EFFECT OF THERMAL TREATMENT ON THE SURFACE CHARACTERISTICS AND HEMOLYTIC ACTIVITY OF RESPIRABLE SIZE SILICA PARTICLES

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ABSTRACT

Thermal and chemical treatment of respirable size silica dusts samples is shown to induce marked changes in their hemolytic activity. The cytotoxicity of crystalline α-quartz (Min-U-Sil), and fumed silica (Cab-O-Sil) particles, as measured by a hemolytic activity protocol, is decreased by calcination and can be related to the dehydroxylation of the surface. The hemolytic activity of β-cristobalite particles of respirable dust size was also determined and found to be lower than that of α-quartz. The change in the surface structure resulting from thermal treatment is detectable by photo-acoustic infrared spectroscopy and zeta-potential measurements. The absorption band in the 3200–4000 cm^{-1} frequency region of both Cab-O-Sil and Min-U-Sil disappears upon heat treatment while a sharp band, identified with single silanol groups, at 3750 cm^{-1} increases in intensity. The zeta potential-pH profile, in the pH range of 4.0–7.0, of the calcined, siloxane surfaced particles is more negative than that of material with a silanol surface.

The cytotoxicity of the crystalline and fumed silica dusts was also found to be strongly dependent on particle size. Fumed silica of large surface area (small particle size) exhibits an initial increase in hemolytic activity upon calcination. This result confirms other experimental observations pointing to a particle size of maximum toxicity.

INTRODUCTION

The toxicity of silica and other mineral particles, as manifested by their role in inducing pneumoconiosis, fibrosis, silicosis and other pulmonary disorders is largely traceable to the characteristics of particle surfaces and to particle morphology. The great wealth of data on the physical and chemical properties of silica is still insufficient for a comprehensive understanding of the specific parameters to be associated with fibrogenic activity and, correspondingly, with the optimum means for characterizing and quantifying the toxicity of dusts, and the design of possible preventive or therapeutic methods.

The great diversity of parameters that have been proposed and tested for correlation with cytotoxicity suggest that more than one mechanism may be involved in the fibrotic activity of silica particles. The crystalline structure of the material (more active tridymite versus less active crisobalite and passive stishovite), freshly formed surfaces and free radicals associated therewith, silicic acid adsorbed onto silica surfaces, the concentration of hydroxyl groups at silica surfaces, particle size and morphology, among other properties, have been investigated and found to correlate in tests of cytotoxicity.

The main thrust of research presented here is to seek more definitive correlations of silica particle properties with cytotoxic activity to identify the mechanism or mechanisms leading to cytotoxic activity and the most suitable instrumental methods for identifying the material properties associated with silicosis and other pathogenic properties of respirable dusts. In the present paper are presented results on the effect of dehydration and dehydroxylation of crystalline and amorphous silica particles on their cytotoxicity as measured by hemolytic activity. One of the prevalent theories on the mechanism of silicosis presumes that such activity is induced by "clean surfaces of crystalline silica, usually quartz,"[1] hence it is deemed of interest to assess the cytotoxic potential of the most common structures of such surfaces.

The main feature of normal, clean quartz surfaces is the degree of surface hydration. The normal, anhydrous surface terminates in -Si-O-Si- (siloxane) groups. Equilibration with water will first result in the formation of —Si—O—H (silanol) groups and, eventually, the physisorption of water onto the silanol groups.[1] Particularly well formed and clean surfaces of quartz may adsorb more than four molecular layers of water.[2] Another type of "clean" quartz surface, but not included in the present study, is that resulting from the fresh cleavage of quartz crystals. Such surface may present broken bonds in the form of free radicals which may exhibit particularly high chemical reactivity.[3] In the present study we compare the cytotoxic activities of quartz and amorphous silica dusts dehydroxylated at different temperatures and re-equilibrated with the atmosphere over prolonged time periods, thus comparing the relative cytotoxicities of surfaces of crystalline and amorphous silica particles covered by adsorbed water, silanol, and/or siloxane groups.

EXPERIMENTAL METHOD

Materials: The materials used in this study were Min-U-Sil crystalline silica dust from two different batches obtained from U.S. Silica Inc. of Pittsburgh, PA, Cab-O-Sil, a fumed silica dust provided by the CABOT Corporation, and respirable size β-cristobalite dust, a standard reference material of National Bureau of Standards. X-ray diffraction analysis showed Min-U-Sil to be essentially pure α-quartz, while the Cab-O-Sil dust exhibited no detectable crystallinity. The Min-U-Sil particle size ranged from 0.4 to 10.0 microns with 98.2% of the particles below 4.7 μm and 85.2% below 1.1 μm; its BET specific surface area was determined as 5.2 m^2/g. The β-cristobalite particle size was in the 2-5 μm range with a specific surface area of 2.5 m^2/g. The Cab-O-Sil material consists of 2 to 40 μm diameter aggregates formed by primary particles of 0.1 to 0.2 μm diameter. The BET specific surface of the M-5 grade Cab-O-Sil used in most of the experiments reported was measured to be 195.4 m^2/g. Other grades of Cab-O-Sil tested had specific surface areas of 100, 255, and 380 m^2/g. The thermal treatment of the materials were conducted in air at temperatures ranging between 100 C and 1095 C for time periods of 48 and 72 hours.

Hemolysis assay: The test protocol developed by Harington et al.[4] was used, with slight modifications (Wallace et al. (4a)), for the present investigation. Dusts were made up to a stock concentration of 2 mg dust per ml of calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS) obtained from Sigma Corp of St. Louis, MO. The dust-saline mixture was stirred in a sonicator bath until the dust was fully dispersed and suspended in the liquid phase. The stock suspension was then diluted to make sample preparations, in duplicate, of 0.04-2.0 mg dust/ml PBS. Sheep blood erythrocytes, supplied by Scott Laboratories of Fiskeville, RI, were washed twice with PBS, centrifuged at 990g and diluted to a 2% by volume suspension in PBS. Equal volumes of dust suspension and the 2% erythrocyte suspension were then mixed to obtain mixed suspensions of 1% by volume of erythrocyte cells and dust concentrations in the range of 0.02 to 1.0 mg dust/ml. These suspensions were subsequently incubated for 30 minutes at room temperature with agitation every 10 minutes and then centrifuged at 990g. The amount of hemoglobin released was determined colorimetrically on a Bausch and Lomb Spectrometer (Spec 20) at a wavelength of 540 nm. Negative controls consisted of 1% suspensions of erythrocyte cells in PBS and positive controls of an equal volume mixture of water and 2% by volume suspension of erythrocytes in PBS.

EXPERIMENTAL RESULTS

The hemolytic activity of untreated Min-U-Sil, β-cristobalite, and Cab-O-Sil, as function of dust concentration, are compared in Figure 1. These results show that on a per unit weight basis the Cab-O-Sil material is about an order of magnitude more active than the crystalline Min-U-Sil. On a surface area basis, however, Min-U-Sil is more toxic than the amorphous material by, approximately a factor of 3 to 4, since the specific surface area of Cab-O-Sil was found to be over 30 times larger than that of Min-U-Sil. The hemolytic activities of Cab-O-Sil and β-cristobalite are, on

the other hand, comparable on a unit area basis. This is in general agreement with other findings on the relative biological activity of various allotropic forms of silica.[1,5,6] However, a test of hemolytic activity conducted on Cab-O-Sil material of different specific surface areas (Figure 2) shows a decrease in activity with increasing specific surface area (or decreasing primary particle size) suggesting that a different mechanism may underlie the toxicity of the fumed silica particles. This latter finding is in qualitative agreement with the particle size dependence of colloidal silica reported by Harley and Margolis.[7]

The effect of calcination on the hemolytic activity of Min-U-Sil and Cab-O-Sil and the change in the specific surface area of the materials on calcination is illustrated in Figures 3 and 4. It is evident from these results that the percent decrease in hemolytic activity exceeds the percent decrease in surface area by sintering, indicating a net decrease in activity on a per unit of surface basis. This decrease in activity coincides with the dehydroxylation of the silica surface reported for various types of silica.[1] The dehydration/dehydroxylation process was followed by photo-acoustic spectra of surface modes of the calcined materials and is illustrated in Figure 5. The untreated sample exhibits a broad absorption band in the 3000-3700 cm^{-1} range which is associated with hydrogen bonded silanol groups and adsorbed water molecules, and the sharp 3747 cm^{-1} band characteristic of free silanol groups. Calcination leads first to the disappearance of the broad band and an increase in the intensity of the free silanol group concentration at temperatures below 800°C and, subsequently, to the disappearance of this band at temperatures above 800°C. It has been found by previous investigators of the dehydroxylation of silica surfaces that at temperatures below approximately 400-450°C less than half of the hydroxyl groups have been removed and thus there is an appreciable concentration of adjacent hydroxyl groups which facilitate rapid rehydroxylation of the surface. As the calcination temperature is increased beyond this range the dehydroxylation process becomes more irreversible until, at around 1100°C, a fully dehydrated, hydrophobic siloxane surface is attained.[8,9] This process appears remarkably well reflected in the observed hemolytic activity of calcined silica particles shown in Figures 3 and 4, which leads to the conclusion that the hemolytic activity of the siloxane surface is much lower than that of the normal, hydroxylated surface, regardless of the type of crystal structure (α-quartz or amorphous) under consideration. It was also observed that the calcined materials, maintained under normal desiccator conditions, recovered their cytotoxicity with time. For fumed dusts calcined at 800-950°C this recovery occurs over a period of 10 to 20 days, as is illustrated in Figure 6. For crystalline materials and calcination temperatures where a fully siloxinated surface is generated, these times were found to be considerably longer. For example, a sample of Min-U-Sil calcined at 1100°C recovered only about 30% of its precalcination activity after a period of 180 days. This observation is in general agreement with hydroxylation rates reported by other researchers.[1,9] The recovery of cytotoxicity by the calcined dusts is accompanied by the reappearance of the I.R. absorption bands characteristic of the hydroxylated surface.

Another method for monitoring the surface structure changes of the silica particles is by the measurement of the electrophoretic mobility or "zeta-potential." This potential measures, if other variables are held constant, the magnitude of the electric charge of the surface double-layer of the particles. It is thus a convenient method for detecting changes in the surface structure of particles. Figures 7a and 7b compare zeta-potential-pH profiles for untreated and calcined Min-U-Sil, and for β-cristobalite and α-quartz (Min-U-Sil), respectively. For the calcined samples it is observed that the zeta potential decreases for calcination temperatures up to 500°C which may be associated with desorption of water from the particle surface and a corresponding increase in the double-layer potential. Further dehydroxylation to form surface silane bonds results in a reduced electrical double-layer potential and, consequently, a more negative zeta potential. Cristobalite particles have a lower zeta-potential than quartz particles and a lower hemolytic activity as well.

The effect of degree of surface hydroxylation was also tested by treating Min-U-Sil samples with alkaline and acidic solutions. It has been reported that the rehydroxylation of partly dehydroxylated silica surfaces is catalyzed by alkali.[10] Min-U-Sil samples calcined for 48 hours at 800°C and which had lost 50% of their hemolytic activity by this treatment, recovered their full hemolytic potential after immersion in a stirred 5% solution of NaOH in water. Similar treatment of non-calcined Min-U-Sil dust resulted in a slight increase

in hemolytic activity while exposure to acid solutions (10% HCl) had no significant effect on hemolytic activity, as is shown in Figure 8a. However, the enhancement of hemolytic activity resulting from the alkaline solution treatment was found not to be permanent and the particles so treated were observed to return to the initial cytotoxic levels over a period of 40–80 days (Figure 8b).

CONCLUSIONS

A test of the effect of dehydration and dehydroxylation on the cytotoxicity of respirable silica dust particles, as measured by their hemolytic activity, suggests that such activity can be correlated with the total concentration of surface silanol groups in the sample. Although this may not be the only mechanism for cytotoxicity of silica, the finding confirms the results of Nash et al. of some years ago.[11] This correlation appears, however, to be independent of the structure of the underlying silica (crystalline or amorphous), contrary to other findings on this issue. The concentration of OH groups on hydroxylated silica surfaces is not a readily definable parameter and may vary with particle size, porosity, degree and type of crystallinity, thermal history, etc.[12,13] which may explain the great variability in cytotoxicity resulting from various surface treatments of the material. In the study reported here, the elimination of hydroxyl groups at calcination temperatures above 500°C coincides well with the observed decrease in cytotoxicity.
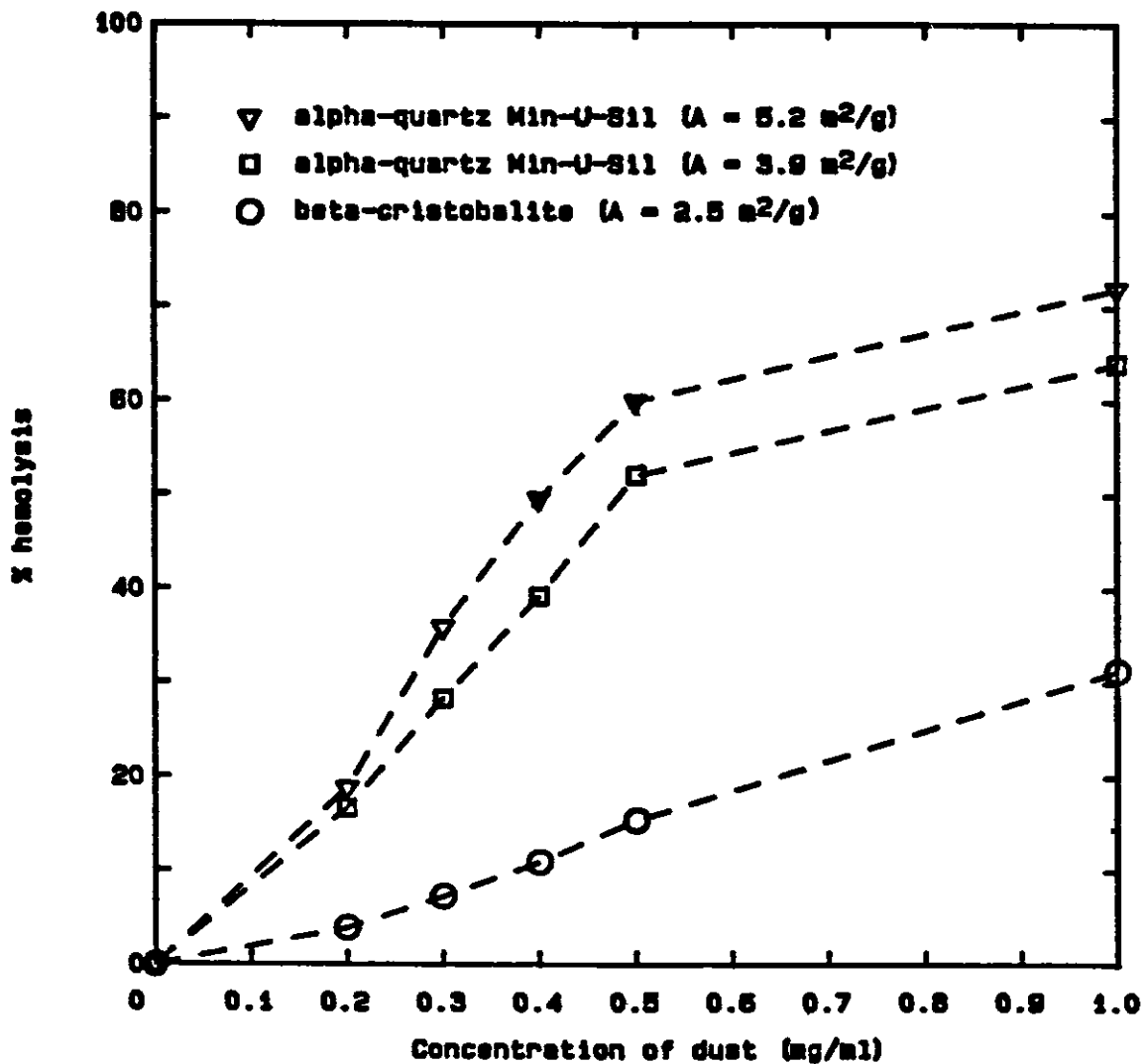
Figure 1a. Cytotoxicity as function of dust concentration for quartz and cristobalite.
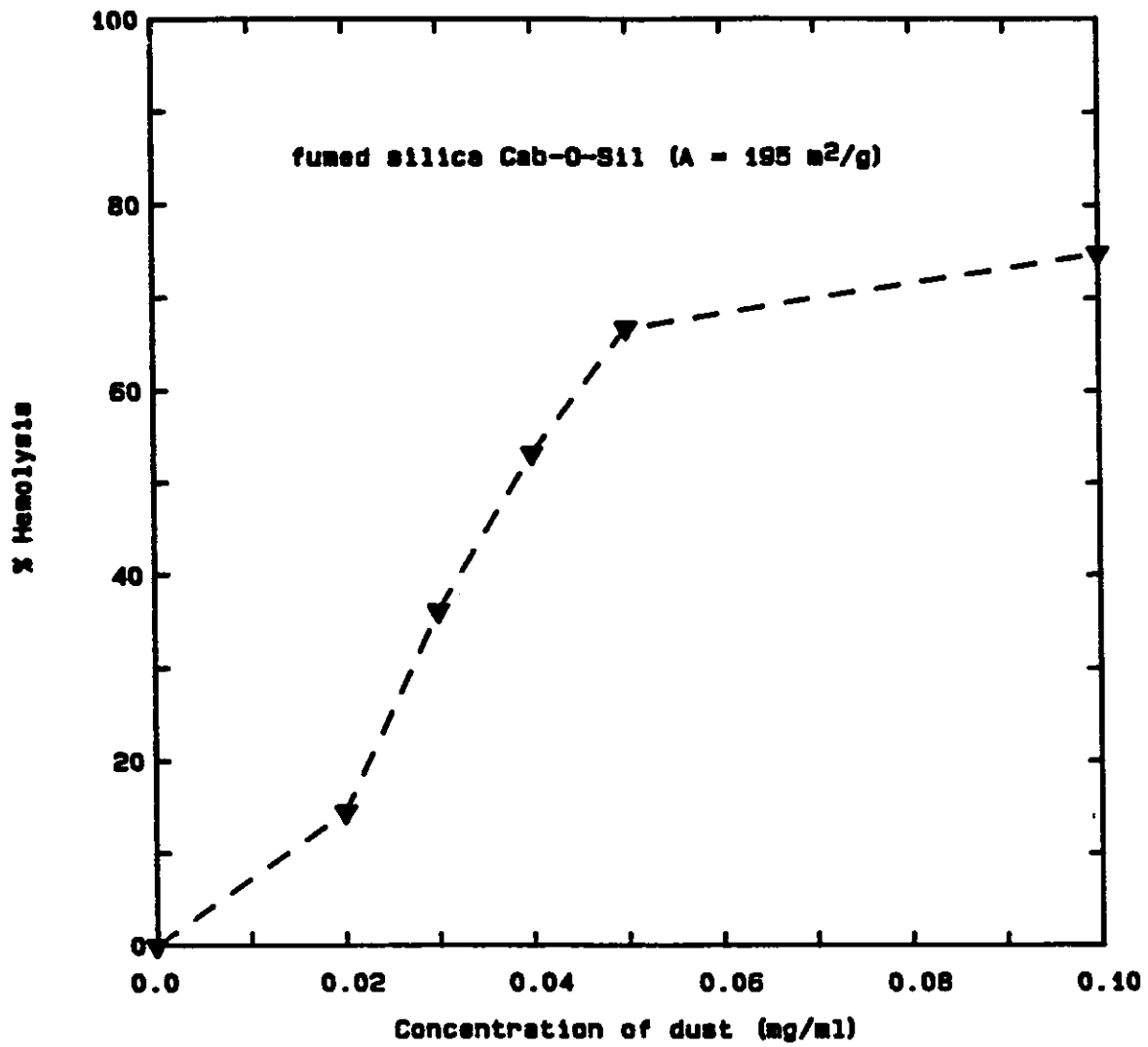
Figure 1b. Cytotoxicity as function of dust concentration for fumed silica (Cab-O-Sil).
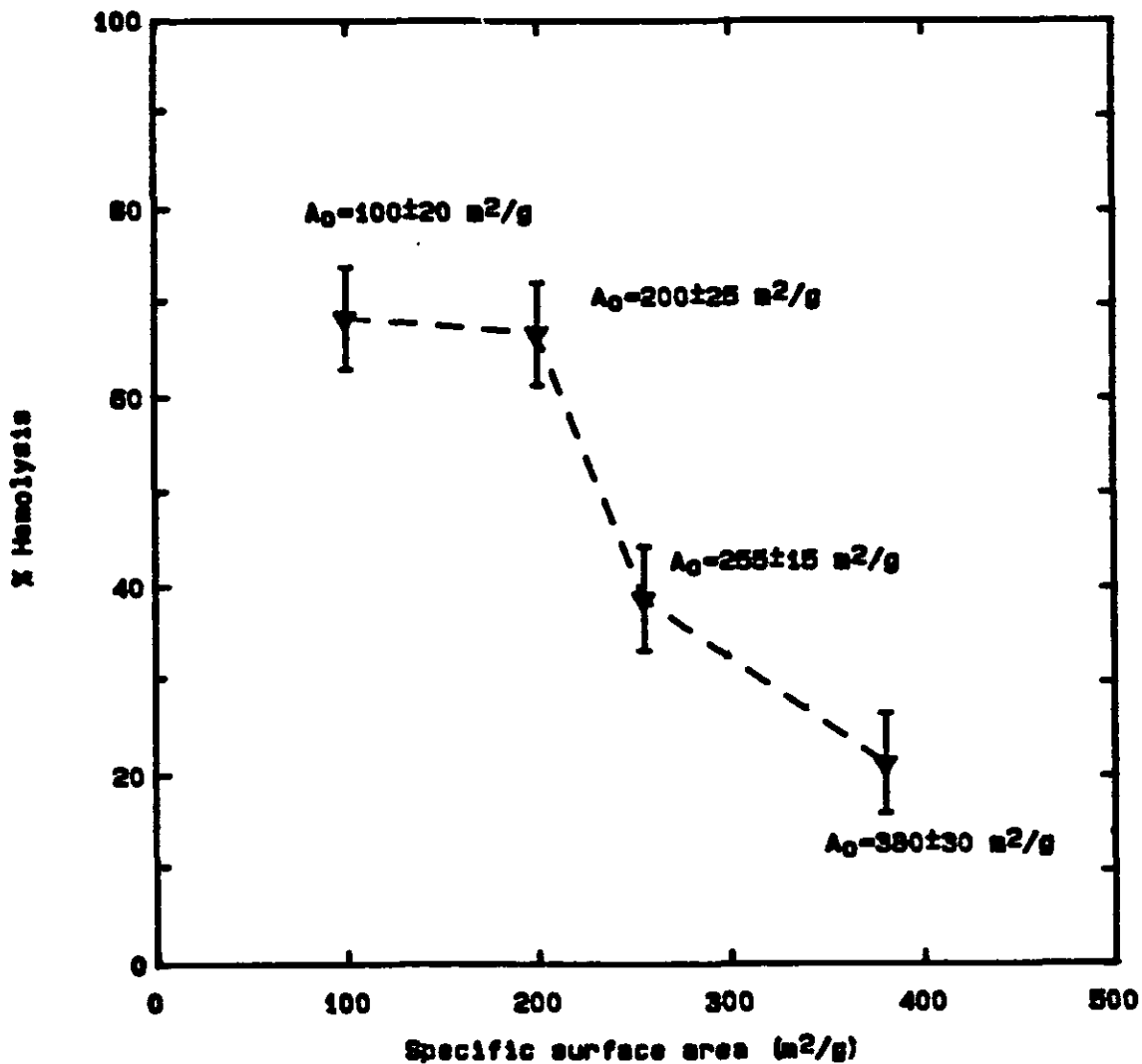
Figure 2. Hemolysis by fumed silicas of different surface areas (tested at dust concentration of 0.05 mg/ml).
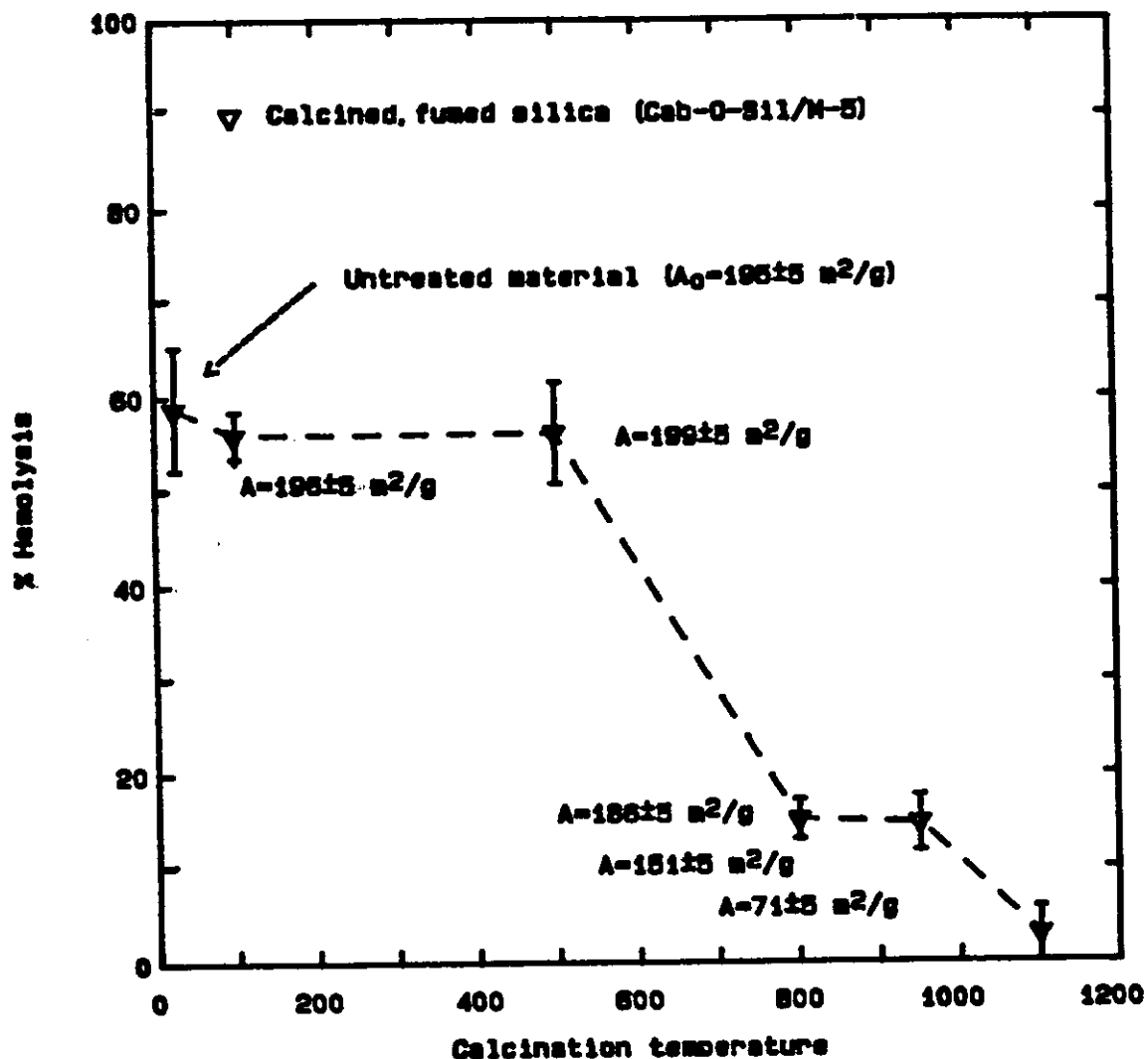
Figure 3. Hemolysis by calcined Cab-O-Sil (tested at dust concentration of 0.05 mg/ml).
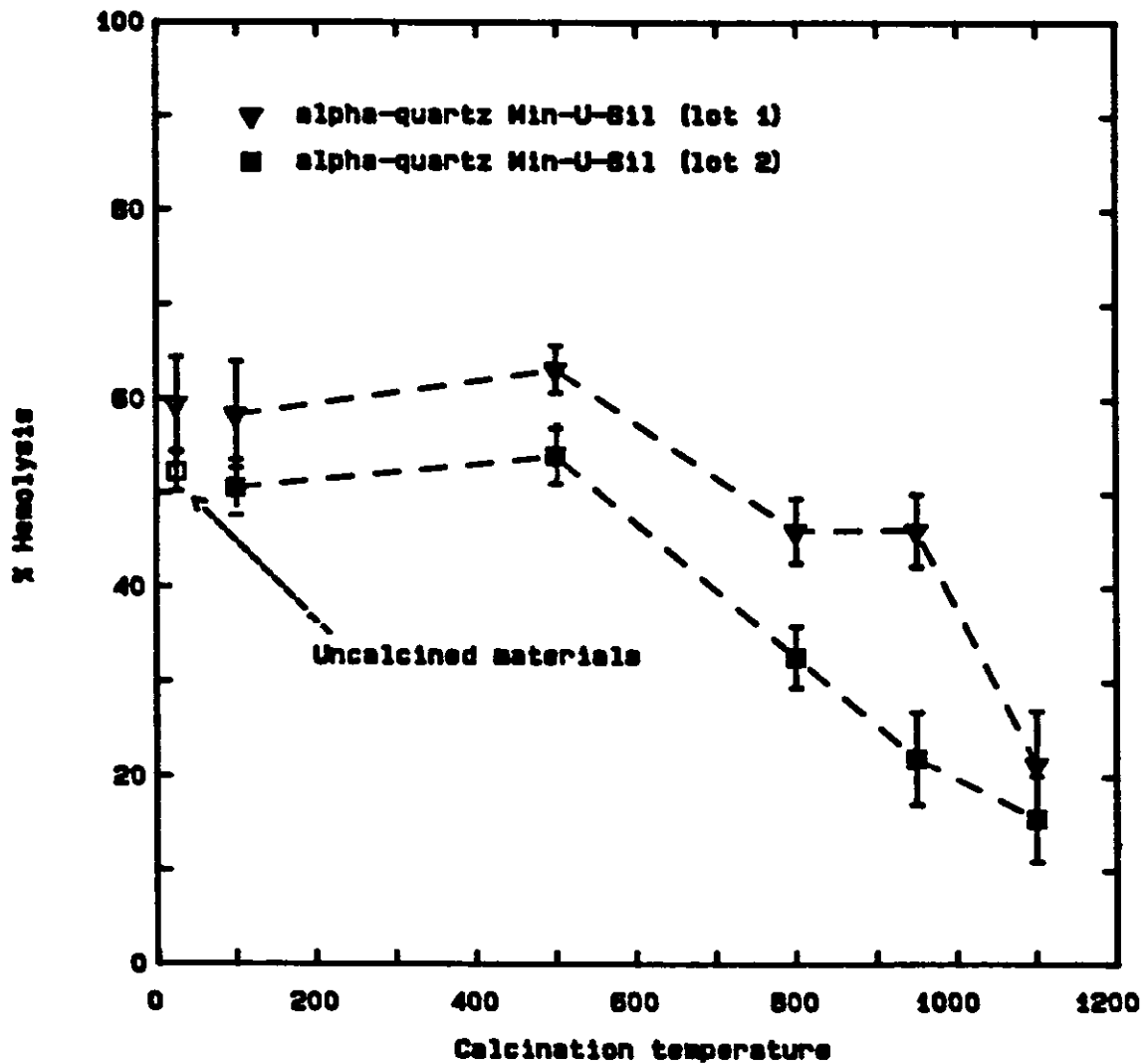
Figure 4. Hemolysis by calcined Mun-U-Sil (tested at dust concentration of 0.5 mg/ml).
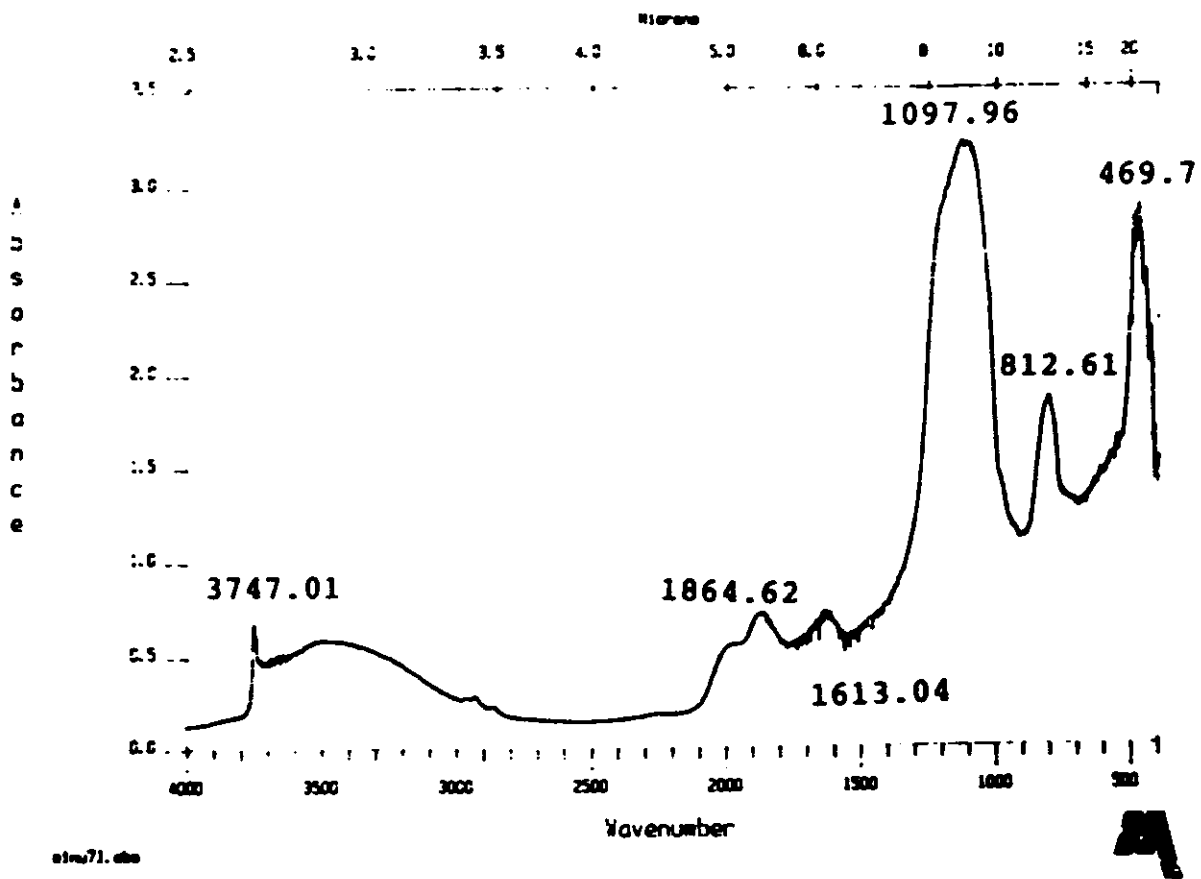
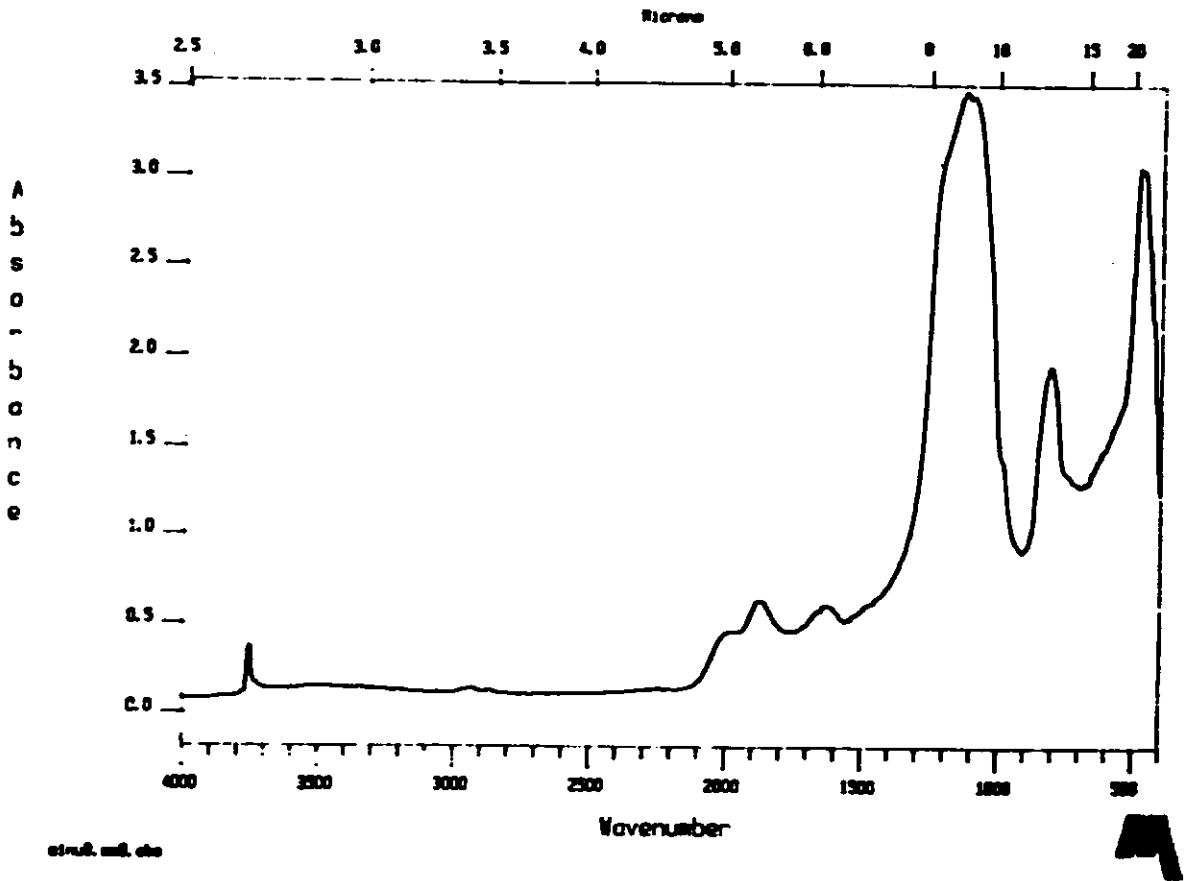Figure 5a. Untreated CAB-O-SIL.
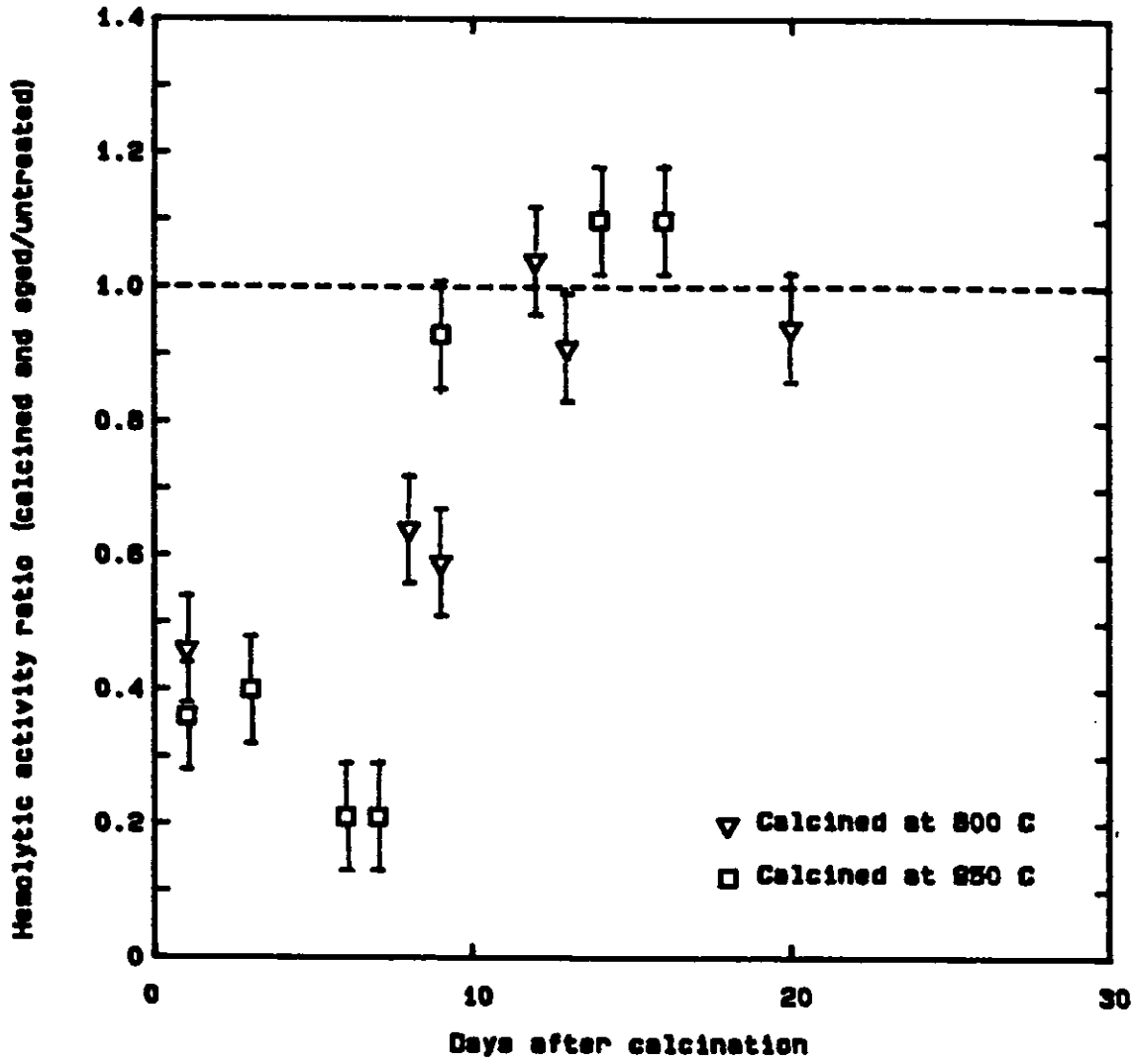
Figure 5b. CAB-O-SIL calcined 800°C.

Figure 6. Hemolytic activity recovery of calcined Cab-O-Sil by aging (tested at dust concentration of 0.05 mg/ml).
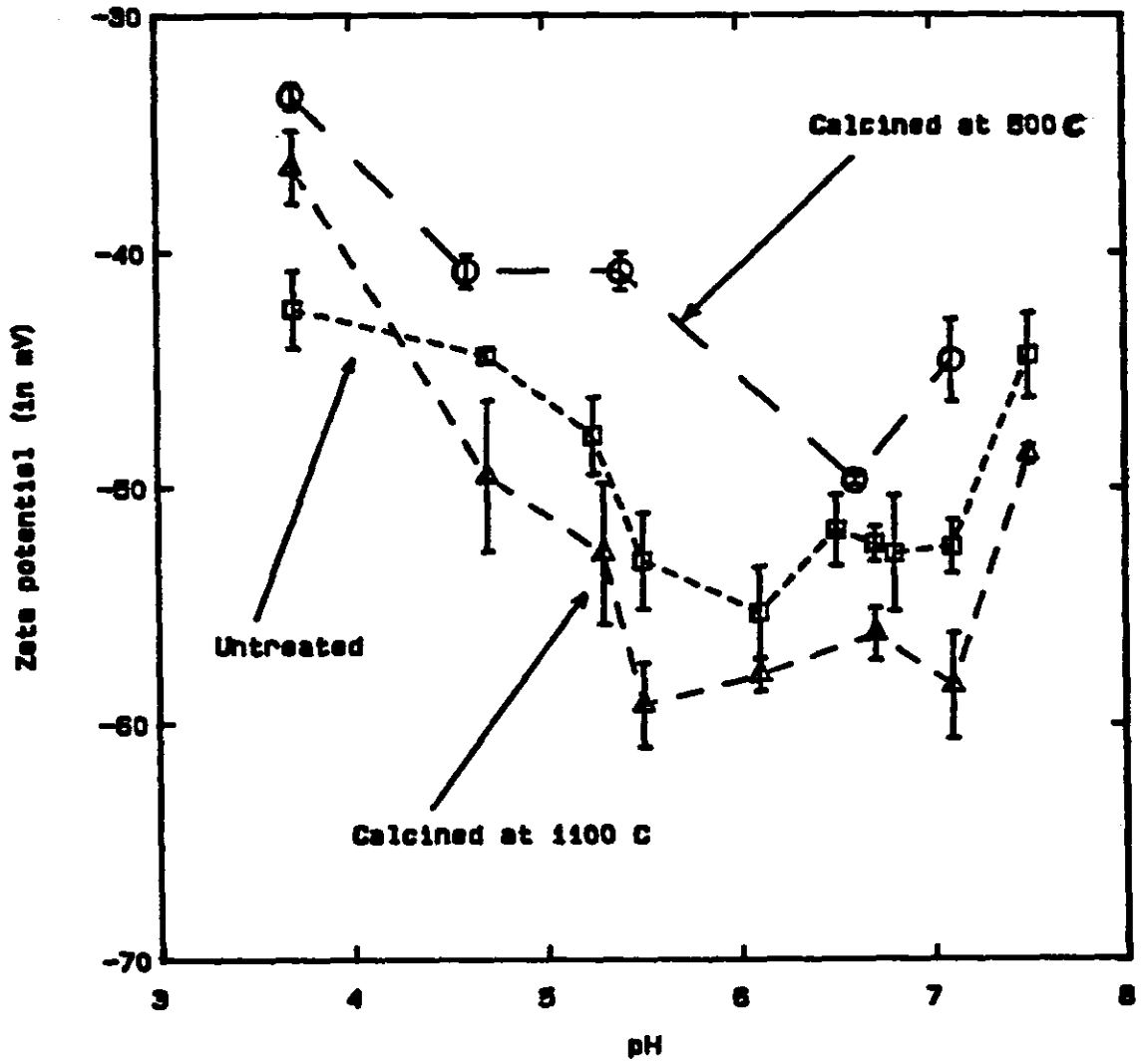
Figure 7a. Effect of calcination on zeta-potential of alpha-quartz (Min-U-Sil) dust in 0.1M KCl.
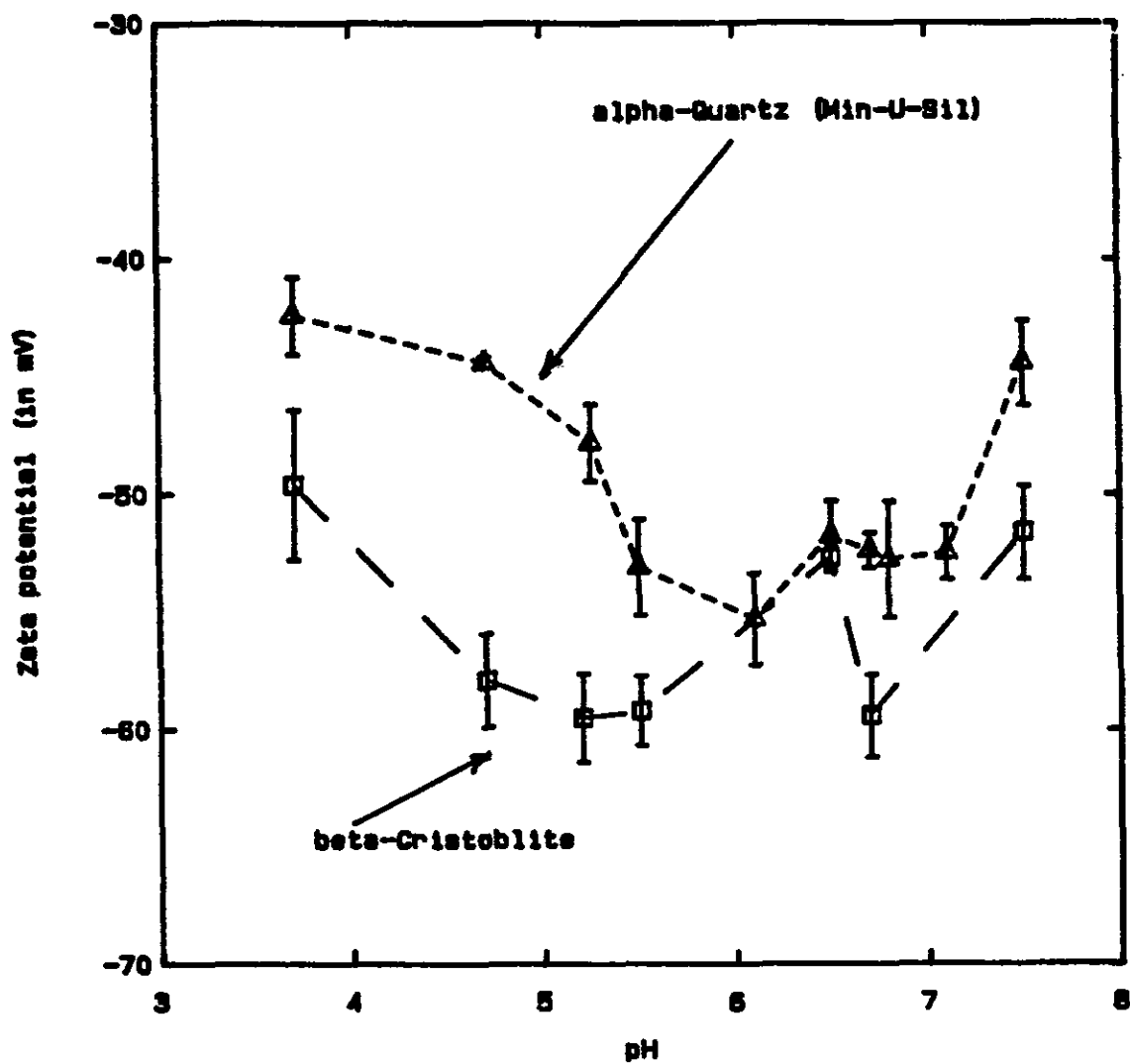
Figure 7b. Effect of crystalline structure on zeta-potential alpha-quartz (Min-U-Sil) and beta-cristobalite (in 0.1M KCl).
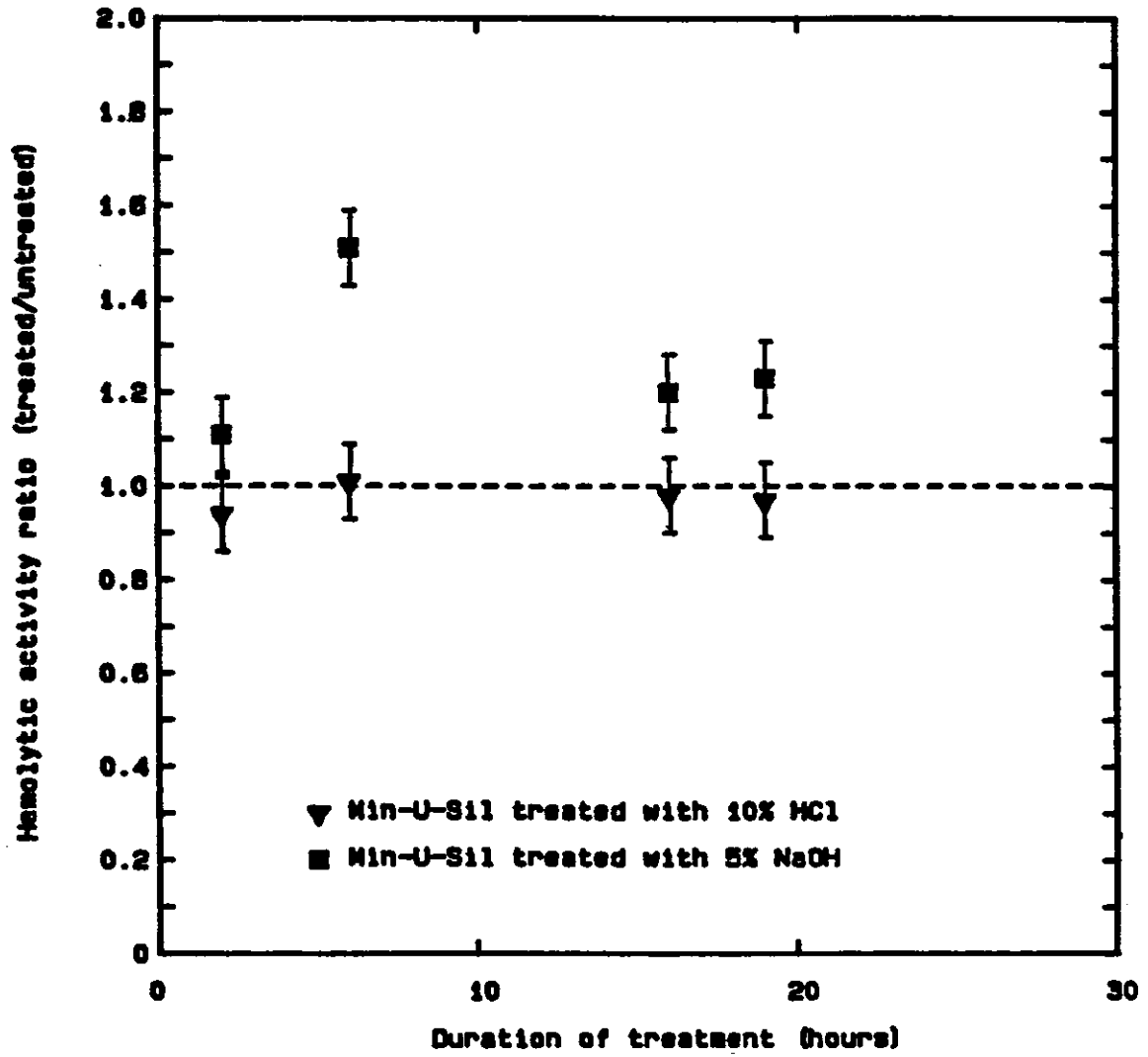
Figure 8a. Min-U-Sil treated with 5% NaOH or 10% HCl (tested at dust concentration of 0.5 mg/ml).
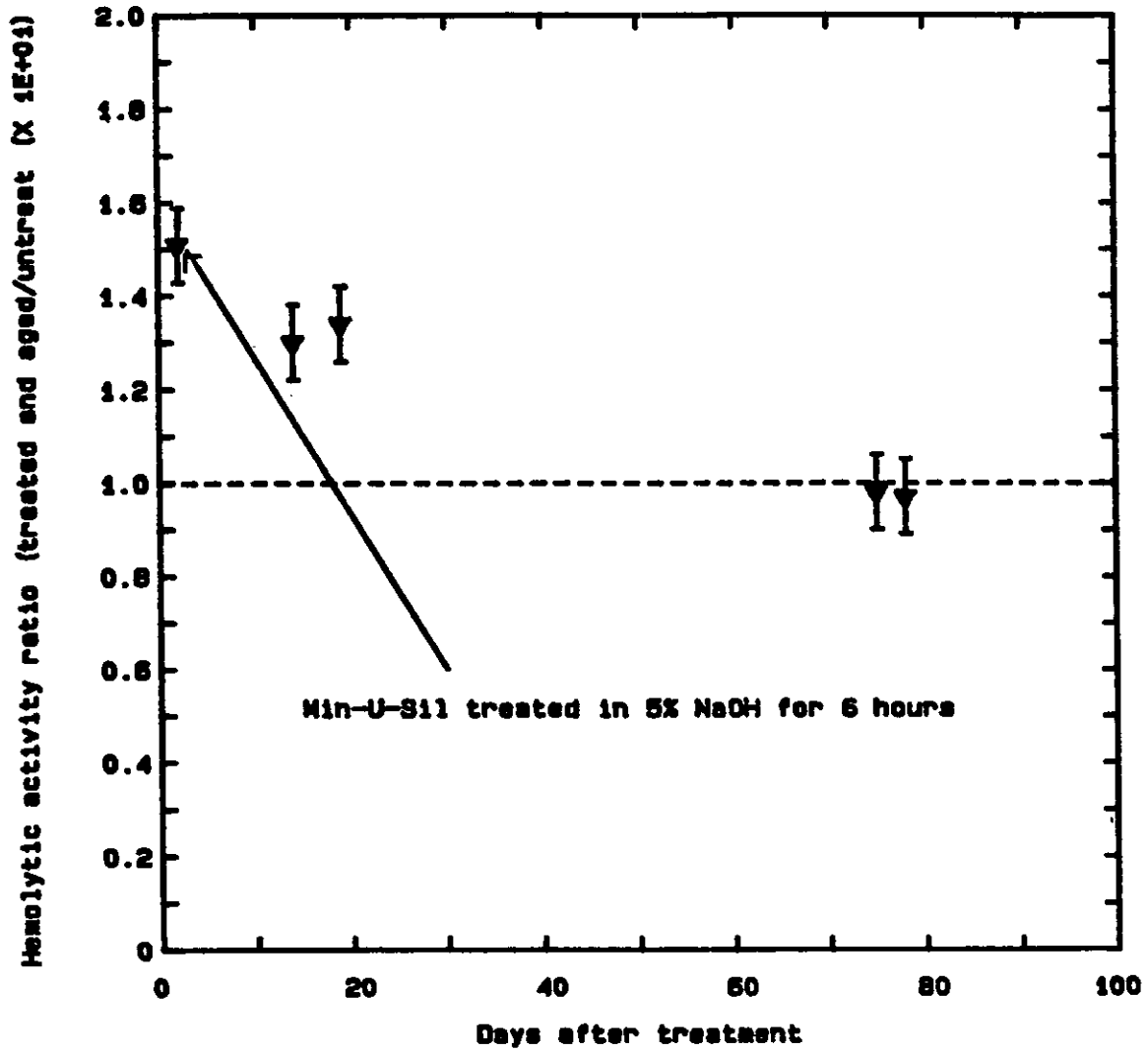
Figure 8b. Hemolytic activity recovery of treated Min-U-Sil by aging (tested at dust concentration of 0.5 mg/ml).

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RESPIRABLE PARTICULATE INTERACTIONS WITH THE LECITHIN COMPONENT OF PULMONARY SURFACTANT

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ABSTRACT

Dipalmitoyl glycerophosphorylcholine (lecithin) dispersed in physiologic saline, a model of the primary component of pulmonary surfactant, is adsorbed by respirable quartz and aluminosilicate dusts. Dust cytotoxicity as measured by erythrocyte hemolysis and pulmonary macrophage enzyme release is suppressed by this adsorption. The degree of suppression of hemolytic potential versus specific adsorption of lecithin from dispersion in saline by respirable quartz and kaolin dusts are compared with dusts' BET specific surface areas to interpret the prophylactic effect of lecithin adsorption. Dust hemolytic potential versus medium pH is presented. Fourier transform infrared spectroscopy and photo-acoustic spectroscopy of lecithin on quartz and of lecithin on kaolin are presented and reviewed with results of studies of the time course of removal of lecithin adsorbed on mineral surfaces by digestion by phospholipase enzyme. Results are discussed in terms of a model of prompt neutralization of respired mineral dusts by pulmonary surfactant, and a gradual re-toxification by digestive processes acting on the adsorbed prophylactic surfactant coating following phagocytosis.

INTRODUCTION

Quartz dust of respirable size is well known to cause fibrotic lung disease, but numerous questions persist in the understanding of the initiation and progression of this disease. Our approach concentrates on physical and chemical aspects of mineral dusts early-on in their interactions with living organisms, and we have chosen simplified models to investigate that interaction.

In the alveolar spaces of the lung, tissue is coated with a surface-active material (pulmonary surfactant), which, among other functions, mechanically stabilizes the lung from collapse by reducing the surface tension of water in the alveolar sacs.[1] This surfactant is also the material that is first contacted by a mineral particle that is transported to an alveolus and is impacted there. This surfactant material has been studied extensively. The primary components are known to be proteins (about 11% in dog lavage fluid), and phospholipids (about 88%).[2] Phosphatidyl cholines constitute roughly 80% of the phospholipid fraction; about 70% of the phosphatidyl choline fraction is dipalmitoyl lecithin (DPL).[2] Respirable aluminosilicate particles are capable of adsorbing dipalmitoyl lecithin from dispersion in physiologic saline, a model for a possible initial event occurring upon deposition of a particle in a pulmonary alveolus.[3]

As may be seen from Figure 1, the DPL molecule has several fixed charges at neutral pH; a positive charge on the trimethylamine (choline) moiety, and a negative charge on the phosphate group. Also evident are the two fatty acid residues of palmitic acid, which are bonded through ester likages to the glycerol segment of the molecule. The fatty acid moieties of phosphatidyl choline make the molecule insoluble in aqueous solutions under normal conditions, but a colloidal unit of aggregated molecules called a micelle is usually formed spontaneously above a certain minimum concentration. Small micellar vesicles are generally formed in the laboratory by using ultrasonic agitation or by solvent evaporation methods.

Our simplified system uses dispersions of DPL in physiological saline as a surrogate pulmonary surfactant, and we have used quartz, a crystalline, fibrogenic dust, and kaolin, an aluminosilicate clay that is not generally considered fibrogenic.[4-10] The approach has been to use *in vitro* cytotoxicity assays (sheep erythrocyte hemolysis and lysosomal enzyme release from pulmonary macrophages) to examine the effects of the surrogate surfactant on mineral dust cytotoxicity.[11]

The first results of the *in vitro* system were that DPL above a certain concentration virtually eliminates cytotoxicity of both dusts;[11] curves of cytotoxicity vs. DPL to dust ratios are shown in Figures 2 and 3. The effect has also been demonstrated with other materials, such as serum proteins and alveolar washings.[12,13] The effect was seen in both cytotoxicity assays, and a dose-response pattern is observed for both dusts.[11] The two untreated dusts are about comparable in cytotoxicity on a BET specific surface basis; the
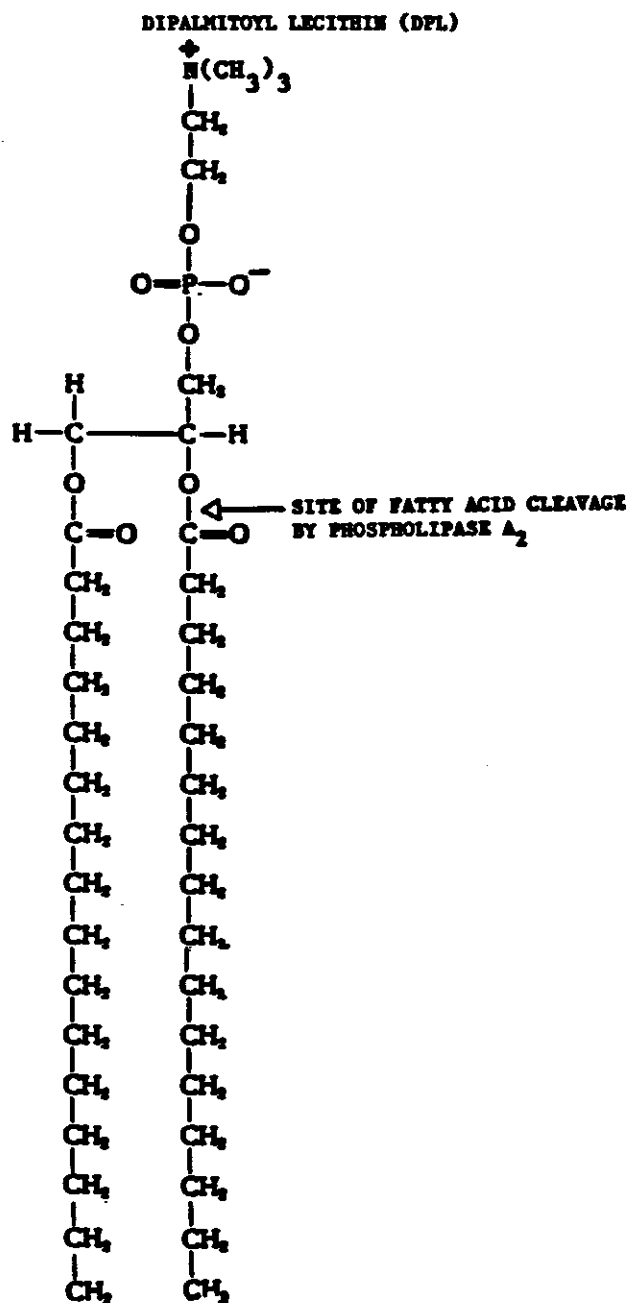
DIPALMITOYL LECITHIN (DPL)

$$N(CH_3)_3$$
$$CH_2$$
$$CH_2$$
$$O$$
$$O=P—O^-$$
$$O$$
$$CH_2$$

H—C———C—H
(with H and CH₂ groups)

$$O \quad O$$
$$C=O \quad C=O$$ ⟵ SITE OF FATTY ACID CLEAVAGE BY PHOSPHOLIPASE A_2

$$CH_2 \quad CH_2$$
$$CH_2 \quad CH_2$$
$$CH_2 \quad CH_2$$
$$CH_2 \quad CH_2$$
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$$CH_2 \quad CH_2$$
$$CH_3 \quad CH_3$$

Figure 1. Structural formula of phosphatidyl choline molecule.

quartz is about 4 m²/g for the less than 5 micron size, and the kaolin is about 13 m²/g for the same size fraction.[11]

While this finding is significant, it is surely not the *in vivo* situation. Quartz is certainly fibrogenic in normal individuals, at least after extended periods. Some of the prevailing theories on silicosis have been recently reviewed;[14] our next approach was to reexamine the current hypotheses on the initiation of fibrosis, and modify them if necessary. Our working hypothesis is shown in Figure 4.

Our principal efforts were directed toward item 5, the

degradation of surfactant coating on dusts by pulmonary macrophages. We have been using a cell-free *in vitro* model to characterize enzymatic digestion of dipalmitoyl lecithin adsorbed on mineral dusts, while developing cellular *in vitro* methods to measure digestion of labelled dipalmitoyl lecithin from phagocytized respirable dusts. In particular, we sought to determine if such adsorption could occur, and if there are mineral specific differences in the rate of such digestion. Our artificial "lysosome" contained the enzyme phospholipase A_2, derived from porcine pancreas, to simulate the phospholipase enzymes found *in vivo*.[15] These enzymes have been identified in many cells, and we have isolated and concentrated phospholipase A activity from rat liver cell lysosomes, but not at sufficient activity levels to allow large scale laboratory use.[15] The use of commercially prepared enzyme of known activity, rather than a cell culture or *in vivo* system, allows the elimination of numerous uncontrollable variables, so that attention can be focussed on differences between dusts.[16-18] Our laboratory protocol is shown in Figure 5.

RESULTS AND DISCUSSION

When the coated dusts are treated with the phospholipase A_2, several things are evident (Figures 6 and 7). For both dusts, for a short period of time, toxicity in the hemolysis assay may exceed that of the untreated dusts. The Figures indicate that this is invariably the case at the 2 hour point. Subsequent assay of lipids indicate that the hydrolysis product lysophosphatidyl choline (lysolecithin) is retained on the dusts. This product results when the fatty acid ester linked to the center carbon of the glycerol chain is hydrolyzed to a free fatty acid, leaving an hydroxyl group; this substance is also highly lytic to cell plasma membranes, thus explaining the exaggerated cytotoxicity. As time progresses, less lysolecithin is found to be associated with the dusts, as seen in Figures 8 and 9.

The most significant finding is that the quartz toxicity returns to essentially its untreated value, even with fairly low enzyme levels relative to the kaolin. Analysis of the retained lipids confirms that the dust is almost free of adsorbed DPL or other lipids, as seen in Figure 10.

The situation for kaolin is quite different; toxicity is not restored except at quite high activity levels, and lipids are retained on the surface to a much greater extent, as seen in figure 11.

The results up to this point raise an important question: what is the basis for a difference in re-toxification of quartz and kaolin dusts? We have looked at several methods to try to clarify this difference, although the case is by no means closed.

In general, enzymatic digestion of substrate molecules is quite dependent on molecular conformation. Because quartz and kaolin surface structure and functional groups differ significantly, we are investigating the possibility that conformational differences between lecithin adsorbed to quartz and to kaolin surfaces might provide differing degrees of steric hindrance to digestive removal, with resultant differences in rates of restoration of surface cytotoxic potential.

To examine this hypothesis, we used Fourier Transform Infrared Spectrophotometry at the West Virginia University Physics Department to look at the spectra of DPL on both quartz and kaolin, and compared the spectral features to the pure DPL spectrum. The DPL-coated quartz and DPL spectra are shown in Figure 12. Samples were prepared as wet films of DPL or coated dusts on a KBr pellet substrate. In the DPL treated quartz, the 3024 cm⁻¹ trimethylamine band disappears, but the 3400 cm⁻¹ band associated with P—O—HOH is not suppressed. For the kaolin, shown in Figure 13, the 3400 cm⁻¹ group has virtually disappeared, and the trimethylamine band is suppressed and shifted. The evidence here is strongly suggestive of a quartz-trimethyl amine association, and a kaolin-phosphate association. There also exists the possibility of a kaolin-trimethylamine association, but the evidence is not as strong. The use of dry or moist samples for IR spectroscopy limits extrapolation of these results to dusts immersed in aqueous media. But the data suggest an association of the phosphate moiety of lecithin with basic aluminol groups on the alumina octahedra portions of the kaolin surface, and a consequent hindrance of enzymatic hydrolysis of the nearby glycerol-to-fatty acid ester.

To consider quartz and kaolin surface functions involved in direct lysis of erythrocyte membrane, in the absence of surfactant coating, we also performed some limited experiments to determine whether pH significantly affected dust cytotoxicity in the hemolysis assay. Quartz would be expected to show only acidic characteristics, due to surface silanol groups, while kaolin may have acidic silanol surface groups, as well as weakly acidic and weakly basic aluminol surface groups. An experimental problem arises here, however: the red blood cells are subject to hemolysis when a hydrogen ion, or other ion, gradient is present across the membrane. We tried to see whether the external osmolarity could be increased to offset this gradient, and the results are shown in Figure 14. The method seemed reasonable down to pH 5, so all blood suspensions were adjusted to 400 mOsm for the pH dependence experiments.

Figure 15 shows the dependence of hemolysis on pH. For both quartz and kaolin, the slope is positive between pH 5 and 7, suggesting that a charge dependent mechanism is involved with hemolysis for both dusts. The acidic character of both dusts suggests an acid-base interaction of the minerals with the trimethylamine group of membrane lecithin. Inter-
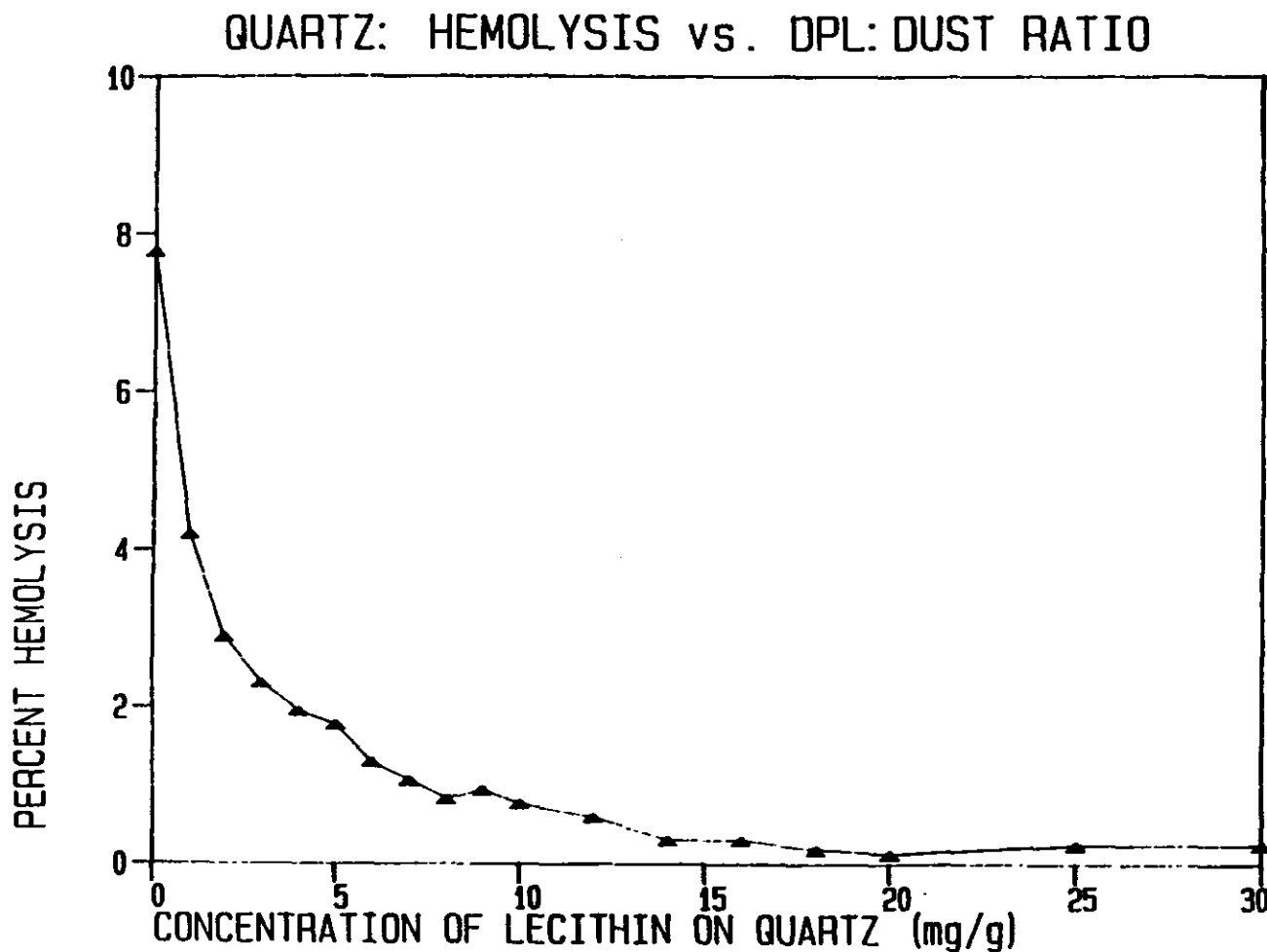

Figure 2. Percent hemolysis vs. DPL: quartz ratio.
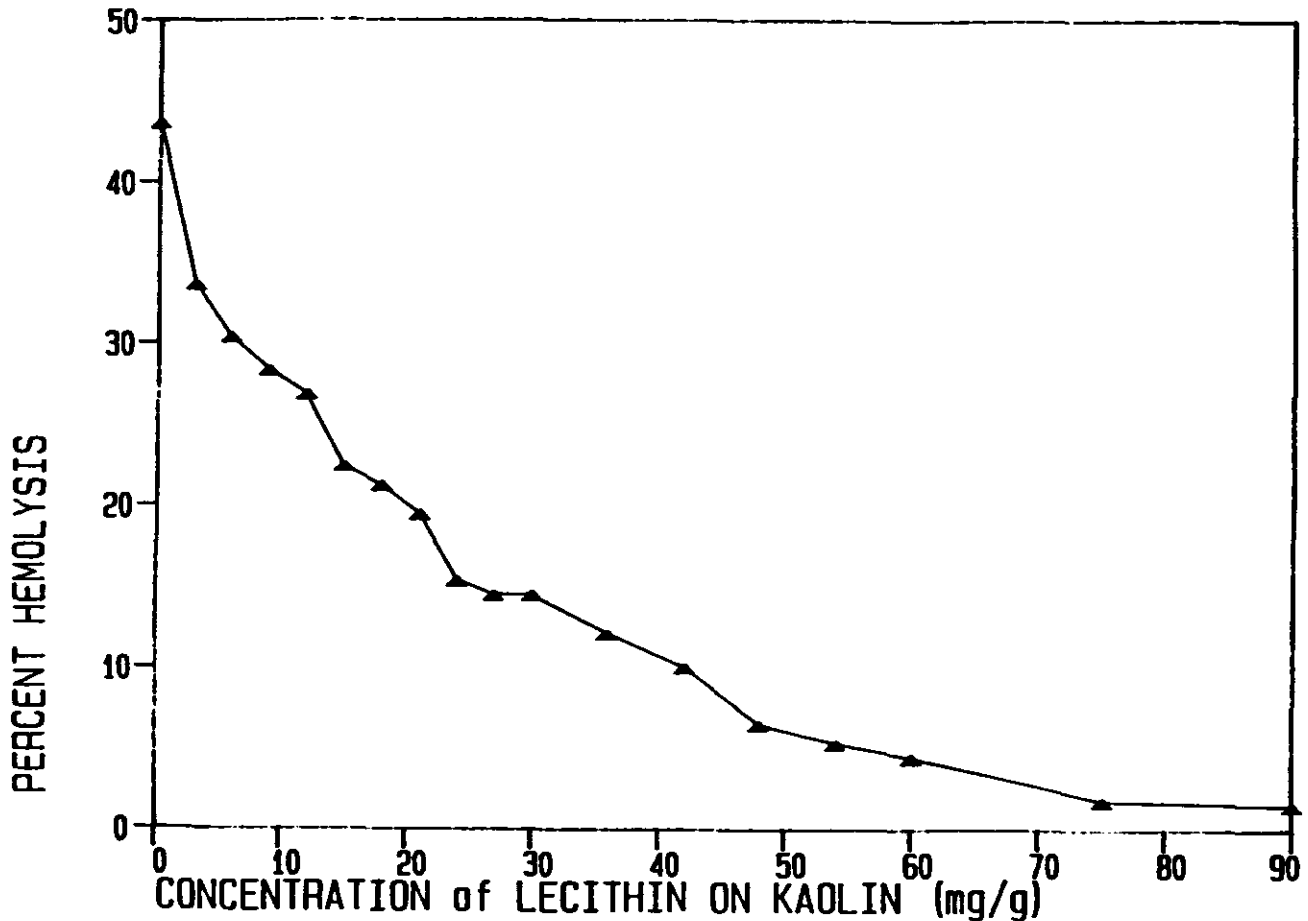
KAOLIN: HEMOLYSIS vs. DPL:DUST RATIO

Figure 3. Percent hemolysis vs. DPL: kaolin ratio.

HYPOTHESIS: EVENTS OF SILICOSIS INITIATION

1. INHALATION OF SILICA PARTICLES TO ALVEOLAR REGION
2. CONTACT WITH AND SUBSEQUENT COATING OF PARTICLE WITH SURFACTANT
3. PHAGOCYTOSIS OF COATED PARTICLE BY ALVEOLAR MACROPHAGE
4. FORMATION OF PHAGOLYSOSOME IN THE MACROPHAGE
5. HYDROLYSIS OF SURFACTANT BY LYSOSOMAL ENZYMES
6. RETOXIFICATION OF DUST
7. DEATH OR DAMAGE OF MACROPHAGE/ RELEASE OF SIGNAL SUBSTANCE TO FIBROBLASTS
8. PROLIFERATION OF FIBROBLASTS AND COLLAGEN SYNTHESIS
9. FIBROSIS

Figure 4. Working hypothesis for silicosis initiation.

LABORATORY PROTOCOL

1. PREPARE DPL DISPERSION IN SALINE WITH ULTRASONIC AGITATION
2. DUST COATED WITH DPL FOR 1 HOUR AT 37 DEGREES C
3. EXCESS DPL RINSED FROM DUST
4. INCUBATE DUST WITH PHOSPHOLIPASE A2 FOR 2 TO 72 HOURS
5. DUST RINSED WITH EDTA BUFFER TO INACTIVATE ENZYME (TWICE)
6. DUST RESUSPENDED IN BUFFER/CYTOTOXICITY ASSAY
7. LIPIDS EXTRACTED FROM REST OF DUST WITH SOLVENT
8. LIPIDS SEPARATED BY THIN LAYER CHROMATOGRAPHY
9. LIPIDS RECOVERED AND QUANTIFIED BY PHOSPHORUS ASSAY

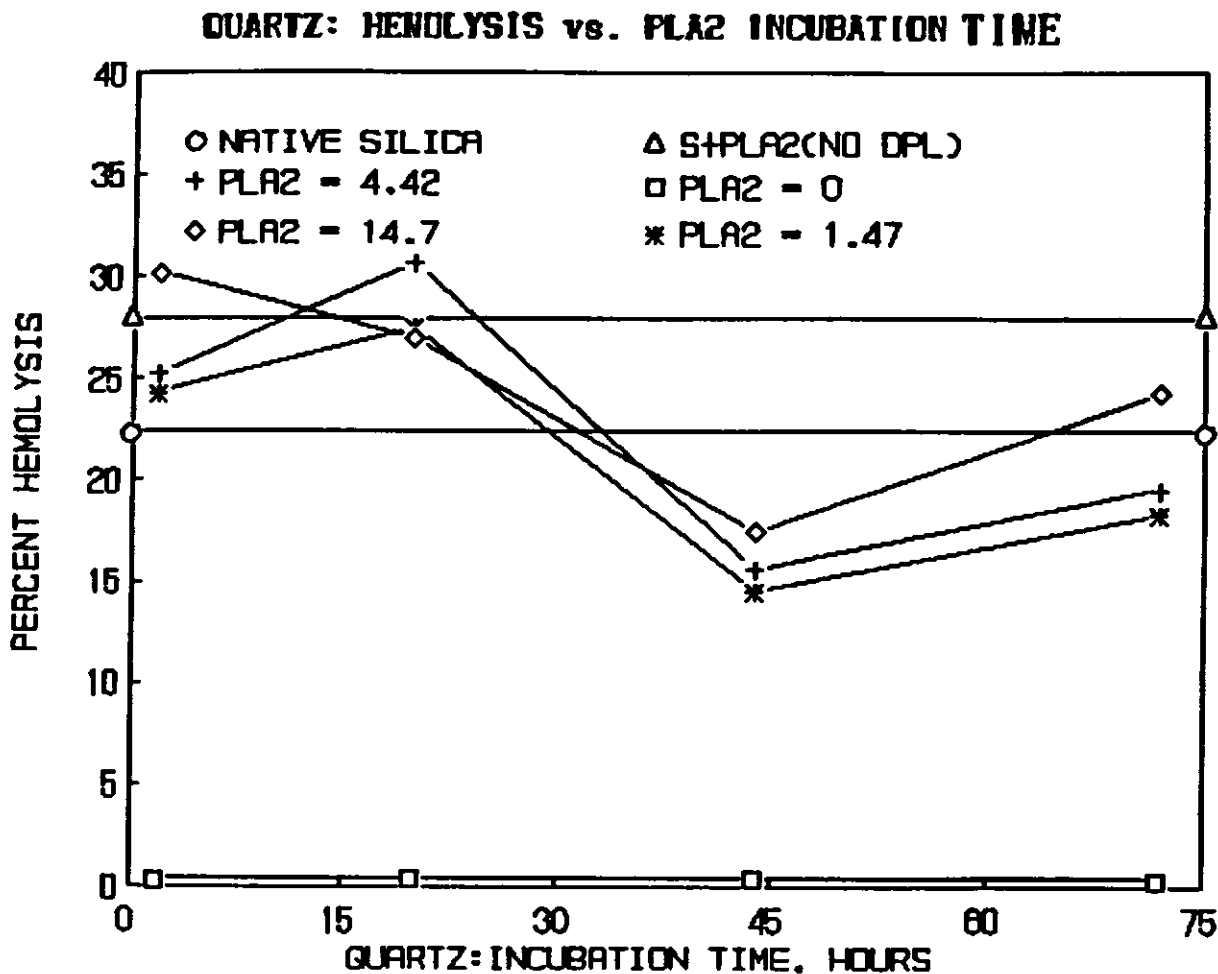Figure 5. Laboratory protocol for *in vitro* cell free system.

Figure 6. Hemolysis vs. time for DPL-coated quartz treated with phospholipase A_2.

pretation of these results on the pH dependence of the lytic potential of uncoated dusts are compromised by questions of the effect of pH on the lytic fragility of the membrane itself.

An overall research hypothesis which presents itself is that native quartz and aluminosilicate dusts can damage cellular membrane by direct interaction with dissociated mineral surface acidic silanol groups; that adsorption of the lecithin portion of pulmonary surfactant masks and thereby passivates these mineral surfaces; that phospholipase enzymatic digestion of lecithin coated dusts following their phagocytosis can remove the protective surfactant coating and restore cytotoxic potential of dusts within the phagocytic cell; and that the rate of this restoration may be affected by conformational differences between lecithin adsorbed to acidic silanol groups on quartz and to acidic silanol and basic aluminol groups on kaolin.

CONCLUSIONS

The surface toxicity both of quartz and kaolin dusts is eliminated in short-term cytotoxicity assays by coating the dusts with DPL.

Lecithin treated quartz is readily re-toxified by phospholipase A_2 in a cell-free *in vitro* system, and is relatively free of retained phospholipids.

DPL treated kaolin is not readily re-toxified at comparable enzyme levels, and retains both DPL and phospholipid degradation products.

The pH dependence suggests that both quartz and kaolin have acidic surface groups that are involved in hemolysis, and also may associate with the positively charged trimethylamine group of DPL.

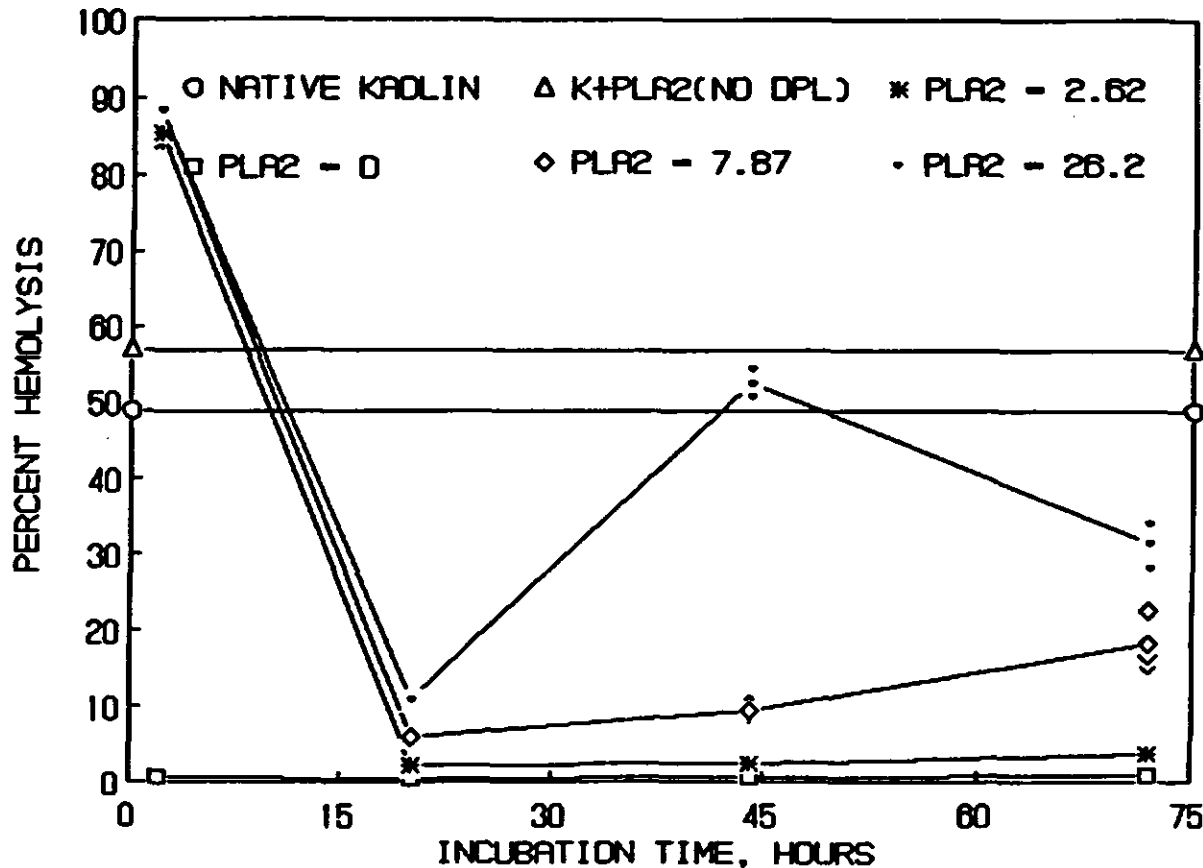Figure 7. Hemolysis vs. time for DPL-coated kaolin treated with phospholipase A_2.
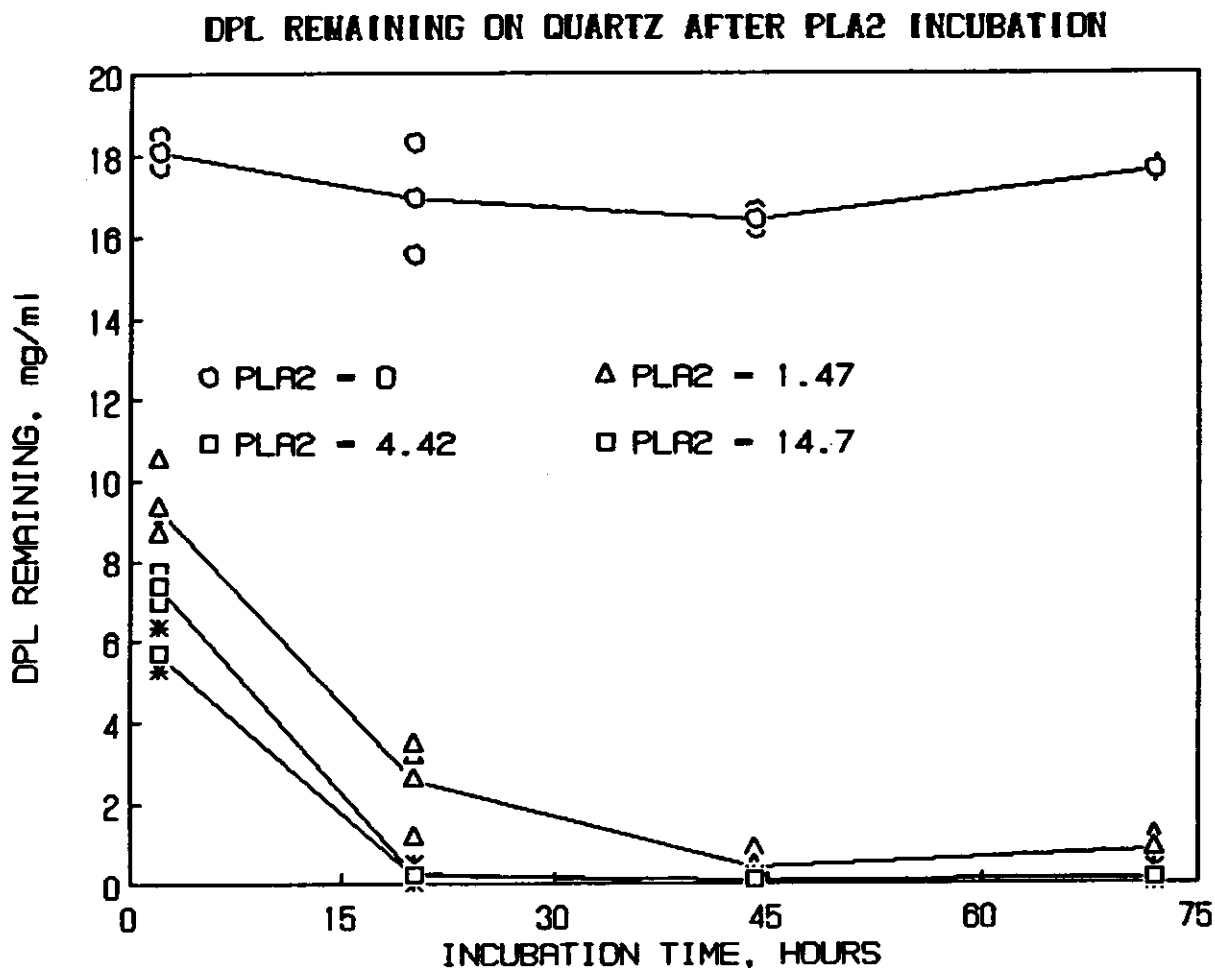
Figure 8. Lysolecithin retained on quartz after PLA₂ incubation vs. time.

FTIR spectra suggest that kaolin probably interacts with the phosphate group of DPL, and both quartz and kaolin probably interact with the trimethylamine group. Thus, there may be a surface chemistry effect in the differing rates of hydrolysis by phospholipase A₂.

DPL REMAINING ON KAOLIN AFTER PLA2 INCUBATION
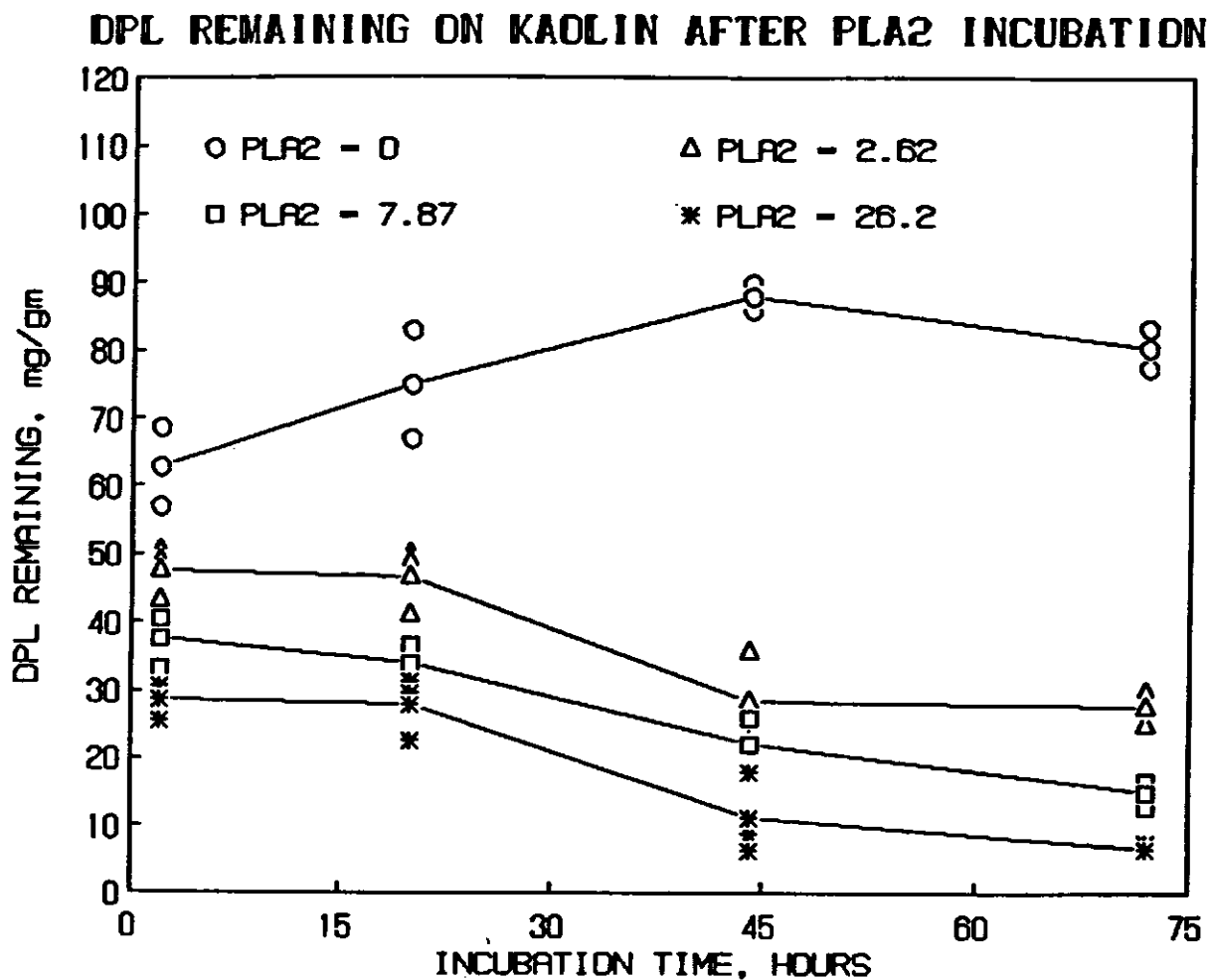
Figure 9. Lysolecithin retained on kaolin after PLA_2 incubation vs. time.

Figure 10. DPL retained on quartz after PLA_2 incubation vs. time.

LYSOLECITHIN REMAINING ON KAOLIN AFTER PLA2 INCUBATION
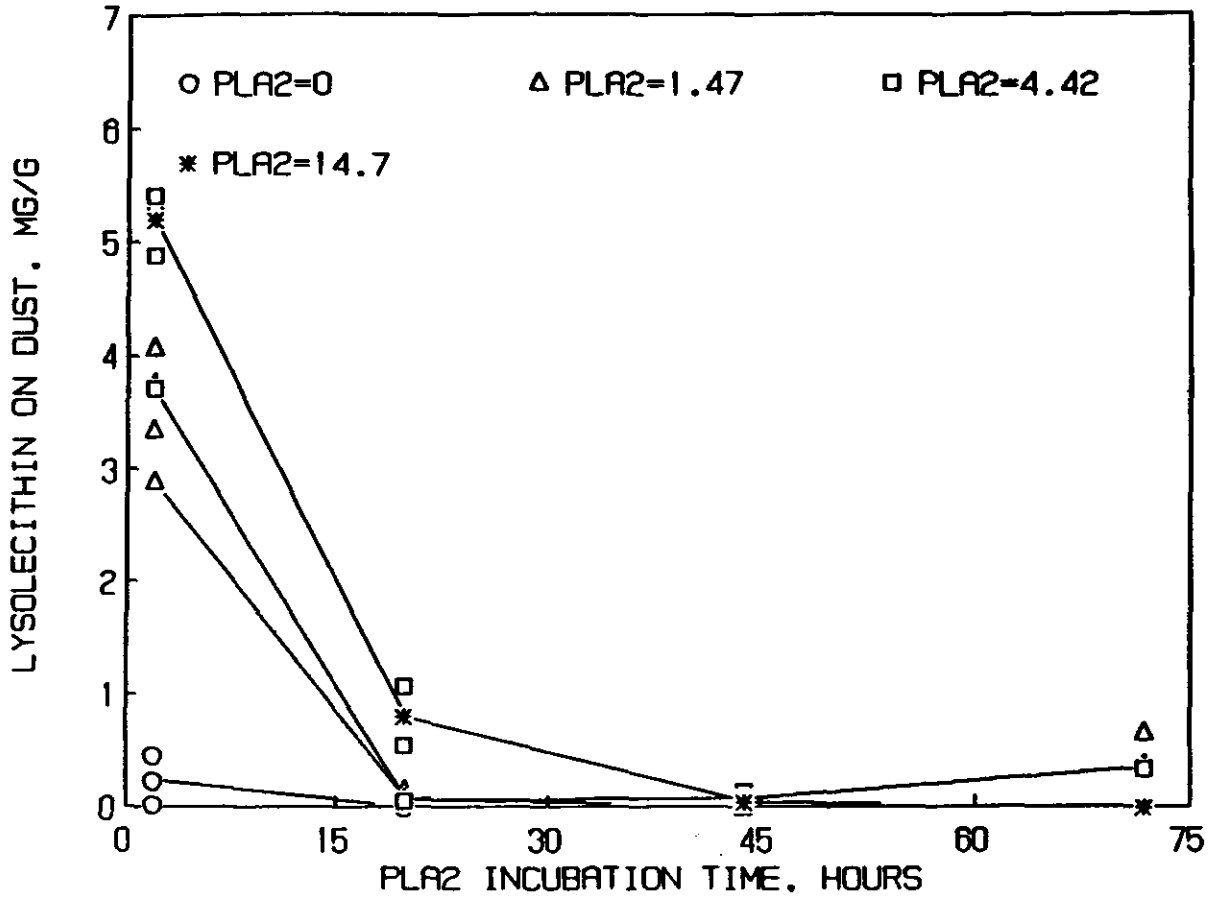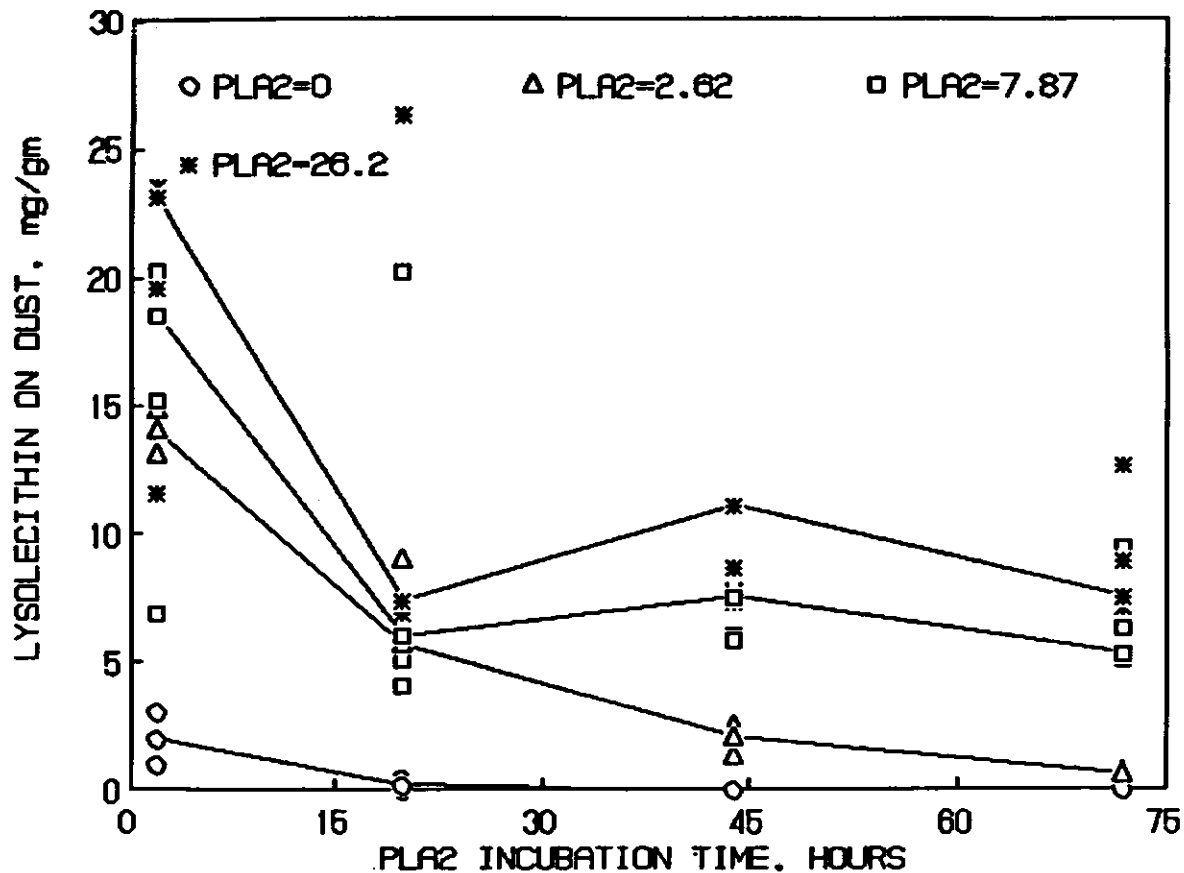

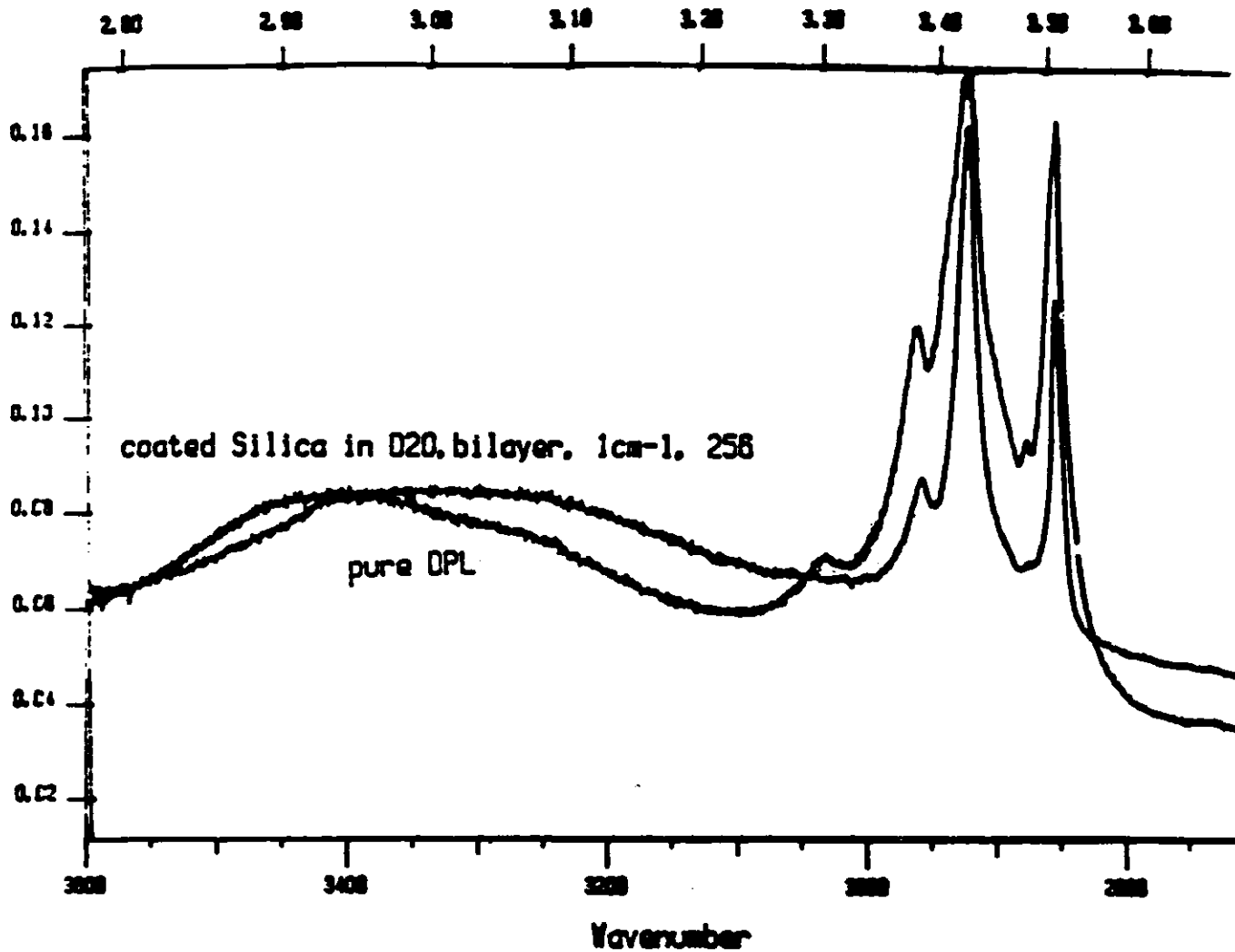Figure 11. DPL retained on kaolin after PLA_2 incubation vs. time.

Figure 12. FTIR spectra of DPL-coated quartz and DPL only, 2750–3600 cm^{-1}.

coated kal in D20 1cm-1 256 scans bilayer.
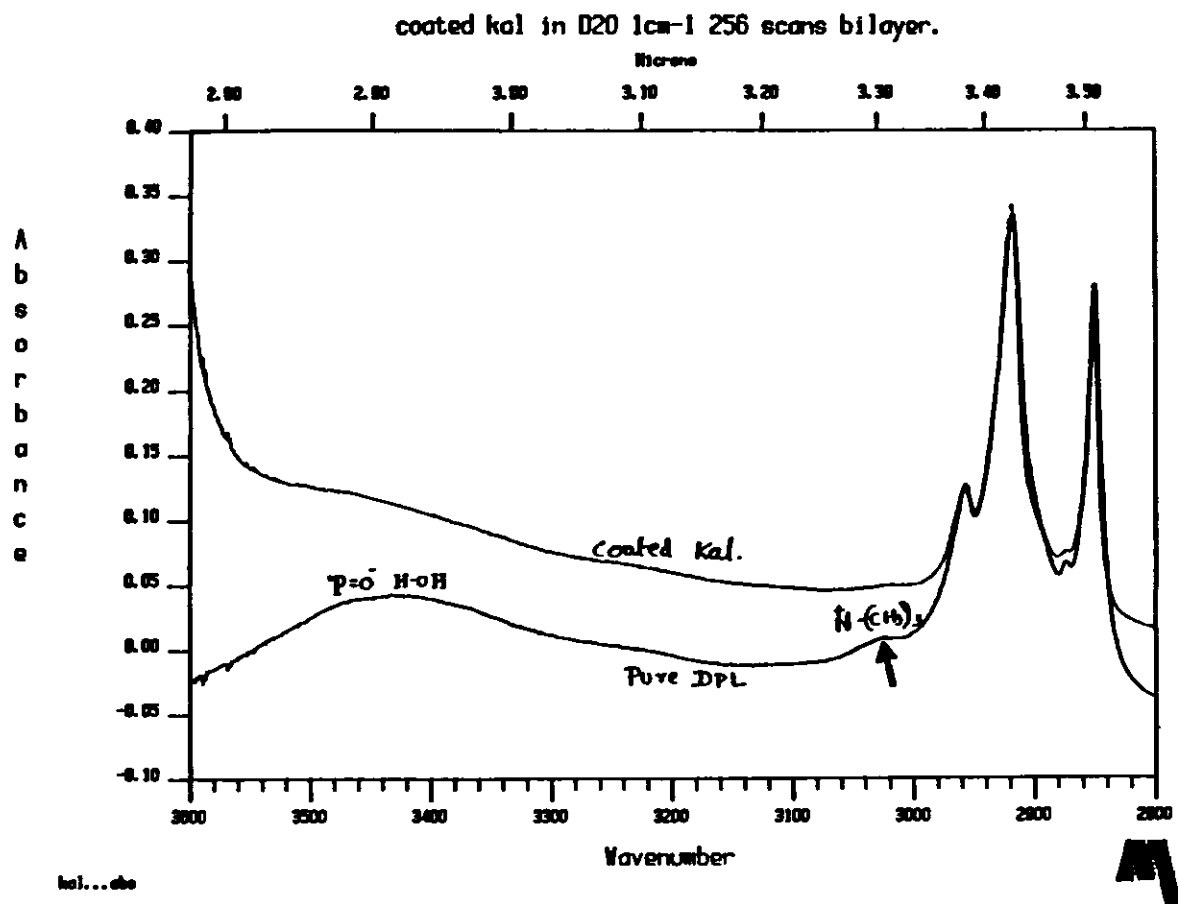
Figure 13. FTIR spectra of DPL-coated kaolin and DPL only, 2800-3600 cm^{-1}.

PERCENT HEMOLYSIS vs. OSMOLARITY FOR RBC'S AT pH 5.0 AND 5.5
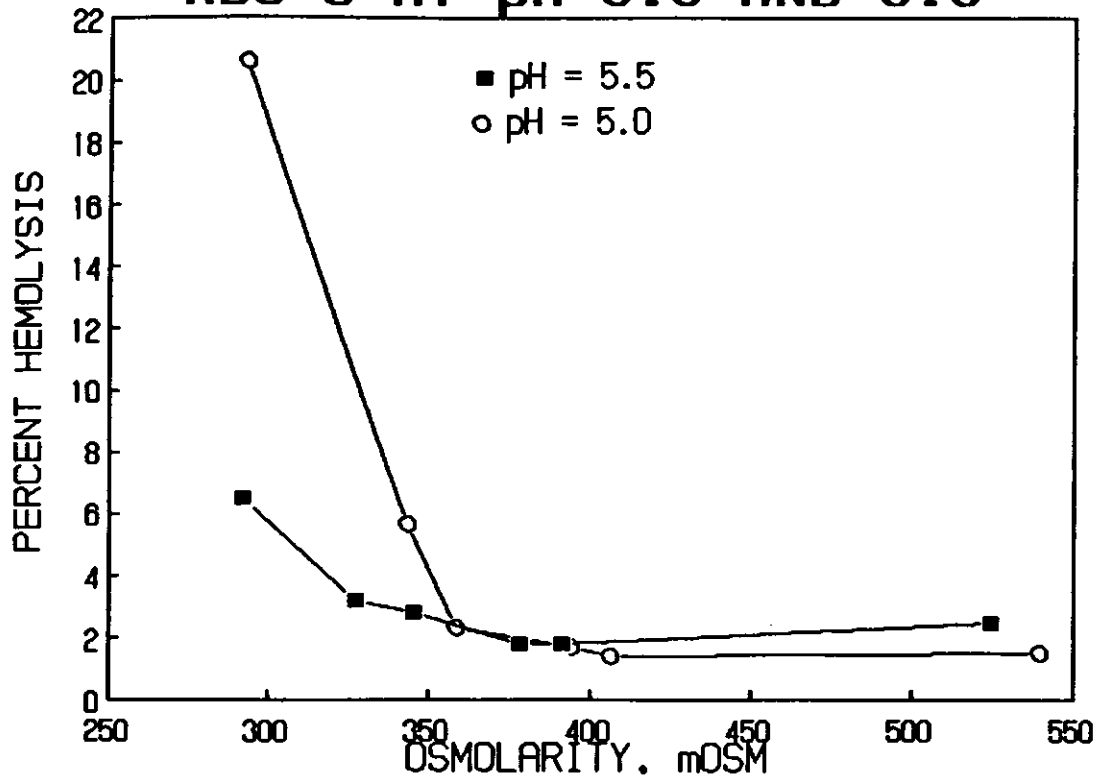
Figure 14. Percent hemolysis vs. osmolarity at pH 5 and pH 5.5.

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PERCENT HEMOLYSIS vs. pH

Figure 15. Percent hemolysis vs. pH for silica and kaolin @ 400 mOsm.

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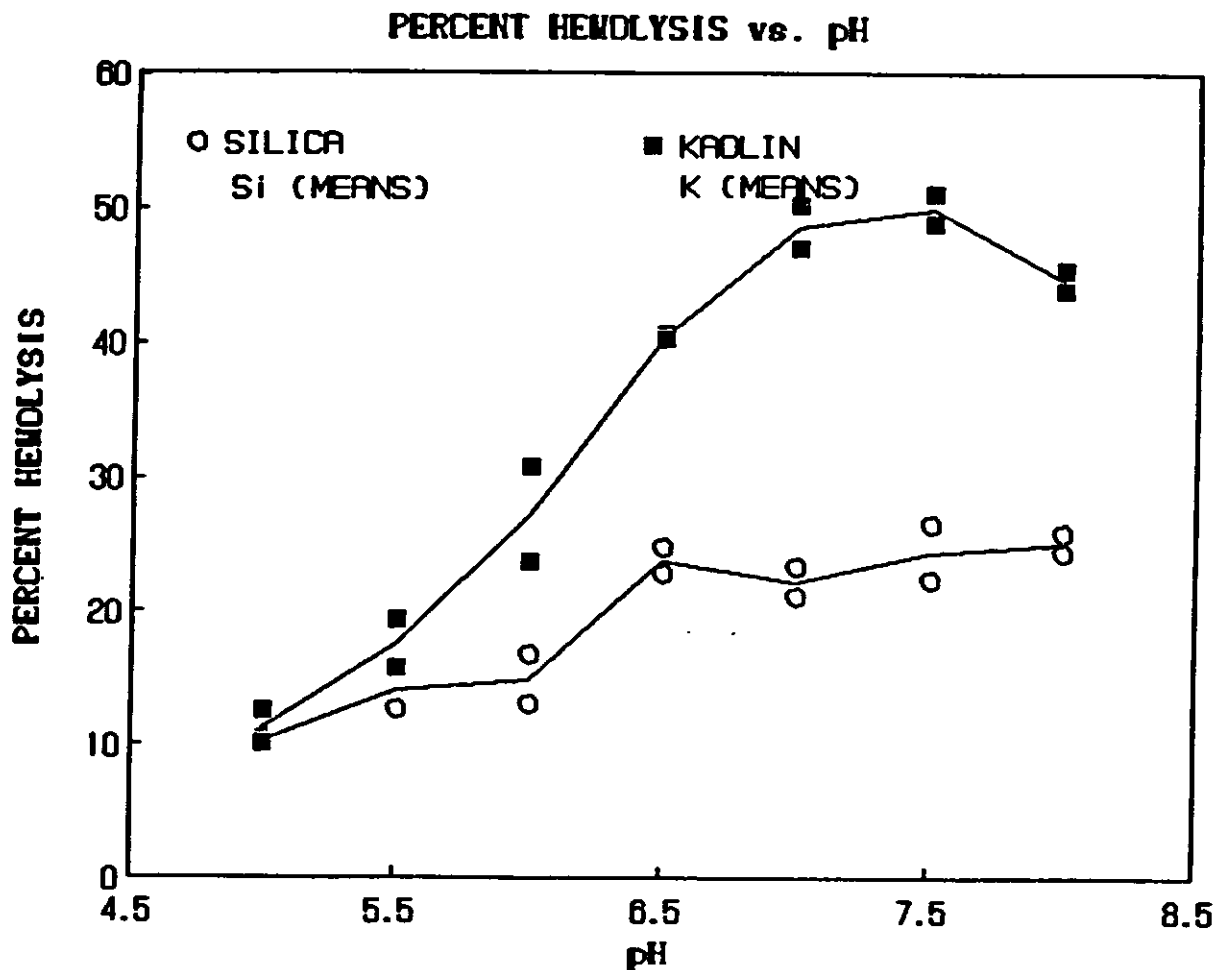
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DUSTS CAUSING PNEUMOCONIOSIS GENERATE ·OH RADICALS AND RED CELL HEMOLYSIS BY ACTING AS FENTON REAGENTS

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ABSTRACT

We hypothesized that dusts can produce toxic hydroxyl radicals (·OH) from lung H_2O_2. Amosite asbestos, silica (Minusil) and kaolin generated substantial ·OH, measured by conversion of 13 mM DMSO to methane with 1 mM H_2O_2 as substrate and 1 mM ascorbate (A) as reductant. Methane generation measured by gas chromatography was prevented by the ·OH scavenger dimethylthiourea (DMTU), or dust preincubation with the iron chelator transferrin (TRAN, 2 mg/ml).

	Methane ppm (mean±SEM)			
	−A	+A	A+DMTU	A+TRAN
Amosite 1 mg/ml	4±.1	1,008±53	4±.2	3±0
Minusil 1 mg/ml	3±2	1,032±46	0±0	1±0
Kaolin 1 mg/ml	3±.2	925±55	4±.3	23±3

Human red cell hemolysis was significantly antagonized by the anion channel blocker 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS, 1 mM), the hydroxyl radical scavenger n-propyl gallate (PG, 6 mM), the H_2O_2 scavenger catalase (CAT, 100 U/ml), or preincubation of dust with transferrin (TRAN, 2 mg/ml).

*p<.001 compared to untreated		%Hemolysis (mean±SEM)			
	Untreated	DIDS	PG	CAT	TRAN
Amosite 1 mg/ml	77±2	30±3.3*	29±.4*	32±1*	2±0*
Minusil 1 mg/ml	62±1	10±.5*	38±.5*	40±.6*	2±0*
Kaolin 1 mg/ml	50±.6	12±.1*	22±.2*	21±.3*	2±0*

Thus, hemolysis is caused by a dust-mediated Fenton reaction with superoxide anions (O_2-) and H_2O_2 from hemoglobin autooxidation as reducing agent and substrate.

No Paper provided.

EFFECT OF METAL ELEMENTS IN COAL DUSTS ON THE CYTOTOXICITY AND COAL WORKERS' PNEUMOCONIOSIS

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INTRODUCTION

It has been proved that many factors could influence the occurrence and progress of coal workers' pneumoconiosis (CWP). Among them, the composition of coal dust is regarded as an important factor contributed to the difference of CWP Prevalence in different coal mine areas. Therefore, the effects of metal elements in coal dust Were concerned gradually. Sorensen et al., stated that the concentration of some metals such as Fe, Pb, Cu, and Ni in the coal from a PA mine having a high incidence of CWP was higher than a sample of coal from a UT mine with low disease incidence.[13] Different contents of some trace metals in the lungs of coal miners from different collieries were also reported.[1] Furthermore. Christian et al. found that the effects of nickel (Ni) and Zinc (Zn) were very important to the pathogenicity of coal dust.

Coal is the principal energy resource in our country and millions of coal workers are being exposed to various kinds of coal dust. In some mines, the concentration of coal dust was tens, even hundreds times higher than the healthy standard (1O mg/m^3. It was also found that the incidence of CWP did not keep balance among different collieries.

In order to explore the factors which contributed to this difference, especially the effects of metal elements, we selected six coal dusts from six coal mines which were typical in our country. The research plan consisted of two parts, in the first one, both epidemiological investigation and laboratory experiments were designed, and in the second part, both *in vitro* and *in vivo* test were included.

MATERIALS AND METHODS

Part One

Animals: healthy, male Wistar rats (200 to 220g wt.) were supplied by the animals centre of our university, and were divided randomly into each group.

Coal dusts: six coal dusts were prepared by the grinding of coal samples which were collected from six coal mines in China. They were numbered 1, 2, 3, 4, 5, and 6, respectively. More than 90% of particles were less than 5 μm in diameter. The content of free silica (SiO$_2$) in coal dust was measured with the pyrophosphoric acid weight method, and

the contents of Zn and Ni in coal dust were determined by the method of Proton Induced X-ray Emission (PIXE).

Cytotoxicity test in vitro: the rat pulmonary alveolar macrophages (PAM) were collected with the method of Myrvik's.[11] The lungs were lavaged with D-Hanks' solution and the lavages fluid was centrifuged (1,500 rpm × 10 min). The concentration the cell suspension prepared with 1640 medium was 1 × 10^6 cells/ml. Each aliquot (2.0 ml) of this suspension was transferred to a culture vassel. The exposure concentration of coal dust was 100 μg/ml in the medium. After exposing to coal dust and heat-killed yeasts for 2.5 hours at 37.0°C, the phagocytic rate and phagocytic index of PAM were measured using a modification of the technique of Graham et al.[8] At 24 hours after exposure, the viability of PAM was determined by the trypan blue exclusion technique, and the necrotic rate of PAM was observed under the light microscope after stained with Giemsa dye. The surface morphology of PAM at 24 hours after exposure was observed with the scanning electronic microscope (SEM) and following three types of changes were described: (1) intense response: the morphology of cell surface changed apparently, the pseudopodia occurred actively, the vaculose in cell membrane and the indistinct cell border were also seen; (2) faint response: cells were round and the microvillion the cell surface were densy and well-distributed; (3) modiate response: lay between above two states.

Epidemiological investigation: according to the occupational history, coal workers (mainly blasting coal workers) were selected in six coal mines. CWP was diagnosed on the basis of the diagnostic standard published in 1983 in China, and the detection rate (numbers of CWP/100 examined coal workers × 100%) was used to indicate the prevalence condition of CWP in each coal mine.

Part Two

In this part, coal dust 2 which contained the highest content of Ni (called nickel-coal dust) was chosen to carry out following tests to explore the antagonistic effect of Zn further.

In vitro test: rat PAM were isolated and collected as described in the first part. Five groups were designed, as showed in Table II. The exposure concentration of nickel-coal dust in the medium was 1OO μg/ml. After exposing to the nickel-

coal dust and different dose of zinc chloride ($ZnCl_2$) for 24 hours at 37.0°C, the ATP levels in PAM were determined with a fluorescence fluorescase enzyme system.

In vivo test: 1.0 ml suspension which contained 50 mg nickel-coal dust and different dose of $ZnCl_2$ were intratracheally installated into rat lungs (see Table III). At fifteen days after installation, lungs were lavaged with D-Hanks' solution and PAM were isolated by centrifugation. The contents of Zn and k ions in the PAM were measured by the method of atom absorption spectrophotometer (AAS).

Statistical Treatment

The statistical significance of differences between each two groups was evaluated by t test $P < 0.05$ was accepted as the level of significance.

RESULTS AND DISCUSSION

The results of the first part of this study were showed in Table I.

1. The results of the cytotoxicity test *in vitro* showed that coal dust 2 was the most cytotoxic, and coal dust 3 was the least one. The sequence of the cytotoxicity was coal dust 2>6>5>4>1>3. That was consistent with the results of epidemiological investigation of CWP in six coal mines.

2. The contents of SiO_2 in six coal dusts were less than 5% except coal dust 2, and it was found that they were not correlative to the cytotoxicity of coal dust and the detection rate of CWP in six coal mines. In recent years, many results from both experimental study and epidemiological investigation proposed that if SiO_2 content in coal dust was less than 5% or 10%, no evidence could be found about its influence on the cytotoxicity and the prevalence of CWP.[9,12,14] The effect of SiO_2 in coal dust is influenced by many interfering factors, and it was regarded that some metal elements may play an important role in this process.[10]

3. Coal dust 2 had the most cytotoxic effect on PAM with the highest content of Ni, while coal dust 3 contained the highest content of Zn and correspondingly, its cytotoxicity was the least and the detection rate of CWP in coal dust 3 was the lowest. So it was concluded that the contents of Zn and Ni in coal dust correlated closely with the cytotoxicity and the detection rate of CWP. Christian et al. found that the leachates from PA coal sample had more cytotoxic effect *in vitro* than that from UT coal sample, and the PA leachates contained more Ni and less Zn than the UT one.[2,3] It is widely known that Zn is one of the essential trace metals and is very important to maintain the structures and functions of living cells.[4] Ni has been proved to be cytotoxic both *in vitro* and *vivo*.[8,16] Therefore, further studies on the

Table I

Results of the Analyses and the Cytotoxicity Test of Coal Dusts
and the Epidemiological Investigation in 6 Coal Mines

Source of Coal Dusts	Composition			Cytotoxicity						Investigation
	SiO_2 (%)	N_i (ppm)	Z_n (ppm)	Viability (%)	Phagocytic Rate(%)	Index	Necrotic Rate(%)	Surface Response	Harmful Sequence	Detection Rate of CWP
2	5.45	155.5	87.8	75.7	30.2	0.44	23.8	intense	5.4	23.60
6	2.23	72.3	82.5	73.8	42.2	0.68	20.0	intense	4.6	4.68
5	1.55	39.4	78.8	75.2	47.7	0.83	20.3	mediate	4.2	2.83
4	1.55	38.6	73.6	73.6	46.2	0.63	18.2	mediate	3.6	2.60
1	2.80	108.5	75.4	76.0	48.7	0.72	16.6	mediate	1.8	1.87
3	3.65	129.4	342.3	80.0	58.2	0.86	17.3	faint	1.4	0.90

Table II

ATP Levels in Rat PAM After Exposing to the Nickel-Coal Dust and Zn for 24 Hours *in vitro*

Groups	ATP levels $(\times 10^3 \text{umol}/4 \times 10^6 \text{PAM})$	P Value
1. nickel-coal dust(100ug/ml) ZnCl$_2$(0.2ug/ml)	3.883±1.270 (n=11)	(1 and 3)*
		(2 and 3)*
2. nickel-coal dust(100ug/ml) ZnCl$_2$(0.4ug/ml)	3.239±1.654 (n=9)	(3 and 4)*
		(1 and 5)*
3. nickel-coal dust(100ug/ml)	0.756±0.331 (n=9)	
		(2 and5)*
4. ZnCl$_2$(0.2ug/ml)	3.194±0.921	(3 and 5)*
5. physiological saline (control)	1.575±0.525 (n=12)	(4 and5)*

*P<0.05

Table III

The Content of K and Zn Ions in Rat BAM in 15 Days After Intratracheal Instillation of Nickel-Coal Dust and ZnCl$_2$

Groups	$K(\text{Mg}/10^{10}\text{PAM})$ $\bar{x} \pm s$	P value	$Zn(\text{mg}/10^{10}\text{PAM})$ $\bar{x} \pm s$	P value
1. nickel-coal dust (50mg/ml) ZnCl$_2$(0.1mg/ml)	26.35±9.77 (n=5)	(1 and 3)* (1 and 5)*	1.12±0.57 (n=5)	(1 and 3)* (1 and 5)*
2. nickel-coal dust(50mg/ml) ZnCl$_2$(0.2mg/ml	23.41±6.04 (n=7)	(2 and 5)* (5 and 6)*	0.93±0.28 (n=7)	(2 and 4)* (2 and 5)*
3. ZnCl$_2$(0.1mg/ml)	17.42±5.07 (n=5)		0.52±0.30 (n=5)	
4. ZnCl$_2$(0.2mg/ml)	18.87±6.60 (n=6)		1.64±0.77 (n=6)	
5. nickel-coal dust(50mg/ml)	11.76±1.53 (n=7)		0.38±0.06 (n=4)	
6. physiological saline (control)	29.64±14.09 (n=7)		0.62±0.33 (n=6)	

*P<0.05

effects of Zn and Ni on the cytotoxicity of coal dust and on the etiology of CWP are needed.

4. Fisher et al. reported that Zn was antagonistic to the cytotoxicity of Ni *in vitro*.[5] Waalkes et al. also suggested that the pre-treatment with zinc acetate could increase the resistance of rat to the toxicity of nickel acetate.[15] In this results, coal dust 3 contained the highest content of Zn and high content of Ni, but its cytotoxicity was the least. Therefore, it is possible that Zn is antagonistic to the cytotoxicity of Ni in coal dust.

In order to explore the antagonistic effect of Zn toward the cytotoxicity of Ni further, we carried out the second part of this study.

1. The effects of $ZnCl_2$ and nickel-coal dust on ATP levels in PAM *in vitro* (Table II). The ATP levels in nickel-coal dust group were significantly lower than that of control group ($p < 0.05$). However, in the nickel-coal dust and $ZnCl_2$ mixed group the ATP levels increased remarkably comparing with that of nickel-coal dust group ($p < 0.05$). It is already proved that ATP is the direct energy resource for cell activities and the reducing of ATP levels in cell is the sensitive index for reflecting the damage of cell structure and function.[6] In this test $ZnCl_2$ increasing the ATP levels in PAM after exposing to nickel-coal dust *in vitro* indicated that appropriate dose of $ZnCl_2$ could antagonize the cytotoxicity of nickel-coal dust.

2. The effects of $ZnCl_2$ and nickel-coal dust on the contents of K ions in PAM *in vivo* (Table III). At fifteen days after instillation of nickel-coal dust, the content of K in PAM in rat BAL was significantly lower than that of control group ($p < 0.05$). However, when certain dose of $ZnCl_2$ was instillated with nickel-coal dust, the K content in PAM increased notedly ($P < 0.05$). The results in Table III also revealed that the content of Zn in PAM in nickel-coal dust and $ZnCl_2$ mixed group was significantly higher than that of nickel-coal dust group. The contents of ions in cell are correlated closely with the permeability of cell membrane which changed significantly in the early stage of cell damage.[7] So the results in this test proved that $ZnCl_2$ could reduce the toxicity of nickel-coal dust to cell membrane *in vivo*.

The prevalence of CWP changed significantly in different coal mine areas in China. The understanding of the relationship between the Zn and Ni content and the pathogenecity of coal dusts will provide a new basis for the explanation of this difference and for getting deeper understanding of the etiology of CWP, and further, for paying more attention to the prevention of CWP in high prevalence coal mines.

In summary, the results of this study proposed that the cytotoxicity of coal dust and the detection rate of CWP did not correlate with the content of SiO_2 ($<5\%$), but correlated closely with the contents of metal elements in coal dust. The cytotoxicity and the detection rate of CWP were higher in coal dust with high content of Ni; on contrast, the cytotoxicity and the detection rate of CWP were lower in coal dust which contained high content of Zn. It was found further that appropriate dose of $ZnCl_2$ was antagonistic to the cytotoxicity of nickel-coal dust both *in vitro* and in vivo.

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DETECTION OF HYDROXYL RADICALS IN AQUEOUS SUSPENSIONS OF FRESH SILICA DUST AND ITS IMPLICATION TO LIPID PEROXIDATION IN SILICOSIS

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INTRODUCTION

Despite considerable effort over the years, the mechanism by which the quartz particles exert their toxic action on cells and the processes by which these actions progress to fibrosis are still not fully understood.[1,2] It is generally thought, nevertheless, that the interaction of the quartz particles with the cell membranes is the starting point of the silicotic process.[3] We felt that the mechanism of the membrane damage by quartz might involve oxygenated free radicals because (a) a suspension of quartz particles in contact with alveolar macrophages has been reported[4,5] to initiate an enhancement of lipid peroxidation, defined broadly as the oxidative deterioration of polyunsaturated components of lipids, and (b) hydroxyl (•OH) radicals are known to be capable of peroxidation by abstracting hydrogen atoms from cell-membrane Lipids[6] and initiating lipid peroxidation in lysosomal membranes.[7] Moreover it is known that exposure of cell membranes, fatty acids and unsaturated food oils to ionizing radiation, which generates •OH radicals, causes rapid peroxidation.[6] Earlier studies of the aqueous chemistry of quartz suspensions have reported detection of H_2O_2,[8] implicating the formation of •OH radicals as transient species, but, we are not aware of any report of the detection of •OH radicals in quartz suspensions and this provided the motivation for the present undertaking. Since it is known that, because of their high reactivity (hence short life time) in aqueous media, the •OH radicals cannot be detected via electron spin resonance (ESR) directly,[9,10] we have used ESR combined with the spin-trap methodology[9] for studying the •OH formation.

MATERIALS AND METHODOLOGY

Crystalline silica with particle sizes of 0.2 to 2.5 mm was obtained from the Generic Respirable Dust Technology Center, Pennsylvania State University, University Park, Pennsylvania. Particles in the range of smaller than 25 microns were produced by hand grinding in air, using an agate mortar and pestle because of the structural similarity of agate to that of quartz. Also a rather mixed particle size, rather than a specific range, was employed, with a view to

roughly approximate the random particle-size distribution in the mining atmosphere. ESR spectra were obtained at X-band (~9.7 GHz) using a Bruker ER 200D ESR spectrometer. For accurate measurements of the g-values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, model ER035M) and the microwave frequency was measured with a frequency counter (Hewlett-Packard, Model 5340A). 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was purchased from Aldrich and used without further purification, since very weak or no ESR signals were obtained from the purchased sample when used by itself. If necessary the background signals were subtracted from those related to quartz by using an Aspect 2000 microcomputer.

RESULTS

Some typical results of the ESR spin-trapping studies are shown in Figure 1. We found that a 0.1 M aqueous solution of the spin-trap DMPO alone, with unground particles or with TiO_2 powder did not give a detectable ESR spectrum. TiO_2 was used as a control because it is known not to be fibrogenic[11] and has a structure resembling quartz (SiO_2). However, when quartz was ground in a 0.1 M DMPO (aqueous) solution or when ground quartz particles were mixed with 0.1 M DMPO (aqueous) solution, an ESR spectrum (g = 2.0059), consisting of a 1:2:2:1 quartet pattern with a splitting of 14.9 G, was observed (Figure 1a). Based on earlier work,[9,12,13] this spectrum was considered to be due to the DMPO-OH adduct.

Two further tests were made to identify the spectrum. First, the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + •OH + OH^-$),[14] known to produce •OH radicals, was used as a standard. The ESR spin-adduct spectrum obtained by mixing 0.085 M H_2O_2, 0.0165 M $FeSO_4$ and 0.1 M DMPO was the same as that of Figure 1a (obtained with ground quartz), thus attesting to the formation of the •OH radical in the quartz suspension.

As a second, confirmatory, test of the •OH radical formation, spin-trap ESR experiments were performed in which ethanol was added as a secondary trap. It has been shown[10,15] that in the presence of ethanol, the intensity of the DMPO-OH signal decreases, because ethanol scavenges some of the •OH radicals to form the ethanolyl radicals[12] which react with DMPO to give the spin-adduct DMPO-
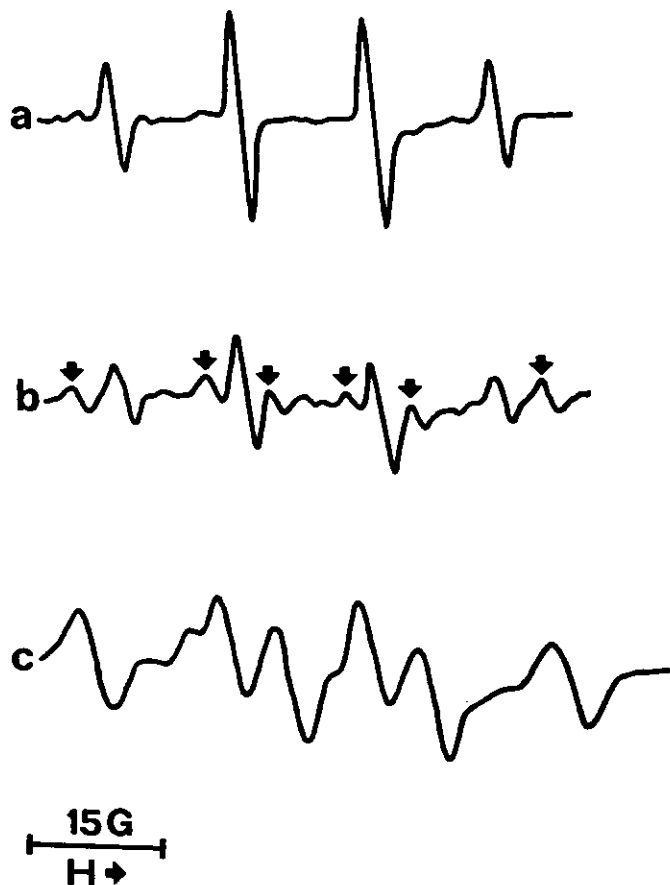
Figure 1. ESR spectra recorded 2 minutes after mixing 100 mM DMPO aqueous solution with (a) freshly ground quartz particles; (b) same as (a) but with 30% ethanol added; (c) same as (a) but with excess ethanol. Receiver gain, 5×10^5; modulation amplitude, 2 G; scan time, 100 seconds; field, 3460 ± 75 G.

$CHOHCH_3$. The ESR spectrum of the spin-adduct DMPO-$CHOHCH_3$ was indeed observed as indicated by arrows in Figure 1b (for 30% ethanol) and more clearly in Figure 1c, obtained in the presence of excess ethanol, thus confirming the •OH radical formation in the quartz suspension.

The intensity of the •OH radical adduct signal increases with the amount of grinding (Table I), thus showing that the •OH radical generation is related to some surface property of the freshly made dust, most likely the silicon-oxygen radical sites known to form on grinding.[16-21] Additional spin-trap measurements as a function of the time of "aging" of the dust after grinding showed that freshly generated quartz dust produces more •OH radicals than that which had been stored in air after grinding (Table II). In order to characterize the kinetics of the dust's aging on its ability to generate •OH radicals, attempts were made to determine whether the reaction was of the first order (a straight line plot for log (con.) vs. time) or second order (straight line plot for (con.)$^{-1}$ vs. time). The analysis indicated the kinetics to be neither first nor second order but of a more complex nature. Thus while it was not possible to define a unique half-life for the decrease in the •OH radical producing potential of the quartz dust on storage after grinding, we note that, on the average, freshly ground quartz dust loses its •OH-generating capacity to about 50% in approximately 1 day.

DISCUSSION

It is clear that the breakage of quartz crystals implies the homolysis of Si-O-Si bonds and the generation of silicon-based radicals ($\equiv$Si • , $\equiv$SiO • , $\equiv$SiOO •)[8,16-21] We have indeed verified that Si • and SiO •-type of radicals are produced by grinding in air, and that the radicals decay as a function of time when the dust is stored in air after grinding,[17] with a half-life of about one and a half day. Earlier workers[16] have reported that the crushing of quartz under vacuum produces SiO •-type radicals whose concentration decreases drastically on exposure to atmosphere with a half-life of about 30 hours.

Table I

Dependence of the ESR Intensity of the DMPO-OH Adduct (i.e., •OH production) on Size (grinding times) of Quartz Particles

Grinding times (minutes)	Relative ESR intensity
0.0	0.0
0.5	0.3 ± 0.3
1.0	1.1 ± 0.6
2.0	2.3 ± 0.7
4.0	3.4 ± 0.8
10.0	5.1 ± 1.2

<div align="center">

Table II

Dependence of the ESR Intensity of the DMPO-OH Adduct
(i.e., •OH production) on the "Aging" of Quartz Dust

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Time after grinding	Relative ESR intensity
5 minutes	5.2 ± 0.8
1 day	3.2 ± 0.8
2 days	1.9 ± 0.7
3 days	1.7 ± 0.8
4 days	1.3 ± 0.7

Following Kalbanev et al.,[8] we suggest that the •OH radical production might involve the following steps:[8]

$$\equiv SiO \bullet + H_2O \rightarrow \equiv SiOH + \bullet OH \qquad (a)$$
$$\equiv SiO \bullet + \bullet OH \rightarrow \equiv SiOOH \qquad (b)$$

Kalbanev et al. have also suggested[8] that the hydrolysis of SiOOH could produce H_2O_2, according to reaction (c):

$$\equiv SiOOH + H_2O \rightarrow \equiv SiO \bullet + H_2O_2 \qquad (c)$$

The yield of H_2O_2, depending on the pH and the temperature of hydrolysis, was reported to be as high as 10^{18} molecules/g quartz particles,[8] enough to be measured by the standard method of wet analytical chemistry, the MnO_4^- reduction:

$$2MnO_4- + 5H_2O_2 + 4H^+ \rightarrow 5O_2 + 2Mn^{2+} + 8H_2O \quad (d)$$
(pink) (colorless)
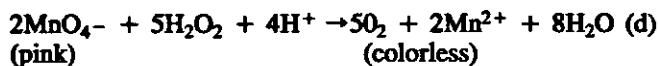
We verified the reducing activity of our quartz particle suspension with respect to $KMnO_4$, although the H_2O_2 yield was measured to be about an order of magnitude smaller for our samples than those of Kalbanev et al.[8] Thus experiments were carried out to examine whether the •OH radical formation was through the Fenton reaction,[14] the Fe^{2+} possibly being a trace impurity. The experiments consisted of spin-trap measurements in which diethylenetriaminepenta-acetic acid (DETAPAC) (0.03 − 3.0 mM) was used as a strong metal-ion chelate. it is known that the iron-DETAPAC complex formation stops the •OH generation from H_2O_2.[9] On adding DETAPAC the •OH radical-related ESR signals showed no variation in either the g value or the observed splitting pattern but only a small (20 %) decrease in intensity even at the high DETAPAC (3 mM) levels. This result, together with the dependence of the •OH radical concentration on time and surface freshness, suggests that the Fenton-type mechanism is not a major contributor to the •OH radical generation in our quartz suspensions.

After this work was essentially complete,[17-20] two significant reports have appeared. In the first, Fubini et al.,[21] have report the formation of Si • , SiO • , and SiO_2 • radicals on quartz particles ground in air, without contact with water. They suggest a possible role of these radicals (or some other surface property) in the mechanism of quartz-induced fibrosis. Our ESR results on the silicon-based radicals,[18,19] agree with Fubini's.[21] We further show that the concentration of the Silicon-based radicals is time dependent[17] and that their reaction with aqueous media generates (perhaps)

an even more potent species,[18-20] the •OH radicals. The second paper, by Gulumian and Van Wyk,[22] reported the detection of •OH formation in aqueous suspension of glass and quartz fibres *in the presence* of H_2O_2, and the scavenging of the generated •OH radicals by the prophylactic agent (polymer) polyvinylpyridine N-oxide (PVPNO). They suggest that the therapeutic efficacy of PVPNO in silicosis might be related to its scavenging effects on •OH radicals. Our work shows that the grinding process itself causes the quartz surface to be a source of •OH radicals in aqueous media and that this activity decreases with the aging of the dusts.[19,20] This higher toxicity of fresh dust must be taken into consideration in the future *in vitro* or *in vivo* laboratory (e.g., animal exposure) studies of quartz and related mineral dusts.

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