# 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 phospbolipid 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

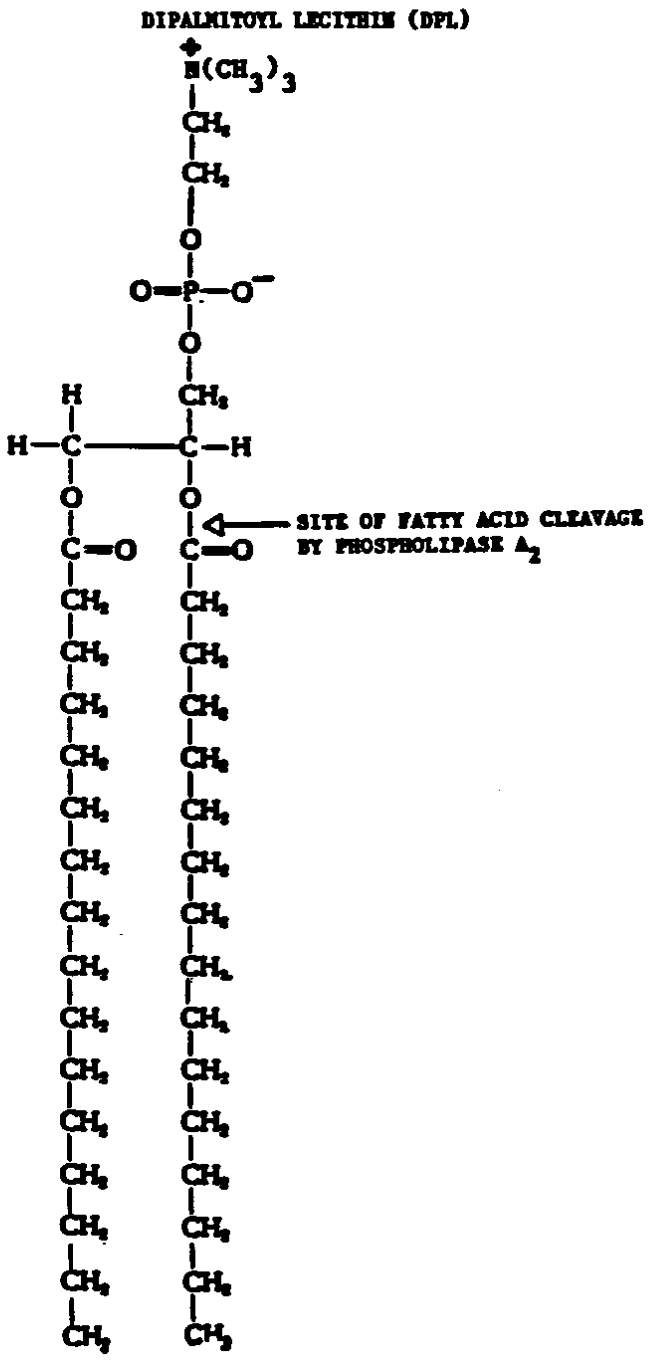


Figure 1. Structural formula of phosphatidyl choline molecule.

quartz is about 4 $m^2/g$ for the less than 5 micron size, and the kaolin is about 13 $m^2/g$ 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-

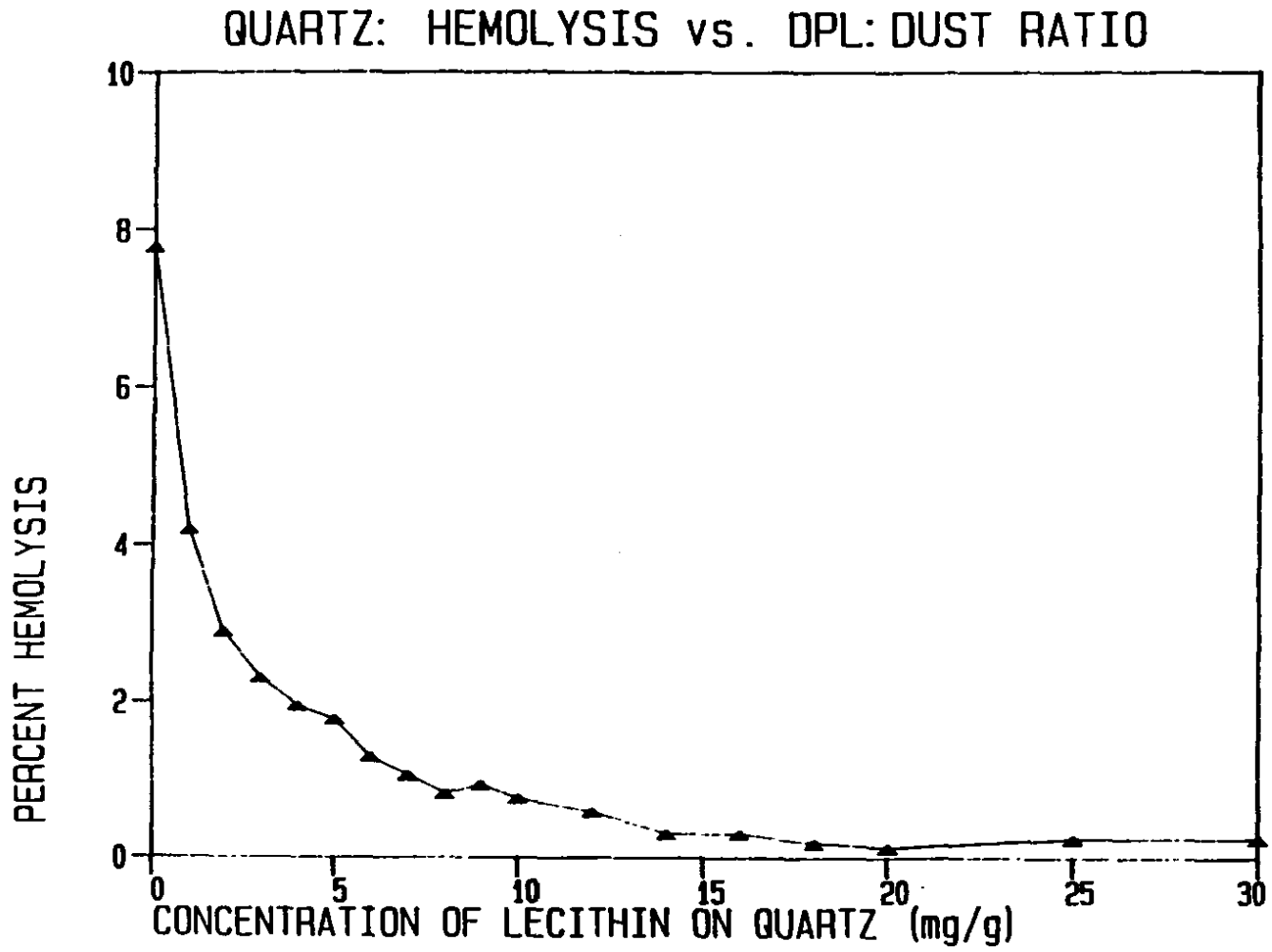


Figure 2. Percent hemolysis vs. DPL: quartz ratio.

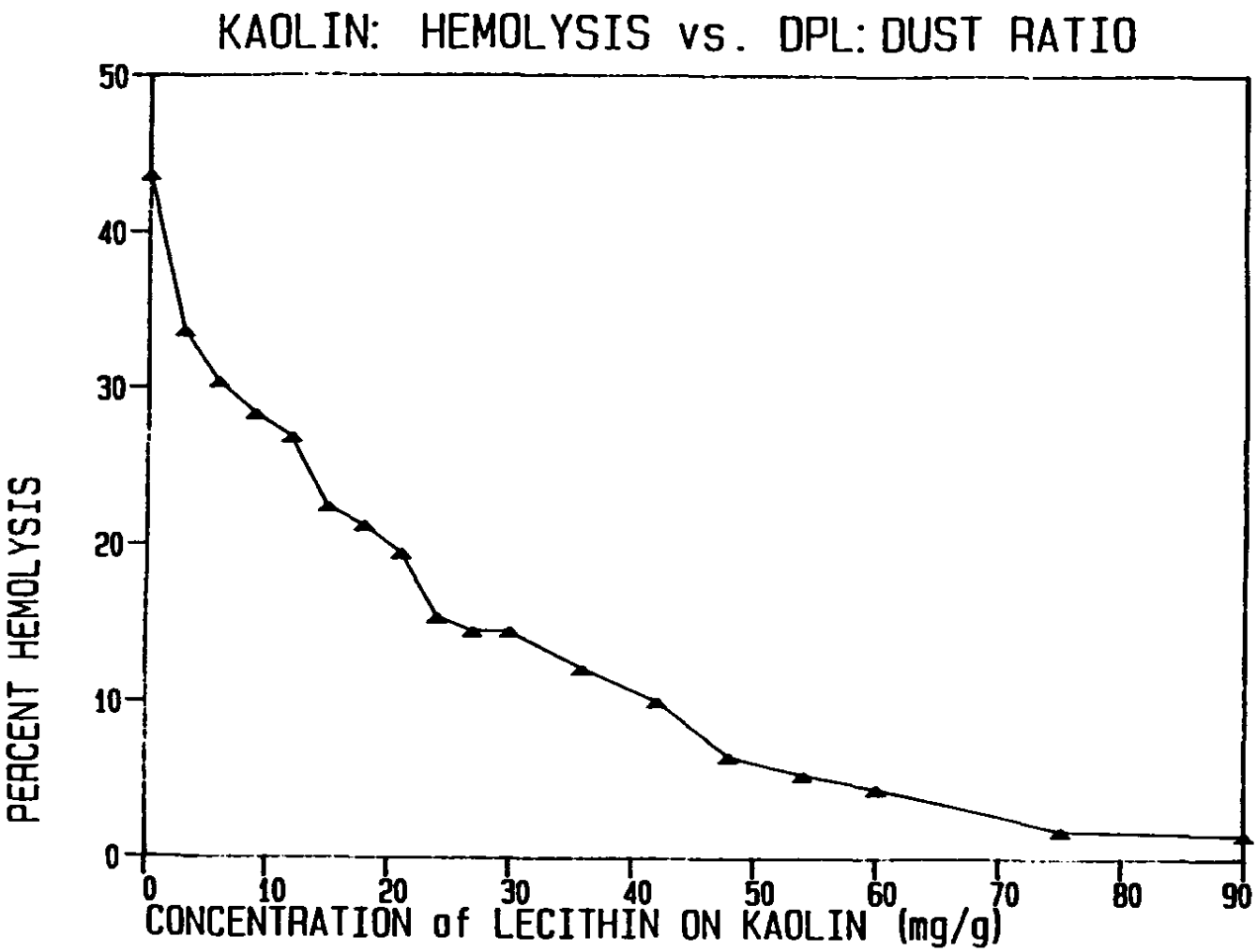


Figure 3. Percent hemolysis vs. DPL: kaolin ratio.

## 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.

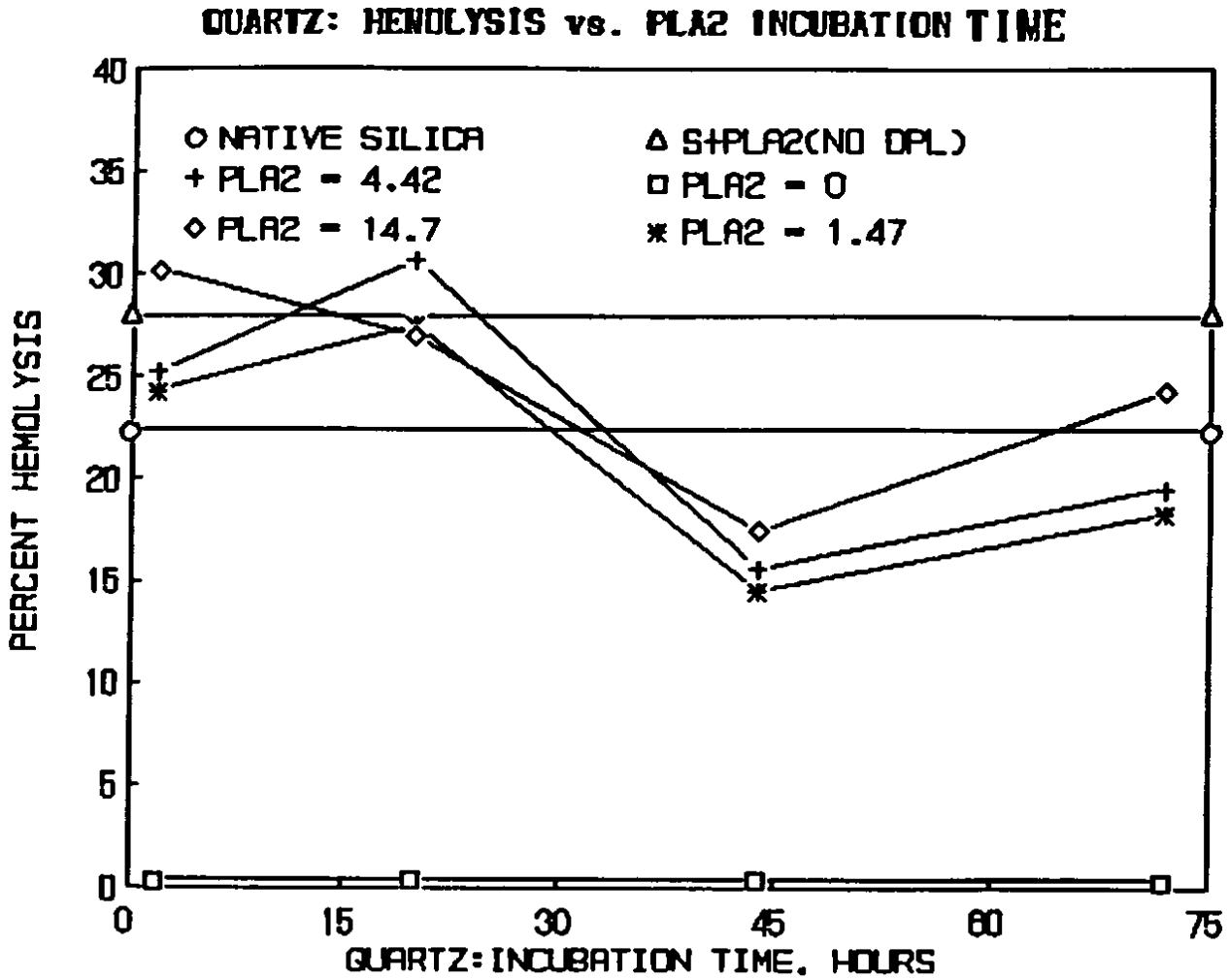


Figure 6. Hemolysis vs. time for DPL-coated quartz treated with phospholipase $A_2$.

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.

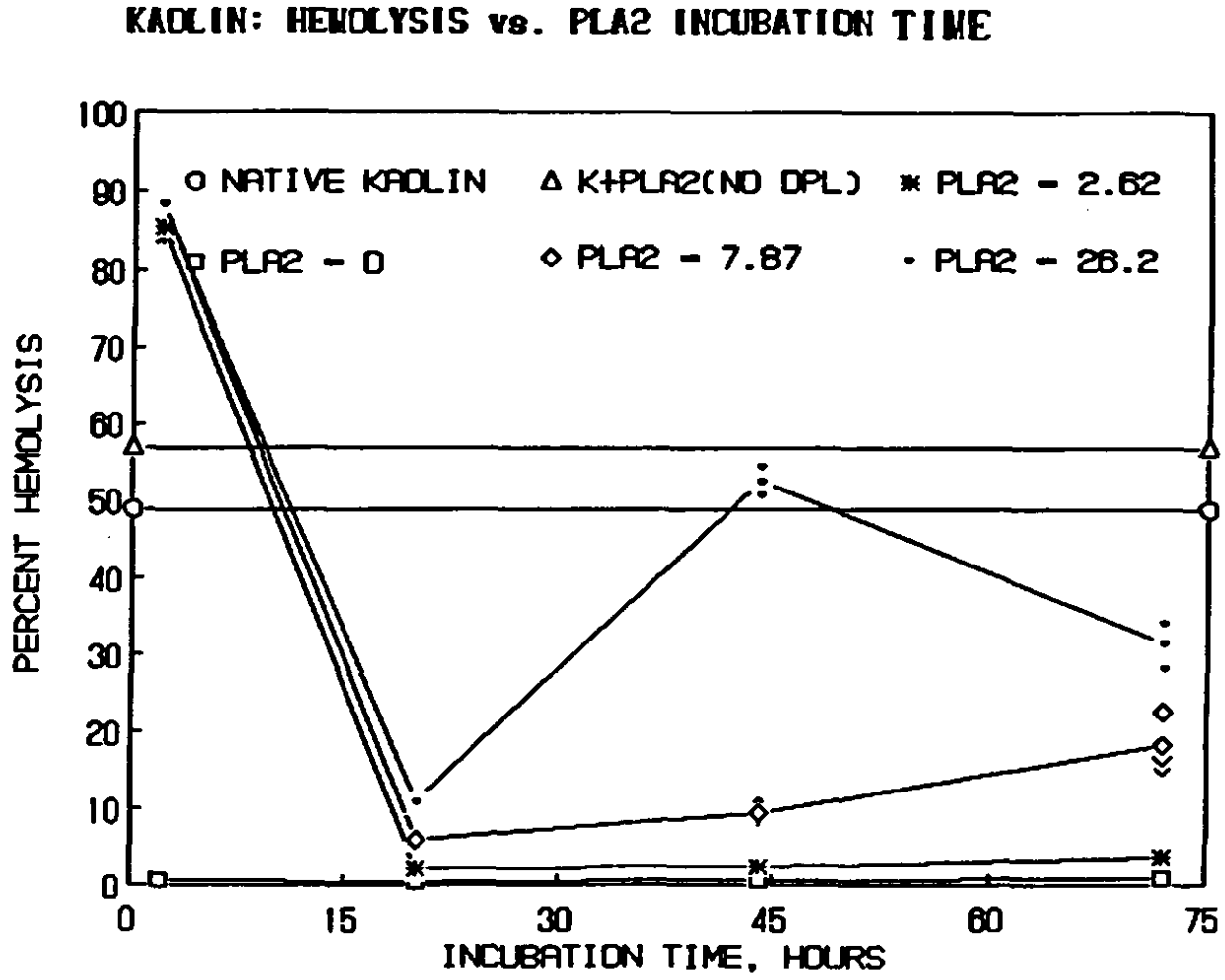


Figure 7. Hemolysis vs. time for DPL-coated kaolin treated with phospholipase $A_2$.

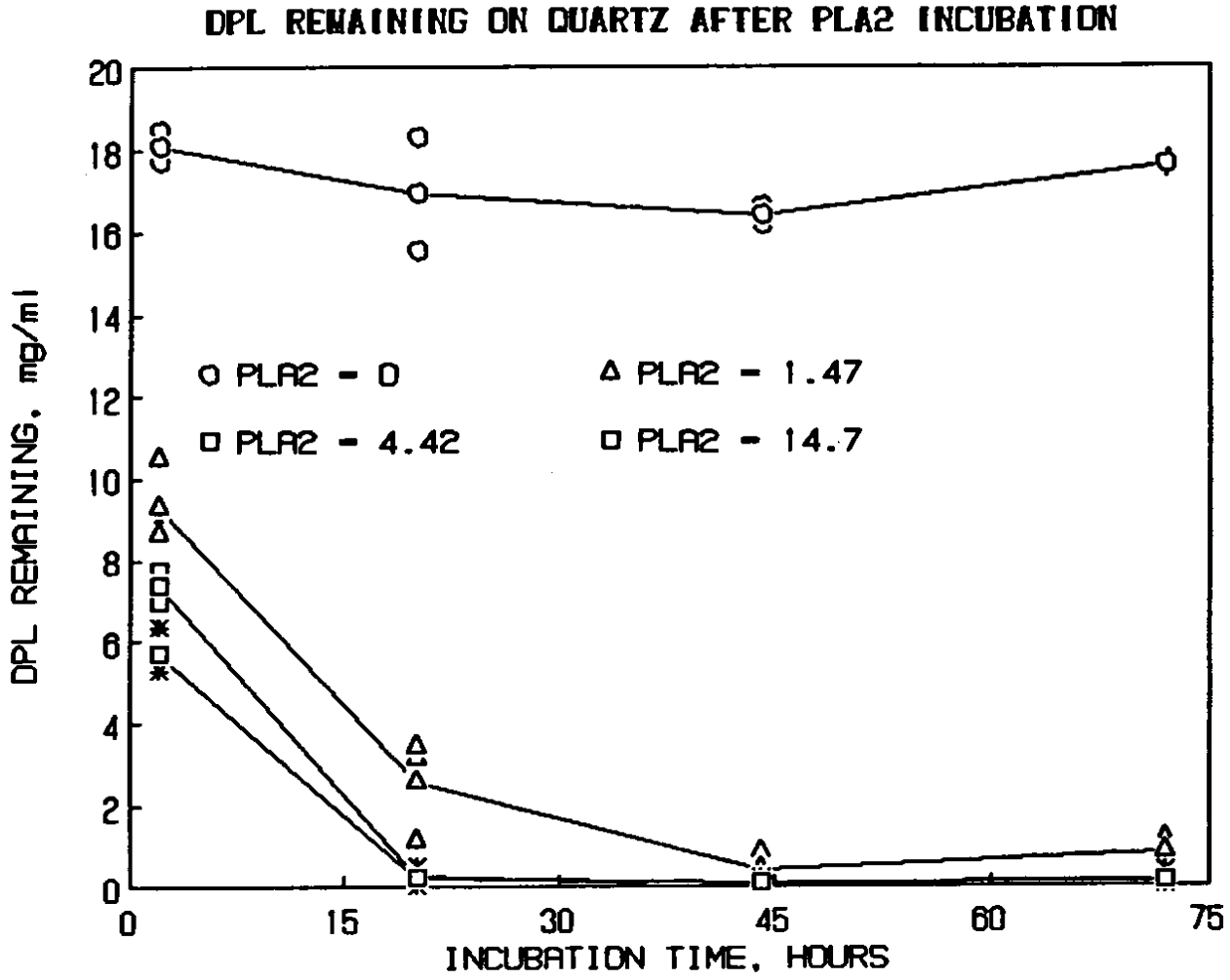


Figure 8. Lysolecithin retained on quartz after $PLA_2$ incubation vs. time.

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 $A_2$.

ACKNOWLEDGMENT: This research has been supported by the Department of the Interior's Mineral Institute Program administered by the Bureau of Mines through the Generic Mineral Technology Center for Respirable Dust under Grant number G1135142.

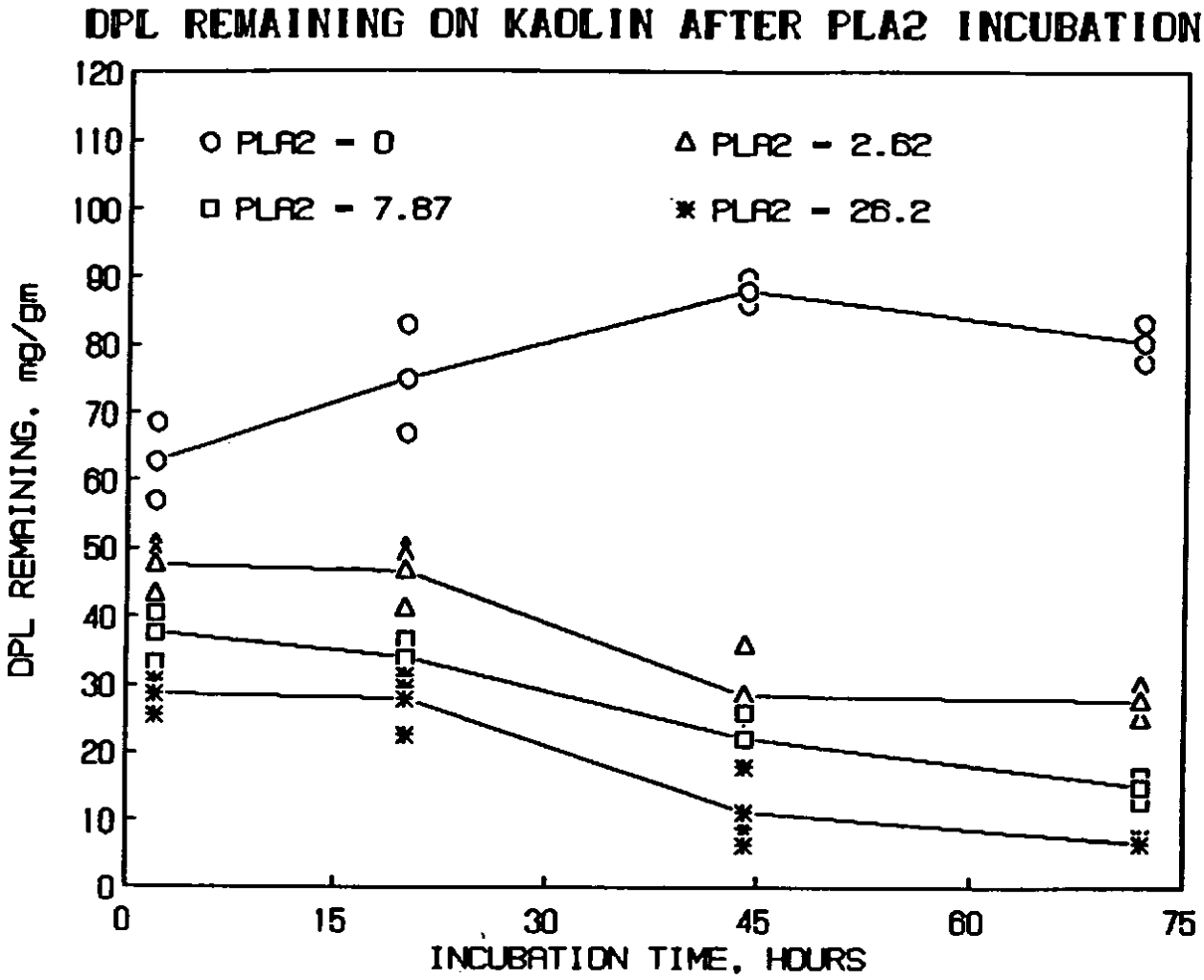


Figure 9. Lysolecithin retained on kaolin after $PLA_2$ incubation vs. time.

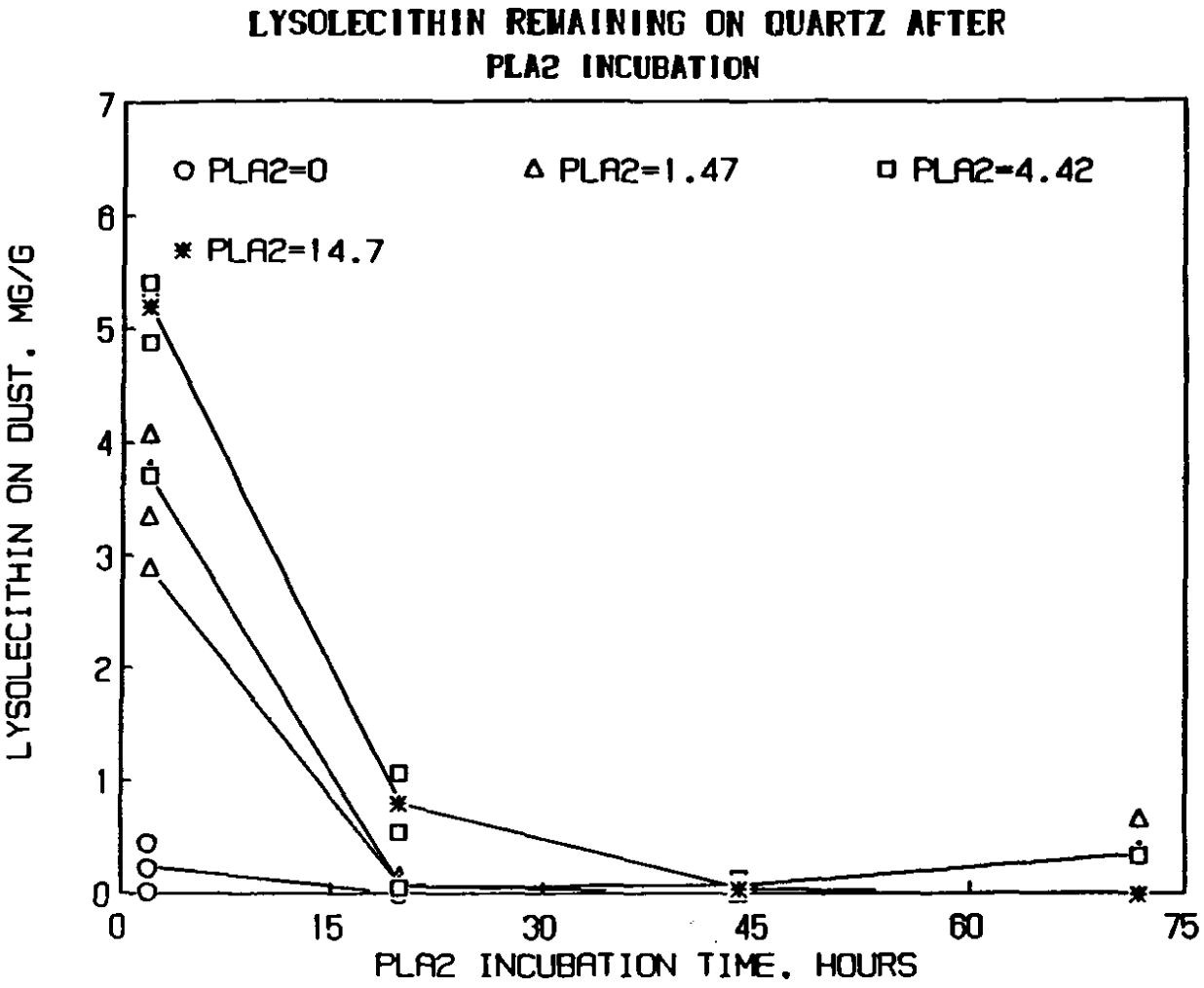


Figure 10. DPL retained on quartz after $PLA_2$ incubation vs. time.

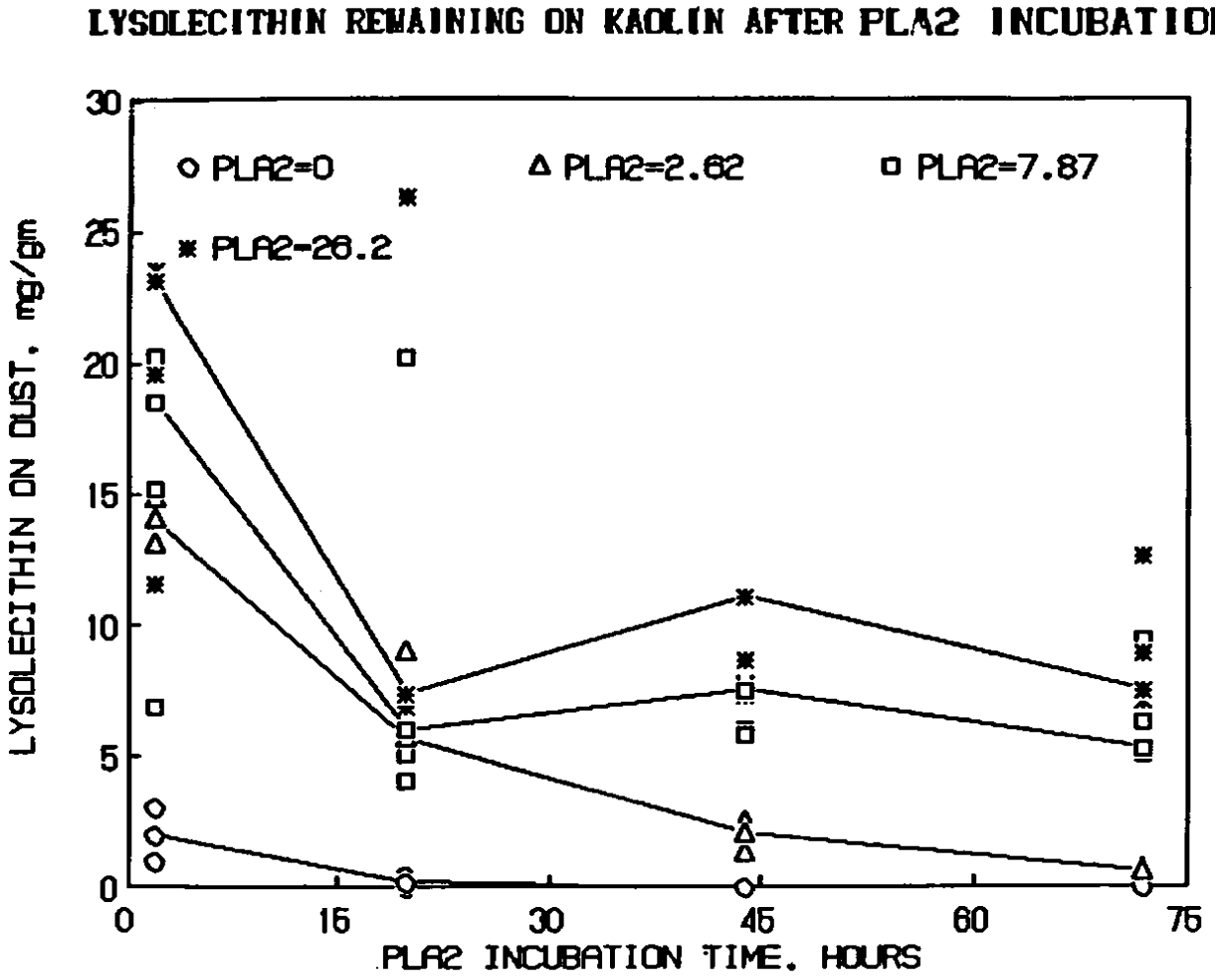


Figure 11. DPL retained on kaolin after $PLA_2$ incubation vs. time.

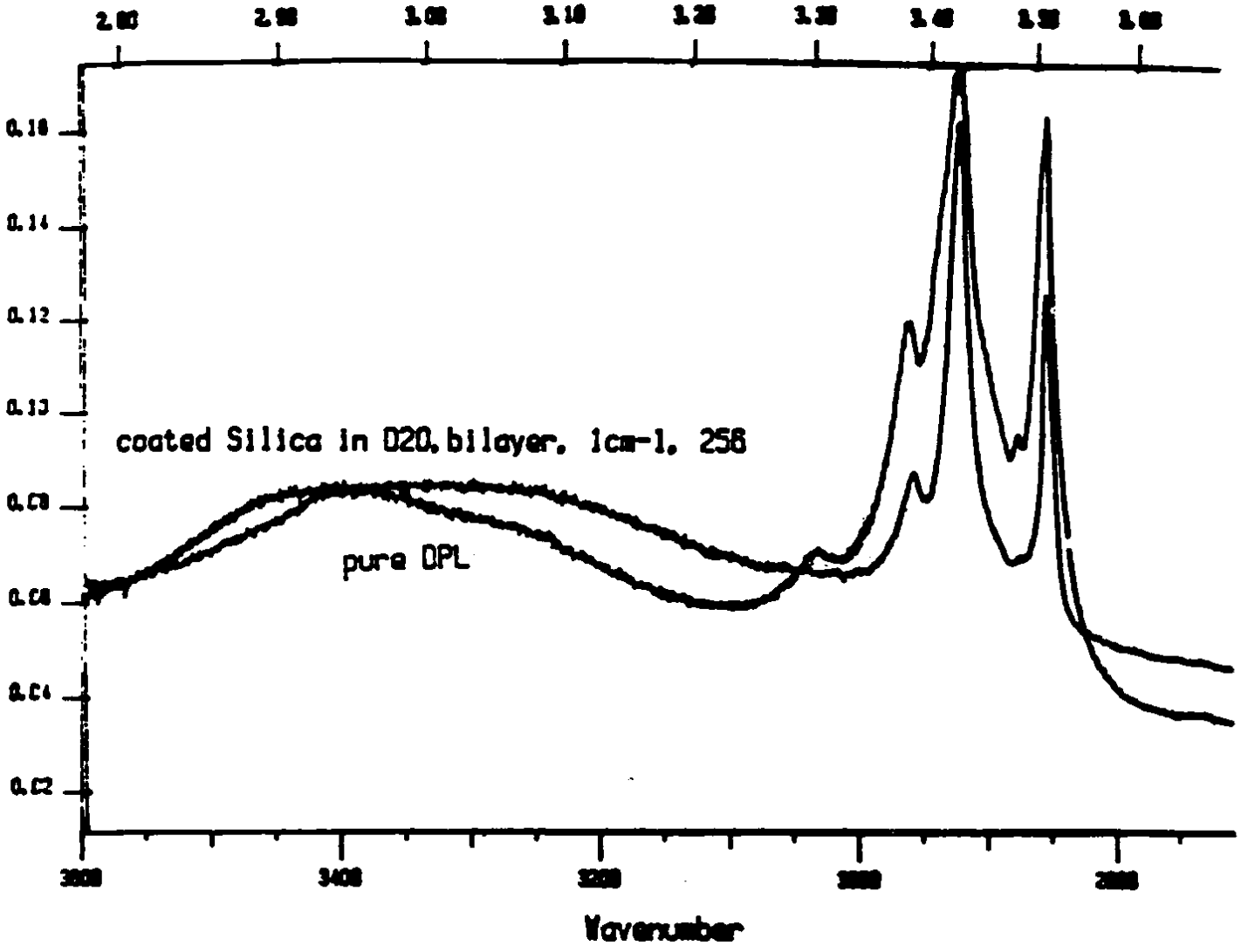


Figure 12. FTIR spectra of DPL-coated quartz and DPL only, 2750–3600 $cm^{-1}$.

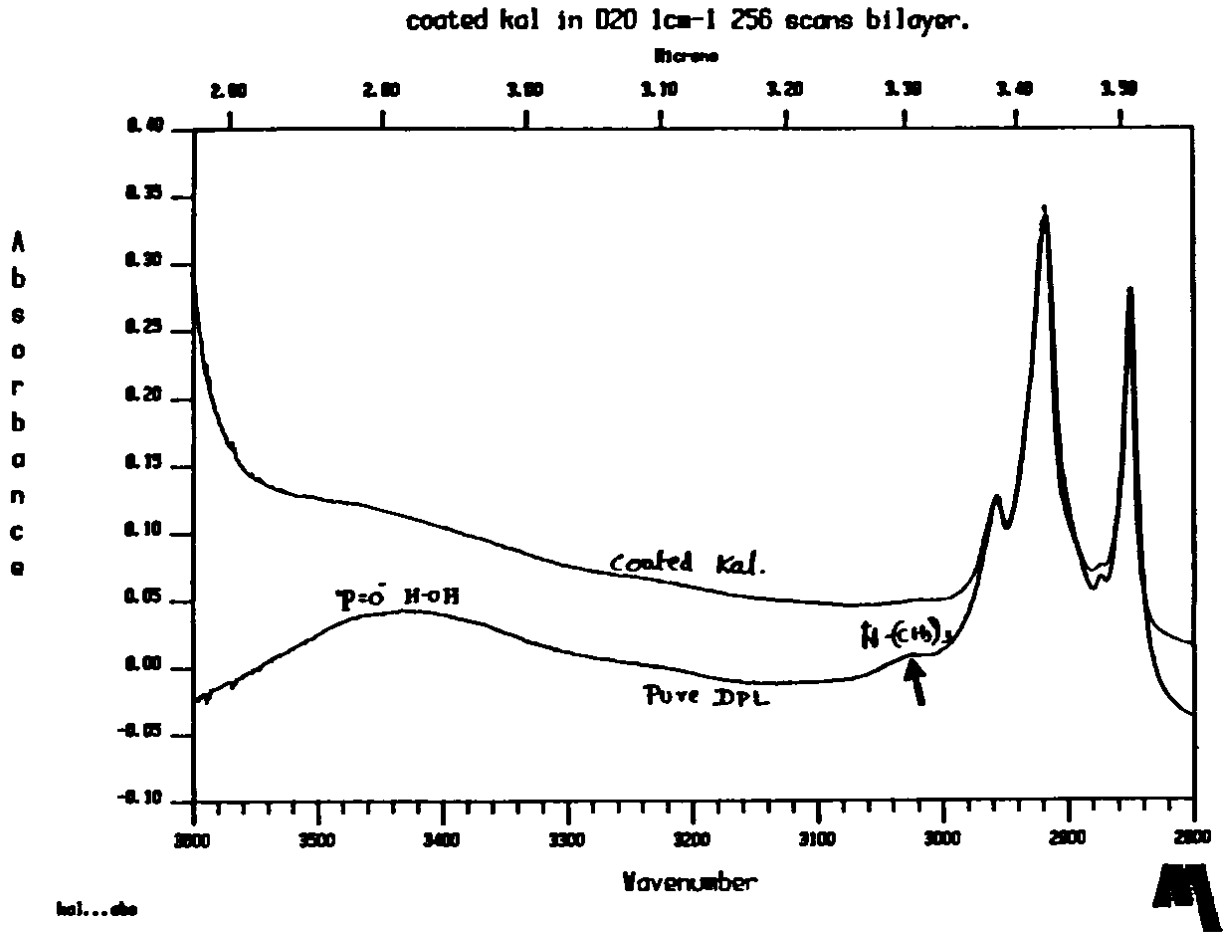


Figure 13. FTIR spectra of DPL-coated kaolin and DPL only, 2800–3600 $cm^{-1}$.

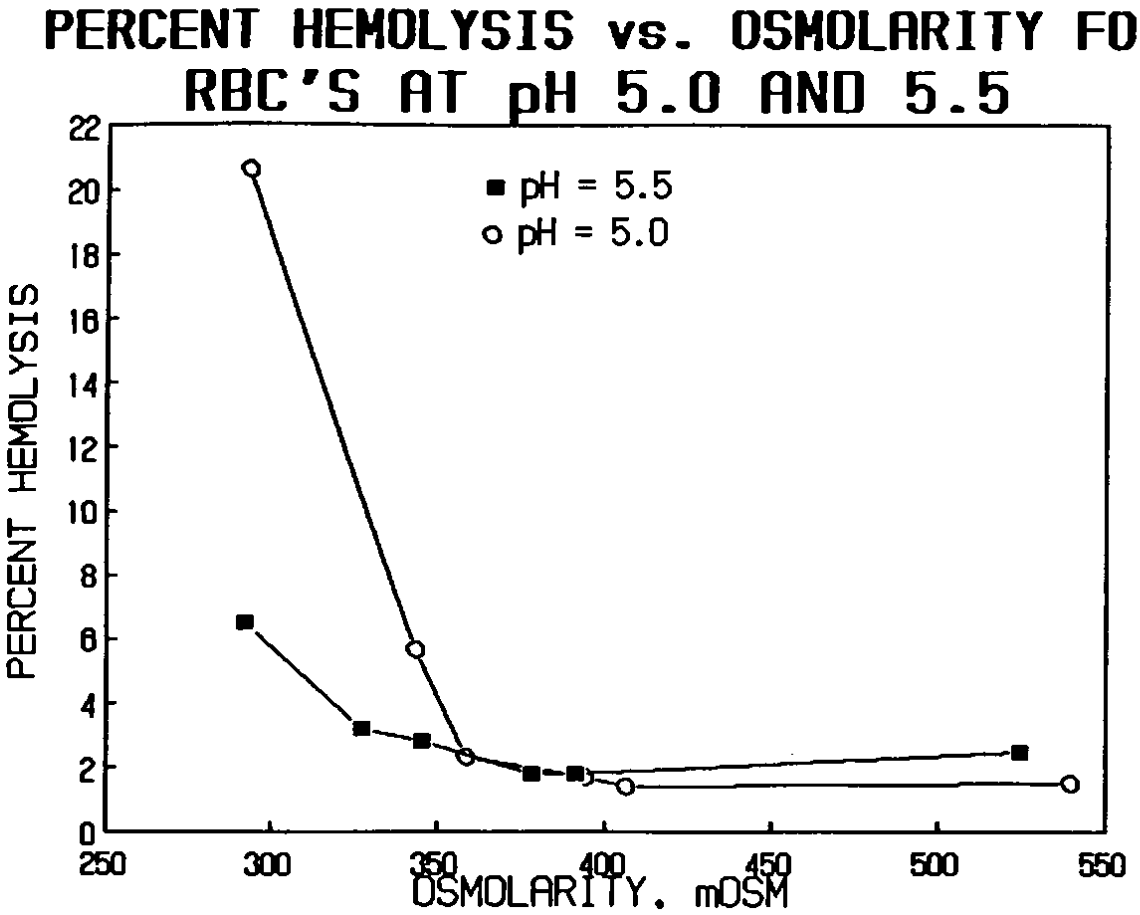


Figure 14. Percent hemolysis vs. osmolarity at pH 5 and pH 5.5.

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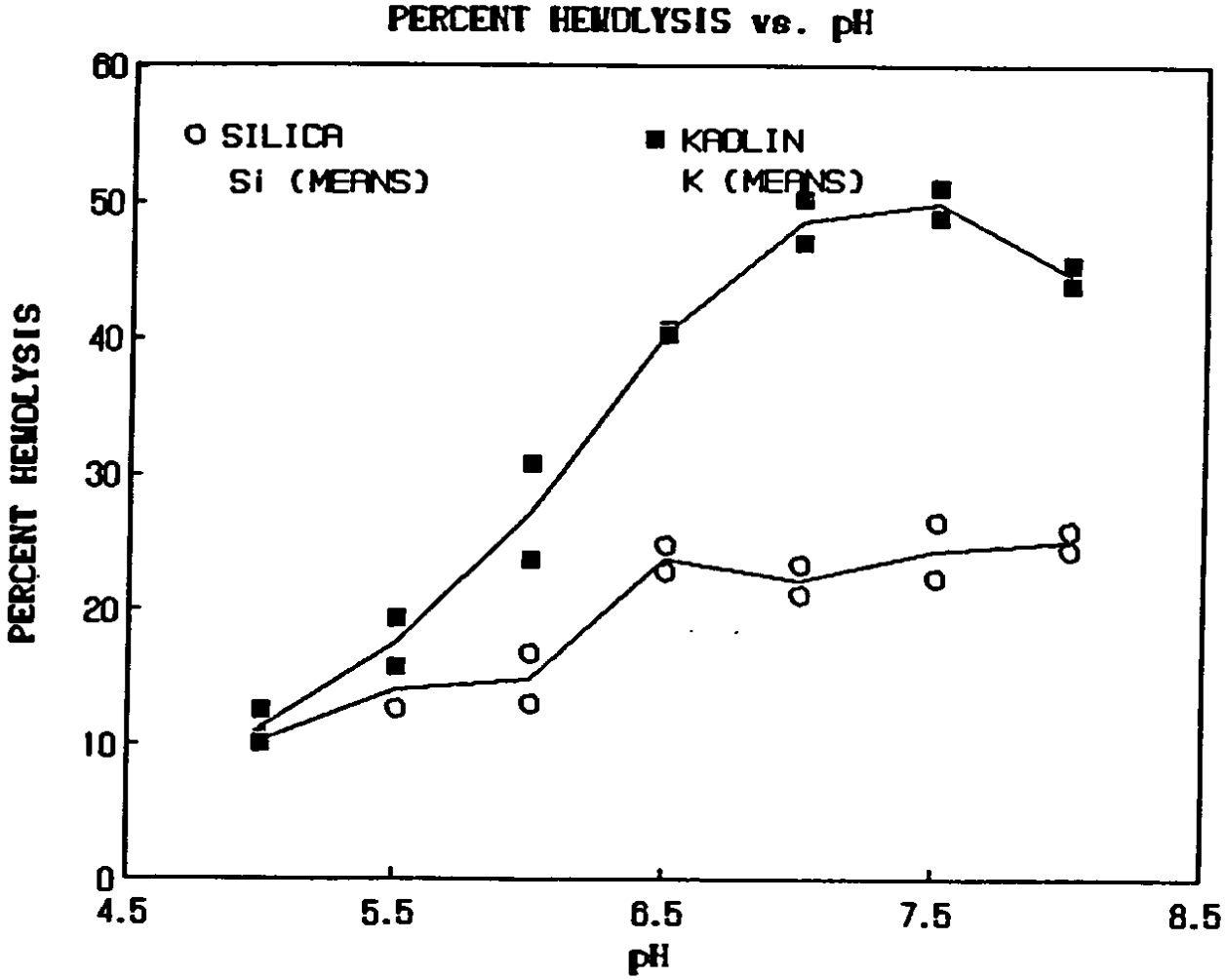


Figure 15. Percent hemolysis vs. pH for silica and kaolin @ 400 mOsm.


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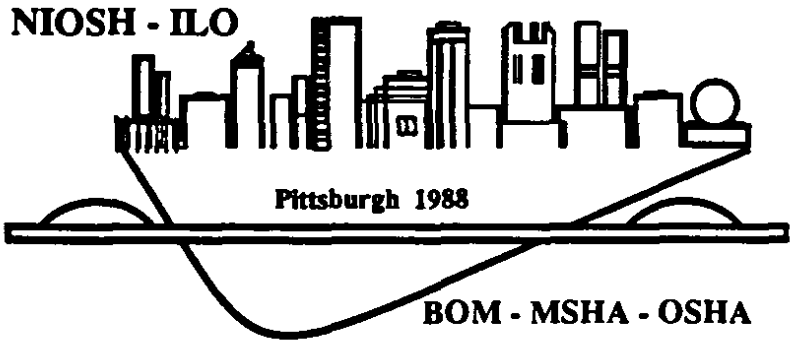


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**DHHS (NIOSH) Publication No. 90-108 Part I**