PULMONARY SURFACTANT INTERACTION WITH RESPIRABLE DUST

By
W.E. Wallace, Jr., M.J. Keane, V. Vallyathan, T-M. Ong, V. Castranova
National Institute for Occupational Safety and Health
944 Chestnut Ridge Road
Morgantown, WV

Inhalation of certain forms of silica, asbestos and some other respirable dusts can result in pulmonary fibrosis, characterized by destruction of the surfaces of alveoli and respiratory bronchioles. Fibrous thickening of the alveolar septa decreases the lung's capacity for gas exchange.

Pulmonary macrophage damage by respired dust may initiate the fibrotic response. In one proposed mechanism, dust particles interact with the plasma membrane of a macrophage. This damages the cell or induces some hyperactivity of the cell, resulting in excessive release of lysosomal enzymes and reactive forms of oxygen (1). These reactive enzymes or radicals then damage the alveolar surface epithelial cells. A second mechanism has also been proposed (2,3) in which dust damage to the macrophage causes the release of mediator substances which induce pulmonary fibroblast cell proliferation and increased collagen synthesis. This produces fibrous tissue in the terminal air spaces (4,5).

Mineral surface interactions with plasma membrane lipoproteins have been proposed as a mechanism of lytic damage of pulmonary cells by silica dust in numerous models (6,7). The significance of dust surface properties has been further revealed in studies showing significant changes in dust induced cytotoxicity as a function of dust surface crystallinity and organic or inorganic contamination or coating of mineral surfaces (8,9). Such modification of the mineral surface might interfere with surface sites responsible for the toxicity of dusts for pulmonary macrophages, thus interrupting the early stages of the process of fibrosis.

In vitro cellular assay systems using erythrocyte hemolysis or the release of macrophage cytosolic or lysosomal enzymes following dust challenge, have been used to analyze the initial mechanism of dust damage to cells. However the ability of these assays to predict the pulmonary disease producing potential of various dusts is imperfect owing to some "false positive" results. These assays as performed are therefore questionable models for the initial lesion in pneumoconiosis or silicosis. In particular, kaolin frequently is found to have comparable biological activity to silica quartz in these assays (10,11).

Silica and kaolin have distinctly different fibrogenic potentials. Silica is highly fibrogenic, resulting in both acute and chronic silicosis (12). Some epidemiological data and animal experimentation data have indicated that long term exposure to kaolin can result in pneumoconiosis; however, kaolin is relatively benign as compared to silica (13-18). Kaolin is also of interest because it is one of the major mineral inclusions in Eastern U.S. bituminous coals. We have observed that exposing respirable sized native silica or kaolin for two hours to pulmonary macrophages results in a greater enzyme release for kaolin exposures, on a dust mass basis. Exposure of erythrocytes to the dusts also results in greater levels of hemolysis by kaolin for equal mass respirable dust doses. Therefore, these results do not correlate positively with known disease inducing potentials of the two dusts.

Characterization of physical and chemical surface properties of dusts involved in occupational exposures, and characterization of the alteration of those surface properties which are likely to occur upon deposition of the dust in the lung, should improve the identification of respiratory disease hazards.

Inhaled dust particles deposited in the lower respiratory tract will come in contact with pulmonary surfactant. This surfactant forms a surface film on and emulsion in the liquid hypophase coating of the alveoli and respiratory bronchioles. The primary constituent of this pulmonary surfactant is the lipid diacyl glycerophosphorylcholine, lecithin (19). Because this lipid is also a major component of cell membranes, it is possible that surfactant can interact with mineral

dust to alter its interaction with macrophages and other pulmonary cells and, thus, its pathogenicity.

Adsorption of dipalmitoyl lecithin from emulsion in physiological saline by kaolin, a layered alumino-silicate, does occur. (20) In addition, silica also exhibits the capacity to absorb surfactant-like material.(21)

Our earlier research quantified the adsorption of dipalmitoyl lecithin emulsion in physiological saline at 37°C by a kaolin respirable sized dust of 26 m^2/g specific surface area. In the concentrated emulsion range, up to 18 weight % lecithin to kaolin was adsorbed.

It has been suggested that such adsorption of lipids may alter the cytotoxicity of dusts (22,23,24). We have been studying this question, using dipalmitoyl lecithin in physiological saline to pretreat dusts to model the initial contact of respired dust with pulmonary surfactant. First, we monitored cytotoxicity using two assay systems. The release of three enzymes from pulmonary macrophages were used as indicators of cell death or damage following dust exposure in vitro. Lactate dehydrogenase (LDH) and two lysosomal enzymes, beta-glucuronidase (β-GLUC) and beta-N-acetyl glucosaminidase (β-NAG) , were measured in the external cell medium following dust exposure and compared with total intracellular levels for unexposed controls. Second, hemolysis of erythrocytes was used as a specific indicator for external cell membrane lysis by dusts. These assays were used with native silica and kaolin dusts in contrast to experiments using lecithin treated silica and kaolin.

We present here some representative data obtained in studies of silica and kaolin respirable sized dusts. Complete data will be reported elsewhere.

Crystalline silica (Min-U-Sil) was fractionated using a Donaldson classifier and the 80% < 5 μm particle diameter fraction collected. The silica was at least 98.5% pure as determined by x-ray energy spectrometric (XES) analysis. It had a median area equivalent diameter of 1.24 μm. The specific surface area of the size fractionated silica was 2.97 m^2/g as determined by BET Nitrogen adsorption.

Kaolin from Georgia Kaolin Mills was similarly size fractionated to obtain a fraction 90% < 5 μm particle diameter. This kaolin was at least 96% pure and contained no crystalline silica as determined by XES analysis. The specific surface area of this kaolin fraction was 13.25 m^2/g, as determined by BET nitrogen adsorption.

Stock dipalmitoyl lecithin (DPL) emulsions of 10 mg of DPL (Calbiochem) per ml of physiological (0.165M) saline were made by sonication. Silica and kaolin dusts were exposed to DPL by vortexing the sized, untreated dusts into DPL emulsion at 7.5 mg dust per ml emulsion and incubating the mixtures for one hour at 37°C. This provided ample DPL for kaolin surface coverage as estimated from the adsorption isotherm data extrapolated to the surface area of the kaolin used. Silica and kaolin controls were similarly incubated in physiological saline without DPL. Following incubation the mixtures were centrifuged for ten minutes at 990 xg and each dust resuspended in Dulbecco's phosphate buffered saline (PBS); this procedure was performed twice. The stock suspension of 2 mg dust per ml PBS was diluted to make sample dust suspensions used. Dust suspension and DPL emulsification did not change the osmolarity of 296 ± 1 mOsm or the pH of 7.3 of the saline system.

As one index of cytotoxicity, hemolysis by the native and treated dusts was measured. Sheep blood erythrocytes were prepared as a 4% by volume suspension in PBS after three washes in PBS with centrifugation at 990 xg. Aliquots of this suspension and the dust suspensions were mixed in equal volumes to make samples of 2% by volume erythrocytes with treated or native dust concentrations from 0.1 to 1.0 mg/ml. Hemolysis assays were performed using the method of Harrington et al. (25), with minor changes. Native silica or kaolin and DPL emulsion-treated silica or kaolin suspensions with erythrocytes were incubated at 37°C for one hour, and then centrifuged at 990 xg for ten minutes. Negative controls contained only erythrocytes in PBS. Standard lysate controls were made by lysing erythrocytes in PBS with 0.5% Triton X-100. All samples were read at 540 nm on a spectrophotometer against distilled H_2O.

Alveolar macrophage enzyme release studies were carried out using alveolar macrophages harvested from male Sprague Dawley rats weighing 250-275 grams. Following sacrifice,

lungs were lavaged repetitively (10-12 times) with calcium and magnesium free Hank's balanced salt solution. Macrophages were sedimented by centrifuation at 990 xg from the pooled lavages and suspended in HEPES buffer at pH 7.4. Cell counts were made by trypan blue dye exclusion test(26). From 85 to 90 percent of the cells counted were viable, based on this test. Approximately 95% of the cells obtained by lavage were alveolar macrophages.

Suspensions of native or DPL-treated silica or kaolin were mixed with the macrophage suspension to produce 2 x 10^6 cells per ml of suspension and dust concentrations of 1 mg per ml of suspension. All samples were incubated for 2 hours at 37°C in a shaking water bath. Following incubation all samples were centrifuged at 500 xg for 10 minutes and total and released activities of 3 enzymes were determined in duplicate tests. For estimation of total enzyme released, one set of controls of cells without dust were lysed with 0.2% Triton X-100 at the end of incubation. LDH activity was determined according to the method of Reeves and Fimignari (27). ß-GLUC activity was measured using p-nitrophenyl--D-glucuronide as the substrate, according to the method of Lockart and Kenedy (28). ß-NAG was assayed according to the method of Sellinger et al. (29). Percentages of enzymes released were calculated relative to the Triton lysed samples.

Representative hemolysis data shown in Figure 1 indicate that the silica and kaolin have comparable cytotoxicities for erythrocyte hemolysis on a dust mass or specific surface area basis. However, the cytotoxicity of both silica and kaolin is almost completely suppressed by incubation of the dust with lecithin emulsion in physiological saline.

Representative data on the release of three enzymes from pulmonary macrophages are shown in Figure 2. Both dusts show comparable cytotoxicity for pulmonary macrophages as indicated by comparable macrophage release of the three enzymes. Once again, lecithin pretreatment reduces the macrophage cytotoxicity, for these two hour incubations of treated dust with the macrophages, to background levels.

Suppression of the toxicities of both dusts by lecithin pretreatment in these assays may represent a prompt *in vivo* physiological response. No fibrosis occurs in the early stages of silica exposure. To the extent that such lecithin treatment is a representative model of one interaction of respired dusts *in vivo*, the assay results imply that the pulmonary surfactant system may constitute a defense system against prompt lytic damage to pulmonary cells by respired dusts. This is not inconsistent with the *in vivo* phenomena of lipidosis, an early response of the lung to silica exposure, in which alveolar type II cells release greater than normal amounts of pulmonary surfactant (30).

The comparable short-term suppression of the cytotoxicity of both dusts by lecithin does not correlate with known relative dust pathogenicity since silica quartz is highly fibrogenic. If relative pathogenicities of silica and other silicates originate in dust damage of the macrophage, then, that damage must manifest itself over a longer time period, that is, after phagocytosis of the dust by pulmonary macrophages. If a dust particle does not promptly lyse and kill the macrophage it will be phagocytized and taken into a phagolysosome. There it will be exposed to lysosomal enzymes, among which are lipases capable of digesting lipids. Pulmonary macrophage phagolysosomal digestion of serum protein from the surface of phagocytized silica has been reported (31).

We are investigating a research premise that after phagocytosis and formation of the secondary lysosome, the protective surfactant surface coating on silica may be enzymatically digested, re-toxifying the dust within the macrophage. As a corrollary, such processes may be quantitatively or qualitatively different for dusts which are not so pathogenic as silica *in vivo*, i.e., kaolin. As a first step we are studying the *in vitro* ability of a specific lipid active enzyme, phospholipase A_2, known to be present in macrophage lysosomes, to affect a lecithin coating on silica quartz, and on kaolin.

Representative data for the restoration of cytotoxicity of lecithin treated silica and kaolin dusts by phospholipase A_2 treatment are shown in Figure 3; the assay used was erythrocyte hemolysis. Commercial phospholipase A_2, obtained from *Crotalus adamanteus* venom, was dissolved in PBS supplemented with 1 mM $CaCl_2$, and made up to 2 mg/ml, equivalent to 400 units/ml. This solution was diluted 1:4 and 1:400. Enzyme solutions were kept on ice until used.

As additional controls, dusts which had not been lecithin treated, were subjected to phospholipase A_2 treatment.

Silica and Kaolin dusts were first incubated with dipalmitoyl lecithin for 15 hours, following the same procedure as discussed above. Then they were washed and incubated *in vitro* with phospholipase A_2 for one hour at 37° with lipase concentrations adequate to provide enzyme activities ranging from 2 unit-minutes to 44000 unit-minutes. The high activity point in Figure 3 is an exception to this; incubation time was 22 hours using the same enzyme concentration used for the 2000 unit-minute point. Dusts were then incubated with erythrocytes for one hour. Representative data are shown for silica in Figure 3.

The Figure 3 ordinate shows the phospholipase A_2 restored cytotoxicity of lecithin treated silica or kaolin as a precentage of native (non-lecithin treated) silica or kaolin cytotoxicity. The abscissa shows the activity of phospholipase A_2 used in the one hour incubations. The final point is the "theoretical equivalent" activity of the 22 hour incubation.

The data indicate that treatment with phospholipase A_2 can restore the cytotoxicity of lecithin pre-treated silica to levels approximately equal to native or bare-surface silica levels, shown by the "100% retoxification" level. This implies that the lecithin component of a pulmonary surfactant defense system against the lytic effects of respired particles, may be undone by the pulmonary macrophage phagocytic defense system against bacterial challenge. Similar studies using full pulmonary surfactant and pulmonary macrophage lysosomal enzymes are required to further investigate a research premise, that the deleterious effects of respired silica begin after phagocytosis when the protective surfactant coating is digested and desorbed by lysosomal enzyme activity.

The results for kaolin retoxification are qualitatively different from the silica results, as shown in Figure 3.

Using the lecithin-kaolin adsorption isotherm data extrapolated by specific surface area to the kaolin used here, and using the homogenous phase enzymatic activity of the phospholipase A_2 used in these studies, the phospholipase activity between 20 unit-minutes and 200 unit-minutes is the approximate activity in homogeneous phase reaction needed to digest the amount of lecithin coating the kaolin. Figure 3 shows that in this activity region the restored hemolytic cytotoxicity goes from near the totally suppressed levels for lecithin coated kaolin to levels greater than the cytotoxicities of native (non-lecithin treated) kaolin. The restored cytotoxicity, therefore, may not be due to digestion and removal of the lecithin from the kaolin. That is, unlike the case with silica, the phospholipase treatment may not bare the kaolin surface. Treating kaolin with lysolecithin and using this dust in erythrocyte hemolysis assays results in cytotoxicity levels comparable to the lecithin-phospholipase treated kaolin levels. Treating silica with lysolecithin results in hemolysis assay results comparable to those using lecithin-phospholipase treated silica.

Further research is underway to determine if lysolecithin, with palmitic acid the product of phospholipase A_2 digestion of dipalmitoyl lecithin, remains bound to the kaolin surface following phospholipase treatment of lecithin coated kaolin, and if phospholipase stays associated with the kaolin surface. Preliminary thin layer chromatographic analyses of chloroform elution of the lecithin and phospholipase treated kaolin show that lysolecithin is associated with the kaolin surface after phospholipase digestion.

These preliminary studies indicate a quantitative and qualitative difference in the restoration of hemolytic potency of lecithin treated silica and kaolin following phospholipase digestion *in vitro*. The results indicate that the lecithin coating on silica may be digested and desorbed by lipase, restoring silica toxicity. Further research using mixed component pulmonary surfactant in longer term macrophage exposures and studies using full macrophage lysosomal enzyme treatment of full surfactant treated dusts is required to determine if similar digestion and desorption within macrophage phagolysosomes might occur to retoxify silica within the macrophage.The lecithin protective coating on kaolin may be partially hydrolyzed but may be removed incompletely; and therefore kaolin may not be restored to its original toxicity during phagocytosis. This

may correlate with the different pathogenicities of silica and kaolin, if kaolin coated with lecithin and lysolecithin or other digestion products is not lytic to the interior phagolysosomal membrane.

These results provide the following research premise which is now under investigation: a) pulmonary surfactant coats silica and aluminosilicate and protects the lung against immediate lytic damage by the dusts; b) after phagocytosis of the dusts by macrophages the lysosomal enzymes digest the surfactant coating; c) this bares the surface of silica which then lyses the lysosomal membrane and thus damages or kills the macrophage; d) some surfactant and products of surfactant digestion by lysosomal enzymes, which are not toxic within the interior of the lysosome, stay bound to the surface of kaolin and continue to suppress its toxicity.

While surfactant may provide a natural defense system to minimize the prompt toxicity of inhaled particles, the emulsifying properties of surfactant may also enhance the toxicity of particles which are coated with or consist of organic compounds, by assisting the in vivo solubilization of these toxic organics. (32-34) The solubilizing effect of pulmonary surfactant may affect the release of toxic materials from respired particles into the alveolar hypophase, the clearance of those solubilized toxicants, and the partitioning of toxicants into alveolar cells. In this area current NIOSH research is directed to studies of the pulmonary surfactant extraction (a simulated in vivo process) of mutagenic materials from respirable diesel exhaust particulate. As measured by Ames assay for reverse mutation in Salmonella typhimurium (35), extraction of mutagenic materials does occur and results in some cases, in slightly greater mutagenic activity than that of standard organic (dichloromethane) extraction products.

Figure 4 shows representative results of S. typhimurium TA98 histidine reversion mutagenicity assays for a diesel exhaust soot extract. Dichloromethane, physiological saline, and lecithin in physiological saline were used in equal liquid volume to extract the soot. Samples of each of the three extracts were applied to the tester strain, pre-incubated for 90 minutes at 37° C and then plated. Plates were incubated for 48 hours at 37° C. Revertant colonies were counted on an electronic colony counter (ARTEK). As shown, the lecithin extract resulted in somewhat higher activity than the organic solvent.

Results indicate that the surface properties of respirable particles and their interaction with pulmonary surfactant are important considerations for predicting the eventual toxicities of particles to the lung.

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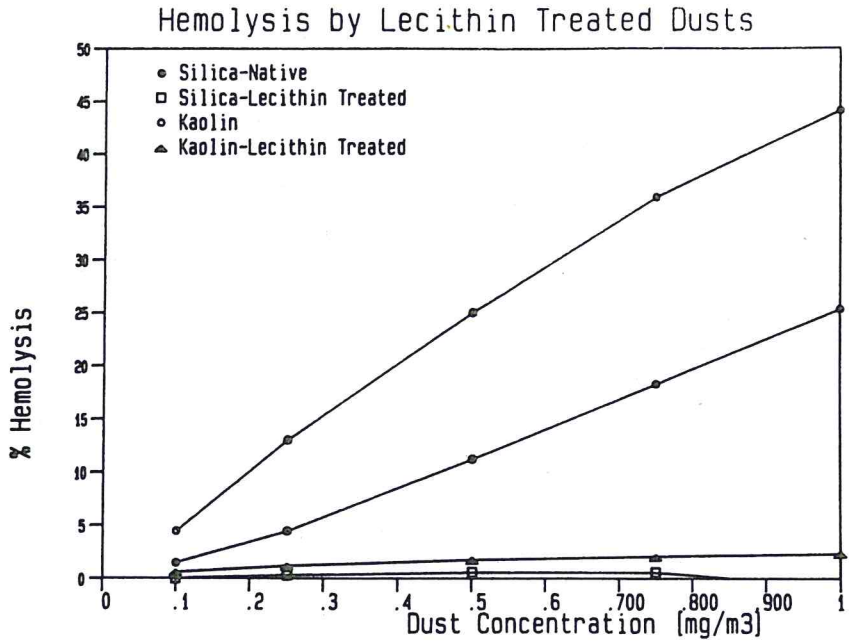

Figure 1 Erythrocyte Hemolysis by Native and Dipalmitoyl Lecithin Treated Silica and Kaolin Dusts

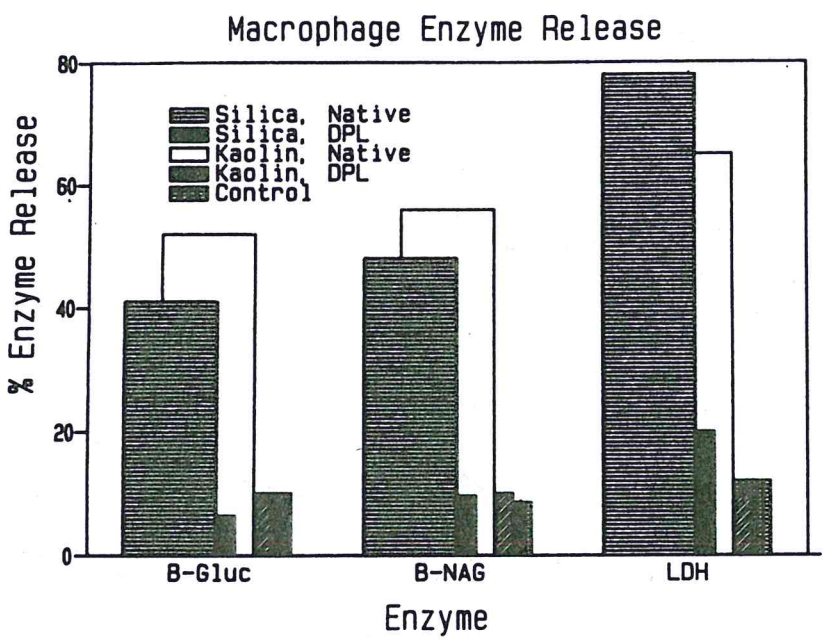

Figure 2 Murine Pulmonary Macrophage Release of β glucuronidase, β-N-acetyl glucosaminidase, and Lactate Dehydrogenase following Exposure to Native and Dipalmitoyl Lecithin Treated Silica and Kaolin dusts

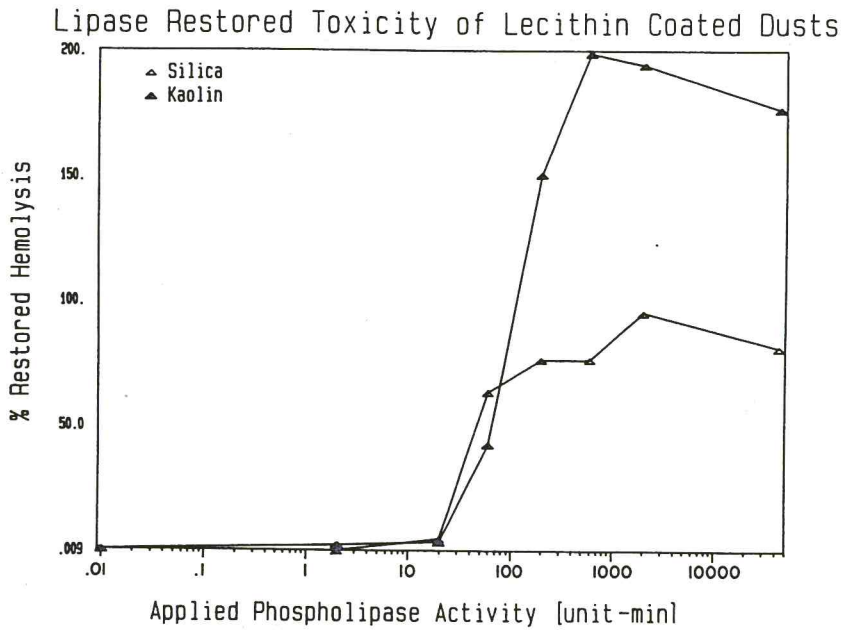

Figure 3 Cytotoxicity (Erythrocyte Hemolysis) of Dipalmitoyl Lecithin Pretreated Silica and Kaolin Dusts Following Incubation with Phospholipase A_2

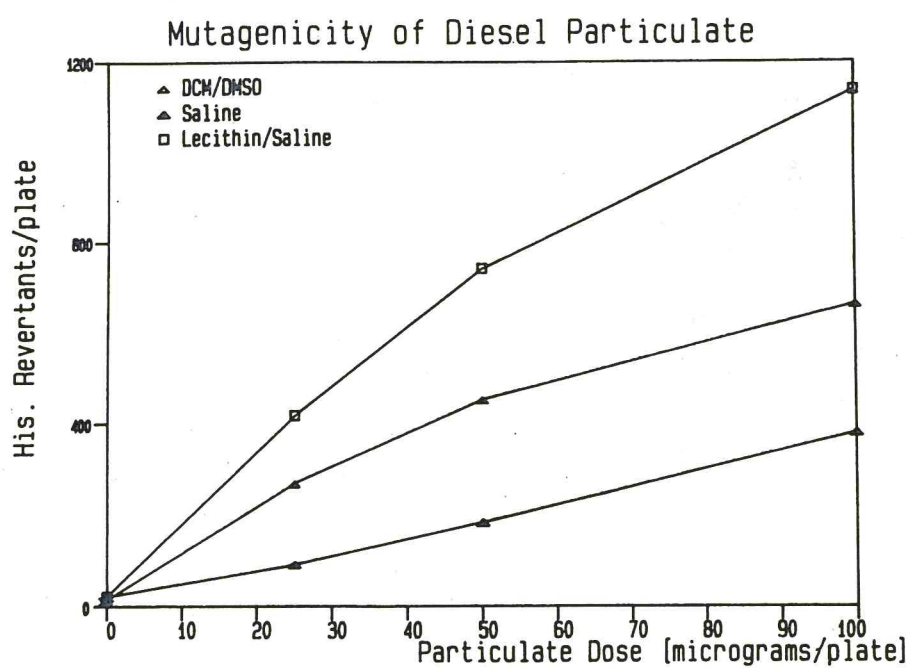

Figure 4 Mutagenicity of Diesel Soot Particulates Extracted or Dispersed in Dichloromethane/ DMSO, Physiological Saline, and Dipalmitoyl Lecithin in Physiological Saline

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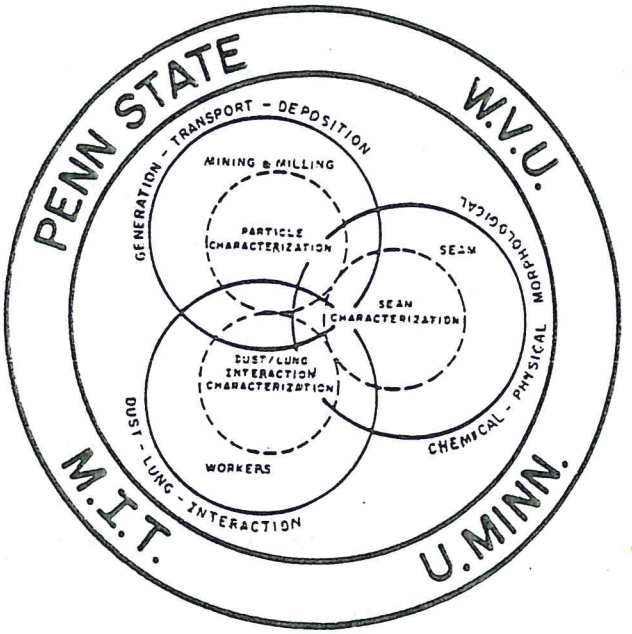

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edited by

SYD S. PENG

Professor and Chairman

Department of Mining Engineering

College of Mineral and Energy Resources

West Virginia University

Morgantown, WV 26506