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LYSIS OF ERYTHROCYTES BY SILICATE MINERALS[1]

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Abstract—*In vitro* studies of the destruction (lysis) of bovine red blood cells (erythrocytes) by some silicate minerals showed the reaction to be complete in less than 1 hr and very destructive to the cell membrane. The activity as lysing agents was found to be in the order smectites > silica > palygorskite ≃ sepiolite > chrysotile > kaolinite. Different compositions (Fe, Al, Mg, Li, vacancy) of the octahedral sheet of the smectite and fibrous clay minerals did not appreciably alter their hemolytic activity. The most active particle size range for kaolinite and montmorillonite was 0.2–2 μm. Structural folding of palygorskite reduced lysis suggesting that edge surfaces and silanol groups are important in this process. Aluminum oxides and hydroxides caused no lysis, and coatings of positively charged aluminum-hydroxy polymers on montmorillonite, silica, palygorskite, and kaolinite significantly reduced lysis.

Key Words—Chrysotile, Erythrocytes, Hemolysis, Kaolinite, Lysis, Palygorskite, Red blood cell, Sepiolite, Smectite.

INTRODUCTION

The destruction (hemolysis) of red blood cells (erythrocytes) by minerals provides a simple and rapid method to study the effects of minerals on a biological membrane. *In vitro* hemolysis has been observed with silica (Harley and Margolis, 1961; Charache *et al.*, 1962; Nash *et al.*, 1966; Macnab and Harington, 1967), chrysotile (Secchi and Rezzonico, 1968; Schnitzer and Pundsack, 1970), sepiolite and palygorskite (Hayashi *et al.*, 1969), kaolinite (Manyai *et al.*, 1969, 1970), and montmorillonite and illite (Manyai *et al.*, 1969).

Silica has been one of the most intensively studied biologically active materials. Harley and Margolis (1961) found that silica particles with a diameter of 300 Å at a concentration of 1 μg/ml caused about 50% hemolysis in a 2% suspension of erythrocytes. Their surface area calculations indicated that contact of silica with a very small portion of the surface of the erythrocytes was sufficient to produce hemolysis. They also showed that (1) silica particles <30 Å in diameter blocked the effects of larger particles but had no direct effect on red blood cells, (2) 30–70-Å particles caused agglutination, and (3) larger particles produced hemolysis.

Nash *et al.* (1966) found that silica particles damaged cell membranes because silanol groups formed on the silica surface through interaction with water; they suggested that these groups were mostly non-ionized at the buffered pH 7.2 of the isotonic saline solutions used and, therefore, acted as strong hydrogen-bonding agents. Interactions were postulated between the silanol groups and secondary amide groups of membrane proteins or between the silanol groups and phosphate ester groups of membrane phospholipids.

Chrysotile, the most hemolytic form of asbestos, has been shown to be active in concentrations as low as 200 μg/ml (Beck *et al.*, 1972). The degree of "opening" of the chrysotile fiber appears to be important; Schnitzer and Pundsack (1970) defilibrated crude asbestos by air-jet milling and increased hemolytic activity more than 40-fold. The more highly opened material had fibers with smaller diameters and greater surface areas. Langer *et al.* (1978) observed that vigorous dry ball-milling of chrysotile reduced its crystallinity as determined by X-ray powder diffraction and changed its surface chemistry as evidenced by IR spectroscopy and diphenylpicrylhydrazyl reduction. Hemolytic potency was reported to have markedly decreased with milling time.

The activity of chrysotile has been related to its magnesium content and surface charge. The relative inertness of forms of asbestos low in magnesium led Harington *et al.* (1971) to suggest that this ion *per se* might be responsible for the hemolysis of red blood cells. Considerable circumstantial evidence supports this hypothesis (Allison, 1973). Surface charge on chrysotile as represented by zeta potential has been highly correlated with degree of hemolysis (Light and Wei, 1977). Harington *et al.* (1975) proposed a mechanism for lysis by chrysotile that involved clustering of glycoproteins of the cell membrane. Clustering apparently increased passive permeability of small ions and caused a Donnan redistribution of ions with an accumulation of Na^+ and H_2O inside the cell which likely resulted in osmotic rupture of the cell.

Hayashi *et al.* (1969) found that sepiolite and palygorskite at a concentration of 1 mg/ml caused hemolysis when incubated with red blood cells. Prior heat treatments of the minerals decreased the hemolytic ac-

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tivity; hemolysis was negligible for samples that had been heated to 600°C. The decreased activity with heating was ascribed to structural changes or to the decrease in the surface area of the fibrous silicates. Schnitzer and Pundsack (1970) also reported sepiolite and palygorskite to be strongly hemolytic. Handcut crude samples of the fibrous minerals were not hemolytic, suggesting that the degree of fiberization or the surface area of the mineral is an important factor in hemolysis.

Heat treatment also affects the hemolytic properties of kaolinite (Manyai *et al.*, 1969, 1970) and montmorillonite (Manyai *et al.*, 1969). These authors found that dehydration of kaolinite to metakaolinite at 500°–650°C was accompanied by a complete loss of hemolytic activity. When the samples were heated at 800°–950°C, the hemolytic effect increased as mullite and cristobalite were formed. Manyai *et al.* (1969) found that montmorillonite also lost its hemolytic activity at 500°–600°C, but unlike kaolinite, the hemolytic activity did not increase when the samples were heated to higher temperatures. These workers found that ~1 and ~2 mg of montmorillonite and kaolinite, respectively, caused 50% hemolysis when incubated with 1 ml of a 2% suspension of erythrocytes, if the diameter of the mineral particles was less than 5 μm. In addition, they reported that the degree of hemolysis was proportional to the surface area of the mineral. In contrast, Macnab and Harington (1967) found finely ground kaolin to be only weakly hemolytic.

It is known from both human pathology and animal experiments that different clay minerals cause different histological reactions (Timar *et al.*, 1966). Erythrocytes can serve as models in studying cell reactions with clay minerals, and hemolysis has been shown to correlate with other cell toxicity tests (Mossman *et al.*, 1980). These data, however, cannot be extrapolated directly to pathological reactions caused by different minerals. The present study was designed to determine the relative hemolytic activity of four clay minerals and one type of noncrystalline silica. Each material was selected for its extreme difference in surface area, surface charge, and morphology. By comparing the general characteristics of each of these materials, some clarification may be obtained regarding the relationships of mineralogical parameters to the hemolysis phenomenon.

MATERIALS AND METHODS

Minerals

Montmorillonite (Upton, Wyoming) and kaolinite (Bath, South Carolina) were obtained from Ward's Natural Science Establishment, Inc., Rochester, New York. Palygorskite (PFl-1, Attapulgus, Georgia) was obtained from the Clay Minerals Repository of The Clay Minerals Society. The minerals were dry crushed to pass through a 300-mesh sieve and the <50-μm size separate was used for all studies. The chrysotile from the Thetford Mines, Quebec, Canada was from Ward's Natural Science Establishment. The crude chrysotile fibers were partially separated by hand and then ground for 2 min in a Janke and Kunkel mechanical grinder. The silica was synthetic, noncrystalline material sold by the Cabot Corporation under the tradename of Cab-O-Sil.

Table 1. HC_{50} values[1] of the four minerals and silica.

Mineral	HC_{50} value[1] (mg/ml)
Montmorillonite	0.006
Silica	0.03
Palygorskite	0.06
Chrysotile	0.1
Kaolinite	0.6

[1] mg of mineral per ml of the mineral-erythrocyte-buffer mixture.

Separation and dispersion of the materials were assured by sonicating the suspensions for 5 min at 80 watts. The <0.2-, 0.2–2-, 2–5-, 5–20-, and 20–50-μm size separates of montmorillonite and kaolinite were obtained using the procedure of Jackson (1975).

Cells and media

Bovine blood was drawn by venipuncture directly into a 60-ml polyethylene tube coated with Heparin and was stored at 4°C. The erythrocytes were washed 3 times with isotonic saline solution buffered with phosphate (0.1060 M NaCl, 0.0303 M $Na_2HPO_4 \cdot 7\,H_2O$, and 0.0080 M KH_2PO_4). After the final wash, a 3% (v/v) suspension of erythrocytes was prepared.

Phosphate buffer (pH 7.3) was used throughout except for the chrysotile system. Macnab and Harington (1967) reported that phosphate ions inhibit the hemolytic activity of chrysotile, an observation confirmed in this laboratory. Without the phosphate buffer, however, the chrysotile erythrocyte system remained at a pH of 7.2 for the concentrations used.

Hemolysis technique

The reaction mixture consisted of adding 1 ml of a mineral suspension, 3 ml of phosphate buffer saline solution (unbuffered saline solution alone for the chrysotile system), and 1 ml of the erythrocyte stock suspension to glass test tubes and sealing the tubes with Parafilm. Four milliliters of phosphate-buffered saline solution and 1 ml of the erythrocyte stock suspension served as control. The tubes were rotated end over end at 10 rpm for 1 hr at room temperature. The cells and minerals were sedimented by centrifugation at 1500 × *g* for 5 min. The hemoglobin content in the supernatant solutions was determined spectrophotometrically at 541 nm against the control sample. The degree of hemolysis was expressed as percentage of a sample totally lysed by ultrasonic treatment. All trials were replicated 4 times.

To facilitate meaningful comparisons between the minerals used, the concentration of the mineral that caused 50% hemolysis when mixed with 1 ml of a 3% suspension of red blood cells in a total volume of 5 ml was determined; hereafter, this concentration will be referred to as the HC_{50} value.

Infrared (IR) and X-ray powder diffraction (XRD) analyses

One milligram of the various particle size fractions of montmorillonite and kaolinite was thoroughly mixed with 300 mg of KBr and pressed into a transparent disk. The spectra were obtained on a Perkin-Elmer 180 spectrophotometer. Two-milliliter aliquots of a suspension (1 mg/ml) of the various particle size fractions of the two minerals were dried on glass microscope slides producing oriented samples. Each film was scanned for the minerals' respective 001 spacing with a Sie-

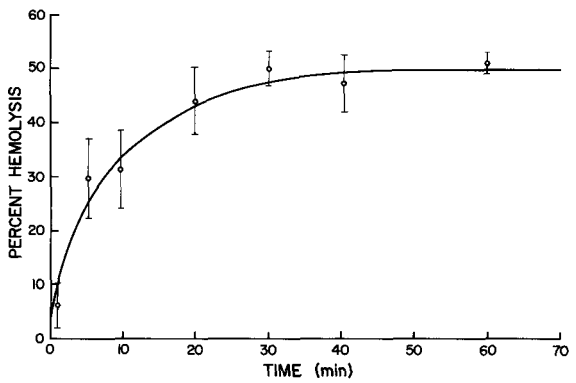

Figure 1. Percentage of hemolysis produced by montmorillonite as a function of time (HC_{50} value of montmorillonite was used) shown as mean ± SD, where n = 4.

mens Type F, X-ray diffractometer equipped with a diffracted-beam graphite monochromator using CuKα radiation.

Aluminum-hydroxy compounds

Aluminum-hydroxy interlayers in montmorillonite and coatings on other minerals were prepared by adding the appropriate amount of $Al(NO_3)_3 \cdot 9\ H_2O$ to 200 ml of a suspension containing 5 g of montmorillonite, silica, palygorskite, or kaolinite. A concentration of 6 meq/g was used. Additional samples of montmorillonite were also prepared using 4 and 16 meq Al/g of mineral. The suspensions were neutralized by adding 200 ml of NaOH solution containing an appropriate amount of NaOH to yield a final OH/Al ratio of 1.5 for all samples. The NaOH solution was added at 1 ml/min with continuous stirring. The resulting suspensions were diluted to 1 liter and aged for 10 days. Samples were dialyzed against distilled water for 5 days to remove excess salts, dried, resuspended in water, and sonicated for 5 min at 80 watts.

The cation-exchange capacities (CEC) of the treated and untreated montmorillonite samples were obtained using Jackson's procedure (1975). The procedure was modified by using Mg and Ca as the saturating and displacing cations, respectively.

Scanning electron microscopy

Immediately after the 1-hr reaction period, drops of the mineral-erythrocyte or erythrocyte suspensions were placed on glass cover slips and fixed in 1% gluteraldehyde in saline solution at 4°C. After 24 hr, the slides were taken through graded dehydrating solutions of ethanol and then dried by the critical point method in a Technics CPA II. Samples were coated with platinum-gold in a Technics Sputter Coater Hummer I, and examined in an AMR 1200 scanning electron microscope.

RESULTS AND DISCUSSION

HC_{50} values

Montmorillonite was the most active mineral of the five tested in causing hemolysis (HC_{50} = 0.006 mg/ml), whereas kaolinite was the least active (HC_{50} = 0.6 mg/ml)—approximately 100 times less active than montmorillonite. The hemolytic activities of silica, palygorskite, and chrysotile were intermediate between those of montmorillonite and kaolinite (Table 1). Woodworth *et al.* (1982) also found montmorillonite to have greater hemolytic activity than either chrysotile or kaolinite; however, they reported silica to be less active than kaolinite.

Nontronite and hectorite, iron and magnesium smectites, respectively, were also found to have hemolytic activities similar to those of montmorillonite (HC_{50} ~ 0.005 and 0.008 mg/ml, respectively). The present results indicate that the nature of the ion in octahedral coordination has little effect on the hemolytic properties of the minerals in the smectite family, i.e., magnesium varieties are no more active than iron or aluminum varieties. Inasmuch as nontronite and montmorillonite are dioctahedral and hectorite is trioctahedral, the degree of octahedral occupancy also appears to have had little effect on the hemolytic activity of the smectite minerals. Likewise, sepiolite, a Mg trioctahedral fibrous clay, had a hemolytic activity (HC_{50} = 0.08 mg/ml) only slightly greater than palygorskite, a Mg-Al dioctahedral fibrous clay (HC_{50} = 0.06 mg/ml).

Time course of hemolysis

At HC_{50} concentration of the minerals, the 5 minerals destroyed red blood cells at about the same rate. As shown for montmorillonite in Figure 1, the hemolytic process was rapid; more than half of the potential hemolysis occurred within 10 min, and the process was essentially complete after 40 min. In addition, minerals which had reached a plateau in their hemolytic activity during an initial trial were inactive in subsequent trials with fresh erythrocytes. Similar results for the kinetics of hemolysis of human red blood cells were reported for chrysotile and crocidolite by Jaurand *et al.* (1979) who concluded that hemolysis by chrysotile was a self-inhibiting process. The lack of additional hemolysis with time or after prior hemolytic equilibrium shows that the reactive sites on the clays were blocked by cellular constituents (Morgan *et al.*, 1977).

Particle-size effects

Harley and Margolis (1961) showed that hemolysis induced by silica depended on particle size. To determine the dependence of hemolysis on the particle size of other minerals, the hemolytic activity of the following size separates of montmorillonite and kaolinite was determined: <0.2, 0.2–2, 2–5, 5–20, and 20–50 μm. (Because of the inherent small size of montmorillonite particles, insufficient 20–50 μm size material was available for the experiment.) Because HC_{50} concentrations of each of the minerals were used, all size fractions should have given 50% hemolysis if particle size had no effect. The 0.2–2-μm separates were the most hemolytically active for both montmorillonite and kaolinite (Table 2). For kaolinite the <0.2-μm particles were completely inactive, even at relatively high concentrations.

Table 2. Percentage of hemolysis produced by various particle size fractions of kaolinite and montmorillonite.[1]

Particle size (μm)	Kaolinite (%)	Montmorillonite (%)
<0.2	nd[2]	43 ± 4[3]
0.2–2	75 ± 2	60 ± 5
2–5	58 ± 4	3 ± 2
5–20	20 ± 4	nd
20–50	3 ± 1	—

[1] 0.6 and 0.006 mg/ml of the size fractions were used for kaolinite and montmorillonite, respectively.
[2] No detectable hemolysis.
[3] Mean ± SD (n = 4).

Table 3. Relative X-ray powder diffraction intensities of various size fractions of kaolinite and montmorillonite.

Particle size (μm)	Kaolinite (7.2-Å spacing)	Montmoriillonite (14.7-Å spacing)
<0.2	<5	34
0.2–2	100	100
2–5	100	15
5–20	100	<5
20–50	100	—

The IR spectra of the <0.2-μm separate from kaolinite (Figure 2) show that very little kaolinite was present, as evidenced by the near total absence of the characteristic 3695, 3670, 3655, and 3620 cm^{-1} bands of kaolinite. The near absence of kaolinite was confirmed by the weak XRD peaks of this mineral in the pattern of the <0.2-μm material (Table 3). The two >2-μm separates gave strong intensity 001 diffraction peaks at 7.2 Å similar to that obtained for the 0.2–2-μm fraction. The XRD pattern showed that the <0.2-μm fraction of kaolinite was largely noncrystalline material. Thus, the hemolytic inactivity of the <0.2-μm separate of kaolinite was due to a predominance of what appears to be inactive impurities, whereas the decreasing hemolysis observed with increasing particle size above 0.2 μm was indeed a particle-size effect.

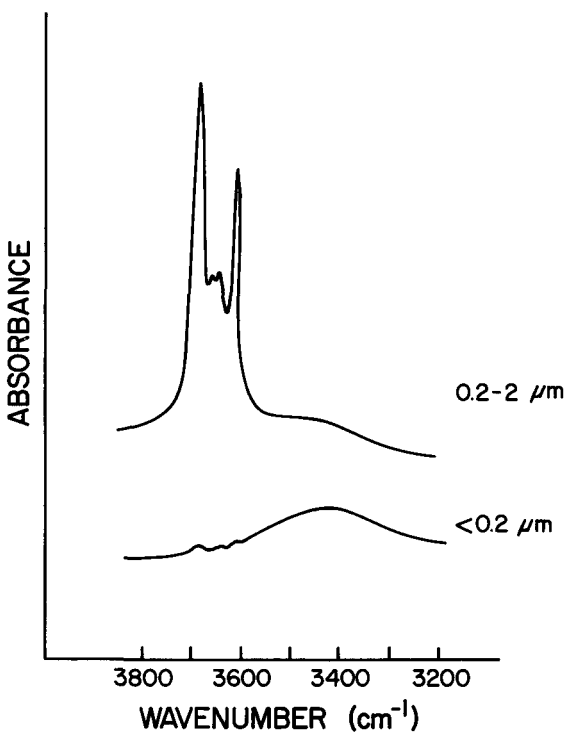

Figure 2. Comparative infrared spectra of the <0.2- and 0.2–2-μm size separates of kaolinite.

The 0.2–2-μm size separate of kaolinite was the most effective in producing hemolysis. Inasmuch as the diameter of the bovine erythrocyte is ~4–9 μm (West and Todd, 1955), plate-shaped kaolinite particles >2 μm may have difficulty contacting enough of the surface of the erythrocyte which is shaped like a biconcave disk (Figure 4).

The results for montmorillonite are more difficult to interpret. Alexiades and Jackson (1966) reported that the <0.2-μm fraction of Upton montmorillonite contains no noncrystalline material. The difference in XRD intensities for the <0.2- and 0.2–2-μm separates (Table 3) is, therefore, likely due to particle-size effects. XRD intensity decreased as crystallite size and order decreased, and small particles appeared amorphous to X-rays (Jackson, 1975). Therefore, as the particle size decreased, the diffraction intensity decreased even though the sample contains no noncrystalline material. On the other hand, the decreasing diffraction intensities for the 2–5- and 5–20-μm separates of montmorillonite were likely due to increasing amounts of impurities because montmorillonite tends to crystallize into particles of predominantly <2-μm size, and the main impurity in the Upton montmorillonite is quartz.

The IR spectra of the four size separates of montmorillonite (Figure 3) show an absorption peak at 3620 cm^{-1}, the OH-stretching mode characteristic of montmorillonite; however, the water absorption band (3400 cm^{-1}) increased with decreasing particle size, indicating that more water was associated with the smaller fractions. Consequently, proportionately less montmorillonite was present in the 1-mg, air-dry sample used for the IR analyses in the smaller particle size separates. Thus, the effect of particle size of montmorillonite was confounded by the increasing water content of the samples in the finer fractions (Figure 3) and by the increasing impurity of the coarser fractions (Table 3).

These studies indicate that particle size has a real effect on hemolysis by montmorillonite; the 0.2–2-μm particles were the most active, whereas larger and smaller particles were less active. The data are confounded, however, by changes in mineralogical purity and the tendency of different minerals to be concen-

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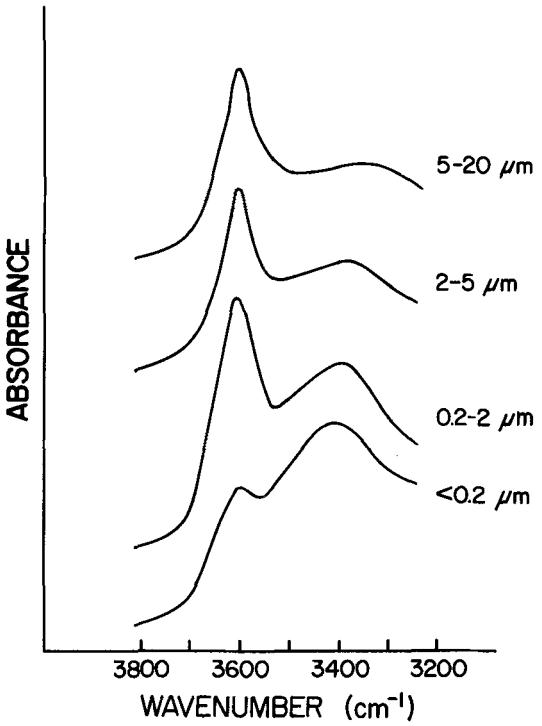

Figure 3. Comparative infrared spectra of the <0.2-, 0.2–2-, 2–5-, and 5–20-μm size separates of montmorillonite.

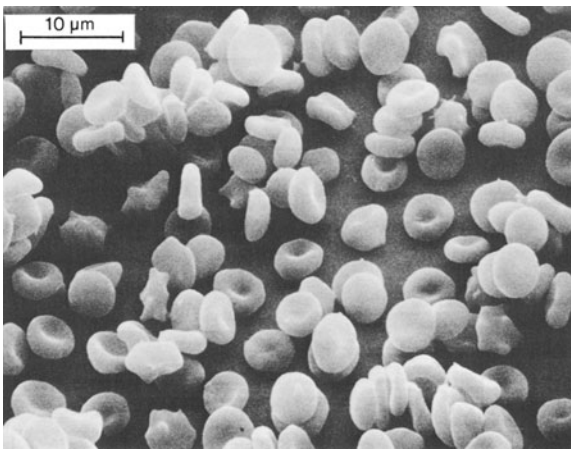

Figure 4. Scanning electron micrograph of red blood cells.

trated in different crystal sizes. Nevertheless, the results are in general agreement with those of Harley and Margolis (1961) who found that the hemolytic activity of silica increased as the particle size increased from 0.003 to 0.03 μm.

Heated palygorskite

Palygorskite is an excellent mineral for studying the possibility of hydrogen bonding between mineral edges and the erythrocyte membrane. Many hydrogen-donating sites in the form of SiOH groups are present on the surface of the untreated mineral (Serna *et al.*, 1977). In the folded structure some of the SiOH groups are perturbed by interacting with neighboring amphibole chains (Van Scoyoc *et al.*, 1979). The folded, dehydroxylated structure should have steric problems for an interaction between the SiOH groups and the membrane.

Heating palygorskite to 250°C caused little, if any, decrease in hemolysis, whereas heating to 400° and 500°C resulted in a decrease in hemolysis from 50% to 27% and <5%, respectively. After aging the heated (500°C) palygorskite in deionized water, however, it became increasingly more hemolytic, and after 10 months its hemolytic activity had returned to about one third that of the unheated material. These data can be explained by the results of Van Scoyoc *et al.* (1979) which show that palygorskite heated at lower temperatures partially dehydrates and folds with rapid rehydration and unfolding in the presence of water. Heating between 400° and 500°C, however, produces dehydroxylation. Palygorskite in the latter state reverts very slowly toward its original structure. Thus, hydrogen bonding between palygorskite and the erythrocyte membrane is likely.

Aluminum-hydroxy compounds

Montmorillonite is an exceptionally potent hemolytic mineral. In view of its high negative surface charge, an attempt was made to correlate the hemolytic activity with a reduction in surface charge. The surface charge was reduced by adding an Al-hydroxy polymer to a suspension of montmorillonite. Al-hydroxy polymers form interlayers in montmorillonite with a concomitant decrease in CEC (Meyers and Ahlrichs, 1972; Barnhisel, 1977).

The CEC of the montmorillonite treated with 16, 6, and 4 meq of Al as the hydroxy polymer per gram of mineral was 28, 52, and 60 meq/100 g, respectively, compared to 90 meq/100 g for the untreated sample. Hemolytic activity was nearly eliminated (HC_{50} > 2 mg/ml) when montmorillonite was treated with 4 meq of Al as the hydroxy polymer. HC_{50} values >3 mg/ml were obtained at high Al-hydroxy polymer levels.

Furthermore, silica, palygorskite, and kaolinite treated with 6 meq of Al as the hydroxy polymer per gram of mineral exhibited greatly reduced hemolytic properties; however, HC_{50} values were not determined. It seems likely, therefore, that when Al-hydroxy compounds were added to a suspension of montmorillonite, silica, palygorskite, or kaolinite, the minerals acquired a surface coating of the Al compounds which blocked the surface sites that cause lysis.

For montmorillonite, the reduction in charge when Al-hydroxy compounds were added may be a partial explanation for the decreased activity; however, the

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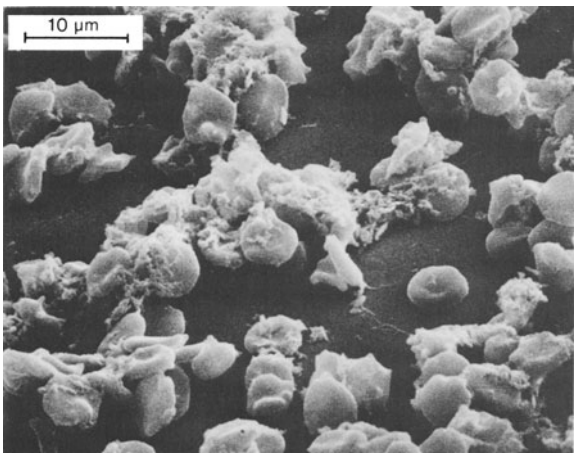

Figure 5. Scanning electron micrograph of a mixture of kaolinite and red blood cells.

physical coating of the silica sheets of montmorillonite with Al-hydroxy compounds was likely the primary factor causing the decrease in hemolysis. The coating could possibly have prevented the silanol groups on the edges of montmorillonite from contacting the erythrocyte membrane by a reaction such as proposed by Boehm (1966):

$$\equiv Si\text{–}OH + Al(OH)_3 \longrightarrow \equiv Si\text{–}O\text{–}Al(OH)_2 + H_2O.$$

The hypothesis of Al-hydroxy coating on montmorillonite as opposed to charge reduction as cause of the decreased hemolysis is strengthened by the following observation. The addition of 6 meq of Al as a hydroxy polymer resulted in a CEC of 52 meq/100 g, a value about half of the original value of 90 meq/100 g, yet the hemolytic activity was reduced 500-fold from HC_{50} = 0.006 mg/ml to >3 mg/ml. Not only was the Al-hydroxy coating material hemolytically inactive but other aluminum compounds (gibbsite and alumina) showed no activity at >5 mg/ml.

Scanning electron microscopy

Figure 4 is a scanning electron micrograph of intact erythrocytes. Kaolinite at an HC_{50} level incubated with a suspension of cells destroyed the structural integrity of some of the cells (Figure 5). Moreover, at a concentration of the mineral that caused 100% hemolysis, intact erythrocytes were not found. The interaction of montmorillonite, palygorskite, and chrysotile with erythrocytes gave the same results, i.e., the minerals destroyed the structural integrity of the cells.

Simple osmotic lysis of erythrocytes does not destroy the cell form, but rather produces "ghost" cells emptied of the cellular constituents yet appearing much like a normal cell (Dodge *et al.,* 1963). The minerals completely destroyed the structural integrity of the cell membrane making the osmotic lysis mechanism proposed by Harington *et al.* (1975) suspect, inasmuch as this mechanism should have produced ghost cells. The observed reaction was much more destructive and left no morphological semblance of the original cell.

Hemolytic activities of other materials

In addition to the five hemolytically active compounds studied in detail, a survey of numerous other materials showed that sepiolite (Valencia, Spain), nontronite (Garfield, Washington), hectorite (Hector, California), opaline phytoliths, and Syloid 224 (porous synthetic silica) were also active and had HC_{50} values of <1 mg/ml.

CONCLUSIONS

Under the conditions of this study, hemolytic activities in the order smectites > silica > palygorskite ≃ sepiolite > chrysotile > kaolinite were found. *In vitro* lysis of erythrocytes by mineral surfaces was rapid and complete in less than 1 hr, and the mineral surfaces became saturated with cellular components and lost their lytic activity. Lysis by minerals destroyed the structural integrity of the erythrocyte membrane in contrast to simple hypotonic hemolysis. Moreover, the nature of the octahedral cation (Mg vs. Al vs. Fe) and the degree of octahedral occupancy (di- vs. trioctahedral) in smectites and fibrous clays did not greatly affect the hemolytic activity, and the 0.2–2-μm particle size fraction of montmorillonite and kaolinite was the most active in lysing red blood cells.

Aluminum-hydroxy polymers coated and largely deactivated montmorillonite, silica, palygorskite, and kaolinite. The fibrous morphology of palygorskite, sepiolite, and chrysotile did not appear to be relevant, i.e., the lysis of erythrocytes by these minerals was primarily related to the minerals' surface physicochemical properties rather than to their particle shape.

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