of the respiratory system. By means of electron microscopy, Lee et al. (1976) sized the particulate of PTFE heated to 450°C to be in the range of 0.02 to 0.04μ. Therefore, penetration of the respiratory system by these particulates is likely.

In the respiratory system, filtration of large particles in the inhaled air is the function of the nose (Guyton 1976). In addition to the hairs at the entrance of the nostril which remove large particles, the main removal mechanism in the nose is turbulent precipitation. This means that inhaled air must travel through a labyrinth of passageways where the direction of flow is constantly and abruptly changed. Particles suspended in the inhaled air have such momentum that they cannot make the abrupt change in air direction and impact against the pharyngeal wall. The obstructing vanes within the nasal passageways are also coated with a thin layer of mucus which traps inhaled particles. In addition, the epithelium of the nasal and tracheobronchial passageways are ciliated. The cilia beat towards the pharynx so particles entrapped in the mucus are slowly

carried toward the pharynx where they are cleared by swallowing or expectoration (Guyton 1976). It was reported by Goldstein et al. (1976) that 90% of the particles deposited in the ciliated portion of the lung are removed within hours. The mucociliary system, which extends from the nares to the terminal bronchioles, can propel the mucus sheet at rates of 10 to 20 mm/min. This allows for quick clearance from this portion of the lung.

Another mechanism of clearance in the lung is the cough reflex (Goldstein et al. 1976). The bronchi and trachea are very sensitive to any type of foreign material or irritation. This irritation induces the cough reflex which, by means of coordinated muscle contractions, expels air at velocities up to 100 miles per hour. It was shown that with such high velocities most foreign matter was carried along with the air and removed from the trachea and bronchi (Guyton 1976).

Particle distribution and clearance in the lung is determined by many factors (Goldstein et al. 1976). These factors not only include the diameter of the air passageways, but also size, shape, velocity, and inertia of the particles. Other factors which involve lung clearance are tidal volume and frequency of respirations. It was shown by researchers that particles with a diameter greater than 10μ tend to plate out early in the nasal or pharyngeal cavity (Goldstein et al. 1976, Guyton 1976). Smaller particles in the range of

2.0 to 10.0μ in diameter were shown by Green (1970) to settle in the tracheobronchial tree. Particles in the size range of 0.5 to 2.0μ were influenced by Brownian motion and tend to be suspended in the inhaled air. These particles pass through the tracheobronchial region and settle in the alveolar section of the lung.

As earlier stated, the distribution of particles in the lung is dependent on many factors. Morrow (1964) best demonstrated this in a graphic presentation of particle distribution in the lung (Figure 2).

Clearance from the alveolar portion of the lung is relatively slow compared to the mucociliary system, however maintenance of a sterile airway is not compromised. It was shown by Laurenzi et al. (1963) that bacteria was 65% cleared or by definition of his assay, non-viable, after six hours post exposure. Laurenzi et al. (1963) also demonstrated that hypoxia, cigarette smoke and alcohol caused a reduction in the animal's ability to clear the bacteria. Green and Kass (1964a) carried this experiment one step further. They radiolabeled bacteria with P^{32}. This method enabled the researchers to not only determine viability of the bacteria, but also determine their rate of mechanical removal. It was shown that after four hours 80 to 90% of the bacteria was non-viable. This compares with the results of Laurenzi et al. who showed an 88% reduction in bacterial viability after four hours. The radioactivity however, as determined by

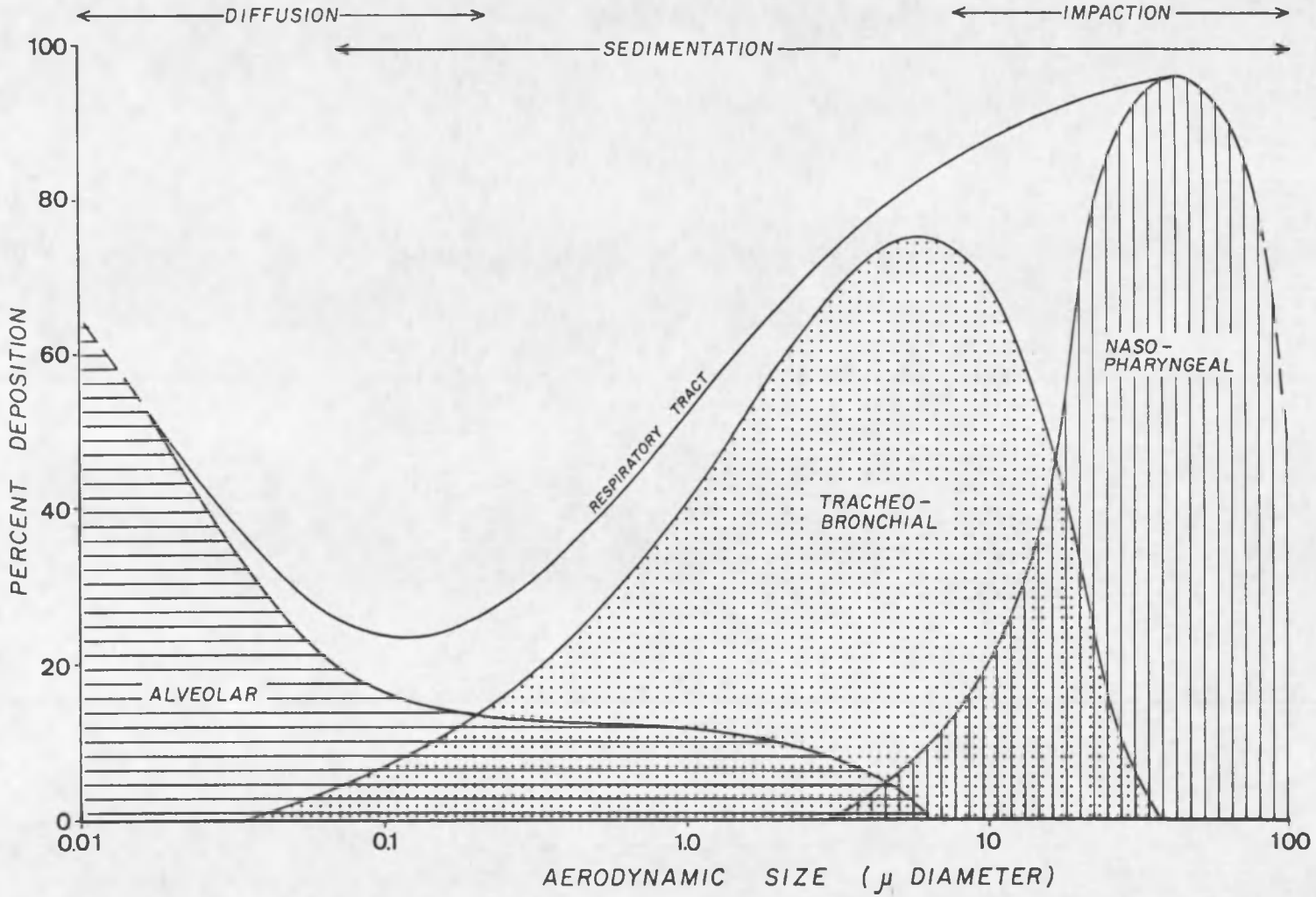

Figure 2. Particle Distribution in the Lung (Morrow 1964)

Green and Kass (1964b) declined by only 14 to 20%. This showed that although bacteria were killed relatively quickly, they were not physically cleared. Therefore, the bactericidal action of the lung was shown to dominate over mechanical removal in accomplishing bacterial clearance. It was concluded by Green and Kass (1964a) that the bactericidal action observed was mainly due to the phagocytotic activity of the pulmonary alveolar macrophages. Metchnikoff, in the 18th century, was the first to recognize the importance of the macrophage in the uptake of foreign material (Carr 1973). He was also the first to distinguish these phagocytes from other circulating cells. Since then macrophages distributed thoughout the body have been studied. The pulmonary alveolar macrophage, also known as PAM, plays an important role in the lungs. It has been suggested that the macrophage in the lung constitutes the primary line of defense against inhaled material (Brain et al. 1978, Biggar, Buron and Holmes 1976, Brain 1970, Rylander 1970, Brain and Frank 1968, Green and Kass 1964b). These cells have an essential role in the maintenance of a sterile surface for gas exchange and are largely responsible for removal of dust pathogens and debris from the nonciliated portion of the lung.

Although there has been much debate over the origin of the alveolar macrophage, it has now been confirmed. The monocyte is derived from precursor cells in the bone marrow (i.e., promonocyte) and migrate to the tissue to become

macrophages (Sbarra et al. 1976, Carr 1973). Monocytes after formation, mature in the bone marrow for one to three days. They then leave the bone marrow to circulate in the blood for a 24 to 36 hour period before migrating into the tissue to become macrophages. All tissue macrophages (i.e., Kupffer cells; peritoneal, alveolar and skin macrophages) are thought to be derived from the monocyte circulating in the peripheral blood (Sbarra et al. 1976). Macrophages, like other phagocytic cells, migrate to injury sites in the body by a mechanism called chemotaxis (Goldstein et al. 1976, Guyton 1976, Sbarra et al. 1976, Carr 1973). Chemotaxis is a process where leukocytes move toward or away from a chemical substance. These chemical substances which cause this cellular reaction can be degenerative products of inflamed tissues (e.g., tissue polysaccharides). Substances generated from inflamed tissue release a substance known as leukocytosis promoting factor (LPF). LPF increases the number of granulocytes in the blood (especially neutrophils) and likewise increases production in the bone marrow. The neutrophil is the primary defense mechanism in the first 6 to 12 hours of an infection. After that time large numbers of monocytes, which change into macrophages, become the principle defense mechanism. Besides the capability of the macrophage to ingest far greater amounts of material than neutrophils, the macrophage can survive in the acidic environment of a chronic infection. Neutrophils cannot live under acidic conditions.

As mentioned earlier, when microorganisms interact with the host tissue they liberate substances that attract phagocytes. Many polypeptides generated from different sources can produce a chemotatic reaction. However, the major mechanism by which bacteria cause chemotaxis is by activating the immune complement complex. When an antibody combines with an antigen the complement is activated (Sbarra et al. 1976, Guyton 1976, Carr 1973). The complement system is a group of nine enzyme precursors generally found in an inactive state in the plasma. These activated enzymes attack the invading organism, but also produce a chemotaxis of neutrophil and macrophages.

Chemotaxis depends on a concentration gradient of a substance. Therefore, the concentration on the side of the cell facing the substance is greater than on the side away from the substance. The greater concentration on one side causes the cell to extend pseudopodia toward the chemotatic agent and consequently move in that direction (Guyton 1976).

The process of phagocytosis basically involves three processes; recognition and attachment, ingestion, and digestion (Sbarra et al. 1976, Carr 1973).

Recognition of foreign material by the macrophage to be non-self is an important part of the immune system. If this recognition was not made, certain tissue or perhaps the entire individual would "self destruct". Loss of this recognition can sometimes result in older people after large

amounts of tissue destruction. This is commonly known as autoimmunity. In general however, recognition of pathogens by the body is facilitated by serum factors called opsonins (Sbarra et al. 1976, Carr 1973, Vernon-Roberts 1972). The opsonin alters the bacterial surface so it is recognized and ingested by the phagocyte. The most important of these opsonins in the serum are activated complement 3 (C3) and Immunoglobulin G (IgG).

Other factors which determine if a material will be phagocytized are texture and electronic surface charge (Guyton 1976). The likelihood of phagocytosis is increased if the surface of a particle is rough. Similarly, if a material has an electropositive surface charge there is an increased chance that it will be phagocytized. Natural substances in the body have an electronegative surface charge as do the phagocytes; therefore, they repel one another. However, dead tissue and foreign material often have an electropositive surface charge, so attraction and subsequent phagocytosis is increased (Guyton 1976). It is thought that on the surface of the monocyte or macrophages are receptor sites for attachment to particles, antibodies or complements (Carr 1973). The process of recognition of the material as foreign or self occurs when the substance first adheres to the phagocyte. If adhesion occurs the material is subsequently phagocytized. If no adhesion takes place there is obviously no phagocytosis. Therefore, the differentiation

between self and non-self is determined by whether or not the phagocyte adheres to the material (Carr 1973).

Ingestion is dependent upon the size of the particle to be engulfed. Macrophages ingest small materials (less than 2μ in diameter) by extending cytoplasmic processes around the particle and encasing them in a vacuole which is passed deep into the cell. With particles larger than 2μ, single macrophages will attempt to engulf the material or if it is too large, a group of macrophages will wall off the substance. This is accomplished by the macrophages becoming very closely apposed to the material and adhering to one another by interlocking cell processes. During this process fine fibrils can be seen in the cytoplasmic flap which engulfs the substance. In addition there appears to be a shearing movement of the surface cytoplasm over the rest of the cell. The vacuole movement is another notable process of the ingestion phase of phagocytosis (Carr 1973).

After ingestion, the vesicle containing the engulfed material combines with one or more lysosomes which contain several lysosomal enzymes. This allows for the delivery of highly active enzymes to the ingested material without endangering the cell's own cytoplasm (Goldstein et al. 1976). These lysosomal enzymes include acid phosphatase, lysozyme, acid ribonuclease, β-glucuonidase, cathepsin, and lipase (Carr 1973). Sbarra et al. (1976) suggests that the bactericidal capability of the phagocyte may be explained by the

peroxidase activity of the lysosome. Peroxide (H_2O_2) is produced as a by-product of NADPH and NADH oxidation. When sublethal concentrations of H_2O_2 and peroxidase combine with halide (i.e., I^- in the macrophage) in an acidic environment, bactericidal activity is observed (Sbarra et al. 1976). The pH inside the phagocytic vesicles has been shown to be acidic and therefore allows for the optimum condition for the H_2O_2-peroxidase-halide antimicrobial system. Evidence shows that the peroxidase-H_2O_2-halide system is normally latent in the cell while particulate content or entry activates it. Although peroxidase activity in the macrophage is low compared to PMNs and lymphocytes, this may be a possible bacterial killing mechanism (Sbarra et al. 1976).

The pulmonary alveolar macrophage plays an important role in host defense against removing foreign particles from the non-ciliated portion of the lung. In fact, it has been shown that there is a positive correlation between clearance rate and the number of phagocytic cells present (LaBelle and Briegar 1960). After digestion the alveolar macrophages are removed to the ciliated portion of the lung. The speed is determined by macrophage migration and mouthward flow of alveolar cell secretion.

Macrophages in the lung appear to be metabolically different from other macrophages found in the body. In a comparative study of the metabolic activities, Karnovsky (1962) and other researchers have demonstrated real

differences between alveolar and peritoneal macrophages (Ouchi, Selvaraj and Sbarra 1965, Oren et al. 1963). It was shown that alveolar macrophages take in more O_2 than do peritoneal macrophages. In addition, with anaerobic conditions, phagocytosis was inhibited with alveolar macrophages, but not with peritoneal macrophages. This suggests that differences in metabolic activity may be relevant to their anatomical location. The alveolar macrophage has a constant source of O_2 and therefore derives its energy from the aerobic means of oxidative phosphorylation. The peritoneal macrophage however, must survive in relatively anaerobic conditions so energy is derived primarily from glycolysis (Vernon-Roberts 1972).

The pulmonary alveolar macrophage (PAM) is approximately 15 to 25μ in diameter (Klika et al. 1975). The surface of the PAM has cytoplasmic processes and blunt pseudopodia which protrude from the periphery of the cell. The cell also contains large numbers of mitochondria since oxidative phosphorylation is thought to be its main source of energy (Vernon-Roberts 1972). The macrophage also contains a single nucleus which occupies a large portion of the cell. As would be expected of a phagocytic cell, other outstanding ultrastructural features include golgi apparatus, endoplasmic reticulum, and primary and secondary lysosomes.

It has been shown that large quantities of pulmonary alveolar macrophages can be isolated from the lung by lavage

methods (Myrvik, Leake and Fariss 1961). Therefore, since these free cells are readily available and a principle defense mechanism of the lung, they are ideal for the toxicological evaluation of gases and aerosols.

The purpose of this investigation was to determine the effect of inhaled pyrolysis products of polytetrafluoroethylene (PTFE) on the phagocytic capabilities of the pulmonary alveolar macrophage. Phagocytosis was determined for PAM of rabbits exposed to Teflon^{R} pyrolysis products generated at different temperatures. Parameters of lung toxicity besides macrophage phagocytosis included, differential cell counts, lung histopathology, and viability and macrophage yield.

White rats also used in this investigation were exposed to: (1) compare susceptibility of different animals to PTFE fumes, (2) compare data with other researchers who used different types of Teflon^{R}, and (3) determine if a kidney effect was demonstrable.

CHAPTER 2

METHODS AND MATERIALS

Material and Thermal Decomposition Characteristics

Polytetrafluoroethylene (PTFE) known by the trade-name of Teflon^{R} was obtained from E. I. du Pont de Nemours and Company. The PTFE resin was an opaque white sheet with a thickness of approximately 1 mm.

To determine thermal decomposition characteristics, small samples of PTFE weighing approximately 5 to 15 mg were burned in a Perkin-Elmer TGS-1 thermobalance. Thermal gravimetric analysis gives information regarding weight loss of sample versus temperature increase. This equipment was calibrated by heating metal alloys of alumel, nickel, mumetal, nicoseal, perkalloy, and thermo grade iron which degraded and lost weight at 163°C, 354°C, 393°C, 438°C, 596°C, and 780°C, respectively.

Samples and standards were run in an air atmosphere at heating rates of 5°C, 10°C and 20°C per minute. In addition, PTFE samples were kept at constant temperatures of 400°C, 425°C and 450°C for six hours to determine if a weight loss resulted.

Exposure Chamber and Equipment

Samples were placed in a stainless steel pyrolysis boat which was likewise placed in a steel tube and sealed at both ends with brass plates (Figure 3). A metal pipe for air flow into the tube and a thermocouple to monitor tube temperature were drilled and extended into the tube through the brass plate in one end. The tube was heated by means of an electric Lindberg Tube Furnace which entirely surrounded the tube. The thermocouple within the tube was an Omega chromel-alumel (nickel chromium vs. nickel-aluminum) and situated so it was directly above the heating sample. An identical chromel-alumel thermocouple was placed in an ice bath and wired in series to the one in the tube. This reference thermocouple was used to correct the voltage output to 0°C. This electrical series was connected to a chart recorder which constantly measured voltage output throughout the exposure. Corresponding oven temperatures were determined from these millivolt readings by extrapolating these data from voltage reading of known temperatures.

Samples of PTFE were placed in the tube furnace only after the desired combustion temperature was reached. This was done in an attempt to limit the amount of pyrolysis products generated at lower temperatures and attain a more exact index of toxicity versus temperature.

Air was filtered through $CaSO_4$ (Drierite) and a molecular sieve to remove moisture and contaminants and then

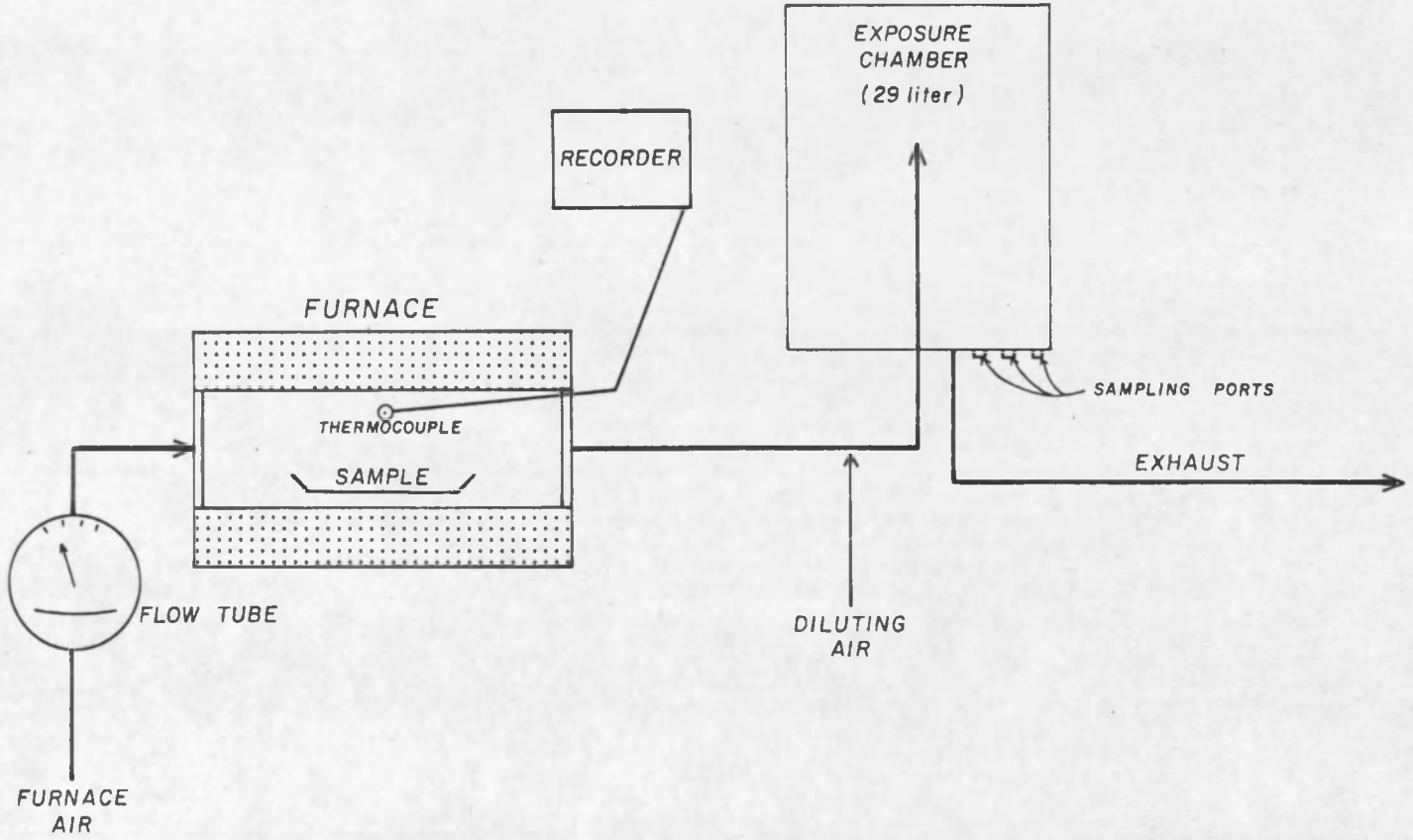

Figure 3. Block Diagram of Pyrolysis Oven and Exposure Chamber

metered through a flow tube to determine air rate. Six liters per minute were delivered through the pyrolysis tube within the furnace. This air carried the pyrolysate from the tube furnace, out a side opening, through stainless steel tubing to the exposure chamber. The 29 liter exposure chamber was made of glass and fitted with a metal plated wooden lid with openings for gas sampling, temperature monitoring, and entrance and exit ports. Percent oxygen (O_2) was determined by means of a constant monitoring I.B.C. differential oxygen analyzer. Parts per million of carbon monoxide (CO) was estimated by means of a MSA (Mines Safety Appliances) pump and colormetric indicator tubes. CO was recorded at 15 minute intervals. The temperature of the chamber was monitored by extending a thermometer into the chamber.

Test Procedure Involving Alveolar Macrophage Phagocytosis

One New Zealand white rabbit weighing between 1.5 and 3.0 kg was exposed for one hour to pyrolysis products from heated samples of approximately 20 gms of PTFE. Samples were heated in the range of 350°C to 450°C. The rabbit was either sacrificed immediately after the exposure or allowed to recover for one week. The rabbits were sacrificed by injecting an air bolus of 20 to 30 mls in a marginal ear vein (Figure 4). The trachea was exposed and clamped to prevent infiltration of red blood cells. This

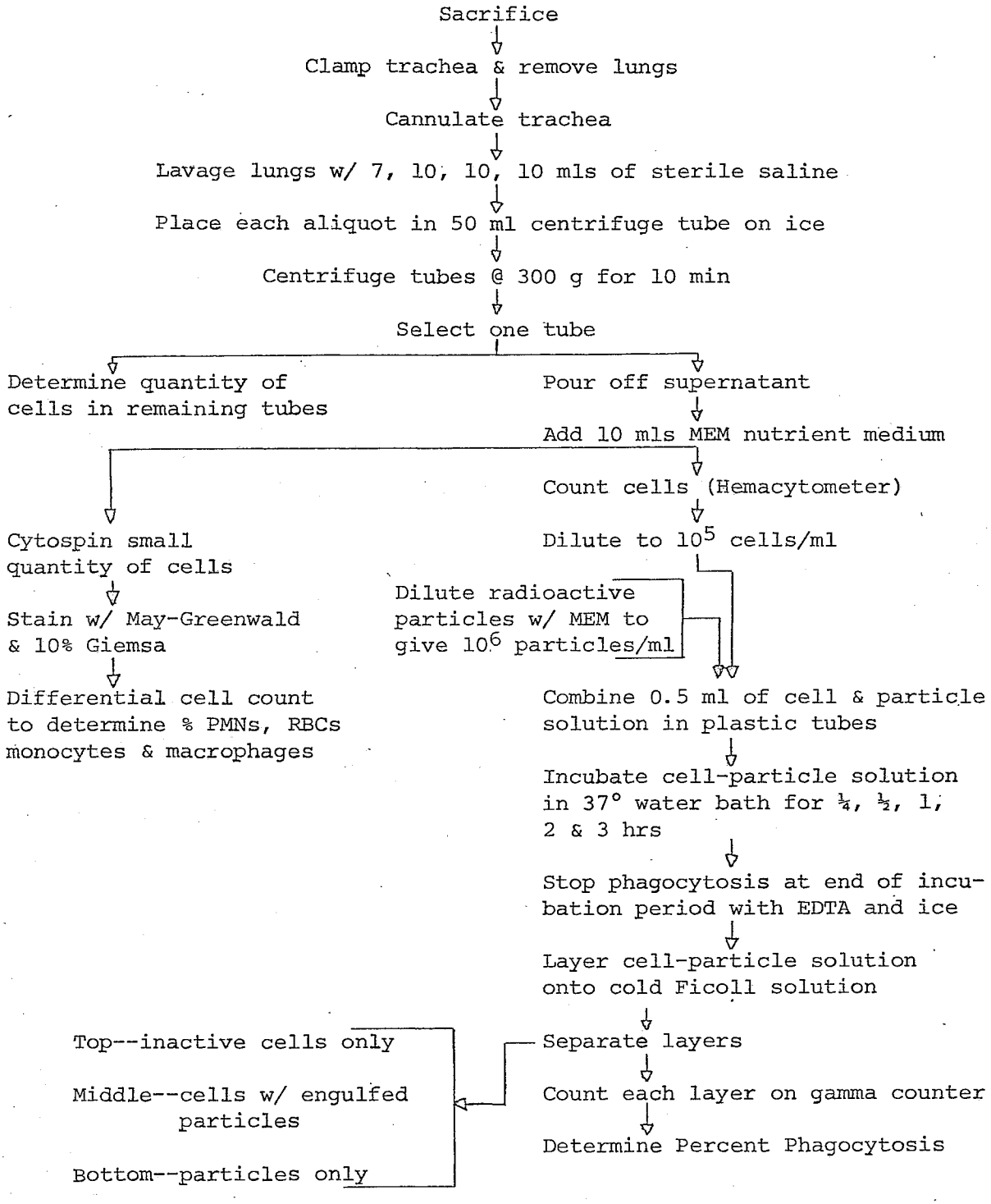

Figure 4. Pulmonary Alveolar Macrophage Assay

method of animal sacrifice resulted in the least amount of blood contamination and damage to the lungs. Following a necropsy, the lungs were excised and lavaged with warmed (37°C) sterile physiological saline (0.5 M NaCl) as described by Hahn et al. (1973). Lunges were lavaged four times with 7 mls, 10 mls, 10 mls, and 10 mls of saline, respectively. Cell suspensions were centrifuged at approximately 1000 RPMs (300 g's) for ten minutes to separate supernatant from cells. The supernatant was poured off and the cells were resuspended in 10 mls of a nutrient medium. This medium consisted of 88% Eagle Minimum Essential Media (MEM), 10% heat inactivated fetal calf serum, 1% L-glutamine and 1% penicillin G/streptomycin. The pH was adjusted to 7.0 by adding drops of sterile 8% (w/v) $NaHCO_3$ which resulted in a cherry red color change. Cells were counted in a hemacytometer to determine yield and red blood cell contamination. Viability of the macrophages was determined by a 1% (w/v) trypan blue dye exclusion method (Lillie 1969). The number of stained (dead) cells per 100 total cells were noted.

Cell populations of the lavages were determined by cytospining the cells onto a glass slide and differentially staining them with May-Greenwald and 10% Giemsa. The percent of each cell type was determined by the number per 100 total cells. An aliquot of the cell suspension, whose concentration was determined by a hemacytometer count, was

diluted with media to give a concentration of 1 X 10^5 cells/ml.

Likewise, tracer microspheres (3M Company) 2 to 4μ in diameter, labeled with ^{85}Strontium, which is a gamma emitter were prepared in media to give a concentration of 1.0 X 10^6 particles/ml. These black inert particles, originally suspended in sterile saline, had a specific activity of 10 microcuries/gm. One half milliliter of both the cell and particle suspension were added together in sterile disposable 15 ml centrifuge tubes to give a particles to macrophage ratio of 10:1.

Triplicate samples were allowed to incubate in a 37°C water bath for .25, .5, 1, 2, and 3 hours. At the end of each incubation period, .3 ml of .03 M EDTA were added to each tube, and then placed in ice to inhibit further phagocytosis. Tubes containing particles only, served as controls. After a three hour incubation period, each deactivated cell-particle solution was carefully layered on top of a 1 ml density medium of cold 30% ficoll (w/v). This was spun on a centrifuge at 1000 RPMs (300 g's) for three hours to separate free particles from engulfed particles. The tubes were divided into three separate layers; (1) 0.5 ml -- supernatant, (2) 0.9 ml -- macrophages with engulfed particles, and (3) 0.9 ml -- free particles. Sample tubes and controls were counted on a Beckman Gamma Counter for ten minutes each. From the results of triplicate samples the percent

phagocytosis was calculated by the following formula:

$$\text{Percent Phagocytosis} = \frac{(Mx - Tx) - (Mo - To)}{Tx + Mx + Bx} \times 100$$

Where:

Mx = Net counts/minute obtained from middle fraction

Tx = Net counts/minute obtained from top fraction

Bx = Net counts/minute obtained from bottom fraction

Mo = Net counts/minute obtained from middle fraction of the zero hour tube

To = Net counts/minute obtained from top fraction of the zero hour tube

The first component of the numerator (Mx - Tx) represented the macrophages that engulfed particles. The second component (Mo - To) represented soluble radioactive particles in the solution. The denominator (Tx + Mx + Bx) represented the total radioactivity in each tube. Percent phagocytosis was determined for each incubation period (i.e., .25, .5, 1, 2, and 3 hours) for each PTFE-burn exposure. To determine if a dose response effect existed, percent phagocytosis was plotted against temperature-concentration.

Other Animal Models

In addition to the rabbit exposures, Sprague-Dawley and Fischer 344 white rats were also exposed to pyrolysis products of PTFE. The purpose of this was threefold. First, to compare the toxicities of pyrolysis products between different animal species. Second, to compare the pyrolysis toxicities of "new" PTFE with earlier work done with Teflon^{R}

samples. In this regard, there was no change in the basic carbon-fluoride chain of PTFE, however there have been apparent changes in the method of fabrication and/or catalysts used. This may have caused the changes in thermal stability and toxicity reported by Waritz and Kwon (1968). And third, to determine if pyrolysis products caused kidney damage which was determined by measuring enzymes and urine concentration, and by means of microscopic pathological examination of the kidney.

Methods for White Rat Exposures

Rats were exposed in the same chamber using the accompanying equipment that was described earlier for rabbit exposures. Rats were exposed in groups of six unless otherwise noted. Air flow was maintained between three and six liters per minute. The time of exposure varied between one and three hours. Rats were exposed to the same temperature range as were the rabbits (i.e., 350°C to 450°C). Animals were either sacrificed immediately to note tissue lesions or allowed to recover and observed for up to two weeks. Animals were weighed daily and observed for signs of toxicity or stress throughout the pre and post exposure period.

During this observation period and a pre exposure conditioning period of three to four days, some animals were placed in metabolism cages where water intake and urine output were noted. Three rats were placed in each cage where

daily observations were made. In addition to measuring body weights, water intake and urine output, the urine was also measured for concentration, inorganic fluoride and lactic dehydrogenase.

Urine was centrifuged for ten minutes at 2500 RPM to remove food particles which may have fallen into the urine during the 24 hour collection period. Concentration of the urine was determined by an Advanced Instruments Advanced Osmometer. Concentration was given in milliosmoles per kilogram by determining the freezing point of the urine. Inorganic fluoride concentration was determined by millivolt measurement through fluoride electrodes attached to a Corning Digital 110 pH meter.

Microscopic Pathology

Microscopic pathology was conducted by a veterinary pathologist at the Veterinary Reference Laboratory in Salt Lake City, Utah. Lung and kidney tissues from animals exposed to various concentrations and temperatures of PTFE were fixed in 10% buffered formalin and sent for examination. All samples were coded so pathology was completely unbiased.

Lactic Dehydrogenase Assay

Lactic dehydrogenase (LDH) activity was determined by the following method as described by the Sigma Chemical Company (1977). Urine was dialyzed for 1.5 hours to remove inhibitors of LDH. The dilution after dialysis was

determined and recorded for use in the calculation of LDH activity. 2.7 mls of potassium phosphate buffer and 0.2 ml of urine, were added to a preweighed Sigma Chemical Company NADH vial containing 0.2 mg NADH. This solution was allowed to equilibrate at room temperature for 20 minutes. Added to this solution was 0.1 ml of sodium pyruvate solution, which was immediately mixed and read on a Beckman Acta CIII spectrophotometer. Absorbance was read and recorded at 20 second intervals for three minutes at an ultraviolet wavelength of 340 nm using a 1 cm lightpath cuvet. A linear change in absorbance during the three minutes was used in calculation of the LDH activity (ΔA/min). The urinary LDH activity was determined by the following formula:

$$\text{URINARY LDH (units/mls)} = \frac{\Delta\text{A/min} \times \text{TCF} \times \text{D.F.} \times \text{mls urine}}{0.001 \times 0.2 \times \text{LIGHTPATH (cm)} \times 24 \text{ hours}}$$

Where:

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

0.2 = Urine volume (ml) in cuvet

TCF = Temperature Correction Factor (1.2 at 22°C)

D.F. = Dilution Factor

One unit of LDH activity is defined as the activity which will cause a decrease in absorbance at 340 nm (A_{340}) of 0.001 per minute at 25°C in a 3 ml reaction mixture in a cuvet of 1 cm lightpath.

CHAPTER 3

RESULTS

General Observations

In all the tests conducted with heated PTFE samples, no smoke or fumes were observed in the chamber. The oxygen content did not drop below 18.5% and carbon monoxide was not detectable with indicator tubes with a sensitivity of 10 PPM. The chamber temperature did not rise more than four degrees centigrade above room temperature (average room temperature was 25°C).

In order to fit into the pyrolysis boat, samples of the 1 mm thick sheet of PTFE were cut into two strips, 3.1 by 11.9 cm. Since samples overlapped within the pyrolysis boat, the surface area of PTFE exposed to air was 62 cm^2. At temperatures between 400°C and 430°C, there was an overall length reduction of 0.9 cm which corresponded to a 7.5% change. At these temperatures the appearance and texture of the cooled sample were the same as the original material. Between 440°C and 450°C however, the material showed severe signs of degradation including pitting, brittleness and shrinkage.

Rabbits exposed to concentrations of PTFE fumes above 1.0 mg/l (i.e., 450°C) exhibited different degrees

of respiratory irritation throughout the exposure and post exposure period. Initially, animals sneezed and pawed at their nose. This was followed by mucus and saliva accumulation around the nose and mouth. During this time, the rabbit was often passive, almost lethargic, with eyes partially or totally closed. The culmination of respiratory irritation began with labored breathing and ended with dyspneic gasping and death.

Animals exposed to concentrations above 1.0 mg/l for one hour usually died within 24 hours post exposure. If animals survived this period they would usually recover from respiratory distress and then gain weight equivalent to control animals. In this regard, animals (including rats) would initially lose weight the first day post exposure, then gain weight in parallel amounts to control animals. Specifically, rabbits exposed to concentrations above 1.0 mg/l (i.e., 450°C) of PTFE fume for one hour would show weight losses of 20 to 100 gms the first day post exposure. Rabbits would then gain weight the following days at rates of 25 to 100 gms/day, which is equivalent to control values. Rats showed a similar effect but at a much lower concentration. At concentrations of .03 mg/l and above (i.e., 380°C), rats exposed for three hours to PTFE fumes would show weight losses the first day that averaged 1.5 gm. These weights would then return to pre-exposure values of 1 to 5 gm of weight gained per day.

The temperature and concentration associated with lethality are summarized in Table 2. Temperatures above 400°C caused a 100% mortality in rats, while PTFE heated to 450°C was the temperature which caused partial lethality in rabbits. When rats were exposed to fumes of Teflon^{R} heated to 450°C which were filtered with a 0.2μ Metricel filter, there were no deaths. This occurred even though the heated sample's weight loss indicated that the potential concentration of the chamber was over 22 times a probable lethal concentration. Upon examination of the filter, it was observed that the entire surface was covered with a fine white powder. On microscopic examination, individual particles were indiscernible indicating their diminutive size.

Thermal Decomposition Characteristics

As described in the methods section, samples of PTFE were analyzed by thermal gravimetric analysis (TGA) at heating rates of 5°C, 10°C and 20°C per minute. In all experimentation involving TGA, an air flow of 60 ml/min was delivered to the thermobalance. Data from these experiments are represented in Table 3. It appears from the uncorrected data that the material might decompose at different temperatures. Daniels (1973) however, has shown that decomposition is heating rate dependent. When these data were corrected to the standards of known temperature decomposition they approached one another.

Table 2. Summary of Temperature and Concentration Associated with Lethality

Animal	350-400°C (0-.1 mg/lit) Observations	400-445°C (.1-.9 mg/lit) Observations	445-450°C (1.0 mg/lit and above) Observations
Rat Sprague-Dawley	375°C and above Dyspnea Clear secretions at nose and mouth Petechial hemorrhage in lung ALT	400°C and above Dyspnea Clear secretions at nose and mouth Massive hemorrhage in lung 100% Mortality	450°C Same as at 400°C *
Rat Fischer-344	375°C No apparent effects All organs within normal limits	400°C Dyspnea Hemorrhage in lung Partial Mortality (66%)	None Tested
Rabbit New Zealand	No apparent effects	Nasal irritation (pawing nose and sneezing) Lethargy Slight dyspnea High amounts of RBCs in lavage fluid No deaths	Respiratory irritation Wet muzzle Sneezing Dyspnea Massive hemorrhage in lungs 450°C is the temperature which produced partial mortality #

* When a .2 filter was placed in the pyrolysis air stream between the exposure chamber and oven, all rats survived the exposure and showed no side effects.

When a .8 filter was placed in the pyrolysis air stream, rabbits survived the exposure with no toxic signs demonstrated.

Table 3. Temperatures Associated with Weight Loss Recorded from Thermal Gravimetric Analysis

HEATING RATE (0°C/min)	WEIGHT LOSS TEMPERATURE (0°C)	CORRECTION (°C)	CORRECTED WEIGHT LOSS TEMPERATURE (°C)	MEAN -- WITH PLUS OR MINUS STANDARD DEVIATION
5	581	-5	576	
5	579	-4.5	574.5	577.4 ± 2.8
5	586	-5	581	
5	583	-5	578	
10	613	-8.5	604.5	
10	605	-8	597	600.4 ± 3.9
10	611	-8	603	
10	605	-8	597	
10	612	-8	604	
20	615	-26	589	
20	617	-27	590	591.1 ± 2.4
20	618	-27	591	
20	622	-27.5	594.5	
		MEAN OF ALL CORRECTED WEIGHT LOSS TEMPERATURES =		590.7 ± 10.6

At heating rates of 5°C/minute, the PTFE samples were totally consumed at approximately 580°C. The samples run at 20°C/minute vaporized at more that 10°C higher (i.e., 591°C) than the slowest heating rate. At 10°C/minute the corrected values were the highest at 600.4°C.

The smallest correction factor determined from standard values was at 5°C/minute. This would indicate that at a 5°C/minute heating rate, the average decomposition temperature of 577.4°C would be the most accurate. However when all corrected values were averaged, the mean (i.e., 590.7°C) and standard deviation (i.e., 10.6°C) indicated that the values at different heating rates were relatively close.

At all heating rates, TGA showed only one weight loss. Therefore at these heating rates, significant amounts of pyrolysis products were evolved at only one temperature range and not at multiple intervals. This indicated that the PTFE sample vaporized at a single temperature range, and had a high thermal stability.

To determine how much weight loss resulted at temperatures below the thermal decomposition temperature, small samples of PTFE were kept at constant temperatures of 400°C, 425°C and 450°C for six hours. These temperatures were chosen because they were used for animal exposures (e.g., 450°C is the temperature at which partial lethality occurs in rabbits). Weight losses observed during the six hour

heating period were 0.23%, 1.55% and 10.2% for temperatures of 400°C, 425°C and 450°C, respectively.

During the first hour of heating, which would correspond to an animal exposure period, the samples lost 0.051% at 400°C, 0.16% at 425°C, and 1.25% at 450°C. These data are summarized in Figure 5.

Phagocytic Index

Photographs of macrophages and carbon particles used in this assay are shown in different phases of phagocytosis in Figures 6 to 8. In Figure 6 the cell is in the initial phase of phagocytosis. Having attached to the particle, the phagocyte has begun to encase it with cytoplasmic processes. The following photograph (Figure 7) clearly shows the pseudopodia used in attachment. The last photograph (Figure 8) shows macrophages with numerous ingested particles. This last figure represents macrophages which had been incubated with particles for three hours. Percent phagocytosis for each incubation period was derived from averages obtained from triplicate samples. The chamber concentration in this dynamic exposure system was determined nominally as decribed by Silver (1946). At each range level of exposure (Figures 9 through 13), it can be observed that in the first hour of incubation the increase in percent phagocytosis was greatest. Between one and two hours the change was less dramatic and

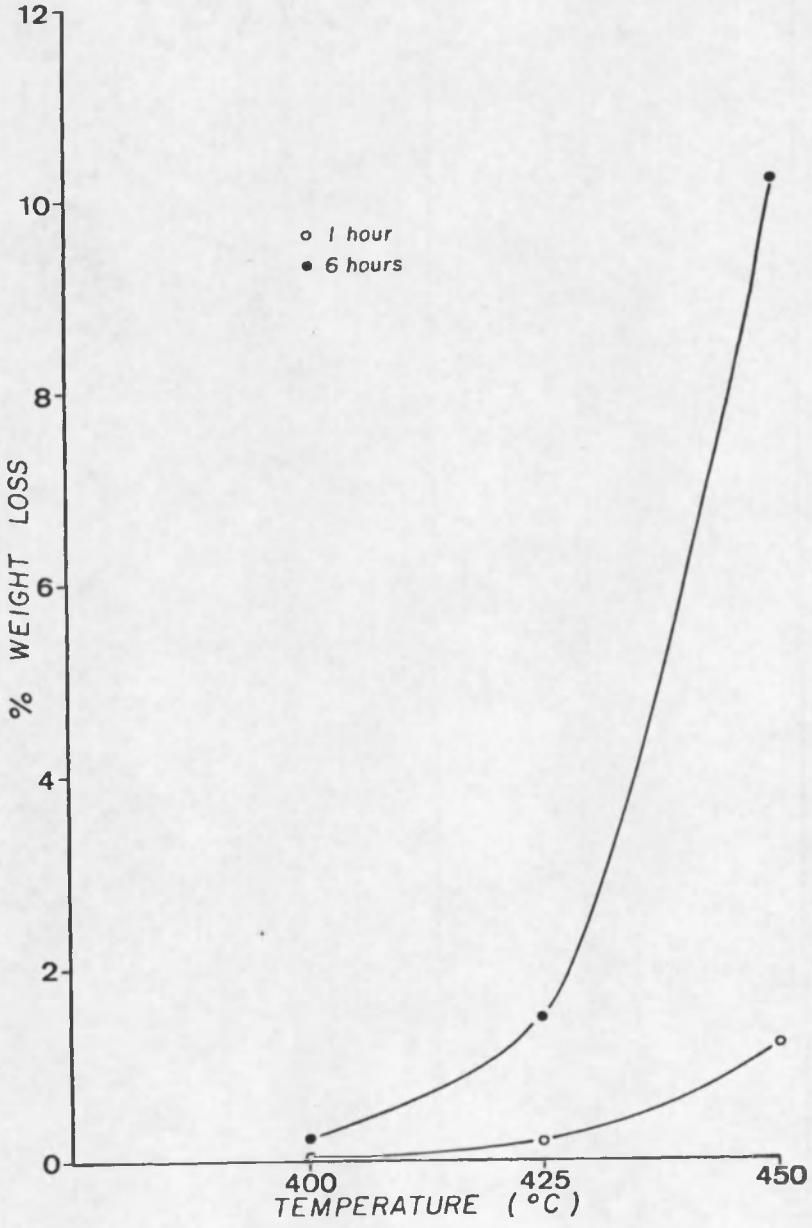

Figure 5. Constant Temperature Polytetrafluoroethylene (PTFE) Weight Loss Using Thermal Gravimetric Analysis (TGA) with 60 mls Air Flow per Minute

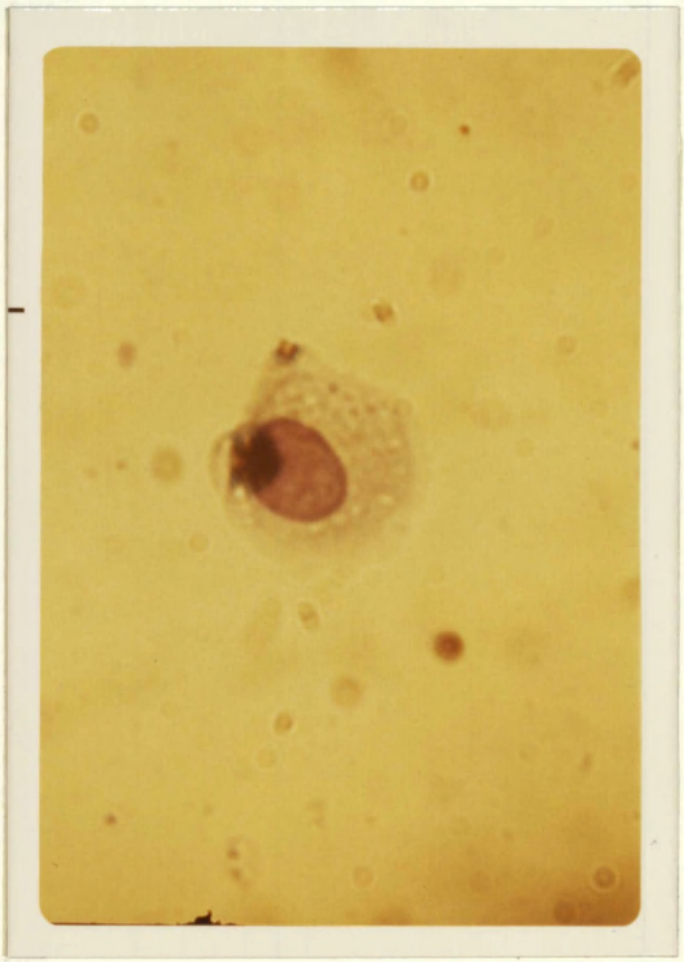

Figure 6. Macrophage in Initial Phase of Phagocytosis of Carbon Particle

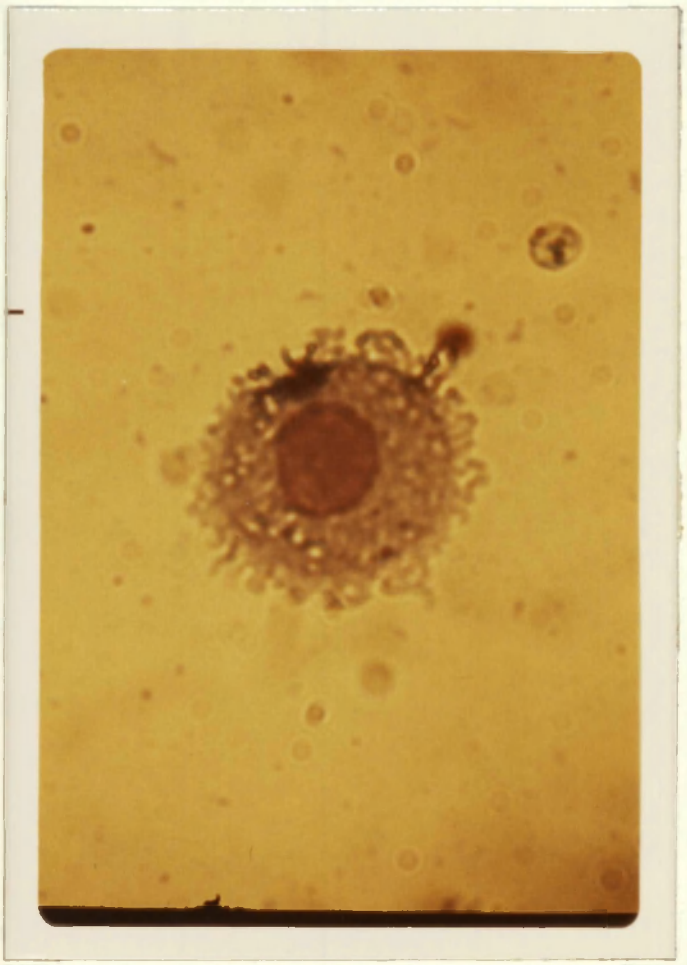

Figure 7. Macrophage Showing Pseudopodia Used in Attachment to Particle

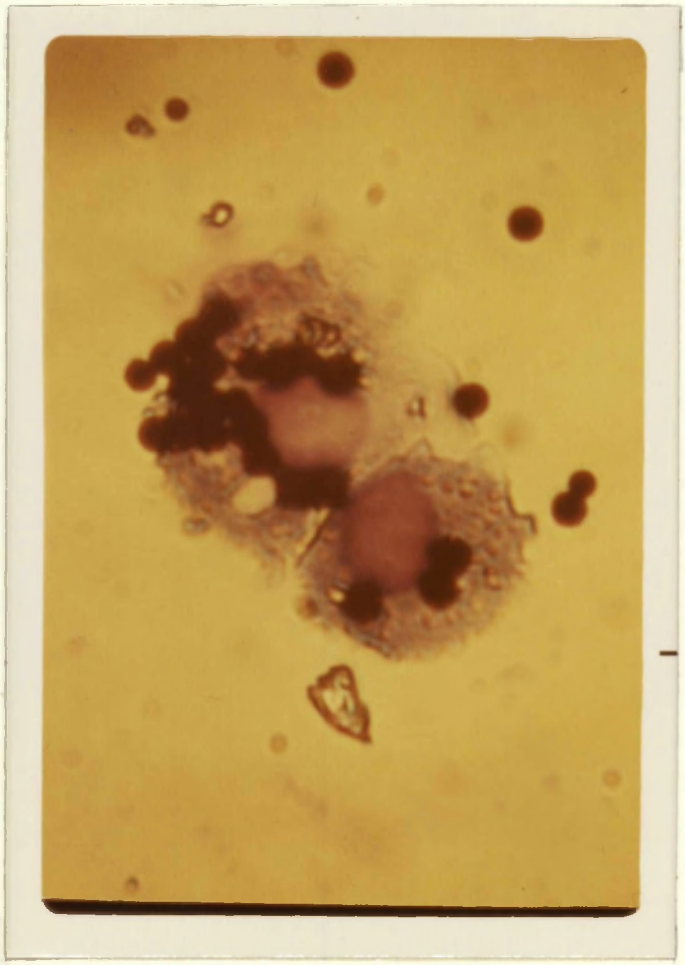

Figure 8. Macrophages with Numerous Ingested Carbon Particles

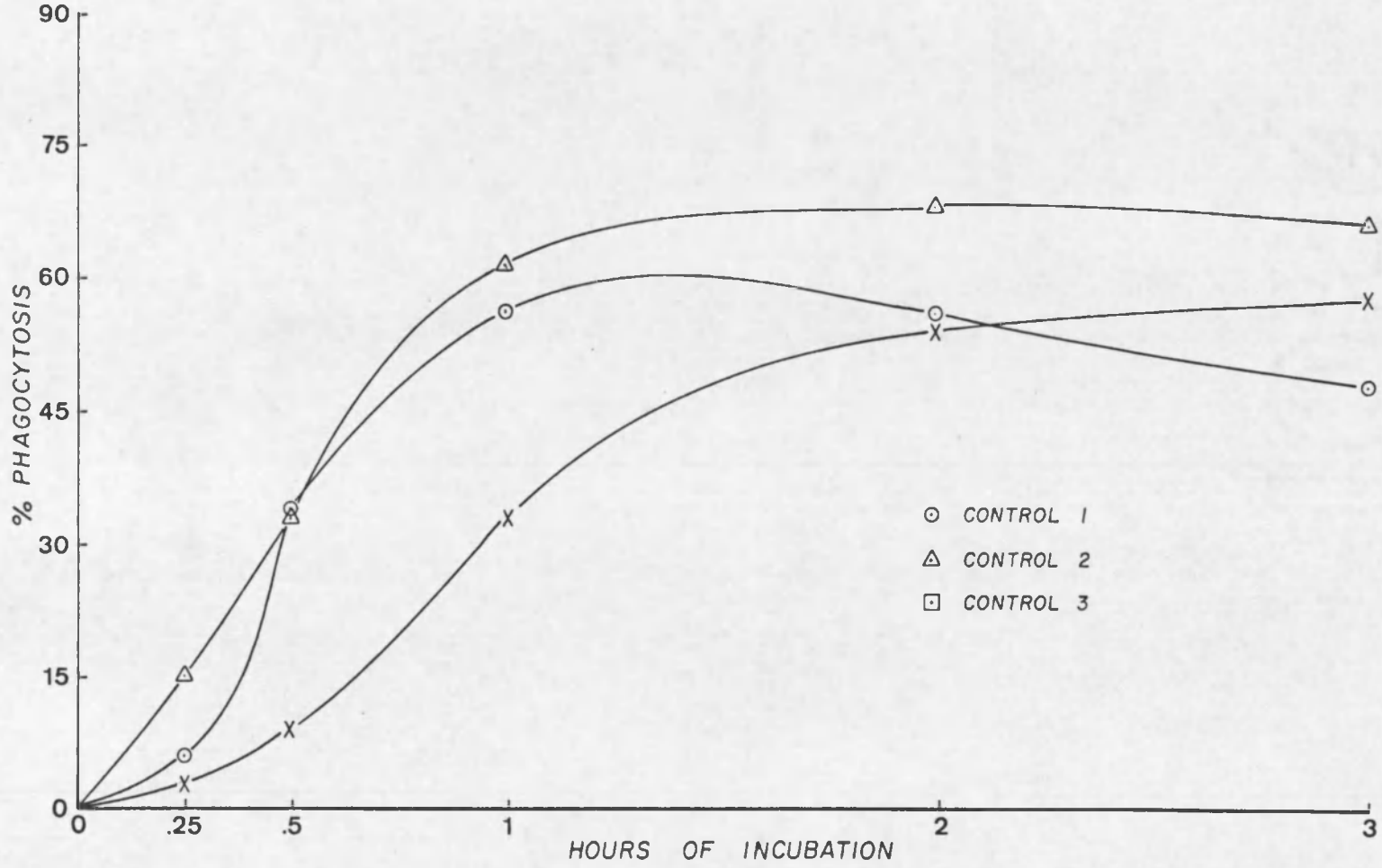

Figure 9. Phagocytic Activity of Control Macrophages Incubated for Three Hours with ^{85}Strontium Labeled Particles

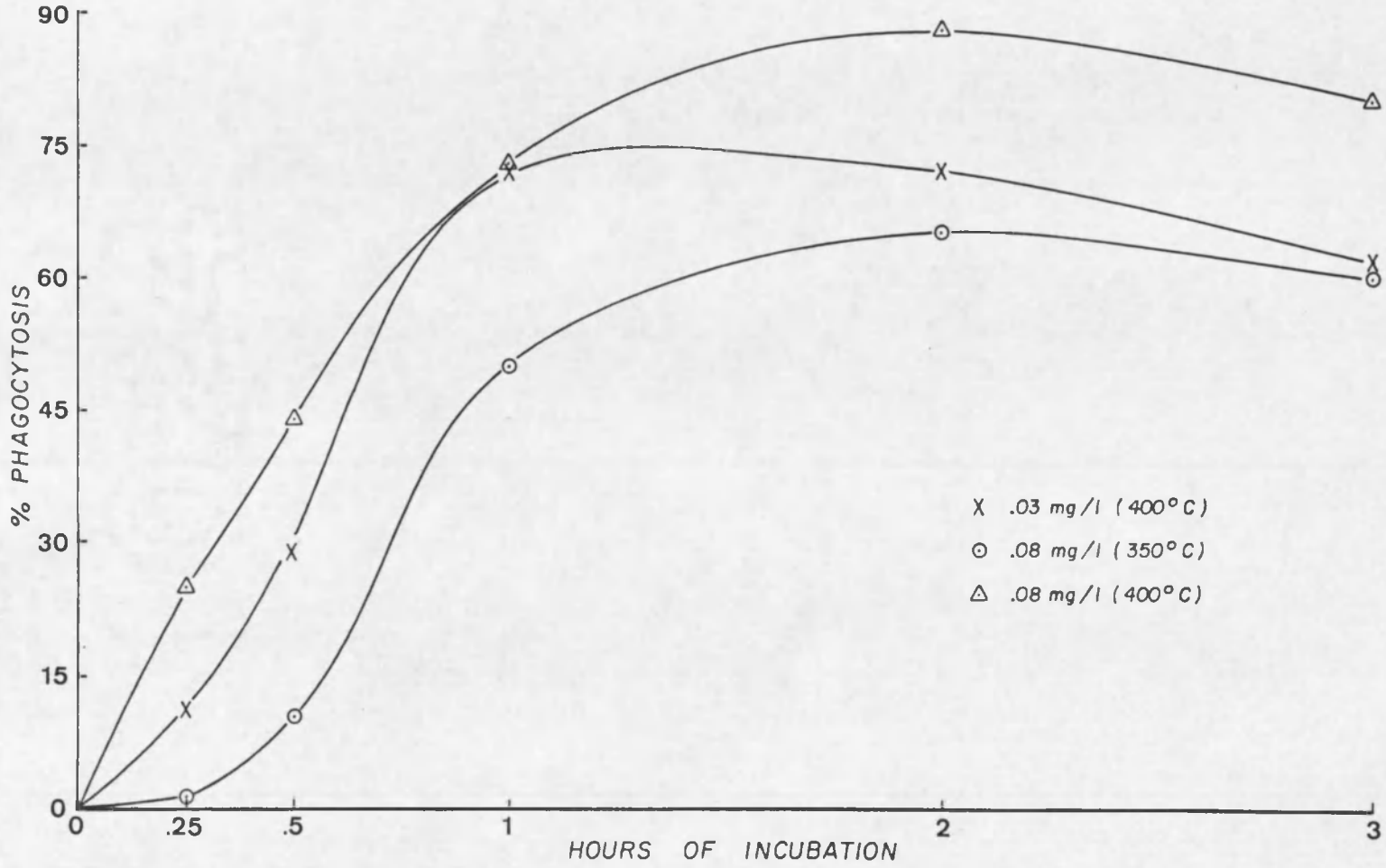

Figure 10. Phagocytic Activity of Lung Macrophages Taken from Rabbits Exposed for One Hour to PTFE Fumes of a Concentration Ranging from .01 to .1 mg/l

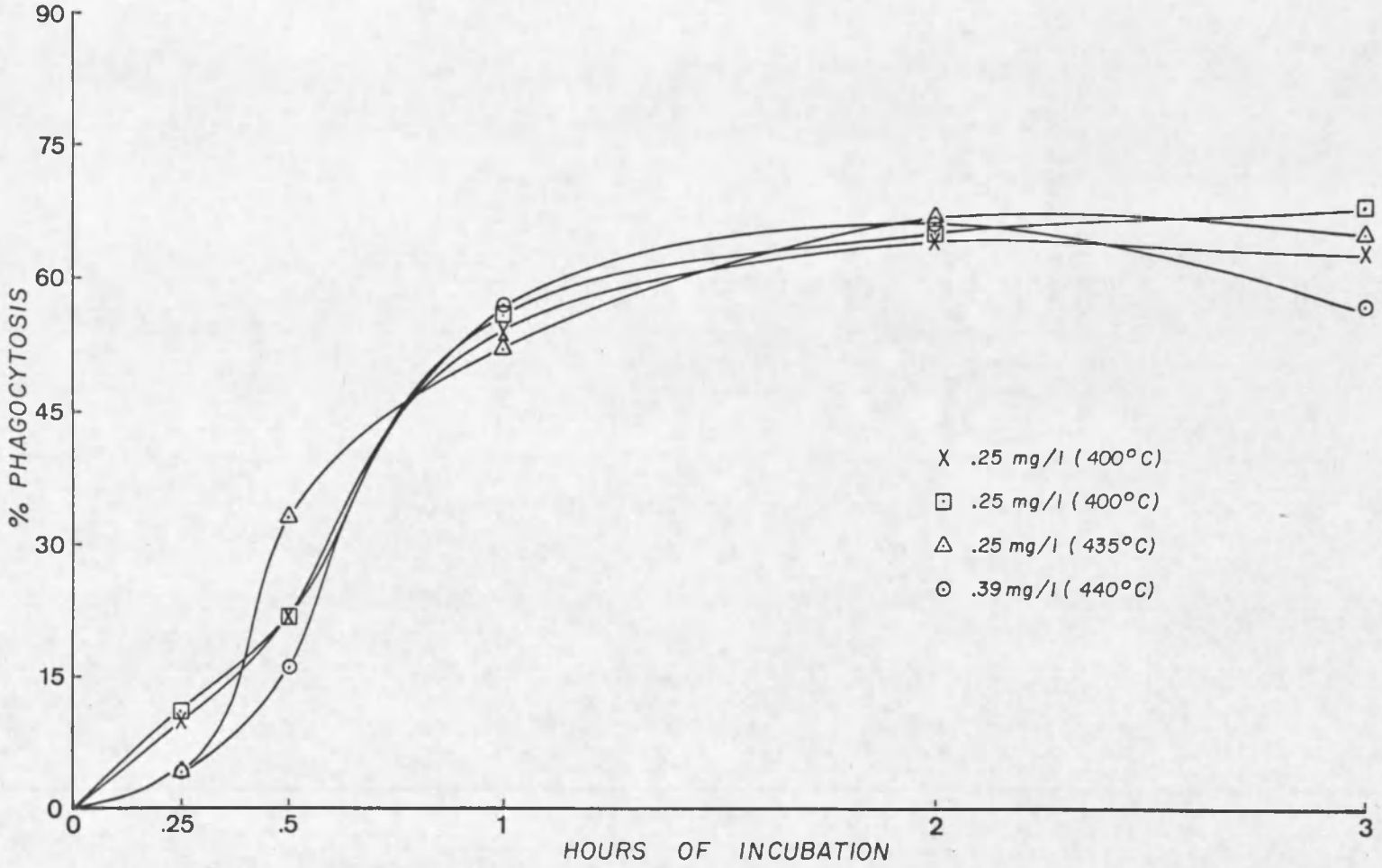

Figure 11. Phagocytic Activity of Lung Macrophages Taken from Rabbits Exposed for One Hour to PTFE Fumes of a Concentration Ranging from .1 to .4 mg/l

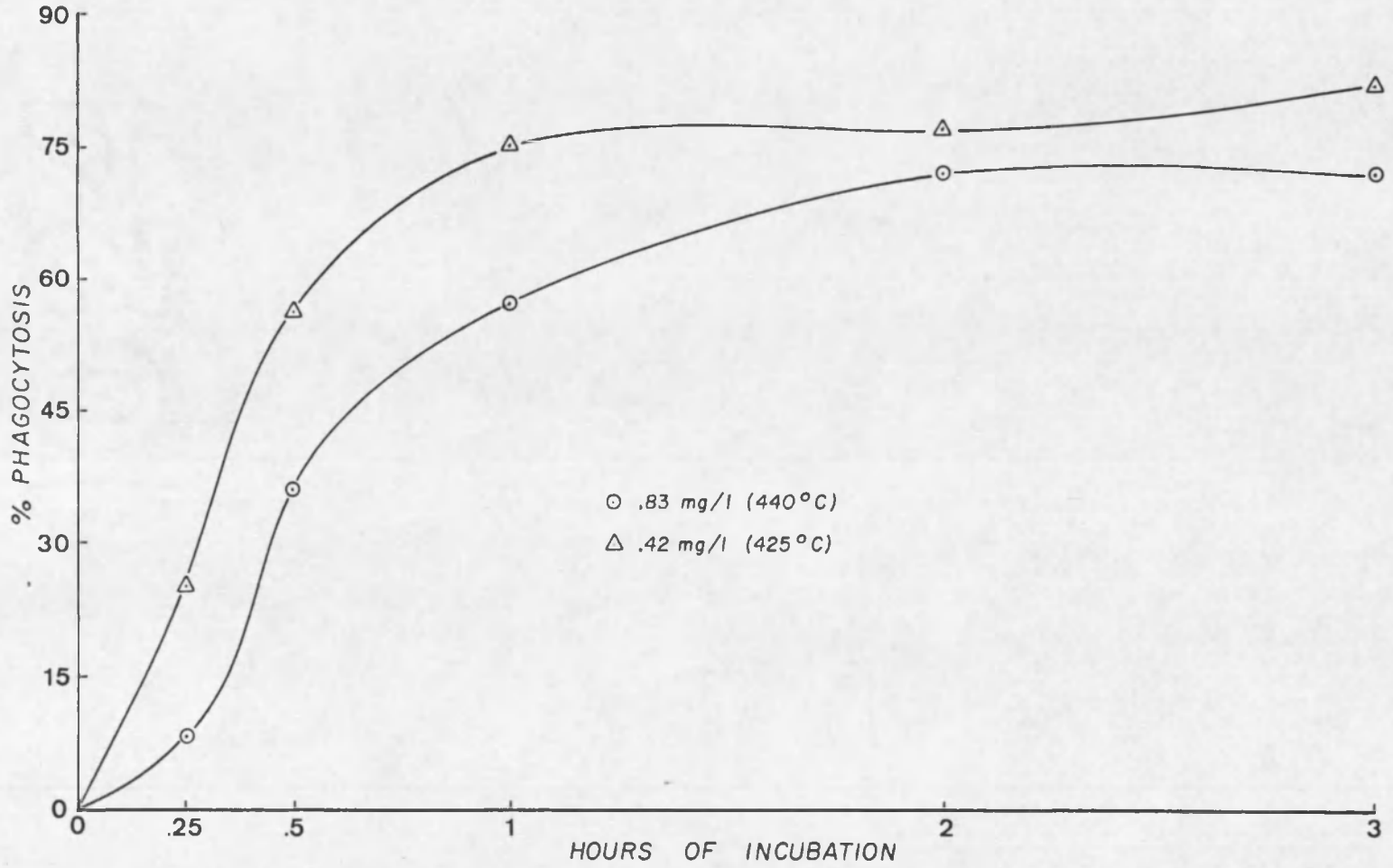

Figure 12. Phagocytic Activity of Lung Macrophages Taken from Rabbits Exposed for One Hour to PTFE Fumes of a Concentration Ranging from .4 to .9 mg/l

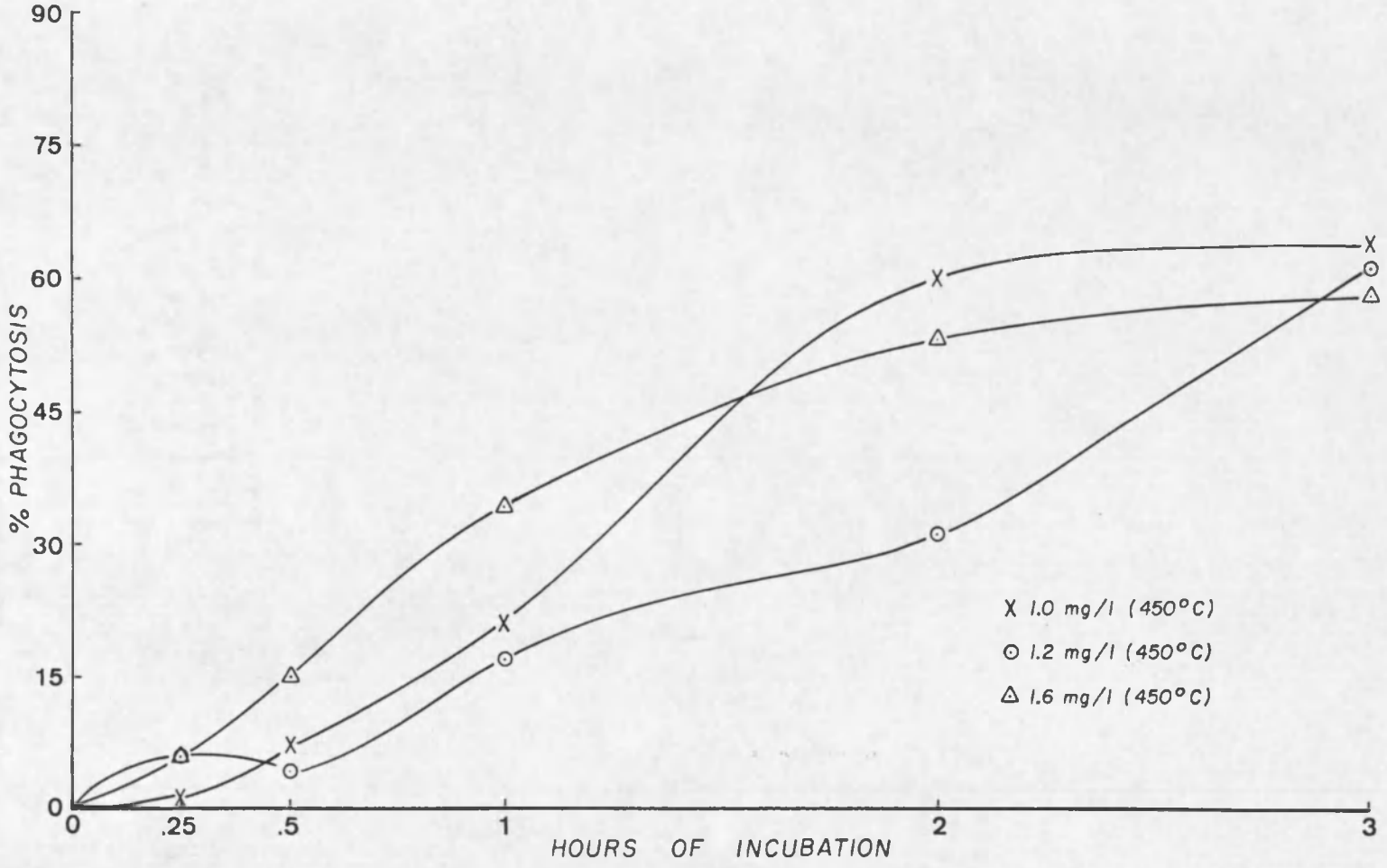

Figure 13. Phagocytic Activity of Lung Macrophages Taken from Rabbits Exposed for One Hour to PTFE Fumes of a Concentration Above 1.0 mg/l

after two hours, percent phagocytosis had leveled off or in some cases, decreased.

To determine if a dose-response effect existed, percent phagocytosis was plotted versus concentration for the incubation periods of .5 and 1 hour. The graphic representations of these data are presented in Figures 14 and 15.

Although a dose-response effect was not apparent there was a noticeable depression at concentrations above 1.0 mg/l. This effect was most noticeable at incubation times of .5 and 1 hour. At two hours, these higher exposure levels appeared to return to control values.

At concentrations below 1 mg/l, there appeared to be no significant difference between the phagocytic index of these values and controls.

As was expected, the concentration depended on the temperature of the pyrolysis oven. From the range of temperatures tested by this investigator (i.e., 350°C to 450°C) there was a positive correlation between temperature and chamber concentration. This is seen in Figures 14 and 15, which have the temperatures adjacent to the plotted points. As mentioned earlier (Figure 13), at a high temperature of 450°C, or high concentration above 1.0 mg/l, there appeared to be a reduction in the uptake of particles by the macrophage in the first two hours. The temperature where this effect was noticed approached the temperature (450°C) at which fumes were produced that caused partial lethality among

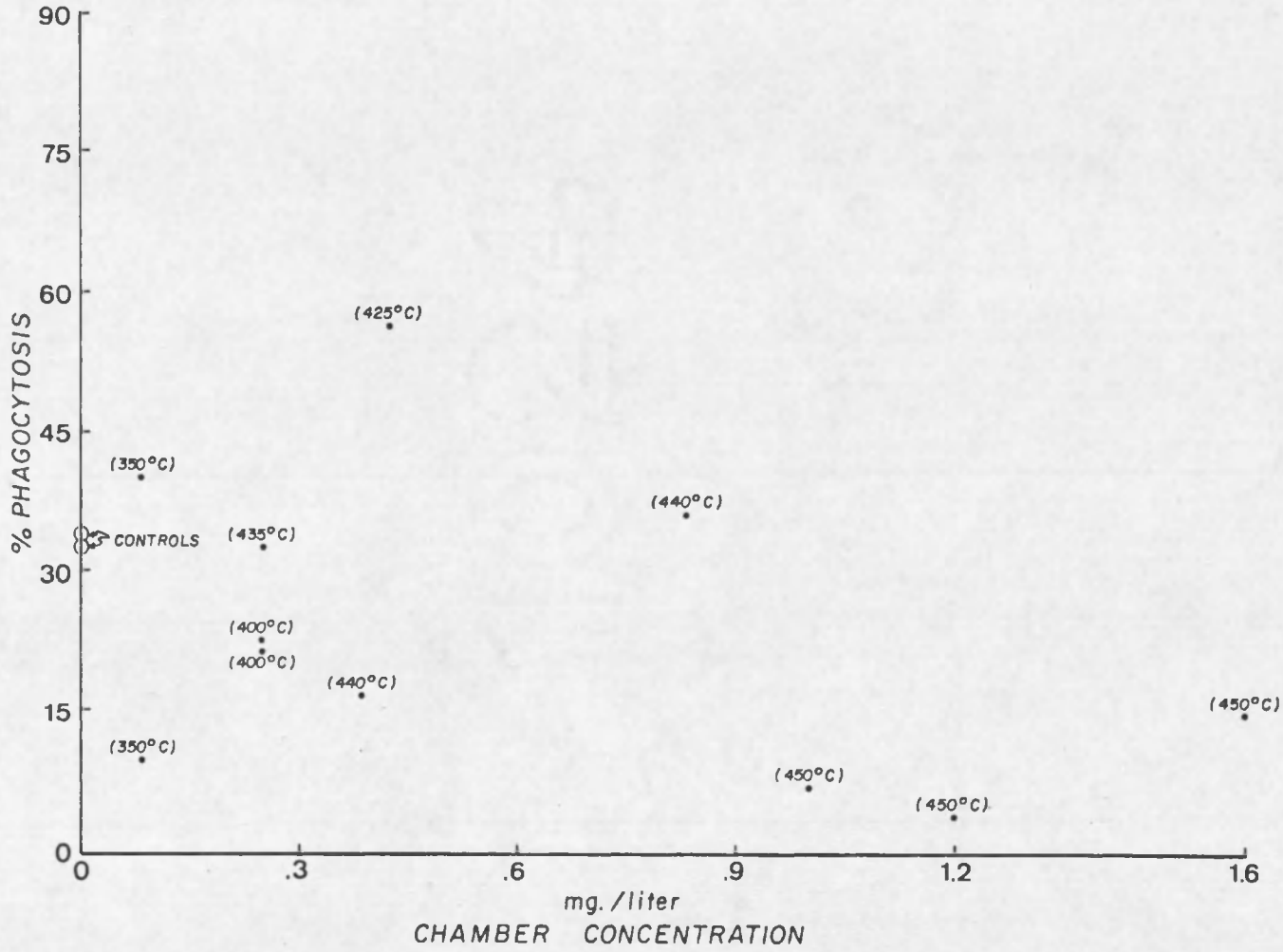

Figure 14. Chamber Concentration versus Phagocytic Activity of Macrophages Incubated with ^{85}Strontium Labeled Particles for .5 hour

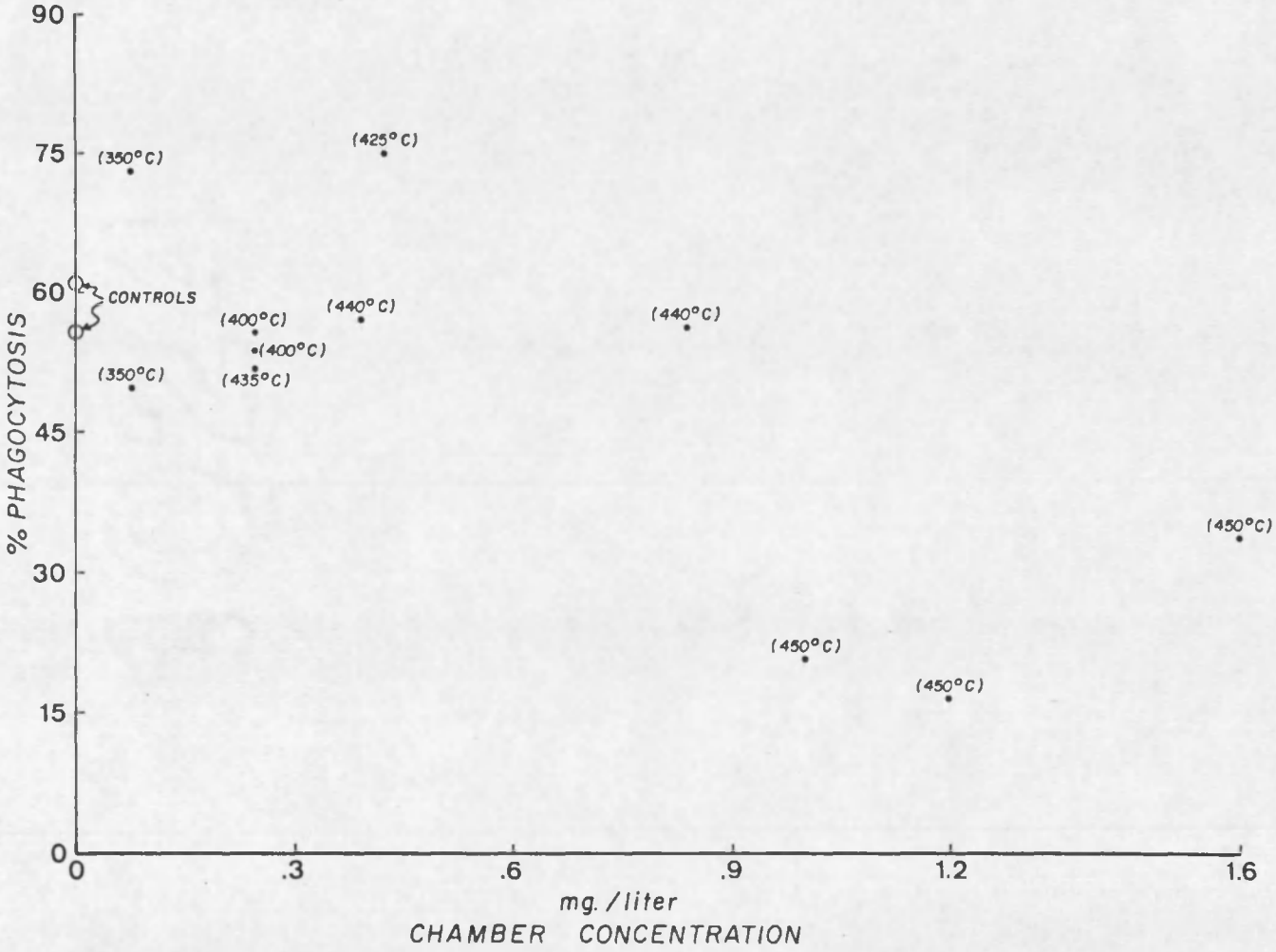

Figure 15. Chamber Concentration versus Phagocytic Activity of Macrophages Incubated with ^{85}Strontium Labeled Particles for 1 Hour

the exposed rabbits. At this temperature it was difficult to conduct the macrophage assay. After an exposure at 450°C, the rabbit usually had labored breathing. When the animal was sacrificed immediately after exposure, there was massive hemorrhage in the lung. Upon lavage of the lung, the red blood cell (RBC) concentration was overwhelming. Besides large amount of RBCs in the lavage fluid, there was a poor yield of macrophages. Since the macrophage yield was so low making it impossible to remove RBCs from the lavage fluid, the assay was not attempted. It was concluded that if the RBCs were lysed to remove them from the fluid, the cell debris would influence the ability of the macrophage to engulf particles for the assay. In addition, the yield of macrophages was too low to conduct the assay.

When it was determined that it was impossible to conduct an assay immediately following a 450°C exposure, the animals were allowed to recover for a short period after the exposure. Fumes from 450°C heated PTFE caused many animals to die within 24 hours of pulmonary edema and hemorrhage. Of the animals that survived the post exposure period, a macrophage assay was conducted after one week. Two animals that survived this period showed a slow initial uptake of particles which returned to control values after two or three hours of incubation time. The highest concentration where an assay was conducted (i.e., 1.6 mg/l) was completed after a three day post exposure period. Although there may

be a slight initial decrease in the phagocytic index, there was not a significant difference from controls.

Figure 16 represents those data and experiments where there was a one week post exposure period. This clearly demonstrates the slow uptake of particles at concentrations above 1.0 mg/l or temperatures of 450°C. This is a significant difference with a 95% confidence between the two sets of values at one hour incubation.

Other parameters measured during the macrophage assay were RBCs, differential cell count and total macrophage yield and viability. In those cases where the macrophage assay was conducted, RBC contamination in the lavage fluid did not correspond to temperature or chamber concentration of the exposure. Likewise, no trend was observed in the number of different cell types or viability of the macrophages. The large majority of cells in the lavage were macrophages which had a viability of 95 to 99%. Similarly, there appeared to be no trend in the macrophage yield. Total yield from four lavages ranged from 1.0×10^7 to 7.7×10^7 macrophages with no dose-response effect apparent.

Necropsy

In rabbit lungs there was no gross pathological change in those animals exposed to pyrolysis products of PTFE heated to 430°C and below. In rabbits exposed to fumes

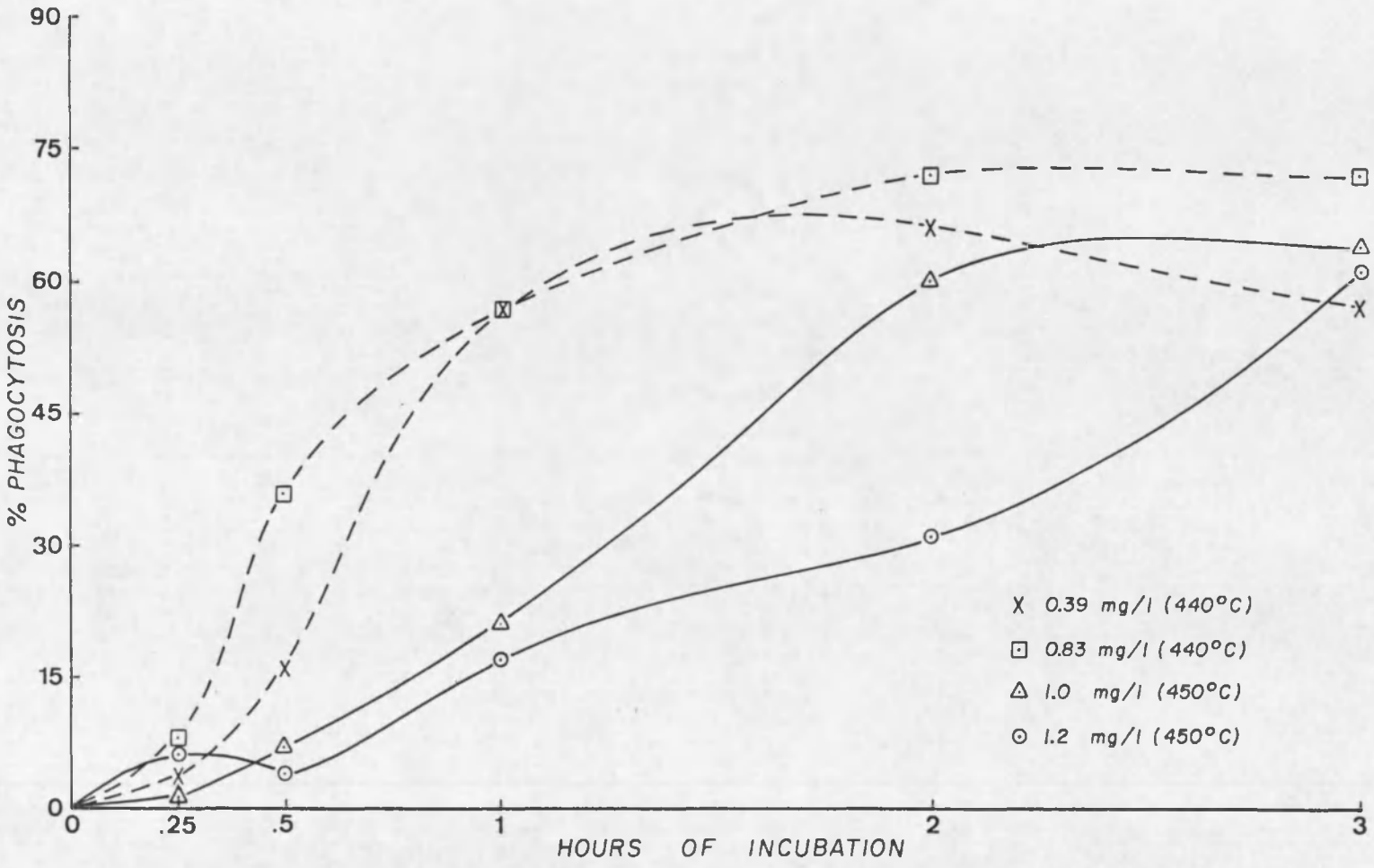

Figure 16. Comparison of Phagocytic Activity of Macrophages Taken from Rabbits One Week Post-exposure

of PTFE heated between 440°C and 450°C however, the lungs demonstrated severe tissue damage. Lung damage ranged from numerous petechiae to massive hemorrhage. When lungs of such animals were lavaged with saline, the resultant fluid was typically overwhelmed with red blood cells. At those higher temperatures, concentrations above 0.8 mg/l correlated with the pulmonary hemorrhage described above.

White rats of both Sprague-Dawley and Fischer 344 strains were also exposed to pyrolysis products of PTFE. White rats of both strains exhibited similar lung pathology as the rabbit. The petechial hemorrhage in the lung of rats was observed at a much lower temperature and concentration than in the rabbit. Toxic effects were first observed at temperatures of 375°C, while fumes produced at 400°C caused definite lung hemorrhage, and resulted in lethality. Lethal chamber concentration which corresponded to a one hour exposure to 400°C PTFE fumes, was approximately 0.1 mg/l. It appeared that rats were more susceptible to PTFE fumes than rabbits, with a tenth of the rabbit lethal chamber concentration (i.e., 1.0 mg/l) resulting in rat lethality (i.e., 0.1 mg/l).

Although Fischer 344 rats were not extensively tested in mortality studies, their susceptibility appeared to be similar or slightly less than that of Sprague-Dawley. Fischer 344 rats were exposed to demonstrate an effect of the concentrating ability of the kidney. The results of

these tests are explained in a later section; however, upon gross examination kidneys from these animals appeared to be normal.

The kidney and liver in both rats and rabbits appeared within normal limits after exposure to all concentrations resulting from heating PTFE between 350°C and 450°C.

Microscopic Pathology

Tissues from rats which died during a one hour exposure to PTFE fumes of a concentration of 2.3 mg/l showed mild emphysema and hemorrhage in the lung. The lungs also indicated mild hyperemia or congestion. Kidneys of these animals appeared to have been unaffected.

The main reason these microscopic examinations were conducted was not to observe the lethal tissue effects, but to observe the sub-lethal effects and recuperative abilities of the animal. Mild edema, congestion and inflammation were manifested in the lung of some animals exposed for three hours to sub-lethal concentrations of PTFE fumes of .03 mg/l. While the kidneys of some rats demonstrated slight congestion and proteinuria at three hour exposures of .05 mg/l PTFE fumes, there was no glomerular or renal tubular damage. These sub-lethal effects were insignificant in comparison with control tissues which also showed lesions similar in type and severity to exposed rats. This demonstrated the

animal's recuperative abilities and/or lack of deleterious effects from sub-lethal concentrations and temperatures.

Urinary Assays

Rats were placed in metabolism cages for several days before the exposure to stabilize the animals. Rats were removed from the metabolism cages for exposures and body weight measurement, then immediately returned to the cages. Water intake and urine output were measured every 24 hours for the pre and post exposure period. Average water intake per rat per 24 hours was 22.4 mls with a standard deviation of 2.7 mls. These data included control as well as exposed rats, indicating no significant difference in water intake between exposed (between .03 mg/l and .05 mg/l for a three hour period) and unexposed animals. Similarly, urine output did not significantly differ between control and exposed (same exposure as above) animals. During 24 hour periods, the mean urine output per rat was 6.17 mls, with a standard deviation of 0.9 mls.

From collected urine, solute and fluoride concentrations were also measured. The solute concentration was measured in milliosmoles per kilogram. Again, the same exposed and control animals showed no significant differences. The mean solute concentration was 2482 milliosmoles/kg with a standard deviation of 220. Likewise, urinary fluoride concentrations in all animals averaged 4.0 μM per rat per 24

hours, with a standard deviation of 0.5. If fluorides were being excreted, a rise in urinary concentration would have been expected within 24 to 48 hours post exposure (Wisemann 1970). However, no significant increase in urinary fluorides were observed in exposed animals.

After urine dialysis, a lactic dehydrogenase assay was also performed. This assay determined the amount of NADH present in the urine. NADH is absorbed at 340 nm, and is directly proportional to the amount of LDH. In this way, LDH activity was established for a three minute period. As mentioned in the Methods Section, NADH, Pyruvate and buffer were added to the dialyzed urine. The basis of this assay is shown in the reaction below:

$$\text{NADH} + \text{PYRUVATE} \xrightarrow[\text{LDH}]{} \text{LACTATE} + \text{NAD}^+$$

Therefore NADH, which is absorbed at 340 nm and a catalyst for LDH, was depleted in proportional amounts to LDH. When a change in absorbance per minute was measured, a relative LDH activity was demonstrated.

LDH activity, which was given in LDH units per rat per 24 hours, had a range of 218.2 to 664.2 LDH units. If renal tubular damage was present, an elevated LDH activity should have been apparent (Sigma Chemical Company 1977). However, the high value recorded (i.e., 664.2) was from control animals. There was no significant difference between

exposed and control animals. Since LDH activity is an indicator of renal tubular necrosis, it appeared that no such damage resulted.

CHAPTER 4

DISCUSSION

General Observations

The general observations cited by this investigator are similar to those reported by other researchers.

In regard to particulate evolution from heated PTFE, Lee et al. (1976) observed a white fume in the glass flow tube when the Teflon^{R} sample was heated to 550°C. At temperatures below 550°C no visible fume was observed, even though rat mortality resulted when the polymer was heated to 450°C for four hours (Lee et al. 1976). These data support my results in that there was never a visible smoke or particulate evolved at temperatures between 350°C and 450°C during my experimentation.

Besides the similarities between Lee et al. (1976) and I in the observation of a visible particulate, equivalent results in rat mortality were demonstrated when the pyrolysis stream was filtered. When a 0.2μ pore size filter was placed in the pyrolysis stream to filter the particulate a significant change resulted. When the polymer was heated to 450°C, a condition which normally produced fumes which were lethal to rats, the filter trapped the particulate and no rat deaths resulted. Filtering the air stream was also

conducted by Clayton et al. (1959), who similarly reported a reduction in mortality. By means of electron microscopy, Lee et al. (1976) sized the particulate evolved at 450°C to be in the range of 0.02 to 0.04μ in diameter.

These data confirm that the particulate is the toxic species when PTFE is heated to 450°C. When animals were subjected to fumes from PTFE heated to 450°C, it was shown that respiratory tract injury ensued which was specifically demonstrated by necrosis of the tracheobronchial epithelium and pulmonary edema, congestion and hemorrhage (Lee et al. 1976). My results correlated with Lee et al. (1976) in that rabbits exposed to fumes produced at 450°C suffered pulmonary hemorrhage and alveolar necrosis which was characterized pathologically as emphysema.

The pathology and general observation of those animals subjected to sublethal concentrations or temperatures of PTFE (i.e., no permanent effects), clearly attests to the thermal stability and low toxicity of the polymer.

In 1955, Zapp et al. reported that when PTFE was heated to 300°C, products lethal to rats were evolved. In 1976, Lee et al. reported that this same phenomenon resulted at 450°C. This comparison of data over the years is included to demonstrate that PTFE resin has changed. My results differ from Lee et al. in that I observed rat mortality when rats were exposed to fumes produced at 400°C, while Lee et al. (1976) showed partial mortality at 450°C.

However, generally our results correlated and perhaps discrepancies in observations could have been the result of differences in the PTFE resin, equipment or procedure.

Thermal Decomposition

From thermal gravimetric analysis (TGA) conducted in an air atmosphere by other investigators, weight losses occur between 500°C and 600°C (Barrow, Alarie and Stock 1978, Light, Fitzpatrick and Phaneuf 1965). My results indicate that when the initial results were corrected by standards, the PTFE samples were totally volatilized by 600°C. Differences in results in regard to TGA have been explained by other investigators (Daniels 1973, Paciorek et al. 1973). Daniels (1973) stated that there may be a lag between furnace temperature and true temperature by more than 30°C, depending on operating conditions and increasing with the rate of heating. Paciorek et al. (1973) indicated that ignition of the polymer is partially dependent on the amount of oxygen present. Therefore, differences in decomposition temperatures may be influenced by air flow, equipment or rate of heating. Similarly, different PTFE resins have been developed and produce toxicity at different temperatures (Waritz and Kwon 1968). These resins have a different thermal stability, so in addition to differences in procedure and equipment, samples may also be responsible for the slight discrepancies observed.

Besides observing the weight loss versus heating rate, the TGA equipment was utilized to determine sample weight loss when kept at a constant temperature. These results showed a 0.051%, 0.16% and 1.25% weight loss for one hour at 400°C, 425°C and 450°C. These results correlate with the toxic effects at each of these temperatures.

Phagocytic Index

The results showed that when rabbits were exposed to fumes of PTFE heated to 450°C, there was an initial decrease in the in-vitro uptake of particles by macrophages. The greatest difference in percent phagocytosis between macrophages from animals exposed to fumes of 450°C PTFE and control macrophages was shown by the macrophage assay at one hour incubation time. The initial slow uptake of particles by exposed macrophages returned to control values by the end of the incubation period (i.e., three hours), however, the initial inhibition of the macrophage phagocytosis may be more than just a temporary effect. Work done by Lockard and Kennedy (1976) indicated that macrophages from rabbits subjected to traumatic shock only slightly impaired bacterial ingestion. However, the cell's ability to destroy the ingested bacteria was significantly diminished. After rabbits were subjected to traumatic shock by spinning them 400 times at 30 RPM in a Noble-Collip drum, the animals were sacrificed and alveolar macrophages were collected in a

modified version of the method described by Myrvik et al. (1961). Results showed that macrophages from traumatized rabbits released increased amounts of lysosomal enzymes without intracellular replenishment of the depleated enzymes. Therefore, the enzyme released from the macrophage was not immediately replaced so the ingested bacteria were unaltered morphologically with no evidence of degradative changes as shown in photomicrographs by Lockard and Kennedy (1976).

In this regard, even though the trauma associated with my experiments was passive in comparison to the shock treatment of Lockard and Kennedy, unseen effects may have resulted. My exposures, besides showing an initial decrease in macrophage phagocytosis, may have also shown biochemical effects on the macrophage which would not have been demonstrated upon phagocytosis of inert particles.

In addition, the only percent phagocytosis deviation observed were macrophages from animals exposed to fumes of Teflon^{R} heated to 450°C (which is the temperature at which the polymer gives off products which cause partial lethality). This all or nothing effect observed may be due to the narrow range at which the particulate was evolved (Waritz and Kwon 1968). Since the sample size was kept relatively constant, the variable which determined concentration was the pyrolysis oven temperature. Thus, if the oven was kept at a constant temperature of 450°C and the sample size was allowed to fluctuate, then different

concentrations of the particulate formed at 450°C could have possibly demonstrated a dose-response relationship. However since the sample size was kept constant, the only indication of this dose-response relationship at 450°C was from the two experiments conducted at that temperature. The higher concentration at 450°C (i.e., 1.2 mg/l) showed a slight decrease in macrophage phagocytosis in comparison to the lower concentration (i.e., 1.0 mg/l).

I contend that at temperatures lower than 450°C the products evolved from heating PTFE are either chemically different from those formed at 450°C or are in insignificant quantities to elicit a response. The particulate as shown by Clayton (1962) and other investigators, is the toxic etiological agent at 450°C (Lee et al. 1976, Waritz and Kwon 1968). Therefore, one would not expect to see similarities between macrophages from animals exposed to a particulate and those exposed to other gases. In fact, since the primary purpose of the pulmonary alveolar macrophage is to clear particles and bacteria from the alveoli by means of ingestion, one would not expect to see an effect on these cells by gaseous exposure (Biggar, Buron and Holmes 1976, Green 1970, Ferin, Urbankova and Vlckova 1965, LaBelle and Briegar 1960). Unless these gases were in such high concentration that hypoxia interfered with oxidative phosphoylation which provides energy for phagocytosis, gases in small quantities should have no effect on macrophage phagocytosis

(Ouchi et al. 1965, Green and Kass 1964b, Oren et al. 1963). In fact, of all the gases tested by Weissbecker et al. (1969) only ozone (O_3) effected cell viability. In general, Thorpe and Marcus (1964) attested to the macrophage's hardiness by showing that they were still phagocytically active after 72 hours of in-vitro incubation.

Even though Tyler, McLaughlin and Canada (1967) suggested that other animals may be more suitable in inhalation studies to compare and extrapolate to the human effects, the pulmonary alveolar macrophage may be the preferred means of eliciting subtle effects which may not be demonstrated physically for months or even years. In addition, Brain et al. (1978) suggested that the macrophage may be a useful guide in the development of cellular pharmacology.

Interpretations of the significance of the results observed in this investigation are preliminary. However, with more research the alveolar macrophage could be a useful tool in toxicological or clinical evaluations.

Urinary Assay

Renal damage in mice observed by Lucia et al. (1978) following exposure to pyrolysis products of polytetrafluoroethylene demonstrated that the effect of these fumes are not necessarily isolated to the pulmonary area. Mazze et al. (1973) and Wisemann (1970) agree that inorganic fluoride can cause the abnormalities in the kidney's urine concentrating

ability and renal tubular damage observed by Lucia et al. (1978). At the temperature which Lucia et al. (1978) conducted these experiments (i.e., 550°C) the principle product was carbonyl fluoride (COF_2) (Scheel, Lane and Coleman 1968).

At the temperature range where my experiments were conducted (i.e., 350 to 450°C) COF_2 was not evolved at a significant concentration (Waritz and Kwon 1968). The main material was an acidic particulate at 450°C. My results confirm the absence of carbonyl fluoride at temperatures below 450°C because there was no apparent effect on the kidney's urine concentrating ability or renal tubules. Furthermore, there was no evidence of an increase in urinary fluoride or lactic dehydrogenase.

Comparison of Animal Models

From the results of my study it cannot be clearly concluded that the rabbit is more resistant to the pyrolysis products of PTFE than the rat. Even though the chamber concentration which caused lethality in rabbits was 1.0 mg/l and with rats 0.1 mg/l, there is also a ten-fold difference in weight between the rat and rabbit. Therefore, the dose received in mg per kg of body weight would be equivalent. A theory of equal susceptibility presumes that the breathing rates and the material inhaled are similar. However as stated earlier, PTFE gives off different products at different temperatures (i.e., 400°C -- Hydrolyzable fluoride and

450°C -- Acidic Particulate) (Waritz and Kwon 1968). The only way an accurate comparison could be attempted would be to keep a constant temperature and vary the size of the heated sample.

Both rabbits and rats exhibited similar toxic signs of respiratory irritation and initial weight losses in the first 24 hours post exposure.

In addition, pathology indicated lung damage in both rats and rabbits exposed to lethal concentrations of PTFE fumes.

In comparing the two strains of rats, Sprague-Dawley and Fischer 344, no significant differences were observed from the experiments conducted by this investigator.

Differences in species' susceptibility in regards to PTFE fume inhalation is still unclear. When PTFE is heated between 350°C and 450°C and the dose is kept constant there may be no difference. Clayton (1967) exposed rats, mice, guinea pigs, rabbits, and dogs to fumes of PTFE heated to 300°C. He observed an effect only among the rats (i.e., slight deep respiration) and concluded that the hazard of breathing fumes of that temperature was negligible.

The biggest difference in effect among animals exposed to PTFE fumes is between man and all other mammals. Man is the only mammal which demonstrates the symptoms of polymer fume fever (Milby et al. 1968). Until the

uniqueness of this symptom is explained, no one can definitely determine the effect on man of breathing PTFE fumes.

Summary

In summary, macrophages from rabbits exposed to the particulate evolved from PTFE heated to 450°C caused an initial decrease in the ability of the cell to ingest particles. At temperatures below 450°C there was no effect on macrophage phagocytosis or lung morphology. Rats exposed to fumes from PTFE heated below 450°C demonstrated no adverse kidney effects. In conclusion, the particulate evolved from PTFE at 450°C may inhibit macrophage phagocytosis in rabbits.

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