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Chemically Sensitized Erythrocytes for Hemagglutination Reactions

REFERENCE: Harrington, J. J., Gordon, R. E., Ross, J. P., and Kobilinsky, L., **"Chemically Sensitized Erythrocytes for Hemagglutination Reactions,"** *Journal of Forensic Sciences*, JFSCA, Vol. 35, No. 5, Sept. 1990, pp. 1115–1124.

ABSTRACT: The use of chemically modified indicator erythrocytes for hemagglutination reactions can result in increased sensitivity. Treatment of erythrocytes with polyvinylpyrrolidone (PVP) or dextran T40 (10% weight/volume) induces changes in the cell surface in the form of extensions and blebbing, thereby increasing the surface area. These sensitized cells can be used in forensic science when detection or quantitation of erythrocyte surface reacting antibodies is important. The effect of altering membrane lipid fluidity on erythrocyte surface antigens has also been investigated. Treatment of cells with a reagent that increases the membrane ratio of cholesterol to phospholipid results in enhanced hemagglutination capacity despite the lack of extensive spiculation.

KEYWORDS: forensic science, serology, hemagglutination, antibody, antigen, bovine serum albumen (BSA), dextran, erythrocytes, forensic serology, hemagglutination, polymer, polyvinylpyrrolidone (PVP), zeta potential

Much of the serological testing relevant to forensic science is conducted on samples that are exceedingly small and that are often in less than optimal condition. Because of these constraints, commonly available indicator erythrocytes sometimes prove inadequate, in terms of sensitivity, for use in testing based on hemagglutination reactions. A means of increasing the sensitivity of these indicator cells for agglutination would thus be of interest to the forensic serologist.

Hemagglutination is an easily observed phenomenon in which specific cross-linking antibodies form a lattice network when combined with the appropriate erythrocytes. The network structure is constructed when single antibody molecules bind to, and form a bridge between, two adjacent cells bearing the same cell surface antigen. The cross-link can form more easily when the antibody is of the immunoglobulin M (IgM) class rather than the immunoglobulin G (IgG) class because of the higher valence of the former.

Part of this research was performed in partial fulfillment of the requirements for the M.S. degree in Forensic Science, John Jay College of Criminal Justice, City University of New York, by James J. Harrington.

Received for publication 20 June 1989; accepted for publication 19 Sept. 1989.

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However, cross-linking can also readily occur when IgG antibodies, providing that the antigenic determinants on the cell surface are in close proximity, for example, less than 120 Å apart [*1,2*]. It is reasonable to assume that cross-linking would be facilitated by alteration of the conditions which normally maintain a separation between cells. Erythrocytes are normally kept physically separated by the envelope of negative charge that covers their surfaces, resulting in an electrostatic (zeta potential) repulsion between cells. Hemagglutination should therefore be significantly affected by (*a*) lowering the zeta potential that exists at the cell surface, (*b*) changing the dielectric constant of the cell suspension medium, (*c*) increasing the surface area of these cells, (*d*) physically bringing the cells into closer proximity, and (*e*) modifying the orientation or distribution, or both, of proteins at the cell surface. Of these five possibilities, the first two have been investigated by Brooks [*3*]. Thus, suspension of erythrocytes in a medium containing the polymer dextran would result in a decrease in erythrocyte zeta potential and a corresponding increase in the ability of these cells to approach one another. For aqueous media surrounding the cells, the value of the dielectric constant is essentially unaffected by the presence of dextran. The influence of the various physicochemical factors (supra, *c*, *d*, and *e*) on hemagglutination has been studied by various investigators [*4–6*]. The effects of changing cell shape, intracellular distance, extracellular colloid-osmotic pressure, cell surface hydration, and the zeta potential were investigated by van Oss et al. Factors affecting intracellular distance and spiculation of the cell surface were shown to be the two major parameters that effect hemagglutination. Treatment of the cells with dextran resulted in increased extracellular colloidosmotic pressure, decreased cell surface hydration, and alterations in the cell membrane in the form of blebbing. In addition to the physicochemical studies, the effects of various classes of antibody on hemagglutination have been studied by scanning electron microscopy (SEM) [*6*]. It was noted that anti-A blood grouping serum, which is composed of a combination of IgM and IgG, produced agglutinates in saline consisting of strongly spiculated cells. The IgG fraction, prepared from this antiserum, caused similar effects when added to Type A erythrocytes. Lectin, specific for the A_1 antigen, also caused the same effect. Anti-B antiserum effected Type B erythrocytes in the same manner, resulting in spiculation.

The cell surface extensions appeared to have formed as a result of the interaction between the cell surface antigens and the antibody combining sites. No such extensions of the cell membrane were found upon combining "incomplete" antibody to the Rh factor "D" with "D" positive cells. However, it was noted that addition of 15% bovine serum albumin (BSA) to the extracellular medium helped stimulate the agglutination reaction.

The effects of changing lipid fluidity on erythrocyte membrane proteins have also been investigated [*7,8*]. Human erythrocytes were incubated in media having varying molar ratios of cholesterol to phospholipids. The effect of the incubation medium on the position of the membrane proteins was determined by means of a fluorescent sulfhydryl reagent. It was demonstrated that, as lipid fluidity increased due to larger values of the cholesterol to phospholipid molar ratio, an increase in protein exposure to the aqueous surroundings was observed. This finding suggests that membrane proteins may be vertically displaced (outwards with respect to the cell membrane), resulting in a decrease in distance between antigenic sites on adjacent cells to approximately 120 Å, thus increasing the possibility of agglutination if specific antibodies of the appropriate class are present [*1,2,4*]. The use of such chemically treated erythrocytes as "sensitized" indicator cells has been reported by various investigators. Vitullo used bromelin treated indicator cells for ABO grouping of bloodstains, employing the absorption-elution method [*9*]. Seven stains that gave no results with untreated cells could be successfully grouped using the enzyme-treated cells. Coombs and Dodd used papain-treated O cells for the detection of bound anti-H lectin, using the mixed agglutination method [*10*]. Also, it has been found that anti-H lectin will usually be assigned a higher titer with papain-treated Type O indicator

cells than with untreated ones [*11*]. Desensitization of erythrocytes can also occur. Moskowitz and Carb found that treating Type A cells with formalin, a cross-linking reagent and preservative, rendered them nonagglutinable with anti-A antiserum [*12*]. The antigenic receptors were apparently not damaged by this treatment, however, since the formalin-treated cells absorbed the anti-A from the reagent as affectively as did fresh cells. In addition, the pH and ionic strength of the suspension medium can have an effect on hemagglutination reactions. Hughes-Jones et al. found that the rate of association between anti-D antiserum and Rh positive erythrocytes was increased by a reduction in ionic strength of the suspending medium [*13*]. Also, the optimum pH was found to be in the range of 6 to 8, with a reduction of the association constant outside this range.

More recently, the use of sensitized erythrocytes for salivary hemagglutinin studies has been described by Harrington et al. [*14,15*]. Indicator cells were treated with enzymes (bromelin and papain), protein (albumin), polymers (dextran), and other reagents such as polyvinylpyrrolidone (PVP). These sensitizing reagents were applied singly or in combination, with promising results. As a result, we began the present study to determine the feasibility of using such sensitized erythrocytes in bloodstain analysis, and also to attempt an explanation of the sensitization mechanism. This paper describes further studies of chemically induced changes in erythrocyte membranes that result in enhanced hemagglutination.

Materials and Methods

Erythrocytes

The erythrocytes used in this study were obtained from several sources. Commercial (pooled) cells were purchased from both Organon Teknika (West Chester, Pennsylvania) and from Ortho Diagnostic Systems (Raritan, New Jersey). Each bottle of pooled cells contained a two-donor pool of human red blood cells from a particular blood group. The cells were prewashed and suspended in a phosphate-buffered diluent which contained neomycin sulfate (0.1 mg/mL) and chloramphenicol (0.25 mg/mL) antibiotics. Whole blood was obtained by venipuncture from volunteers blood-typed in the ABO and Rh systems. Blood was collected in vacutainer tubes lacking anticoagulant for immediate use or in vacutainer tubes containing ethylenediaminetetraacetate (EDTA) for later use.

Whole blood was centrifuged for 15 to 30 s at 1000 × *g* to separate the cells from the plasma. The cells were then washed four times by repeatedly suspending them in phosphate-buffered saline (PBS), pH 7.0, followed by centrifugation at 1000 × *g*. The cells were subsequently treated chemically as described below.

Reagents

Dextran T40 was obtained from Pharmacia (Piscataway, New Jersey); bovine albumin was obtained from Gallard-Schlesinger Chemical Manufacturing Co. (Carle Place, New York); PVP was obtained from Sigma Chemical Co. (St. Louis, Missouri); and Mod-U-Cyte VIII was obtained from Miles Diagnostics (Kankakee, Illinois).

Chemical Treatment of Erythrocytes

The erythrocytes were suspended in PBS (0.5% v/v) containing dextran T40, PVP, albumin, or Mod-U-Cyte VIII in various concentrations ranging from 1 to 50% (w/v or v/v), and incubated for various lengths of time ranging from 15 min to 24 h. After incubation in a treatment solution, the cells were again pelleted by centrifugation as described above in a refrigerated centrifuge, washed, and resuspended in PBS.

Scanning Electron Microscopy

Prior to SEM examination, the cells were fixed, dried, and sputter coated. A number of different fixatives (paraformaldehyde, gluteraldehyde, gluteraldehyde followed by osmium tetroxide, and gluteraldehyde combined with osmium tetroxide) were tested, with no apparent difference in the results. The fixative routinely used in this study was a gluteraldehyde (2%) and osmium tetroxide (0.67%) solution prepared in 0.2*M* sodium cacodylate buffer, pH 7.3. The cells were fixed in this solution for 1 h at 25°C. The cells were then washed in cacodylate buffer, mounted on a specimen stub, air dried, and coated with gold-palladium using a Hummer V sputter coater. The specimens were then observed and photographed using a Cambridge 150R and a Hitachi S530 SEM. A 5-μm reference bar is shown on each micrograph.

Agglutination Studies

Chemically treated erythrocytes of known blood group (ABO and Rh systems) were suspended in PBS to a concentration of 0.5% (v/v). Equal volumes (50 μL) of the cell suspension and the appropriate antiserum were mixed and allowed to stand for up to 45 min at 4°C. The agglutination reaction was read visually using a hand-held magnifying lens or a microscope if agglutination was questionable. Differing degrees of agglutination were observed and scored using a semiquantitative system. Agglutination was graded from "1+" to "4+," with "4+" representing maximum agglutination and "1+" representing weak but visually apparent agglutination. The lack of hemagglutination was scored as "−."

Results

Rh (D) positive erythrocytes of known ABO blood type were washed two times in saline and then suspended in saline to a concentration of 0.5% v/v. The cells were placed in a test tube and incubated with various chemical reagents including bovine albumin, PVP, and dextran T40. Cells were also incubated with the enzymes bromelain and papain. In all cases the reagents were used at a concentration of 10%. Control cells were incubated with isotonic saline. The incubation period ranged from 15 min to 24 h as described in the Materials and Methods section. Initially, commercially obtained test cells were utilized, but concern about cell aging, reinforced by electron micrographs of the cell surface, resulted in tests being performed with fresh whole intravenous blood. The cell suspensions were maintained either at 37°C, room temperature, or 4°C for 15 min or 1, 3, 12, or 24 h. The cell suspensions which were maintained at 4°C or at room temperature were constantly agitated on a rotating shaker. At this point, specific antiserum or lectin was then added to the appropriate cell mixture. Aliquots were removed from each test tube and examined visually for signs of hemagglutination. In all cases there appeared visually or microscopically to be some indication of enhanced sensitivity to hemagglutination when compared with the saline control cells. Hemagglutination appeared to be more rapid and stronger. The most dramatic effect occurred when Mod-U-Cyte VIII was used. The results are shown in Table 1. Table 2 illustrates the marked effect that Mod-U-Cyte VIII treatment has on Type O indicator cells. Anti-H lectin was prepared by extraction from ground *Ulex europaeus* seeds and then titered using Type O cells that had been incubated either with bovine albumin or with Mod-U-Cyte VIII. The apparent titer is more than twice as large when the latter indicator cells are used. Similar studies using antisera to the A and B antigens and the corresponding test cells were performed, but there was no apparent enhancement of hemagglutination sensitivity regardless of which chemical reagent was utilized.

Scanning electron microscope studies were also performed on chemically treated cells

TABLE 1—*Treatment of Rh positive erythrocytes with Mod-U-Cyte VIII and the subsequent effect on hemagglutination sensitivity.*

Treatment, Mod-U-Cyte/ Saline	Time	Degree of Hemagglutination
"Neat Moducyte"	1 min, 40 s	+ + + ½
	7 min, 30 s	+ + + +
1:1	1 min, 30 s	+ +
	10 min	+ + +
	15 min	+ + + +
1:2	4 min	+ +
	18 min	+ + +
	20 min	+ + + +
1:3	5 min	+
	13 min	+ +
	16 min	+ + +
	21 min	+ + + ½
1:4	5 min	+
	13 min	+ ½
	18 min	+ + ½
	28 min	+ + +
1:5	5 min	+
	13 min	+ ½
	21 min	+ + ½
	28 min	+ + ½
1:6	5 min	+
	13 min	+
	21 min	+ +
	28 min	+ +

TABLE 2—*Titration of anti-H lectin with Type O erythrocytes treated with either albumin or Mod-U-Cyte VIII.*

Albumin		Mod-U-Cyte VIII	
Dilution of Lectin	Observations	Dilution of Lectin	Observations
1:1	+ + + + / + + + +	1:1	+ + + + / + + + +
1:2	+ + + + / + + +	1:2	+ + + + / + + + +
1:4	+ + + / + + +	1:4	+ + + / + + +
1:8	+ + + / + + +	1:8	+ + + / + + +
1:16	+ + + / + +	1:16	+ + + / + + +
1:32	+ + / +	1:32	+ + / + +
1:64	− / −	1:64	+ + / + +
1:128	− / −	1:128	+ / +
1:256	− / −	1:256	− / −

to ascertain if surface changes had occurred as a result of chemical treatment. It was noted that as commercially obtained cells age, these cells became crenated and developed surface blebs. As a result of these observations, fresh whole blood obtained intravenously was used for all studies. Cells treated with 10% dextran T40 or with 10% bromelin displayed numerous extensions of the cell surface, a phenomenon known as spiculation (Figs. 1 and 2). Cells treated with albumin at concentrations ranging from 2 to 10%

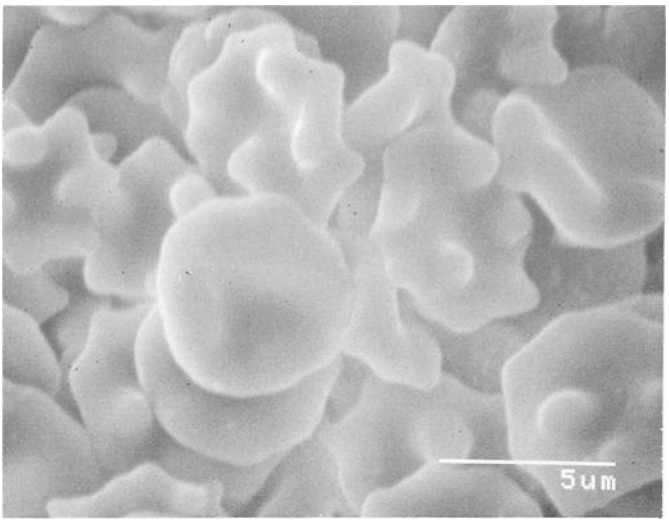

FIG. 1—*Scanning electron micrograph of erythrocytes treated with dextran T40 (10% w/v).*

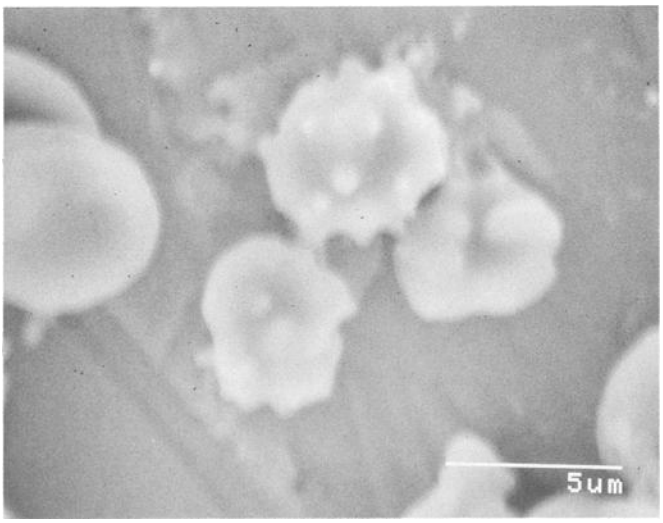

FIG. 2—*Scanning electron micrograph of erythrocytes treated with bromelin (10% w/v).*

developed a cupped appearance, as shown in Fig. 3. Cells treated in an identical fashion with Mod-U-Cyte VIII displayed morphological alterations but did not appear to be spiculated (Fig. 4). Thus, it appears that increased sensitivity to hemagglutination does not require the formation of cell membrane extensions.

Conclusions

From the results of this study it now appears that there may be more than one mechanism resulting in the observed increased sensitivity to agglutination. We consider first

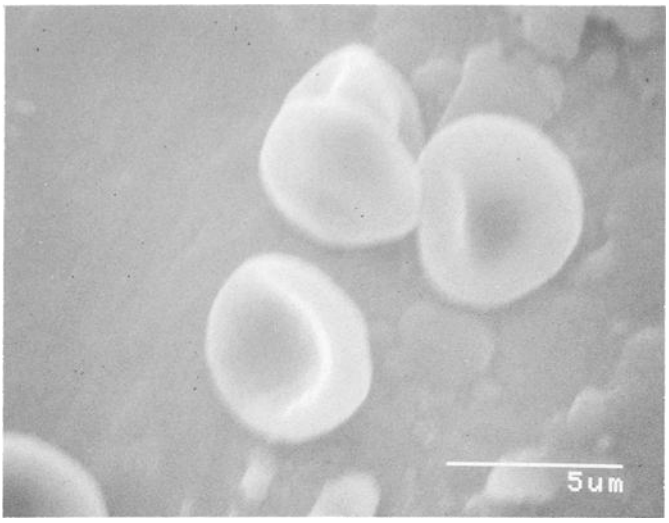

FIG. 3—*Scanning electron micrograph of erythrocytes treated with bovine albumin (10% w/v).*

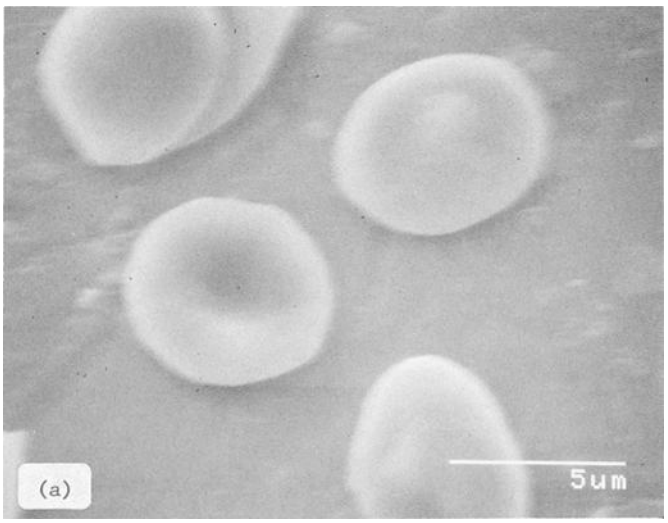

FIG. 4—*Scanning electron micrograph of erythrocytes treated with Mod-U-Cyte VIII at concentrations of:* (a) *1%.*

the case of erythrocytes treated with Mod-U-Cyte VIII, where the sensitization was most pronounced. In this case, there was not the strong surface morphological change (spiculation and blebbing) seen in the case of dextran-treated cells. Thus, although spiculation of the cell membrane may result in the effective separation of the antigenic sites on adjacent cells becoming less than 120 Å (the maximum separation that will allow linking via an IgG molecule [*4*]), clearly this is not the predominant sensitization mechanism here. Mod-U-Cyte VIII is a cholesterol-rich reagent which can increase the ratio of cholesterol to phospholipids in the erythrocyte cell membrane. This has the effect of

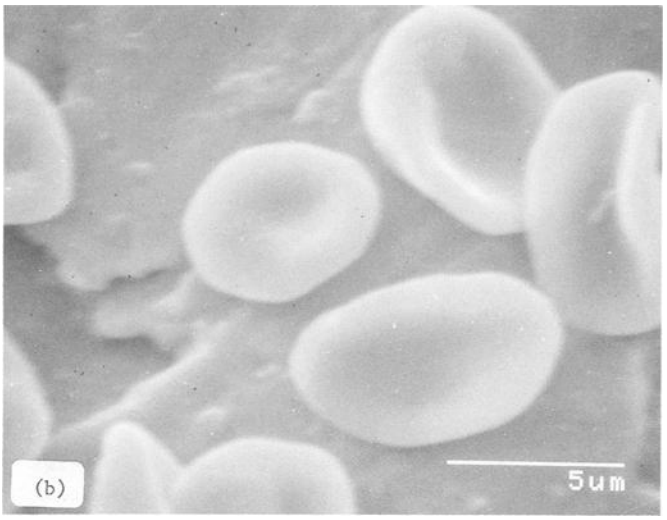

FIG. 4—*Continued:* (b) *10%*.

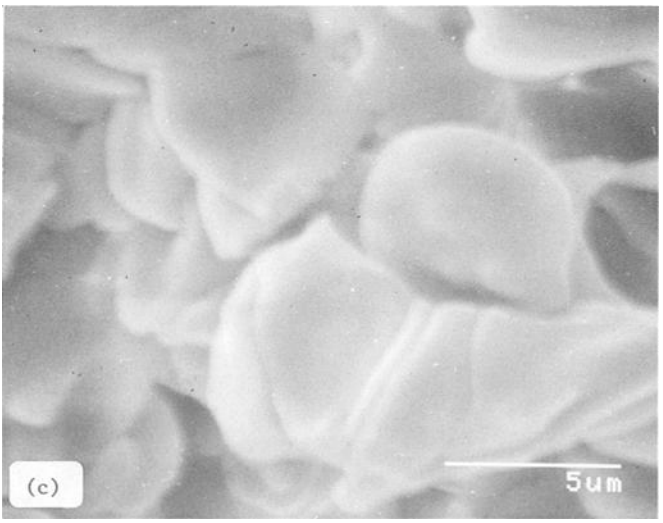

FIG. 4—*Continued:* (c) *50% (v/v)*.

causing membrane proteins to migrate outward toward the aqueous surroundings, in addition to gaining increased lateral and rotational mobility [*7,8*]. Studies using fluorescent antibody labeling show that the D antigen is "unmasked" upon cholesterol enrichment [*16*]. The same study found that the degree of modulation of the A_1 antigen by similar fluidity changes, due to cholesterol enrichment, was only about one fifth as much as that for the D antigen. This is supported by the present work, where the increase in agglutination sensitivity was markedly greater when using anti-D or anti-H reagents than when using anti-A or anti-B reagents with appropriate indicator cells. The present findings

appear reasonable based on the fact that the A and B antigens are well exposed and in large quantity on the untreated erythrocyte surface, whereas the D antigen is present in much smaller numbers. It has been estimated that there are approximately 810 000 to 1 170 000 A_1 antigenic sites on A_1 cells, 240 000 to 370 000 A_2 antigenic sites on A_2 cells, 610 000 to 850 000 B antigenic sites on B cells [*17*], and 110 000 to 202 000 D antigenic sites on D-positive cells [*18*]. Treatment with Mod-U-Cyte may be effective in increasing sensitivity to hemagglutination by indirectly facilitating contact between homologous antibody and surface antigens which have become more mobile.

An increase in sensitivity to agglutination was also noted in the case of erythrocytes treated with water-soluble polymers, such as albumin, dextran, and PVP. Such reagents have been known to enhance agglutination reactions with IgG antibodies, such as anti-D [*5*]. For such polymer-treated cells, the increase in agglutination sensitivity was found to be less pronounced, as in the case of the Mod-U-Cyte VIII-treated cells; moreover (particularly in the case of dextran), rather apparent surface morphology changes were noted in the form of spiculation and blebbing. Thus it is felt that the mechanism operative here is distinct from that in the case of Mod-U-Cyte VIII treated cells, that is, modulation of the membrane proteins. It has been proposed that polymers enhance the sensitivity to agglutination by three mechanisms: electrokinetic, steric, and osmotic [*5*]. The electrokinetic influence can be manifested as a change in the zeta potential (modification of surface charge or surface double layer), or a change in the dielectric constant of the suspension medium, or as a change in both. Dextran, however, has been shown to increase the zeta potential; moreover, the dextran molecule is nonpolar and thus has only a small effect on the dielectric constant of the aqueous suspension medium.

The polymeric molecules employed in this study all possess a high degree of molecular asymmetry. Studies by Brooks [*19*] and Hummel [*20*] have shown that these macromolecules cause cell aggregation due to polymer bridging. Hence, a mechanism for an increase in hemagglutination sensitivity (especially when the small IgG antibodies are the agglutinins) may be the decrease in intercellular separation, due to the polymer bridging. In the case of Dextran, the observed spiculation may be due to polymer bridging between different sites on the membrane of the same cell. As previously mentioned, this can also lead to a reduction in the effective separation between intercellular antigenic sites, and thus potentiate agglutination.

The presence of extracellularly dissolved macromolecules results in a decrease in colloid-osmotic pressure which can cause human erythrocytes to become cup-shaped (stomatocytes) [*21*]. These can approach one another closely with a larger fraction of their surface area, in comparison with the ordinary biconcave disk-shaped erythrocytes. In addition, hydration of the dissolved macromolecules reduces the chemical potential of the intercellular aqueous medium, allowing a decrease in the intercellular separation [*22*]. Hence, the change in colloidosmotic pressure due to dissolved macromolecules can also bring about an increased sensitivity to hemagglutination.

Acknowledgments

This research was conducted with the help of funding obtained from the Professional Staff Congress, City University of New York, Award PSC-BHE 664120, and from the U.S. Department of Education MISIP Award G-008641165.

We wish to thank Charles Cusumano for his help in conducting some of the experiments.

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