Since isosorbide dinitrate is a vasodilator (29), it probably increases the rate of ergotamine absorption by increasing the rate of intestinal blood flow. This conclusion is consistent with the earlier postulate that the rate-determining step in ergotamine absorption is its rate of passage from the membrane into the blood rather than its passage into or through the membrane.

Caffeine is also a vasodilator (30); at a concentration of 2 mg/ml, it increased intestinal blood flow by 166% in anesthetized rats and increased the rate of absorption of tritiated water, urea, and antipyrine at pH 8.0 and of salicylic acid at pH 6 (31). Hence, while part of the effect that caffeine has on increasing the absorption of ergotamine at pH 5.0 may be due to its effect on the blood flow rate, this effect is thought to be minor because it does not affect absorption rates at pH 3, it does not affect absorption rates at pH 5 from adjacent loops that do not contain caffeine, and it exerts an accelerating effect on ergotamine absorption in *in vitro* experiments where no blood supply is present.

CONCLUSION

1. Caffeine increases the rate of ergotamine absorption from the rat small intestine when the pH of the intestinal contents is 5.0 but not when it is 3.0. This effect is independent of the effect that caffeine exerts on the dissolution rate of ergotamine because, in the experiments performed, ergotamine was administered as a stable solution.

2. Although caffeine increases intestinal blood flow, this property only plays a minor role in its absorption-accelerating effects at pH 5.0. Vasodilation and increased blood flow are likely to explain the rate-accelerating effect of isosorbide dinitrate on ergotamine absorption.

3. The rate-determining step in the absorption of ergotamine appears to be transport from the intestinal membrane into the blood.

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Effect of Poly-2-vinylpyridine-*N*-oxide and Sucrose on Silicate-Induced Hemolysis of Erythrocytes

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Abstract □ The biological activity of montmorillonite, palygorskite, kaolinite, chrysotile, and silica was examined using *in vitro* hemolysis of erythrocytes. The hemolytic potency was in the order montmorillonite > silica > palygorskite > chrysotile > kaolinite. The polymer poly-2-vinylpyridine-*N*-oxide inhibited hemolysis caused by montmorillonite, palygorskite, kaolinite, and silica, but it was less effective with chrysotile. The extent of polymer binding to the silicates and red blood cells was measured by UV spectroscopy. When sucrose was substituted for the saline solution as the incubating medium, hemolysis was eliminated in all systems except chrysotile–erythrocyte, where it was enhanced. The results indicate that both hydrogen bonding and ionic interactions between silicate surfaces and the erythrocyte membrane are important in the hemolytic process.

Keyphrases □ Silicates—effect of poly-2-vinylpyridine-*N*-oxide on silicate-induced hemolysis □ Hemolysis—effect of poly-2-vinylpyridine-*N*-oxide, silicates □ Suspending agents—effect of poly-2-vinylpyridine-*N*-oxide on silicate-induced hemolysis

Inhaled silicate particles cause various pathological disorders (1). In addition to pulmonary tissue damage, the particles can potentially damage other body tissues because silicates such as montmorillonite, kaolinite, and palygorskite are used in many pharmaceuticals and cosmetics (primarily as fillers, stabilizers, and suspending

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agents); consequently, silicate particles are ingested in relatively large quantities or applied directly to the skin. Palygorskite (attapulgite) also has a fibrous morphology somewhat similar to chrysotile asbestos.

The effect of silicates on cellular membranes may be studied by *in vitro* hemolysis of erythrocytes. Silica (2, 3), the chrysotile variety of asbestos (4, 5), sepiolite and palygorskite (5, 6), kaolinite (7), and montmorillonite and illite (7) are known to induce *in vitro* hemolysis.

The weakly basic polymer poly-2-vinylpyridine-*N*-oxide (I) is an effective preventative against experimental silicosis (8). It also protects erythrocytes against the hemolytic action of various silica forms (9, 10) but is less effective in preventing chrysotile-induced hemolysis (5). Little is known about the effect of I on hemolysis caused by other silicates.

The purposes of this study were to determine the effect of I and sucrose on hemolysis caused by montmorillonite, kaolinite, and palygorskite and to compare these systems with the silica- and chrysotile–erythrocyte systems, which have been the most extensively studied systems. In addition, the extent of I binding to the silicates and erythrocytes was determined.

EXPERIMENTAL

Silicates—Montmorillonite[1], kaolinite[1], and palygorskite[2] samples were passed through a 300-mesh sieve, and the 50-μm fraction was used for all studies. Crude chrysotile fibers were separated partially by hand and then ground[3] for 2 min. The silica[4] was a synthetic, amorphous material and was used as received. Samples of the silicates[5] were suspended in deionized water and sonicated[6] for 5 min at 80 w to assure separation and dispersion.

Erythrocytes and Media—Bovine blood was drawn by venipuncture directly into a sterile 60-ml polyethylene tube coated with heparin. The red blood cells were washed three times with isotonic phosphate-buffered saline solution (0.106 *M* NaCl, 0.030 *M* $Na_2HPO_4 \cdot 7H_2O$, and 0.008 *M* KH_2PO_4). After the final wash, a 3% (v/v) suspension of red blood cells was prepared.

Phosphate buffer (pH 7.3) was used throughout, except for the chrysotile–erythrocyte system. It was reported (10) that phosphate inhibits the hemolytic activity of chrysotile, an observation confirmed in this laboratory. The pH of the chrysotile–erythrocyte system was monitored closely, however, and remained at 7.2 ± 0.1 for the concentration of chrysotile used.

To study the effect of sucrose on silicate-induced hemolysis, 0.250 *M* sucrose (0.250 *M* sucrose is isotonic with 0.145 *M* NaCl) was substituted for the saline solution as the incubating medium.

Hemolysis Technique—Glass test tubes (10 × 75 ml) were used for all hemolysis studies. The silicate–red blood cell suspensions consisted of the following and were prepared in the order given: 1 ml of a silicate suspension, 3 ml of the phosphate-buffered saline solution (sodium chloride alone for the chrysotile–erythrocyte system), and 1 ml of the 3% suspension of red blood cells. For the study of the effect of I on silicate-mediated hemolysis, the polymer was added to the described mixture immediately after the silicate suspension. The total volume of the mixture was always 5 ml. A sample consisting of 4 ml of the phosphate-buffered saline solution and 1 ml of the 3% suspension of red blood cells served as a control for the possible fragility of the erythrocytes.

The test tubes were sealed[7] and gently rotated end-over-end for 1 hr at room temperature. The suspensions then were centrifuged at 1500×*g* for 5 min, and the absorbance of the supernatant solution was measured spectrophotometrically[8] at 541 nm with the control sample as the reference. The degree of hemolysis was expressed as a percentage of a totally lysed sample.

Table I—Percentage Hemolysis Produced by the Silicates in the Presence of I

Silicate[a]	I Concentration, μM/ml 0.2	1.0
Montmorillonite	23 ± 2[b]	ND[c]
Palygorskite	20 ± 3	3 ± 2
Kaolinite	50 ± 2	20 ± 5
Silica	7 ± 4	ND
Chrysotile	50 ± 1	42 ± 4

[a] The HC_{50} concentrations of the silicates were used (see text). [b] Mean ± *SD*, % ($n = 4$). [c] No detectable hemolysis.

Binding of I—The UV spectrum of I gives a λ_{max} equal to 260 nm in water (11); therefore, the extent of polymer binding to the silicates and erythrocytes can be measured conveniently.

Twenty milligrams of a silicate or 10 ml of a 6% suspension of red blood cells and 1 mg of I were added to 20 ml of the phosphate-buffered saline solution in 100-ml volumetric flasks. The flasks then were brought to volume with deionized water (phosphate-buffered saline solution was used for the erythrocyte–I mixture). After 5 min of periodic shaking, a sample was removed from the flasks, placed in polypropylene centrifuge tubes, and centrifuged at 40,000×*g* for 10 min. A sample of the supernate was transferred to a quartz cell, and the absorbance was measured[9] at 260 nm.

The following formula was used to calculate the percentage of 1 mg of I bound to 20 mg of the silicate or 10 ml of a 6% suspension of red blood cells:

$$\% \text{ bound} = [A_p - (A_s - A_m)/A_p] \times 100 \qquad \text{(Eq. 1)}$$

where A_p is the absorbance of a 0.001% (w/v) solution of I, A_s is the absorbance of the supernate after centrifugation of a mixture of 0.001% I and a 0.02% (w/v) silicate or 0.6% (v/v) erythrocyte suspension, and A_m is the absorbance of the supernate after centrifugation of a 0.02% silicate or 0.6% erythrocyte suspension.

RESULTS AND DISCUSSION

To facilitate meaningful comparisons between the silicates, the concentration of the silicate that caused 50% hemolysis when incubated with 1 ml of a 3% suspension of erythrocytes was determined. This concentration[10] (in milligrams of silicate per milliliter of silicate–erythrocyte-buffer suspension) was as follows for the five silicates examined: montmorillonite, 0.006; silica, 0.03; palygorskite, 0.06; chrysotile, 0.1; and kaolinite, 0.6. The use of the HC_{50} concentration partially compensated for physical as well as chemical differences in the untreated silicates. There appeared to be a correlation between the specific surface area of the silicates [montmorillonite > silica ≥ palygorskite > kaolinite ≥ chrysotile (12–14)] and their hemolytic activity.

It was confirmed that I is very effective in inhibiting hemolysis caused by silica, but it was much less effective with chrysotile (Table I). The polymer also decreased hemolysis produced by montmorillonite, palygorskite, and kaolinite, although its effect was somewhat less than on silica-induced hemolysis (Table I).

Two hypotheses concerning the mechanism of interaction between silica and the erythrocyte membrane have been proposed: (*a*) hydrogen bonding (9) of the silanol groups on the silica surface with the phosphate ester linkages of phospholipids and the amide groups of proteins, and (*b*) ionic interaction (15) between dissociated silanol groups on the silica surface and positively charged species on the erythrocyte membrane surface.

Compound I is a strong hydrogen bonding material; therefore, its effectiveness in preventing silica-induced hemolysis suggests that hydrogen bonding is the predominant mode of interaction between silica and the erythrocyte membrane during hemolysis. The polymer apparently bonds to the silanol groups on the silica surface and thus prevents these groups from contacting the red blood cell membrane.

Because I also inhibits hemolysis caused by montmorillonite, paly-

[1] Montmorillonite (Upton, Wyo.), kaolinite (Bath, S.C.), and chrysotile (Quebec) samples were obtained from the Wards Natural Science Establishment, Rochester, N.Y.

[2] The palygorskite (Attapulgus, Ga.) sample was obtained from the C.M.S. Clay Minerals Repository, Department of Geology, University of Missouri, Columbia, Mo.

[3] Janke-Kunkel mechanical grinder.

[4] Cab-O-Sil, Cabot Corp.

[5] Montmorillonite, kaolinite, palygorskite, chrysotile, and silica will be referred to as silicates.

[6] Model W185, Branson Sonic Power Co.

[7] Parafilm, American Can Co., Greenwich, Conn.

[8] Bausch & Lomb model 20 spectrophotometer.

[9] Beckman model Acta CII spectrophotometer.

[10] Hereafter referred to as the HC_{50} concentration.

Table II—Amount of I Bound to the Silicates and to Erythrocytes

Material	I Bound[a], %
Montmorillonite	100
Palygorskite	97 ± 2[b]
Kaolinite	48 ± 3
Silica	96 ± 1
Chrysotile	37 ± 4
Erythrocytes	4 ± 1

[a] One milligram of I was added to 20 mg of the silicates or 10 ml of a 6% suspension of erythrocytes in a total volume of 100 ml. [b] Mean ± *SD*, % (n = 4).

gorskite, and kaolinite (Table I), a hydrogen bonding mechanism probably is at least partially involved in these systems. The three silicates, montmorillonite, palygorskite, and kaolinite, have many surface hydroxyl groups bound to the cations (predominantly silicon, aluminum, and magnesium exposed at crystal edges) (16, 17); these hydroxyl groups could function as hydrogen donors. Furthermore, montmorillonite has a high cation-exchange capacity (12), and the water of hydration of the exchangeable cations also can function as hydrogen donors (18).

Chrysotile, on the other hand, is a fibrous, tubular magnesium silicate with the silica sheets on the inner side and the magnesium hydroxide sheets exposed at the surface. Since the pK of chrysotile is close to 11 (19), it has a positive surface charge at physiological pH values. Compound I is relatively ineffective in preventing chrysotile-induced hemolysis (Table I), while many anionic species such as phosphate and ethylenediaminetetraacetic acid are effective inhibitors (10, 20). This fact suggests that little hydrogen bonding is involved in the chrysotile–erythrocyte system. An ionic interaction between chrysotile and the red blood cell membrane has been proposed (21).

The effect of I on silicate-induced hemolysis previously has not been clearly interpretable; it could affect the surface of the silicate, the erythrocyte membrane, or both. The data in Table II clearly show that erythrocytes bind very little of the polymer while it is readily bound to montmorillonite, palygorskite, and silica and to a lesser extent to kaolinite and chrysotile. Therefore, I probably exerts its protective action by blocking the lytic sites on the surfaces of the silicates.

When sucrose was substituted for the saline solution as the incubating medium, no hemolysis was observed when the HC_{50} concentrations of montmorillonite, palygorskite, kaolinite, and silica were incubated with erythrocytes. The sucrose molecule has many potential hydrogen-accepting sites, so it can form preemptive hydrogen bonds with the hydrogen-donating surface groups of montmorillonite, palygorskite, kaolinite, and silica. Consequently, these hydrogen-donating groups cannot contact the erythrocyte membrane and hemolysis cannot occur.

In contrast, erythrocytes incubated in sucrose underwent increased lysis (65% ± 4, n = 4) when the HC_{50} concentration of chrysotile was used. If the chrysotile–erythrocyte interaction is predominantly ionic, the interaction would be enhanced in a medium of relatively low dielectric constant such as sucrose, and this reaction is what was observed.

A recent report indicated a correlation between hyperplastic changes in isolated hamster trachea segments and *in vitro* hemolysis of erythrocytes by chrysotile asbestos and montmorillonite (22). Elucidation of the mechanism by which various silicate minerals interact with the erythrocyte membrane *in vitro* may lead to a better understanding of silicate–membrane interactions *in vivo*.

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