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CONTRASTING RESPIRABLE QUARTZ AND KAOLIN RETENTION OF LECITHIN SURFACTANT AND EXPRESSION OF MEMBRANOLYTIC ACTIVITY FOLLOWING PHOSPHOLIPASE A_2 DIGESTION

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Respirable-sized quartz, a well-established fibrogenic mineral dust, is compared with kaolin in erythrocyte hemolysis assays after treatment with saline dispersion of dipalmitoyl phosphatidylcholine, a primary phospholipid component of pulmonary surfactant. Both dusts are rendered inactive after treatment, but the membranolytic activity is partly to fully restored after treatment with phospholipase A_2, an enzyme normally associated with cellular plasma membranes and lysosomes. Phospholipid-coated dusts were incubated for periods of 2–72 h at a series of applied enzyme concentrations, and the adsorbed lipid species and hemolytic activity were quantitated at each time for both dusts. Surfactant was lost more readily from quartz than from kaolin, with consequent more rapid restoration of mineral surface hemolytic activity for quartz. Interactions of surfactant and mineral surface functional groups responsible for the mineral-specific rate differences, and implications for determining the mineral surface bioavailability of silica and silicate dusts, are discussed.

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INTRODUCTION

Mineral particles depositing in the lung acinus will contact an aqueous coating containing surfactant molecules spread on the air–liquid interface and dispersed within this hypophase; the primary components of that surfactant are diacyl lecithin phospholipids (King and Clements, 1972; King, 1982). Adsorption of this surfactant can suppress the cytotoxicity of mineral dusts (Marks, 1957). Suppression of hemolytic activity, a measure of membranolytic potential and an indicator of bioavailability of the mineral surface, of respirable-sized quartz and kaolin clay dusts with the adsorption of dipalmitoyl glycerophosphorylcholine or dipalmitoyl lecithin (DPL) has been measured (Wallace et al., 1985). Such contact and adsorption of pulmonary surfactant in vivo may provide a first defense system of the lung against respired dusts.

Respirable crystalline quartz is a well-documented etiologic agent for pulmonary fibrosis. Reported associations of kaolin, a layered aluminosilicate clay mineral, with pneumoconioses appear to involve relatively prolonged exposures to high concentrations of dust (Lapenas et al., 1984; Lynch and McIver, 1954; Sheers, 1964). However, quartz and kaolin respirable dusts have been found to be of comparable membranolytic potential in vitro on a specific surface area basis, as measured by short-term erythrocyte hemolysis assay, and to be of comparable cytotoxic potential to lavaged pulmonary macrophages as measured by release of lactate dehydrogenase, beta-*N*-acetyl glucosaminidase, and beta glucuronidase. Therefore, these direct short-term bioassay comparisons of prompt cytotoxic potential alone do not reveal the basis for the particular pathogenic activity of quartz. Complete suppression of cytotoxicity of both quartz and kaolin after adsorption of comparable amounts of DPL on a specific surface area basis implies that this surfactant–particle interaction is not the basis for their differing potentials for disease induction. Subsequent processes must permit quartz to manifest some cytotoxic activity in vivo leading to fibrosis.

A respired particle that has adsorbed sufficient surfactant may be phagocytosed without prompt cytotoxic effect to the cell. In that process the coated particle may be subject to enzymatic activities associated with the external cell membrane and with internal lysosomes. Some of those enzymes are active on components of surfactant; in particular, phospholipases can hydrolyze phospholipid surfactants such as DPL (Hostetler et al., 1983). Phospholipase A_2 and phospholipase C enzymes have been found to digest and remove DPL adsorbed to quartz and kaolin in cell-free systems in vitro (Wallace et al., 1988a, 1988b, 1989). Those studies indicated a mineral-specific digestion rate difference between the two minerals, with quartz losing its DPL coating more rapidly than kaolin, and with hemolytic activity being restored in parallel with removal of DPL.

This report compares rates of digestive removal of DPL by a phospholipase A_2 (PLA_2) and restoration of membranolytic activity for a quartz and a kaolin dust. The two minerals were incubated with PLA_2 for 2, 20, 44, and 72 h. At each of these times, measurements were made of the DPL remaining adsorbed, and of adsorbed lysolecithin. Lysolecithin is a product of PLA_2 digestion of DPL. At those times erythrocyte hemolytic activities of the dusts also were measured; those activities are an indicator of the membranolytic potential of the dusts, and provide an indication of the bioavailability of the mineral surface.

MATERIALS AND METHODS

Mineral Dusts

Respirable quartz dust (Min-U-Sil) (U.S. Silica Corporation, Berkley Springs, W. Va.) was determined by automated x-ray diffractometry to be 99.5% alpha quartz. The size fraction <5 μm diameter was used as collected by a centrifugal air flow particle classifier. Ninety-eight percent of the fraction was <5 μm with a median area equivalent diameter of 3.5 μm as estimated by automated scanning electron microscopy analysis, and the fraction was at least 98.5% silica as determined by energy-dispersive x-ray analysis. The specific surface area of the fraction was 3.97 m^2/g as determined by nitrogen adsorption isotherm measurement.

Respirable kaolin dust (Georgia Kaolin Mill) was similarly fractionated and analyzed. Ninety-nine percent of the fraction was <5 μm; the fraction was at least 95% aluminosilicate with no crystalline quartz detected by x-ray diffraction, and had a specific surface area of 13.25 m^2/g.

Chemicals

L-Alpha-dipalmitoyl glycerophosphorylcholine (lecithin) (DPL) obtained from Calbiochem Corporation, was $>99\%$ pure and used without further purification.

Phospholipase A_2 (PLA_2) enzyme, obtained from Sigma Chemical, was from porcine pancreas, a neutral pH optimum phospholipase.

DPL Coating of the Dusts

Stock dispersion of DPL in physiologic saline was made by sonicating 3 mg DPL per milliliter of 0.165 *M* NaCl physiologic concentration salt solution (PSS) with an ultrasonic horn at a power level of 40 W. Sonication was performed on about 40 ml of the mixture for 10 min. Debris and nonemulsified DPL were removed by centrifugation at 1500 $\times$ g for 10 min.

Dry silica or kaolin dusts were vortexed in amounts of 24 mg dust into 3.2 ml of emulsion of 0.75 mg DPL/ml saline for quartz or 1.5 mg DPL/ml saline for kaolin, ratios of 100 mg DPL/g quartz and 200 mg DPL/g kaolin. The dispersions were incubated for 1 h at 37°C, with continuous

agitation of the samples in a rotary drum. These concentrations provide DPL in excess of measured adsorption isotherm values for these two dusts of approximately 60 mg DPL/g quartz and 150 mg DPL/g kaolin (Wallace et al., 1988a, 1988b). After incubation, the dusts were washed twice by centrifugation for 10 min at 1500 × g and resuspended in pH 7, calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS). DPL-coated dusts were finally suspended at a concentration of 2 mg dust/ml PBS.

Digestion of DPL Coating by PLA_2

PLA_2 was dissolved in PSS plus 2 m*M* $CaCl_2$ at a pH between 6.5 and 7.0. DPL-coated kaolin was incubated for periods of 2, 22, 44, and 72 h with applied PLA_2 enzyme activities of 2.67, 7.87, and 26.2 units added to 24 mg samples of dust. DPL-treated quartz was incubated for the same time periods with four activities of 0.147, 0.44, 1.47, and 4.42 units per 24 mg dust. These values were chosen by using preliminary tests to identify levels at which the dusts begin to retoxify after 72 h digestion, and to provide comparisons of comparable applied activities between the two minerals on a basis of units of applied PLA_2 activity per micromole of adsorbed DPL. In terms of adsorbed DPL, these applied activities were 0.815, 2.72, and 8.19 units/μmol DPL adsorbed on quartz, and comparable values of 0.958, 2.90, and 9.66 units/μmol DPL adsorbed on kaolin. Digestions were started sequentially so that all the subsequent hemolysis assays could be performed at the same time. Fresh DPL/PSS emulsion was prepared each day for initial incubations of the dusts. Following incubation with enzyme, samples were centrifuged at 1500 × g and resuspended in PBS containing 2.0 m*M* ethylenediamine tetraacetic acid (EDTA) to stop the enzymatic digestion; this was done twice. Samples were then centrifuged at 1500 × g. Samples for assay of hemolytic potential were resuspended in PBS. Samples for phosphate determinations of residual adsorbed lecithin and lysolecithin were vacuum-dried at room temperature, and subsequently eluted with chloroform/methanol (2/1 by volume).

Hemolysis Assay

Hemolytic potentials of native dusts, DPL-coated dusts, and DPL-coated dusts subsequently incubated with PLA_2 were measured by the method of Harington et al. (1971) with minor modification. Erythrocytes were obtained from fresh sheep blood from the West Virginia University Animal Farm from a sheep maintained on a fixed diet. Blood was washed 6 times by mixing in phosphate-buffered saline (PBS) and centrifugation at 990 × g, and prepared as a 4% by volume suspension of erythrocytes in PBS.

Erythrocyte suspensions prepared as already described were mixed with equal volumes of dust in PBS suspension to result in 2% by volume

erythrocyte suspensions with a dust concentration of 1 mg/ml. The mixtures were incubated at 37°C for 1 h. These dust concentrations and incubation times were selected to result in lysis of approximately half the cells incubated with kaolin, the more lytic of the two dusts on a mass basis. Equal masses of quartz and kaolin were used in the experiments, so quartz produced about 15% hemolysis under the same assay conditions. After incubation with the erythrocytes, the samples were centrifuged at 500 × g and the amount of hemoglobin released into the supernatant was determined spectrophotometrically at a wavelength of 540 nm.

Analysis of Digestion Products

Amounts of phospholipid adsorbed to dust samples were quantitated as DPL or lysolecithin (L-alpha-lecithin, beta-palmitoyl). Sets of samples parallel to those used in hemolysis assays were eluted with chloroform/methanol (2/1 by volume). Aliquots were separated by thin-layer chromotography (TLC) on silica gel G 250-μm plates, using 60 : 30 : 5 chloroform/methanol/water. The bands for DPL and lysolecithin, determined by DPL and lysolecithin standards, were scraped from the plates, digested under 10 *N* H_2SO_4 for 4.5 h, and quantitated for phosphate by the method of Bartlett (1959). In these phospholipid and hemolysis assays controls were included for native dust, dust plus the maximum value of PLA_2, and dust plus DPL.

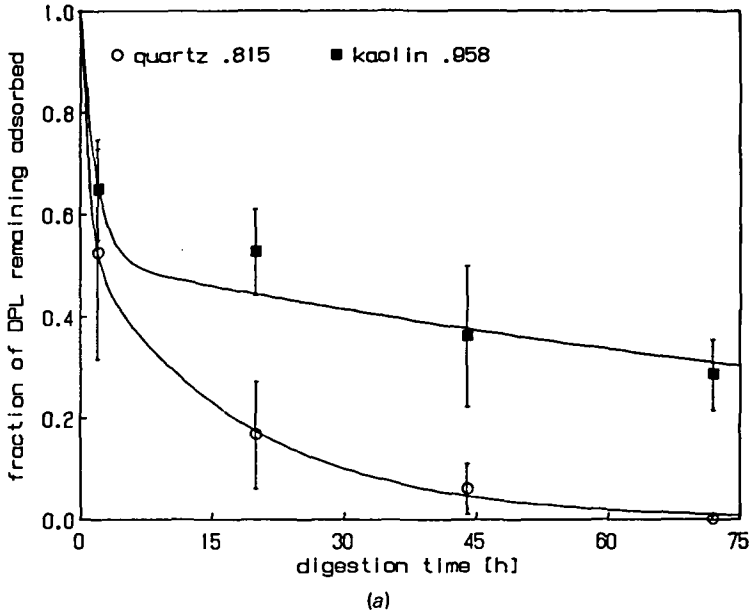
Association of free palmitic acid with particles following PLA_2 hydrolysis of particle-adsorbed DPL was measured at one digestion time by using carbonyl carbon ^{14}C-radiolabeled DPL. A radiolabeled DPL stock solution was made by adding 5 μl of 50 μCi/ml ^{14}C-radiolabeled DPL to 18 mg of nonradiolabeled DPL in 6 ml PBS; 12 mg quartz was mixed with 0.4 ml of this radiolabeled DPL stock dispersion and 1.2 ml of PSS, or 12 mg kaolin was mixed with 0.8 ml radiolabeled DPL stock dispersion of 0.8 ml PSS. Samples were incubated for 1 h at 37°C. Samples were washed once by centrifugation and resuspension in 1.6 ml PSS plus 2 m*M* $CaCl_2$. The resulting pellet was resuspended in 6 ml PSS plus 2 m*M* $CaCl_2$ and PLA_2 was added in the amount of 4.42 units nominal activity for silica and 7.87 units for kaolin. Dusts were incubated with PLA_2 for 2 h at 37°C. The dusts were then rinsed twice by centrifugation and resuspension in PBS plus 2 m*M* EDTA. Surpernatants from all the centrifugation steps were saved. Lipids were extracted from the dusts by 2 : 1 chloroform/methanol. Lipids were separated from the supernatants by mixing 0.5 ml of sample with 10 ml of 2 : 1 chloroform/methanol, shaking, and leaving the mixture to separate for 90 min. Two milliliters of cold 0.1 *M* KCl was added and the tube was shaken for 2 min. The aqueous layer was discarded and the lower layer evaporated. All samples were resuspended in 2 : 1 chloroform/methanol and separated by TLC into DPL, lysolecithin,

and free fatty acid fractions. Bands scraped from TLC plates were counted in a liquid scintillation counter (Beckman).

RESULTS

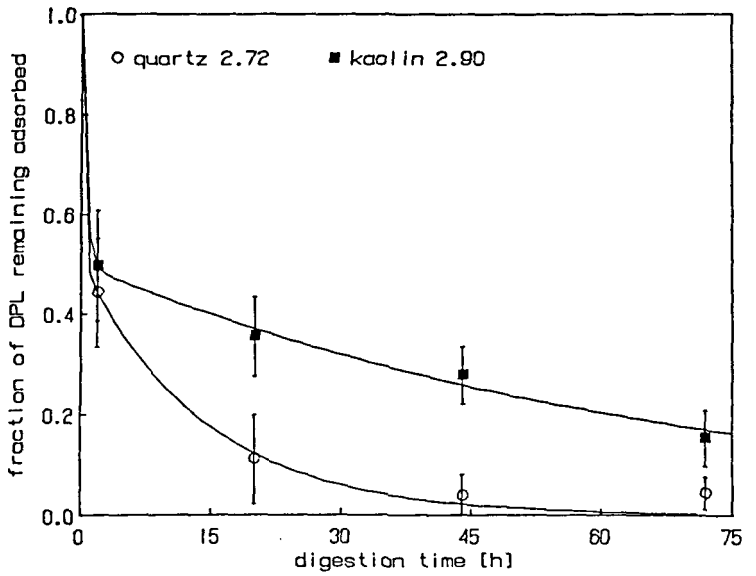
Following incubation in DPL the hemolytic activity of both mineral dusts was completely suppressed. The DPL coating procedure resulted in the retention of a consistent amount of adsorbed DPL on each mineral, as measured by phosphate quantitation. Measurements on 24 DPL-quartz control samples for the phosphate digestion assays gave an average residual adsorbed value of 16.51 mg DPL/g quartz or 5.67 μmol DPL/m^3 of quartz surface; 12 DPL-kaolin controls gave the value of 82.97 mg DPL/g kaolin or 8.53 μmol DPL/m^3 of kaolin surface.

The effects of each of three levels of applied PLA_2 activity on DPL coated quartz and on DPL-coated kaolin are shown in Figure 1. Nominal applied enzyme activities were 0.44, 1.47, and 4.42 units/24 mg coated quartz, and 2.67, 7.87, and 26.2 units/24 mg kaolin. Amounts of DPL remaining adsorbed are shown for each of the applied enzyme levels as measured after 2, 20, 44, and 72 h of incubation. Values are normalized to



(a)

FIGURE 1. Fraction of DPL remaining adsorbed on quartz or kaolin versus time of digestion by PLA_2. The ordinate is normalized to the amount of DPL adsorbed to the quartz or kaolin prior to application of PLA_2: (a) For 0.815 units PLA_2 applied per micromole DPL adsorbed to quartz (open circles) and 0.958 units PLA_2 applied per micromole DPL adsorbed to kaolin (filled squares). The means and standard deviations are shown for nine measurements at each point. The solid curves are the fit of the bilayer digestion model, Eq. (8), to each data set.



(*b*)

FIGURE 1. (*Continued*) Fraction of DPL remaining adsorbed on quartz or kaolin versus time of digestion by PLA_2. The ordinate is normalized to the amount of DPL adsorbed to the quartz or kaolin prior to application of PLA_2: (*b*) For 2.72 units applied to quartz and 2.90 units applied to kaolin.

DPL remaining on parallel samples incubated in PBS only. Data for each mineral for each applied enzyme activity level were taken in three separate experiments, using triplicate points for each time point in each experiment. Thus each point shows the mean and standard deviation for nine values. Data for the lowest enzyme activity applied to each dust and for the maximum value applied to kaolin were illustrated in a previous report (Wallace et al., 1989). Most of the deviation for a specific applied enzyme level and incubation time was found from one separate test date to another, rather than within a single experiment. The smooth curves fit the data with a kinetics model of enzymatic digestion of an adsorbed DPL bilayer, as detailed in the Discussion.

Both dusts showed a rapid loss of adsorbed DPL in the first 2 h of incubation with PLA_2, followed by a slower loss of the remaining DPL. For incubation times greater than 2 h the rate of DPL removal increased with increasing applied enzyme activity levels, and the rates of DPL removal were greater for quartz-bound than for kaolin-bound DPL at each of the three comparable applied enzyme activity levels. In the case of quartz, amounts of DPL remaining after digestion times of 44 and 72 h were within experimental background.

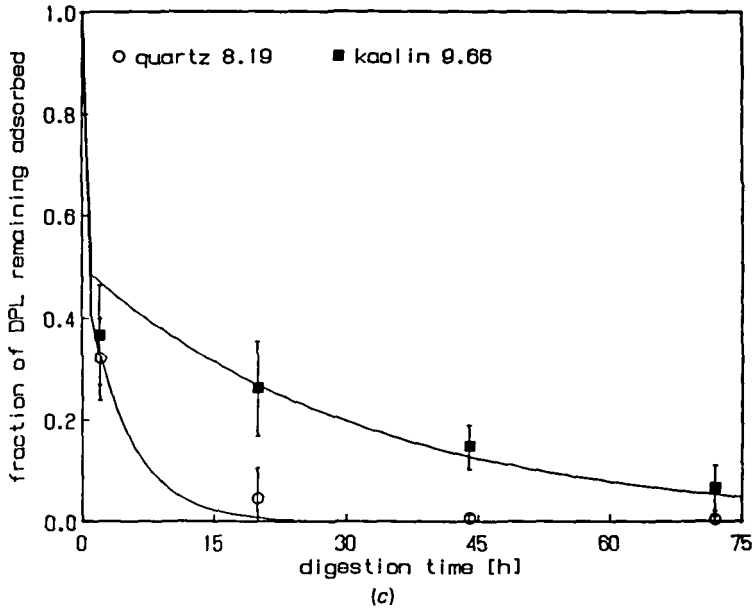


FIGURE 1. (*Continued*) Fraction of DPL remaining adsorbed on quartz or kaolin versus time of digestion by PLA_2. The ordinate is normalized to the amount of DPL adsorbed to the quartz or kaolin prior to application of PLA_2: (*c*) For 8.19 units applied to quartz and 9.66 units applied to kaolin.

Table 1 provides the numerical values for DPL remaining adsorbed at each time, and also shows the results of parallel measurements of quartz- and kaolin-adsorbed lysolecithin, and of parallel measurements of restored hemolytic activity. The first product of DPL digestion by PLA_2 is lysolecithin and a fatty acid residue. This production of lysolecithin must be accounted for in interpreting hemolytic potential measurements because lysolecithin itself is a membranolytic agent. Hemolytic activities of quartz and kaolin incubated with mixtures of DPL and lysolecithin have demonstrated the contribution of lysolecithin to hemolytic activity (Wallace et al., 1988a). A significant amount of lysolecithin was eluted from quartz at the 2- and 22-h time points. Little or no lysolecithin was eluted from quartz at the 44- and 72-h time points. Significant lysolecithin was eluted from kaolin at the 2-h point; this dropped to a lower value for longer digestion times.

Figure 2 shows hemolytic activities of quartz and kaolin versus applied enzyme levels and incubation times, normalized to the hemolytic potentials of native dust controls used in each experiment. For all three enzyme levels, the hemolytic activity of treated quartz was in excess of native quartz activity at 2 and 22 h; at 44 and 72 h the treated quartz

activity approximately equaled that of native quartz. At the 2-h time point the hemolytic activity of treated kaolin was in excess of native kaolin levels. However, unlike the quartz, incubation of DPL-coated kaolin resulted in significantly reduced hemolytic activities for digestion times greater than 2 h for the 2 lower enzyme levels.

Figure 3*a* shows the DPL remaining on quartz following digestion with a half order of magnitude lower level of applied PLA_2 activity, 0.147 units/24 mg quartz, or 0.272 units/μmol DPL adsorbed on quartz. This

TABLE 1. Fractional Adsorbed Lecithin, Lysolecithin, and Hemolytic Activity for Lecithin-Coated Quartz and Kaolin with Applied Phospholipase A_2 Activity and Digestion Time

Applied PLA_2[a]		t = 2 h[b]	t = 20 h	t = 44 h	t = 72 h
			Quartz		
0.147	DPL[c]	0.596 ± 0.175[f]	0.366 ± 0.174	0.154 ± 0.079	0.008 ± 0.014
	LYSO[d]	0.143 ± 0.022	0.111 ± 0.064	0.047 ± 0.053	0.024 ± 0.025
	HEME[e]	0.851 ± 0.243	0.904 ± 0.362	0.570 ± 0.094	0.785 ± 0.155
0.44	DPL	0.524 ± 0.206	0.168 ± 0.106	0.061 ± 0.050	0.002 ± 0.007
	LYSO	0.166 ± 0.029	0.183 ± 0.128	0.026 ± 0.022	0.014 ± 0.017
	HEME	1.217 ± 0.132	1.400 ± 0.462	0.799 ± 0.133	0.971 ± 0.139
1.47	DPL	0.444 ± 0.109	0.112 ± 0.089	0.039 ± 0.044	0.046 ± 0.033
	LYSO	0.177 ± 0.023	0.130 ± 0.144	0.003 ± 0.008	0.007 ± 0.015
	HEME	1.174 ± 0.112	1.223 ± 0.240	0.767 ± 0.087	0.857 ± 0.044
4.42	DPL	0.321 ± 0.080	0.046 ± 0.062	0.006 ± 0.010	0.004 ± 0.008
	LYSO	0.272 ± 0.053	0.202 ± 0.172	0.014 ± 0.023	0.012 ± 0.012
	HEME	1.262 ± 0.119	1.265 ± 0.260	0.807 ± 0.084	0.902 ± 0.034
			Kaolin		
2.62	DPL	0.650 ± 0.100	0.526 ± 0.084	0.362 ± 0.139	0.285 ± 0.067
	LYSO	0.209 ± 0.017	0.057 ± 0.032	0.018 ± 0.008	0.011 ± 0.010
	HEME	1.576 ± 0.136	0.0077 ± 0.0038	0.0048 ± 0.0003	0.0196 ± 0.0205
7.87	DPL	0.498 ± 0.112	0.362 ± 0.073	0.279 ± 0.057	0.154 ± 0.055
	LYSO	0.292 ± 0.032	0.082 ± 0.045	0.070 ± 0.019	0.052 ± 0.013
	HEME	1.762 ± 0.206	0.231 ± 0.172	0.156 ± 0.038	0.446 ± 0.319
26.2	DPL	0.367 ± 0.098	0.263 ± 0.093	0.147 ± 0.043	0.067 ± 0.045
	LYSO	0.361 ± 0.029	0.104 ± 0.050	0.150 ± 0.040	0.092 ± 0.043
	HEME	1.756 ± 0.207	0.465 ± 0.352	1.219 ± 0.128	0.961 ± 0.274

[a]Applied PLA2 activity is in units/24 mg dust.

[b]t is the time in hours of digestion of lecithin-treated dusts by PLA2.

[c]DPL, adsorbed lecithin, is the fractional amount remaining adsorbed at the end of the digestion time, with respect to the amount adsorbed at t = 0.

[d]LYSO, adsorbed lysolecithin, is the amount of lysolecithin eluted from the dust at the end of the digestion time, expressed as a mole fraction of the amount of lecithin adsorbed at t = 0.

[e]HEME, hemolytic activity, is the fractional activity of the dust at the end of the digestion time with respect to the activity of uncoated, untreated dust.

[f]All values are means of nine measurements, from triplicate meaurements in three experiments, and are given ± standard deviation.

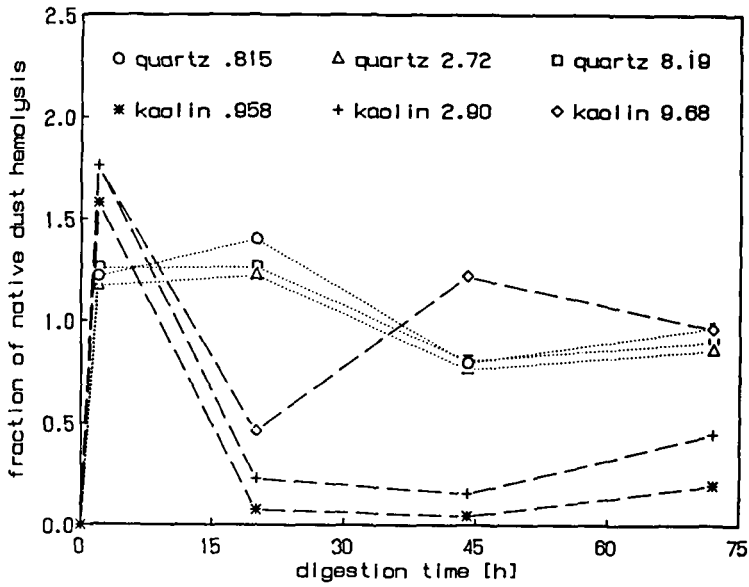


FIGURE 2. Fraction of untreated quartz or kaolin hemolytic activity expressed following incubation of dusts with DPL and subsequent enzymatic digestion versus time of digestion by PLA_2. Data are shown for quartz and kaolin for three different applied enzyme levels each, activities expressed in units PLA_2 applied per micromole DPL adsorbed to quartz or kaolin. Dust samples were from the sets prepared for the DPL measurements illustrated in Figure 1.

additional lower level was used to identify an activity at which quartz still retained a significant amount of adsorbed DPL for digestion times greater than 20 h. Figure 3*a* also shows as a dashed line the amounts of lysolecithin eluted from the quartz samples, given in terms of mole fraction of the initial amount of adsorbed lecithin. The solid smooth curve fitted to the DPL data is the fit of the bilayer digestion model as described in the Discussion. Figure 3*b* shows the parallel hemolysis activities, and the solid line shows for comparison the fraction of mineral surface bared by PLA_2 digestion, as described in the Discussion.

In thin-layer chromatographic separation of DPL and lysolecithin, a band ran at the solvent front and was visible after heating but not after staining for phosphate. Experiments using ^{14}C-radiolabeled DPL were performed to track the disposition of this PLA_2-hydrolysis-released palmitic acid after 2 h of PLA_2 digestion. Figure 4 shows the mean and range for measurements of the fraction of recovered label found in the nonphosphate lipid fraction for quartz (Q + E) and for kaolin (K + E), for both the mineral adsorbed and supernatant phases. Fractions are also shown for DPL-coated quartz and kaolin incubated for an equal period of time without PLA_2 (Q − E and K − E). Three measurements were made on three Q + E samples, and four samples for the other prepara-

tions. The ratios of quartz-bound to free palmitic acid were 0.547, 0.545, and 0.698 for the three runs. Total recoveries of label from quartz sample preparations through final assay of separated fractions were 104.4, 103.1, and 95.4%. The ratios of kaolin particulate-bound to free supernatant palmitic acid were 0.767, 0.709, 0.891, and 0.804. Total recoveries from kaolin sample preparation through final assays were 77.8, 84.2, 87.1, and 86.5%.

DISCUSSION

The quartz and kaolin dusts used in this work are comparably hemolytic to erythrocytes and cytotoxic to lavaged pulmonary macrophages on a surface area basis (Wallace et al., 1985). The amounts of DPL needed to fully suppress these hemolytic activities of the dusts are equal to the amounts of DPL retained on the dusts after incubation and mild rinsing. Maximum suppression of the hemolytic potential, representing essentially complete neutralization, resulted from adsorption of between 15 and 20 mg DPL/g quartz. The corresponding value for the kaolin used is about 75–85 mg DPL/g kaolin. The values for full suppression of the

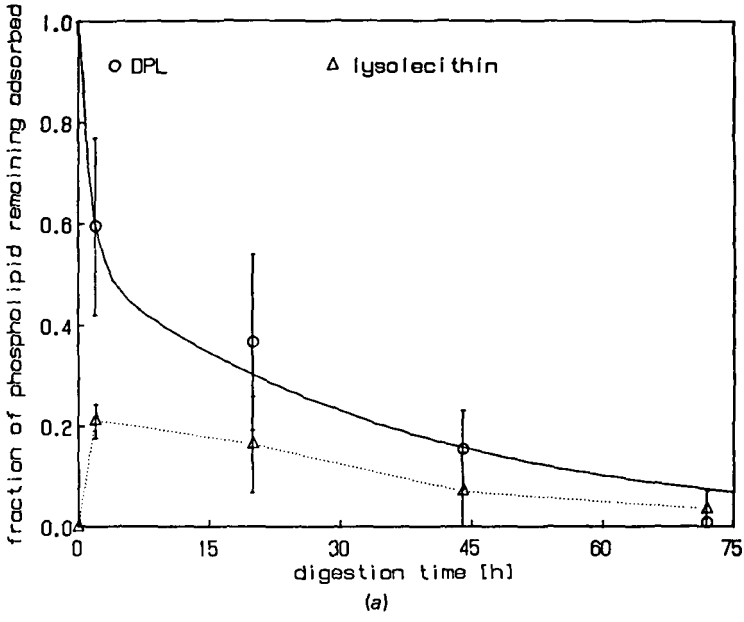


FIGURE 3. Phospholipid remaining adsorbed on quartz and fraction of native quartz hemolytic activity following incubation with DPL and subsequent enzymatic digestion. Applied PLA_2 activity was 0.272 units per micromole adsorbed DPL. (a) Fraction of DPL remaining adsorbed (open circles) and fraction converted to lysolecithin that remains adsorbed (open triangles connected by the dotted straight lines) on quartz versus time of digestion by PLA_2. The solid curve is the fit of the bilayer digestion model, Eq. (8), to the DPL data.

hemolytic potential are well below the adsorption isotherm values for the dusts: This quartz adsorbs about 60 mg DPL/g dust and this kaolin adsorbs about 150 mg DPL/g dust at high lecithin dispersion concentrations.

We interpret these data to mean that both dusts will adsorb multilayers of DPL from dispersion in physiologic saline. With rinsing, most of the outer layers are removed, leaving a bilayer coverage that is sufficient to fully suppress the hemolytic potential. The first layer of DPL molecules, those in direct contact with the mineral surface, are oriented with their zwitterionic head groups toward the surface. These are backed by a second layer of DPL, with acyl tails of the two layers associated and with ionic heads of the second layer pointed outward into the surrounding aqueous medium. Such an arrangement would permit ionic interaction of acidic or basic mineral surface sites with the cationic trimethylammonium or the acidic phosphate moieties of the DPL zwitterionic phosphorylcholine head group, while minimizing contact of hydrophobic acyl groups with the surrounding aqueous media.

Application of PLA_2 to DPL-coated dusts results in the digestion and eventual removal of DPL and its phosphate-bearing products of diges-

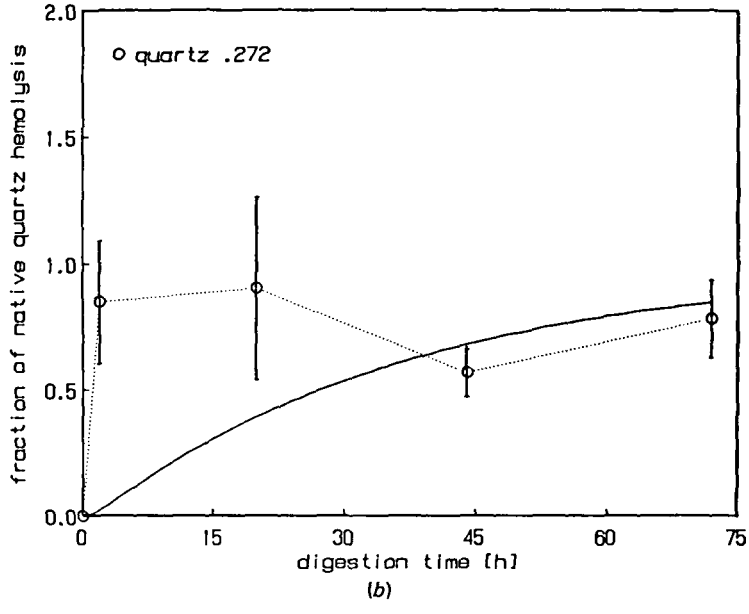


FIGURE 3. *(Continued)* Phospholipid remaining adsorbed on quartz and fraction of native quartz hemolytic activity following incubation with DPL and subsequent enzymatic digestion. Applied PLA_2 activity was 0.272 units per micromole adsorbed DPL. (*b*) Fraction of untreated quartz hemolytic activity expressed by those same DPL and PLA_2 treated sample sets. The solid curve shows the fit of the model for fraction of quartz surface bared of DPL by PLA_2 digestion, for Eq. (9).

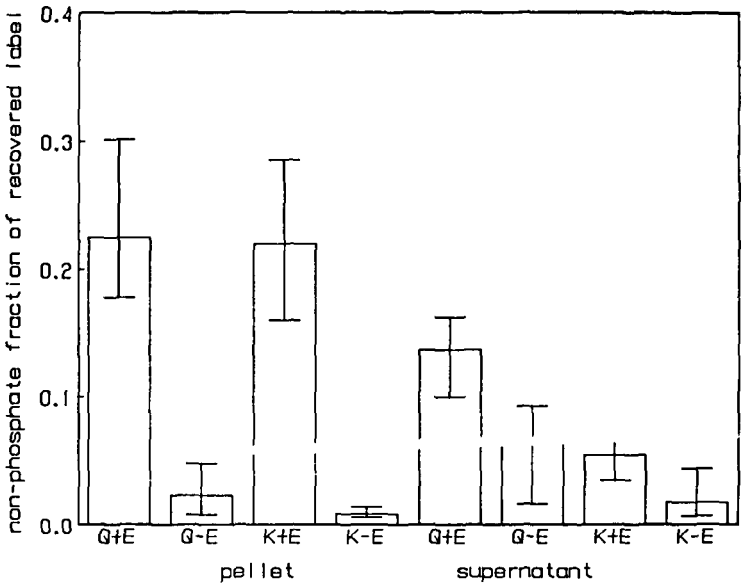


FIGURE 4. Palmitic acid found adsorbed on DPL-treated quartz or kaolin and nonadsorbed with and without 2 h of PLA_2 digestion: Q, quartz; K, kaolin; +E, PLA_2-treated; −E, no PLA_2 treatment; pellet, mineral adsorbed; supernatant, nonadsorbed. The ordinate is the fraction of total radioactivity recovered that was in the nonphosphate chromatographically separated band, equivalent to the mole fraction of initially adsorbed DPL appearing as palmitic acid.

tion. The appearance of two phases in the digestion of mineral adsorbed DPL is consistent with a multi- or bilayer model for adsorbed DPL. A rapid initial rate of digestion seen during the first 2 h of PLA_2 incubation is followed by a slower and mineral specific phase of the digestion. These data have the appearance of two approximately equal populations of DPL that are digested at different rates, the rates being first-order in concentration of each population. Using $S(0)$ for the DPL initially adsorbed to the mineral surface and $B(0)$ for that associated with an outer layer, with disappearance of $B(t)$ proceeding at a fast rate r_B compared to the rate r_S for the surface-bound molecules $S(t)$, gives the descriptive equation:

$$\text{DPL}(t) = B(t) + S(t) = B(0)\exp(-r_B t) + S(0)\exp(-r_S t) \qquad (1)$$

Fitting this descriptive equation to the quartz and kaolin data results in the $S(0)$ and r_S values shown in Table 2. Initially approximately half the lecithin is in each layer. The slower rate r_S increases with applied enzyme level. For a given applied enzyme level r_S is greater for quartz than for kaolin. There is inadequate short-time data, essentially just the 2-h time

TABLE 2. Rate of Digestive Removal of Mineral Surface-Adsorbed Lecithin

Applied PLA_2				
Units/24 mg dust	Units/μmol adsorbed DPL	$S(0)^a$	$r_S{}^b$ (1/h)	$k_2'^c$ (1/h)
	Quartz			
0.147	0.272	0.65	0.033	0.027
0.44	0.815	0.44	0.048	0.055
1.47	2.72	0.51	0.071	0.071
4.42	8.19	0.40	0.110	0.208
	Kaolin			
2.67	0.958	0.66	0.012	0.007
7.87	2.90	0.50	0.015	0.015
26.2	9.66	0.39	0.022	0.031

[a]Apparent fractional population of DPL removed in the slower phase, as fit by Eq. (1).
[b]Apparent overall rate constant for the slower phase removal of DPL, as fit by Eq. (1).
[c]Kinetics rate constant for the slower phase removal of DPL, as fit by Eq. (8).

point, to quantitate or determine if any mineral specificity exists in the fast rate r_B.

We model this process as enzymatic digestion of an adsorbed bilayer, initially with $B(0) = S(0) = 0.5$ of the DPL molecules, where B represents the outwardly oriented DPL molecules of the bilayer and S represents the mineral surface-adsorbed DPL molecules. To account in part for the heterogeneous nature of the system, assume that a surface-adsorbed molecule is not accessible for enzyme digestion until the covering outer bilayer molecule is digested; this reduces the number of S molecules available to the enzyme to $S(t) - B(t)$ at any time t. The heterogeneous nature of the system is also considered by assuming that when the intermediate enzyme-substrate complex, ES or EB, is formed then enzyme access to a number of adjacent substrate molecules, a[ES] or a[EB], is restricted due to the large size of the enzyme compared to substrate DPL molecules. Thus the number of molecules of B available to the enzyme E could be modeled as $B(t) - a[EB(t)]$, and S molecule availability could be modeled as $S(t) - B(t) - a[ES(t)]$:

$$E + (B - a[EB]) \rightleftharpoons [EB] \rightarrow E + \text{lysolecithin} + \text{palmitic acid} \quad (2)$$

$$E + (S - B - a[ES]) \rightleftharpoons [ES] \rightarrow E + \text{lysolecithin} + \text{palmitic acid} \quad (3)$$

For analysis of loss of mineral surface-bound lecithin and consequent restoration of mineral surface membranolytic activity, the interest is in

the longer-term "batch" reaction digestion through complete consumption of the substrate. We simplify the enzyme kinetics description to two coupled first-order reactions with negligible back reaction, and we approximate the enzyme steric hindrance factors to be proportional to the available enzyme and substrate:

$$E + (B - a[E][B]) \xrightarrow{k_1} E + \text{lysolecithin} + \text{palmitic acid} \quad (4)$$

$$E + (S - B - a[E][S - B]) \xrightarrow{k_2} E + \text{lysolecithin} + \text{palmitic acid} \quad (5)$$

so

$$dB/dt = -k_1[E]([B] - a[E][B]) = -k_1[B]([E] - a[E]^2) \quad (6)$$

$$\begin{aligned} dS/dt &= -k_2[E]([S] - [B] - a[E]([S] - [B])) \\ &= -k_2([S] - [B])([E] - a[E]^2) \end{aligned} \quad (7)$$

Subject to the initial condition that half the DPL is adsorbed to the mineral surface and half is in the outwardly oriented half of the bilayer, this model gives an expression for total fraction of DPL remaining adsorbed at time t:

$$\begin{aligned} S(t) + B(t) = {} & 0.5[(2k_2' - k_1')/(k_2' - k_1')] \exp(-k_1't) \\ & + 0.5[k_1'/(k_1' - k_2')] \exp(-k_2't) \end{aligned} \quad (8)$$

where

$$k_1' = k_1([E] - a[E]^2) \qquad k_2' = k_2([E] - a[E]^2)$$

This equation is fitted to each of the sets of data for DPL removal under the action of a specific enzyme level, using k_1' and k_2' as the fitting parameters.

Using a least-squared deviation curve fitting to fit Eq. (8) to experimental data results in the smooth curves in Figures 1 and 3a. The values for the slower phase rates, k_2', are given in Table 2. At the three applied specific activity levels of approximately 1, 3, and 9 units/μmol adsorbed DPL, the ratio of the quartz to kaolin digestion rate constants, k_2' (quartz)/k_2' (kaolin), are 8, 5, and 7, respectively. An average value for k_2 for quartz, in units of reciprocal enzyme unit hours, is obtained by least-squared deviation fitting k_2' versus [E], applied PLA_2 activity, data with the parabolic function $k_2' = k_2[E] - ak_2[E]^2$, as shown for quartz and for kaolin in Figure 5. The values of k_2 and ak_2 for the best fit for each mineral are $0.0342\,[E] - 0.00110\,[E]^2$ for quartz, and $0.00636\,[E] - 0.000331\,[E]^2$ for kaolin. That is, the quartz $k_2 = 0.0342$ in terms of μmol adsorbed DPL digested/(unit · h), and the kaolin $k_2 = 0.006367$ μmol adsorbed DPL

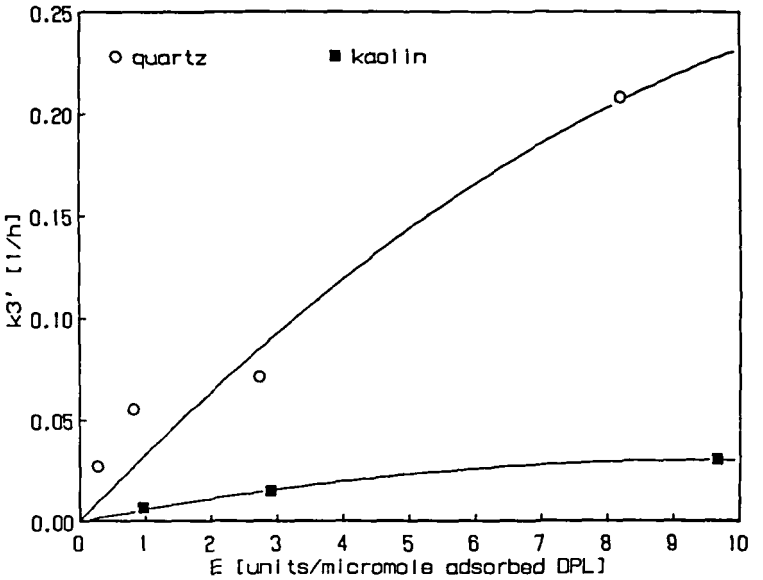


FIGURE 5. Digestion rate constants, k_2', versus applied PLA_2 activity for the slower phase of removal of DPL from quartz or kaolin. Rate constants, in units of 1/h, are those providing the best fit of the bilayer digestion model, Eq. (8), to the data for each applied enzyme level. The solid curves are the parabolic fits of $k_2' = 0.0342[E] - 0.0011[E]^2$ to the quartz k_2' values, and of $0.00636[E] - 0.000311[E]^2$ to the kaolin values. Applied PLA_2 activities were 8.19 units/μmol adsorbed DPL for quartz, and 2.90 units/μmol adsorbed DPL for kaolin.

digested/(unit · h). Thus, on a specific applied PLA_2 activity basis, which removes the effects of the particulate size distribution of a particular sample, there is a fivefold increase in the quartz digestion kinetics rate constant compared to the rate constant for kaolin.

At 2 h of PLA_2 incubation, significant amounts of DPL and lysolecithin remained adsorbed to both dusts at all enzyme levels, and at the same time hemolytic activity approximated or exceeded native dust potential for both dusts. This suggests that the adsorbed lysolecithin was responsible for the membranolytic activity at the 2-h time point. The radiolabel assay for nonphosphate lipid generated after 2 h of incubation with PLA_2 indicates that most of the fatty acid liberated was associated with dust particles in the case of kaolin, and was about equally divided between the adsorbed and nonadsorbed state in the case of quartz.

At 22 h of digestion the histories of the quartz and kaolin began to diverge. For quartz both adsorbed lysolecithin levels and hemolysis activities remained high, indicating at least part of the hemolytic activity at that time was due to adsorbed lysolecithin. For kaolin the low hemolytic activity reflected the loss of lysolecithin and the persistence of adsorbed lecithin.

At 44 and 72 h the quartz hemolytic activity was restored to near the native dust level, and the quartz surface was completely bared of DPL and lysolecithin. Kaolin activity was still significantly suppressed at 44 and 72 h for the lower two applied enzyme levels. At 44 and 72 h, restoration of membranolytic potential appeared to follow removal of DPL from the mineral surfaces. As an example, the solid smooth curve in Figure 3*b* shows the fractional loss of mineral surface adsorbed DPL, given by the bilayer model fit to the DPL digestion data of Figure 3*a*:

$$\begin{aligned}\text{Fractional loss of adsorbed DPL} = 1 - S(t)/S(0) = 1 \\ - [[k_2'/(k_2' \\ - k_1')] \exp(-k_1' t) \\ + [k_1'/(k_1' - k_2')] \exp(-k_2' t)] \qquad (9)\end{aligned}$$

That is, mineral surface membranolytic activity begins to be restored as the mineral surface-bound inner layer of DPL is removed.

DPL is zwitterionic in aqueous media, with a positive charge at the trimethyl ammonium group at the choline tip of the molecule and with an acidic phosphate providing a negative charge adjacent to the glycerol frame of the molecule where phospholipase activity occurs. In aqueous media the quartz forms acidic silanol surface sites and kaolin forms both acidic silanol and amphoteric or basic aluminol sites. Thus a silanol-choline ammonium acid-base association could form for both dusts with DPL. But the kaolin alone could form an aluminol-phosphate acid-base bond. Phospholipase enzymes A_1, A_2, and C act, respectively, at the two fatty acid carbonyl ester linkages and on the phosphate of the phosphorylcholine ester linkages to the glycerol of DPL. Thus, association of the DPL phosphate with the kaolin surface could provide an additional hindrance to phospholipase enzymatic digestion of the adjacent palmitic acid carbonyl ester linkages (Wallace et al., 1988a, 1989). Infrared spectroscopic examinations by grating infrared spectroscopy and by Fourier transform infrared (FTIR) spectroscopy were made on kaolin dusts that had been incubated with DPL in saline, then dried under mild conditions, and prepared in potassium bromide pellets (Keane et al., 1990). These showed suppression of the broad band in the region of the 3400 cm^{-1} attributed to the hydrated acidic phosphate group of DPL. While not definitive because of the use of samples of low water content for IR analysis, this spectral change is consistent with a model of DPL phosphate association with the kaolin surface.

This study has used one component of pulmonary surfactant, and one phospholipase A_2 with a neutral pH optimum activity. Neutralization of dust membranolytic potential in vivo would involve adsorption of a variety of phospholipids and possibly of lipoproteins and other biochem-

ical constituents of the pulmonary alveolar hypophase. Digestion of that surfactant coating could occur by the action of macrophage membrane-associated phospholipase and phagolysosomal phospholipases. Acidic conditions in the phagolysosome could affect the strength or conformation of adsorbed surfactant. Dissociation of acidic silanol surface sites should decrease with decreasing pH, weakening the interaction of lecithin trimethylammonium with negative surface sites; however, silanol groups do not protonate in significant numbers until very low pH values are reached, such as pH 2 (Iler, 1979). Basic aluminol sites on aluminosilicate would remain available for interaction with the acidic moieties of phospholipid surfactants. Use of acidic pH optimum phospholipase is needed to determine if steric hindrance preferentially retards digestion of phospholipids from the kaolin surface under phagolysosomal acidic conditions.

Direct in vitro measurements of cytotoxic potential using pulmonary alveolar macrophages in vitro do not identify quartz as being a particularly pathogenic agent in comparison with clay dusts. Both dusts' prompt cytotoxic potentials are neutralized by surfactant adsorption. For mineral-specific rates of removal of prophylactic surfactant to be important in distinguishing quartz and clay pathogenic potential, it would appear that the digestion rates in vivo must fall on either side of clearance rates of respired dusts. The residence time for macrophages in the alveoli of rats or mice has been estimated to be about 7 d, the time being halved in response to an inhaled particulate load, and with a fraction cleared at a slower rate requiring about 30 d for clearance (Bowden, 1983). Clearance of insoluble particles from the human pulmonary system appears to have two components, with a first-phase half-life of tens of days and a second phase of hundreds of days (Bailey et al., 1985). Studies measuring the rates of digestion of mineral surface-adsorbed surfactant within the cellular phagolysosome and measuring in vivo rates of digestion of surfactant adsorbed on particles subject to different clearance rates (e.g., for dusts taken up by pulmonary alveolar macrophages or by epithelial or interstitial alveolar cells) are needed to determine the time course of mineral surface bioavailability of respirable particles deposited in the lung.

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