Effects of Silicate Polymers on Erythrocytes in Presence and Absence of Complement

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ABSTRACT The effects of silicates upon erythrocytes depend upon the degree of polymerization. Monomeric silicate does not appear to be taken up by red cells. Polymerized silicates are taken up and bound tightly. In the presence of small polymeric forms erythrocytes are lysed by complement. Larger polymers are bound to erythrocytes but do not sensitize to complement hemolysis. Larger polymers, however, are directly toxic and cause hemolysis in the absence of complement. Red cells exposed to complement-active polymers show characteristic alteration in morphology with the assumption of irregular bell shapes. Larger polymers cause the cells to become spherical before spontaneous rupture occurs. Large polymers cause erythrocyte agglutination but this is minimal or absent with small complement-active polymers. Complement-active polymers cause little or no change in osmotic fragility. Increase in mechanical fragility is a sensitive indication of the presence of larger, agglutinating polymers. The conversion of pneumococci from Gram positivity to negativity appears to be caused principally by complement-active polymers. Possible implications of polymer size and complement activity are discussed in relation to production of silicotic lesions by silica-containing ores.

Landsteiner and Jagic (1) reported in 1904 that erythrocytes are agglutinated by exposure to colloidal silica and that lysis takes place when fresh serum is added. Evidence was presented that lysis is caused by complement since it is abolished by heating the serum to 56°C. Subsequently Ponder (2) carried out kinetic studies on the lysis of red cells by serum in the presence of silicates.

In 1956 MacLeod and Roe (3) reported that silicate solutions cause living or heat-killed suspensions of pneumococcus to be converted to Gram negativity. Silicates also exert a lethal effect upon pneumococcus. These silicate
effects are relatively specific for pneumococcus since among many other bacterial species tested they were found to occur in only a few strains of viridans streptococci.

The present studies were undertaken to determine the effects of different polymeric forms of silicate upon red blood cells and to compare these effects with those found in pneumococcus. The nature of the serum components required for serum-induced hemolysis of silicate-treated cells has also been considered.

Use of erythrocytes to study biological effects of silicate polymers has many advantages as compared to the use of pneumococcus. The most important of these is a sharp and easily quantifiable endpoint, namely hemolysis.

MATERIALS AND METHODS

Preparation of Glassware All glassware was acid-washed and then rinsed twenty times in tap water and ten times in distilled water to insure that it was not contaminated by soaps, detergents, or interfering ions.

Red Blood Cells Blood from human donors was drawn usually in dry, sterile 50 ml syringes. When a larger volume was required, a transfusion set was used and the blood allowed to flow by gravity directly into a collecting flask. The blood was mixed with an equal volume of Alsever's solution. Group O human red blood cells were used most commonly. In experiments employing cells of other blood groups, compatible serum was used, usually from the same donor. In general the cells were held at 4°C for 4 days before use and were discarded after 10 days of storage. In occasional experiments, cells were used immediately after blood was drawn and at intervals up to 3 weeks later. No difference in behavior was observed under these varied conditions of storage. Immediately before use cells were washed three times at room temperature in 15 to 30 volumes of 0.15 M sodium chloride solution containing 0.005 M barbital buffer, pH 7.4 (buffered saline). They were resuspended at a concentration of 2 per cent in buffered saline.

Serum Blood was allowed to stand at room temperature for 1 hour. The clot was then rimmed and permitted to retrace for 4 to 6 hours at ice box temperature. Serum was decanted into centrifuge tubes which were spun immediately at 2,000 RPM for 10 minutes to remove red cells. Serum was stored in volumes of 0.5 to 2 ml in CO₂ ice until used. Serum treated in this manner was found to retain its hemolytic activity for silicate-treated red blood cells for at least 1 year.

In most experiments serum was diluted with buffered saline containing 0.00015 M Ca²⁺ and 0.0005 M Mg²⁺. The concentration of serum required for a given amount of lysis varied with the concentration of silicate used to sensitize the erythrocytes. Cells treated with high concentrations of silicate required less serum than those treated with small amounts of silicate. At the silicate concentrations used in most experiments, dilutions of human serum of 1:4 to 1:8 gave 50 per cent hemolysis after 30 minutes’ incubation at 37°C. A serum dilution of 1:2 was used customarily in order to provide excess hemolytic activity.
Properdin Reagents The following reagents were supplied to us by the late Dr. Louis Pillemer:
Eight different batches of RP serum. This is serum from which properdin had been largely removed by treatment with zymosan.
One lot of R3 serum. This is serum lacking the third component of complement.
Three batches of purified human properdin and two of bovine properdin.
Two lots of rabbit anti-human properdin serum, one in fluid state that had previously been absorbed with human O and A cells, and a lyophilized preparation that had not been absorbed.
In addition, multiple lots of RP serum and of R3 serum were prepared in this laboratory by the methods described by Pillemer et al. (4).
RP and R3 sera were stored in CO₂ ice. RP serum retained its activity without apparent change for 18 months. Properdin was kept in a mechanical refrigerator in the frozen state at --20°C as recommended by Pillemer.

Complement Dried guinea pig complement was obtained from Cappel Laboratories. Usually, it was dissolved and diluted on the day of use. Occasional samples after reconstitution were stored in the frozen state at --20°C for 1 to 3 days before use.

Amboceptor (Hemolysin) Amboceptor for sheep red blood cells, prepared by immunization of rabbits, was obtained from Cappel Laboratories. It was stored at 1:100 dilution in 0.15 M sodium chloride solution, and was used for as long as 1 month after dilution.

Sheep Red Blood Cells Sheep cells were obtained from the University of Pennsylvania School of Veterinary Medicine. They were stored in Alsever’s solution at 4°C and were used for as long as 1 month from the time the blood was drawn or until increased hemolysis was observed on washing with buffer. Increased hemolysis appeared abruptly and was usually associated with obvious contamination.

Silicates The silicates used were sodium silicofluoride, Na₂SiF₆ (cp, Fisher Scientific Company) and sodium metasilicate, Na₂SiO₃·9H₂O (Baker analyzed reagent). The activity of these salts was dependent upon the amount of silicate present. Fluoride added to red blood cells in twice the molarity of fluoride present in solutions of the complex Na₂SiF₆ used in these experiments had no demonstrable effects in the test systems used.
The method of preparation of silicate solutions depended upon the type of silicate polymer desired in a particular experiment. For most experiments, a stock solution of 0.2 per cent Na₂SiF₆ was used. The solution was stored at pH 11.4 to 11.7 in a polyethylene bottle¹ for periods up to 1 month, although occasionally it was prepared freshly on the day of the experiment. Aliquots were quickly neutralized with HCl using phenol red as inside indicator, and were allowed to stand at neutrality until the desired polymer had formed. Color reactions of the solutions with molybdic acid provide a useful guide to the rate and per cent of polymerization.

¹ Spontaneous polymerization occurs on prolonged storage in polyethylene containers with accompanying fall in pH of the silicate solution. For this reason solutions at high alkaline pH should not be stored in polyethylene containers for longer than 2 to 3 months.
Immediately prior to addition of red blood cells, the silicate solution was diluted to the required concentration and brought to isotonicity by adding a solution of NaCl. In some experiments silicate was dissolved and stored at neutrality or at acid pH. Sodium silicofluoride at a final concentration of 0.2 per cent dissolves readily at neutrality, but only slowly over periods up to 48 hours at acid or alkaline extremes of pH. A marked buffering effect occurs in the pH range between 5.2 and 5.8.

**Treatment of Erythrocytes with Silicate Solutions** A 2 per cent suspension of washed red cells in buffered saline and an equal volume of silicate solution were mixed rapidly for 1 to 10 minutes (usually 3 minutes) at room temperature. The suspension was then centrifuged at 2,000 RPM for about 1 minute. The sedimented cells were washed once with buffered saline, centrifuged, and resuspended at 2 per cent concentration in buffered saline with Mg++ and Ca++ ions added.

Varying degrees of clumping occurred depending upon the type of polymer used and the duration of centrifugation. When “complement-active” polymer was used, clumping was minimal and aliquots could be pipetted reproducibly. Larger polymeric forms, however, caused marked red cell agglutination, which interfered with quantitative studies of hemolysis.

If excess silicate was not removed from the red cells by centrifugation before adding fresh serum, complement-induced lysis occurred but was partially inhibited, as described by Ponder (5). This is believed to result from inactivation of complement by excess silicate ions present in solution. Inhibition of complement action did not occur when the silicate-treated red cells were washed in buffered saline before use.

**Measurement of Hemolysis of Silicate-Treated Red Blood Cells. Qualitative Method** In test tubes measuring 75 X 10 mm, 0.25 ml of a 2 per cent suspension of silicate-treated erythrocytes in buffered saline containing Mg++ and Ca++ ions was added to 0.25 ml of dilutions of serum in buffered saline containing Mg++ and Ca++. The tubes were placed in a water bath at 37°C and examined at 5 minute intervals in order to estimate the amount of hemolysis. At each reading the suspension was shaken to disperse the cells uniformly. After 30 minutes the cells were sedimented at 2,000 RPM for 5 minutes. The amount of hemolysis was estimated visually and in some cases measured directly in a Beckman DU spectrophotometer at 540 mμ.

**Quantitative Method** 2.5 to 3.0 ml of silicate-treated erythrocytes were added to an equal volume of serum or serum dilution and placed at 37°C. At 5 minute intervals 0.75 ml portions were removed and put into cold tubes in ice water to inhibit further hemolysis. After 30 minutes all tubes were centrifuged and the amount of hemolysis estimated visually. 0.5 ml of the supernatant of each was then mixed with 2.0 ml of cyanmethemoglobin reagent and cyanmethemoglobin was measured in a Klett-Summerson colorimeter employing a 540 mμ green filter.

**RESULTS**

*The Relation of Silicate Polymerization to Complement-Induced Hemolysis*

In preparing solutions of sodium silicofluoride to be used for sensitizing erythrocytes to complement-induced lysis, great difficulty was experienced
initially in obtaining reproducible results from day to day or even at different times on a single day. Similar amounts of the same reagents frequently resulted in marked differences in the amount of hemolysis. The conditions of these experiments suggested that a mixture of different silicate polymers was being formed and that only certain polymers were active in causing complement lysis of treated cells. The effects of different silicate polymers

![Figure 1](Path_to_image)

**Figure 1.** Relationship between silicate polymerization as measured by molybdic acid reactivity and ability to sensitize red cells to lysis by complement. Cells were sensitized by silicate in a final concentration of 0.025 per cent.

were studied therefore by preparing them under conditions which are known to affect polymerization. These conditions include alterations in the pH of the solutions, in the concentration of silicate, in the time of polymerization, and in the presence of contaminating ions or compounds.

**Effect of pH on Silicate Polymerization and Activity in Complement-Induced Hemolysis** At extremes of pH (above 11.0 or below 3.0) silicate solutions tend to exist in an unpolymerized state. As the pH approaches neutrality, polymerization proceeds rapidly. Polymerization can be measured in its early stages by observing the reactivity of the silicate solutions with molybdic acid (6). Monomeric silicate solutions react with molybdic acid within 75
seconds to give a green-yellow color. Solutions consisting of dimers and trimers react with molybdic acid slowly over about 10 minutes. Larger polymers do not cause a color change. Color was measured in a Beckman DU spectrophotometer at 400 m\(\mu\).

Solutions of silicofluoride were held at various acid or alkaline pH values and the amount of polymerization was measured by molybdic acid reactivity. The aliquots were then rapidly neutralized, adjusted to appropriate concentrations, and used to treat erythrocytes. In this way it was possible to correlate the amount of polymerization of a given solution with its ability to sensitize erythrocytes to complement lysis. Fig. 1 shows the results of such an experiment.

A suspension of silicofluoride powder was rapidly brought into solution in distilled water and neutralized by addition of 1 N NaOH. Final concentration was 0.2 per cent. Aliquots of 24 ml were removed and brought to various acid pH values by addition of HCl. The solutions were allowed to stand at room temperature for 4.5 hours before testing for the amount of polymerization by molybdic acid reactivity. A portion of each solution was rapidly neutralized by addition of NaOH. The neutralized solutions were tested immediately at various dilutions for their capacity to sensitize erythrocytes.

As shown in Fig. 1, the polymerization of silicate, as measured by molybdic acid reactivity, is dependent upon the pH at which the solution is held, and the capacity of the solutions to sensitize erythrocytes to complement lysis is related to the amount of silicate present in polymerized form.

The polymerization of silicofluoride solutions held in the alkaline range is also increased as neutrality is approached, and the ability of the solution to sensitize erythrocytes to complement lysis is again related to the amount of silicate present in polymerized form.

Effect of Time on Silicate Polymerization and Activity in Complement-Induced Hemolysis In most experiments, silicate preparations were held at pH 11.3 to 11.7 for at least 24 hours prior to use, in order to disaggregate polymers formed while the powder was being dissolved. The monomeric preparation was then quickly brought to pH 7.4, with a final concentration of silicate of 0.2 per cent. At neutrality polymerization occurs rapidly as measured by the loss of molybdic acid reactivity. The development of the ability to sensitize erythrocytes to complement lysis paralleled the appearance of a polymeric form of the silicate as shown in Fig. 2.

Effect of Dilution on Silicate Polymerization and Activity in Complement-Induced Hemolysis Dilution of silicate solutions decreases the rate of polymerization or abolishes it. At neutrality, a solution of 0.2 per cent Na\(_2\)SiF\(_6\) polymerizes rapidly with maximal erythrocyte-sensitizing activity appearing within 60 to 90 minutes after neutralization. At a concentration of 0.125 per cent poly-
merization occurs slowly and less extensively, whereas at a concentration of 0.1 per cent under similar conditions, polymerization does not occur.  

Fig. 3 shows the ability of silicate solutions of various concentrations held at neutral pH to sensitize erythrocytes to complement lysis. Solutions at the concentrations indicated in Fig. 3 were permitted to stand at neutrality for 90 minutes at room temperature after which aliquots were further diluted to

![Graph](image-url)  
**Figure 2.** Comparison of polymerization of 0.2 per cent silicate solution held at pH 7.4 for various times with its capacity to sensitize red cells to complement lysis. Cells were sensitized by silicate in a final concentration of 0.025 per cent.

0.025 per cent and used immediately to sensitize erythrocytes. A second set of solutions was tested after holding at neutrality for 5 hours. As shown in Fig. 3 the 0.1 per cent solution was inactive in the hemolytic test system. The 0.125 per cent solution showed slight activity at 90 minutes. After 5 hours activity had increased in the 0.125 per cent solution, but none was present in the 0.1 per cent solution even after a 5 hour period. The difference in behavior between the 0.1 per cent and 0.125 per cent solutions has been found to be highly reproducible.

Effect of a Nidus upon Polymerization of Silicate at a Concentration of 0.1 Per Cent  
The observations recorded in Fig. 3 demonstrate that at a concentration of 0.1 per cent silicate solutions do not develop the capacity to sensitize
erythrocytes provided the silicate is all in monomeric form at the time the solution is neutralized. This is in keeping with the inability of silicate solutions at this or lower concentrations to polymerize. On the other hand if polymerization has begun in a silicate solution of higher concentration, and it is then diluted to 0.1 per cent, polymerization continues provided the solution is at neutral pH. In other words, although polymerization does not appear to be initiated in 0.1 per cent silicate solution, if it has already begun, the process can continue at this concentration.

Fig. 4 summarizes the observations on two samples of 0.1 per cent silicate that were held at neutrality under slightly different conditions. In the first sample (1) the silicate solution which had been stored in monomeric form at pH 11.3 was diluted to 0.1 per cent concentration prior to neutralization and did not develop any red cell sensitizing activity upon standing at room temperature for 4 hours. The second sample (2) was neutralized while at a concentration of 0.2 per cent and diluted to 0.1 per cent concentration immediately afterward. During the neutralization process in 0.2 per cent
solution, polymerization began, as shown by the moderate amount of red cell-sensitizing activity present 5 minutes after neutralization and dilution to 0.1 per cent concentration. Both samples were again tested 4 hours later. As shown in Fig. 4, no sensitizing activity appeared in sample (1) which did not contain a nidus for polymerization, whereas the activity of sample (2)

![Figure 4](image-url)

**Figure 4.** Influence of a nidus upon polymerization of silicate at 0.1 per cent concentration. Line (1) represents silicate that was diluted to 0.1 per cent before neutralization. Line (2) represents polymerization of silicate which had been neutralized as a 0.2 per cent solution, immediately diluted to 0.1 per cent concentration, and tested after standing for 5 minutes and again at 4 hours. In all instances the solutions were diluted to a concentration of 0.0125 per cent immediately before addition to the red cell suspension.

which contained a small amount of complement-active polymer at the time it was diluted to 0.1 per cent, continued to increase, indicating progressive polymerization.

*Effect of Continued Polymerization on Behavior of Complement-Active Silicate Polymers*  Upon storage of polymerizing solutions of silicate at room temperature or at 4°C, the capacity of the solutions to sensitize erythrocytes to lysis by complement increases for a short time, and then, as polymerization progresses, gradually declines. With increasing polymerization other effects
upon erythrocytes become apparent which are independent of serum factors. The latter effects are described in a subsequent section of this paper.

The development and decline of complement-dependent activity are illustrated by the results of experiments which are graphed in Fig. 5.

A solution of sodium silicofluoride at 0.2 per cent concentration was neutralized and allowed to polymerize at room temperature over a 2 day period. Aliquots were removed shortly after neutralization and again after 24 and 48 hours to test for their complement-dependent sensitizing activity. Immediately before addition to erythrocytes, the solutions were diluted to the concentrations shown in Fig. 5. When tested at 0.05 per cent concentration the activity remained constant for the first 24 hours and declined during the 2nd day. Tests carried out at lower concentrations of silicate (0.0125 per cent and 0.006 per cent) indicated, however, that a marked loss of complement-dependent activity had occurred during the first 24 hours which was not revealed by testing at a silicate concentration of 0.05 per cent. Concomitant with the loss in complement-dependent activity, there developed spontaneous lysis of the red blood cells in the control tubes to which no serum had been added, revealing a direct hemolytic action of silicate polymers of sizes larger...
than those active in the complement system. When the polymers became sufficiently large, serum could be shown to partially protect cells from the direct hemolytic effect. More lysis was then seen in control tubes than in those to which serum was added, whether or not the serum contained complement activity.

Subsequent studies on the effects of silicate polymers on erythrocytes, especially tests of mechanical fragility, have demonstrated that their activity is in a constant state of change. For this reason, in most of our experiments silicate solutions were used under empirically chosen, standardized conditions. Silicate solutions were stored at 0.2 per cent concentration in polyethylene bottles at pH 11.3 to 11.7. The solutions were quickly neutralized and then allowed to stand at room temperature for 90 to 120 minutes before addition to red blood cells. Under these conditions reproducible results were obtained in sensitizing erythrocytes to lysis by complement.

Polymerized silicate solutions are depolymerized at acid or alkaline reactions. The time required for depolymerization is roughly proportional to polymer size. At high alkaline values the complement-active polymer is completely disaggregated within 20 minutes. At acid pH depolymerization is slower and less complete.

Uptake of Silicate Polymers by Erythrocytes and Effects upon Agglutinability, Morphology, and Osmotic and Mechanical Fragility

Effect of Polymerization on Uptake of Silicate by erythrocytes As previously noted marked variability in capacity to sensitize erythrocytes to complement lysis occurs in silicate solutions depending upon whether the silicate is in monomeric form or in various stages of polymerization. These observations led to a study of the uptake of silicate by erythrocytes, since the differences in hemolysis that were observed might be related to uptake by red blood cells as well as to differences in the properties of the polymers themselves. Both factors are of importance, as will be described.

The observations recorded in Fig. 6 demonstrate that polymerized silicate is removed from solution by erythrocytes but that no uptake of the monomer occurs. In this experiment a monomeric silicate solution (0.2 per cent, pH 11.3) was rapidly neutralized with HCl and at various intervals thereafter, as shown on the abscissa, samples were removed and added to packed erythrocytes. After mixing for 1 minute the cells were spun out. The amount of silicate remaining in the supernate was measured by the molybdic acid reaction following depolymerization by means of NaOH. The lower curve in Fig. 6 shows the amount of polymerization that had occurred in the silicate solutions which were added to the red blood cells at the various times as calculated from the molybdic acid reactivity. It is apparent from the data
shown in Fig. 6 that the amount of silicate taken up by erythrocytes parallels the amount that polymerized over the period of 90 minutes.

Amount of Silicate Taken up by Erythrocytes. In the presence of an excess of red blood cells all the polymerized silicate is removed from solution. This is illustrated in Fig. 7 which depicts the removal of silicate from a partially polymerized, 0.05 per cent solution by various volumes of packed human erythrocytes. With even the largest volume of cells no more than 63 per cent of silicate was removed; the remainder was shown to be in monomeric form by its reactivity with molybdic acid. As the volume of adsorbing red blood cells was reduced so that excess polymerized silicate was present, the amount removed from solution was correspondingly reduced. Addition of the supernates to fresh red cells resulted in further removal of silicate, although never more than the amount that was known to be in polymeric form, in this case approximately 65 per cent.

Erythrocytes that had been exposed to an excess of polymerized silicate and then removed by centrifugation were unable to adsorb more polymer when added to a second polymerized solution. From these and the foregoing observations it is apparent that a given volume or number of cells fixes a
finite amount of polymerized silicate. It was possible therefore to calculate that the number of molecules of silicate adsorbed per human erythrocyte in these experiments was approximately \(3 \times 10^{19}\). However, because the number of silicate molecules in each polymer is unknown, the number of binding sites per cell was not determined.

The present study has not revealed the component or components of erythrocytes that polymerized silicates combine with. The role of lecithin in the cell membrane was studied because silicate combines with and is precipitated by substituted ammonium compounds such as choline and by lecithin itself. The observations of Turner (7) on the lecithin content of erythrocytes of various species provided an indirect means to test whether the presence of lecithin in red blood cells is necessary for uptake of silicate and for lysis by complement. Turner has shown that erythrocytes of certain species of ungulates such as sheep, goat, and ox lack lecithin, whereas it is present in the red cells of other species such as man, rabbit, dog, and guinea pig. When the red cells from sheep, goat, and ox were sensitized by treatment with polymerized silicate and then exposed to serum containing active complement, the hemolytic process did not differ qualitatively from that
observed with human cells. Although quantitative studies might reveal differences among the cells of different species in their reaction to silicate polymers, it is apparent that lecithin is not required for silicate binding or for the subsequent lysis by complement.

Mylius and Groschuff (8) have shown that silicic acid of very low molecular weight does not precipitate egg albumin but that precipitation occurs as polymerization progresses. On the basis of these observations and from our own studies it seems reasonable to assume that polymerized silicate binds to proteins of the red cell membrane and possibly also to lecithin in certain species. However, it cannot be assumed that binding of silicate to protein of the cell membrane is essential for complement lysis since other membrane constituents may be concerned.

**Table I**

<table>
<thead>
<tr>
<th>Time of polymerization</th>
<th>Concentration of silicate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>0 (monomeric)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1+</td>
</tr>
<tr>
<td>24</td>
<td>4+</td>
</tr>
</tbody>
</table>

A 0.2 per cent silicate solution was neutralized and allowed to polymerize for the indicated periods of time. It was diluted to the concentration indicated immediately before adding to an equal volume of a 2 per cent suspension of human erythrocytes.

It should be noted that the presence of hemoglobin is not necessary for the uptake of silicate because it is adsorbed by the thoroughly washed stroma of laked red cells.

Polymerized silicate was found to adsorb to erythrocytes as readily at 2°C as at 37°C.

*Agglutination of Erythrocytes by Polymers of Silicate*  As described originally by Landsteiner and Jagic (1) colloidal silica causes erythrocytes to agglutinate. The present studies show that certain silicate preparations cause agglutination whereas others do not (Table I). As might be expected, monomeric silicate, which is not taken up by red blood cells, does not cause clumping. Small polymers formed within 1 to 2 hours after neutralization (complement-active polymers), although taken up by erythrocytes, do not cause agglutination and the cells show little or no tendency to clump on centrifugation. As polymerization continues the agglutinating effect becomes apparent. In
preparations tested 2 hours after neutralization, as shown in Table I, agglutination begins to appear with certain dilute solutions (0.0125 per cent and 0.006 per cent) although not in more concentrated solutions. After polymerization for 4 hours the same paradoxical effect is seen with the strongest reactions being present in the low concentrations of silicate. Twenty-four hours after neutralization polymerization has proceeded to such a degree that maximum agglutination is produced by all concentrations tested.

**Effects of Silicate Polymers on Erythrocyte Morphology**

The morphology of fresh or stored red blood cells is altered in characteristic fashion by exposure to silicate solutions containing different polymeric forms. In most experiments the cells were stored for several days in Alsever’s solution before use. They were then repeatedly washed in buffered saline solution by centrifugation. When examined in wet preparations under cover slips the cells are spherical but are coarsely crenated (Fig. 8). Exposure to silicate solutions which are active in sensitizing to complement lysis causes the erythrocytes to lose their crenated appearance and to develop a smoother surface outline. They become grossly irregular in form with frequent indentations and the appearance of irregular bell shapes (Fig. 9). These changes are consistent and provide the basis for a simple microscopic determination of the presence of complement-active polymers. When complement is added, the cells tend to round up but do not become completely spherical before lysis.

Erythrocytes exposed to silicate solutions containing larger polymeric forms at first may have the appearance of cells treated with complement-active polymers. After a short time, however, they lose their irregular, indented shape and become spherical before spontaneous lysis occurs. If the silicate polymer is very large, as in the case of ludox SM and HS preparations, in which the polymer sizes are 7 m\(\mu\) and 17 m\(\mu\) respectively, the erythrocytes do not assume the forms seen with smaller polymeric preparations but remain coarsely crenated and burst after brief exposure. The length of time between silicate treatment and spontaneous lysis is roughly proportional to the size and concentration of the polymer used.

Erythrocytes exposed to monomeric silicate preparations are not altered morphologically.

If washed, fresh red blood cells are employed instead of cells stored in Alsever’s solution, the typical biconcave discs with smooth outline are altered by complement-active polymers to abnormal forms indistinguishable from those seen after treatment of stored cells. Upon exposure to larger polymeric aggregations that are inactive in complement-induced lysis, fresh red cells become spherical before bursting.

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2 E. I. du Pont de Nemours and Company, Wilmington, Delaware.
Morphology of wet preparations of human erythrocytes treated with complement-active silicate polymer.

Figure 8. Cells stored in Alsever's solution and washed in buffered saline.

Figure 9. Washed cells treated with complement-active polymer.
Osmotic Fragility of Silicate-Coated Erythrocytes. As might be anticipated, the effect of solutions of silicate upon the osmotic fragility of erythrocytes is also dependent upon the size of the silicate polymer used. Monomer silicate does not alter osmotic fragility. The coating of erythrocytes with complement-active, polymers does not increase osmotic fragility. To the contrary, in many experiments, one of which is illustrated in Fig. 10, a very slight decrease in fragility was observed at higher concentrations of sodium chloride.

![Figure 10. Osmotic fragility of human erythrocytes sensitized with varying concentrations of silicate which had previously polymerized for 90 minutes at neutrality in a concentration of 0.2 per cent.](image)

Cells treated with silicate solutions that had polymerized at 0.2 per cent concentration at neutrality for periods of 2 to 6 hours showed no increase in osmotic fragility. At all concentrations tested, as previously noted, these preparations cause erythrocyte agglutination.

Silicate solutions allowed to polymerize at neutrality for 24 hours or more cause a marked increase in osmotic fragility which parallels the appearance of polymers large enough to cause spontaneous hemolysis.

Mechanical Fragility of Silicate-Treated Erythrocytes. The effect of silicate solutions on mechanical fragility of erythrocytes is directly related to the...
size of the silicate polymer employed. Unlike osmotic fragility, which appears to be independent of the degree of clumping caused by silicate solutions that had polymerized for as long as 6 hours, mechanical fragility of treated cells parallels erythrocyte agglutination.

Mechanical fragility was measured by shaking 5 ml of a 2 per cent suspension of washed human erythrocytes in a 30 ml thick walled test tube containing three glass beads measuring 3.5 mm in diameter. The tubes were agitated for 1 hour in a Kahn reciprocating shaker at a rate of about 275 strokes per minute. The suspension was then centrifuged at 2,000 RPM for 3 minutes and the amount of hemolysis measured by the cyanmethemoglobin method.

No increase in mechanical fragility was observed in cells treated with monomeric silicate solutions nor with complement-active silicate solutions that had polymerized for periods of 60 to 90 minutes. After the solutions had polymerized for 2 to 6 hours a marked increase in mechanical fragility appeared at those silicate concentrations in which clumping of erythrocytes is most pronounced.

**Figure 11.** Mechanical fragility of human erythrocytes treated with a silicate solution that had polymerized at neutrality in 0.2 per cent solution for 4 hours. Polymerized solutions were diluted to indicated concentrations of silicate immediately before being added to red blood cells.
Fig. 11 illustrates the mechanical fragility of erythrocytes treated with dilutions of 0.2 per cent silicate solution that had polymerized at neutrality for 4 hours. At test concentrations of 0.003, 0.006, and 0.0125 per cent silicate, mechanical fragility was markedly increased, whereas at lower or higher silicate concentrations no change was observed.

It would appear from these results that measurement of mechanical fragility of treated red cells provides a sensitive method for determining changes in silicate polymerization within a certain size range.

The paradoxical effect of a greater increase in mechanical fragility at lower silicate concentrations than at higher concentrations is probably related to competition for binding sites on the red cell by the different polymers present in a given solution.

### Table II

**Relation of Complement to Lysis of Silicate-Treated Erythrocytes**

<table>
<thead>
<tr>
<th>Treatment of human serum</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh normal serum at 37°C</td>
<td>++++</td>
</tr>
<tr>
<td>Fresh normal serum at 0°C</td>
<td>-</td>
</tr>
<tr>
<td>Heated at 56°C for 30 min.</td>
<td>-</td>
</tr>
<tr>
<td>Mg⁺⁺ and Ca⁺⁺ removed</td>
<td>+</td>
</tr>
<tr>
<td>Mg⁺⁺ restored</td>
<td>+++</td>
</tr>
<tr>
<td>Ca⁺⁺ restored</td>
<td>±</td>
</tr>
<tr>
<td>Mg⁺⁺ and Ca⁺⁺ restored</td>
<td>++++</td>
</tr>
<tr>
<td>Treated with zymosan to remove C₃</td>
<td>-</td>
</tr>
</tbody>
</table>

**Serum Factors in Lysis of Silicate-Treated Erythrocytes**

**Relation of Complement to Lysis of Silicate-Treated Erythrocytes** The experiments of Landsteiner and Jagic (1) indicated that complement is necessary for the lysis of erythrocytes treated with colloidal silica. The results shown in Table II confirm and extend their observations.

Erythrocytes treated with complement-active silicate polymers are lysed by fresh normal human serum when incubated at 37°C but not at 0°C. Serum treated at 56°C for 30 minutes loses its lytic activity.

Removal of Ca⁺⁺ and Mg⁺⁺ ions by treatment of serum with an equal volume of amberlite IRC-50, sodium cycle, according to the method of Lepow, Pillemer, and Ratnoff (9), greatly depressed the lytic activity. Addition of Mg⁺⁺ and Ca⁺⁺ ions singly to normal levels resulted in partial restoration. When both Mg⁺⁺ and Ca⁺⁺ were added, the full lytic activity of the serum was restored.

Treatment of serum with zymosan to remove C₃ caused a loss of lytic activity, which, however, was not restored by addition of serum inactivated
by heat at 56°C. This result may be due to a combination of the partially inhibitory effects produced by each of these varieties of altered serum. Inhibition can be demonstrated by adding them singly to fresh serum which is used to produce lysis of silicate-treated red cells.

Extensive experiments with various properdin reagents did not indicate that this material has any part to play in the lysis of silicate-treated erythrocytes by fresh serum.

Myrvik has reported that lysozyme is absorbed by zymosan under conditions used to remove properdin (10). To test whether lysozyme is involved in the serum lysis of silicate-treated cells, various amounts of lysozyme were added to normal and to RP sera. No effect of lysozyme was found.

**Effect of Temperature on Serum Lysis of Silicate-Treated Erythrocytes**

Lysis of silicate-treated erythrocytes by serum is temperature-dependent. As shown in Fig. 12, below 20°C, little or no hemolysis occurred within the 30 minute incubation period. At temperatures of 42°C and higher (not shown in Fig. 12) hemolysis is more rapid initially, but after 20 minutes levels off so that at 30 minutes hemolysis is equal to or less than that at 37°C presumably due to inactivation of complement.

![Graph](image-url)

**Figure 12.** Effect of temperature on serum-induced lysis of human erythrocytes treated with complement-active silicate polymer.

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Effect of pH on Lysis of Silicate-Treated Erythrocytes by Human Serum. As shown in Fig. 13, the degree of hemolysis of silicate-treated erythrocytes is dependent upon the pH of the serum.

At pH 6.8 to 7.0 hemolysis is more rapid than at the more alkaline reactions tested up to pH 8.1.

Lysis of Silicate-Treated Erythrocytes by Guinea Pig Serum. Guinea pig serum which was freshly reconstituted from the dried state was used. Guinea pig serum appeared to be less active than human serum at the same level of complement activity in lysing silicate-treated human or guinea pig red cells. In occasional experiments no lysis occurred in the presence of guinea pig serum although silicate-treated erythrocytes from the same batch were lysed readily by human serum. Effects similar to these have also been reported by Peck and Thomas in their studies on the lysis of tannic acid-treated erythrocytes by serum (11). The relatively poor lytic activity of guinea pig serum could not be attributed solely to anticomplementary effects of silicate or silicate-treated erythrocytes.

Addition of high concentrations of guinea pig serum to human serum caused inhibition of the lytic activity of the human serum for silicate-treated human red cells. No inhibition of hemolysis was observed, however, when guinea

![Figure 13. Effect of pH of serum on lysis of silicate-treated erythrocytes.](image-url)
pig serum was added to human serum in the presence of sheep red blood cells treated with rabbit amboceptor.

**Complement Fixation by Silicate-Treated Erythrocytes** To test for complement fixation by silicate-treated cells either two or four 50 per cent units of guinea pig complement were used. Silicate-treated erythrocytes and complement were chilled to 4°C, mixed, and held overnight at 4°C. The suspensions were centrifuged at 2000 rpm for 5 minutes in the cold and the complement activ-

![Figure 14](image.png)

**Figure 14.** Complement activity removed by erythrocytes treated with various concentrations of silicate.

ity of the supernate was measured by addition of sheep cells sensitized with rabbit amboceptor. When human cells were used, hemolysis was minimal on standing overnight in the cold. On the other hand, with silicate-treated sheep cells there was extensive hemolysis so that these cells could not be used to measure complement fixation.

As shown in Fig. 14 the amount of complement fixed is related to the concentration of silicate used to treat the erythrocytes as indicated by the amount of hemolytic activity removed. On occasion, however, cells treated with higher concentrations of silicate (0.1 or 0.05 per cent) fixed less complement than cells treated with lower concentrations (0.025 or 0.0125 per cent silicate).

It should be noted that high concentrations of silicate inactivate comple-
ment when added directly to serum. The amount required to inactivate complement directly is greatly in excess of that bound to the erythrocytes in these experiments. Furthermore, the silicate-treated cells were washed in buffered saline before adding them to the serum in order to remove unadsorbed silicate. For these reasons it seems likely that the loss of complement activity in the presence of silicate-treated cells is due to complement fixation, as in the case of amboceptor-treated cells, and is not due to inactivation of complement by silicate. Furthermore, as noted above, although in general more complement activity is removed from serum by cells sensitized with higher concentrations of silicate, this is not uniformly the case, since at times cells treated with lower concentrations "fixed" more complement.

Effect of Silicate Polymers upon Pneumococci

It has been reported previously that silicates cause conversion of the staining reactions of pneumococcus from Gram positivity to Gram negativity, and also that silicates are lethal for pneumococcus although not for most other bacterial species tested (3). When information had been acquired on the effects of different silicate polymers upon erythrocytes, it was of interest to find out which of the various polymeric forms that are active on red cells caused the pneumococcal effects.

Silicate solutions were stored at acidic and basic pH values as in the experiments described with erythrocytes. The solutions were rapidly neutralized, held for various periods, and then tested simultaneously on erythrocytes and on pneumococci.

Solutions most active in sensitizing red cells to complement lysis likewise are most active in converting pneumococcus to Gram negativity. Conversely, monomeric silicates which are inactive in sensitizing red cells to complement or in altering erythrocyte morphology have little or no effect on the staining reactions of pneumococcus. Silicate solutions containing predominantly large polymeric forms which no longer sensitize red cells to the action of complement, have markedly decreased effects on pneumococcal staining.

It should be noted that under the experimental conditions used pneumococci are more sensitive than erythrocytes to the effect of silicate solutions since conversion to Gram negativity occurs at concentrations too small to cause discernible change in red cells.

Experiments to determine the properties of the polymeric forms responsible for death of pneumococci were inconclusive because changes in polymerization that take place during the prolonged period of exposure of the pneumococcal suspensions could not be assessed.

DISCUSSION

Most of the experiments reported in the present paper deal with the action of silicate polymers upon erythrocytes. The effects of silicates on the staining
properties and viability of pneumococcus that were described earlier (3) appear to be caused by complement-active polymers whose activity can be measured by hemolysis of human and animal red cells. Pneumococcus is not as favorable a subject for study as the erythrocyte, however, because of great technical difficulties in quantifying the effects of silicate polymers upon staining reactions and viability. The use of hemolysis as an indicator of activity in the red cell system enables quantitation to be carried out easily, and at the same time permits study of the action of complement on silicate-treated cells. Additional advantages in the use of erythrocytes are that the effects of different polymeric forms can be correlated with changes in cell morphology, agglutinability, osmotic and mechanical fragility, and to a degree with the uptake of silicate by cells.

Polymerized silicate is tightly bound by intact erythrocytes and by red cell ghosts and is not removed by repeated washings. Monomeric silicate is not bound by the red cell.

The observations that small polymeric forms of silicate sensitize red cells to the lytic action of complement, whereas larger polymers fail to do so but by themselves are directly hemolytic, have been the most intriguing aspects of the present study. We are not aware of observations with other inorganic compounds in which changes in polymerization affect so profoundly their biological effects. It is possible that in this respect silicates are unique.

There can be little doubt that information on the effects of silicates upon red blood cells will be of value in an understanding of the action of complement. One may suggest, for example, that the surface of the red cell possesses active groups which inhibit combination with or interfere with the action of one or more components of complement, and that these groups either are covered up by silicate of the appropriate small polymer size or so modified that the inhibitory properties are annulled. It is also possible that red cells treated with small silicate polymers cause activation of one or more of the components of complement, which in their active form are able to combine with their substrate in the cell membrane. No analysis of the role of the individual components of complement has been attempted.

There has been much speculation over many years on the mechanism by which dusts from silica-containing ores give rise to the silicotic lesions of the lungs and other tissues of man and experimental animals. Gye and Purdy (12, 13) suggested originally that the toxicity of silica may be associated with polymerization of silicic acid. The effect of polymeric form on silicate toxicity in animals has been studied extensively (14, 15). The low solubility of various forms of free silica and the improbability, therefore, that polymerization of silicic acid can occur in the tissues have tended to cast doubt on the “solubility” theory of silica toxicity (16). On the other hand, the recent studies of Holt and Went (17) on the polymerization of low concentrations of silicic
acid in the presence of polyamide films, indicate a mechanism by which polymerization might occur in the tissues.

It is not possible to correlate the toxic effects of polymerized silicates in animals that have been reported with the effects of different polymers on erythrocytes because of differences in the methods of preparation of polymers and the complexities associated with in vivo tests.

That hemolysis can occur in vivo after acute exposure to ethyl orthosilicate was reported by Kasper, McCord, and Frederick in rats (18), and by Smyth and Seaton in guinea pigs and rats (19). In both studies a rapid fall in hematocrit or erythrocyte count occurred without a drop in hemoglobin. On the basis of these reports it is not possible to determine whether hemolysis was of the directly toxic or complement-dependent type.

In recent years several reports have appeared on the metabolic effects produced in vitro by silica-containing compounds. Silicic acid or silicates have been shown to have effects on a wide variety of enzyme systems (20–22) which vary with the polymeric form of the silicate used. Collagen synthesis has been shown to be stimulated or inhibited in tissue culture depending upon silicate concentration (23).

The effect of silicate polymer size on hemolysis of erythrocytes was not considered in the earlier work of Landsteiner (1) and Ponder (2, 5). Presumably, some of the difficulties reported by Ponder (2) in the reproducibility of certain experiments and in the occurrence of spontaneous lysis in the absence of complement may have been due to the presence in his silicic acid solutions of a mixture of complement-active and directly hemolytic polymers. Recently Harley and Margolis (24) have described the effect of various preparations of silicates on erythrocyte agglutination. Polymers of different estimated sizes were prepared. In the range of estimated size of 3.5 to 7 mμ agglutination occurred. Polymers below 3.0 mμ did not cause agglutination but were able to partially block the agglutinating activity of the larger forms. The findings of Harley and Margolis are consistent with observations in our laboratory of the effects of various concentrations of mixtures of silicate polymers on erythrocyte agglutination and mechanical fragility.

The possibility of an immune mechanism in the development of silicotic nodules in patients with silicosis has been postulated by several workers (25–28). There is no evidence that complement-induced hemolysis of silicate-treated erythrocytes has an immunological basis.

It is possible that none of the phenomena observed in the study of red cells has relevance to the means by which some, although not all, silicate-containing minerals cause the characteristic fibrotic lesions of silicosis. It is our opinion that reexamination of the nature of the polymeric forms that may be expected to occur in tissues and consideration of a possible role of complement in tissue toxicity are indicated. It is possible that both the small, complement-
dependent and the larger, complement-independent polymers play a part in the etiology of the disease.

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