Mechanisms of Agglomeration of Red Cells by High Polymer Compounds[1]

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Introduction

It is well-known that erythrocytes agglomerate each other under various conditions. This erythrocyte agglomeration is devided into two types, *i.e.*, specific and non-specific ones, the former being observed in the immunological reaction while the latter practically in all other cases. The increase in sedimentation rate of erythrocytes, which often becomes the object of clinical examination for various diseases, is considered to be due to the agglomeration of erythrocyte. Recently, the mechanism of immunological (specific) agglomeration has been elucidated by the physicochemical studies carried out by Pauling[2] and his colaborators. The present author's paper deals with the results of experiment performed with the purpose of elucidating the mechanism of the non-specific agglomeration caused by the addition of several substances of known structure. First of all the action of high molecular substances has been investigated expecting that this series of substances has different mode of action from that of the low molecular.

In 1921 Fahraeus[3] reported that gum arabic and other macromolecular substances produced pseudoagglutination. Later, this work has been argued by many workers and Meyer[4] and his associates in 1945 proposed the view that probably all highly asymmetrical molecules of large size will cause an increase in the sedimentation rate when added to normal blood. The reasons for this phenomenon, however, are not definitely known. In order to elucidate the mechanism of this phenomenon, the following experiments were carried out.

Experimental

The quantitative expression of the degree of agglomeration was made by sedimentation experiment. To 0.5 ml. of packed red cells which have been washed with isotonic saline were added 1.5 ml. of colloid saline solution of *p*H about 7, and after sufficient agitation, the sedimentation rate was measured by the Westergren method. As has been reported a period of constant rate of falling was observed to follow an induction period. As the sedimentation rate in the constant rate period can be reproduced with sufficient accuracy and characterizes the sedimentation system under investigation, this value will be employed for further discussion. With the assumption that the sedimentation obeys a modified Stokes' law, the relation between the size of agglomerate and sedimentation rate may be expressed by the following equation

$$r = C\sqrt{\eta v}$$

where C is a constant determined by sedimentation system, r, the mean radius of agglomerate, η, the relative viscosity of medium, v, the sedimentation rate in constant rate period.

The present author observed the agglomeration effect of various polymers upon the human red cells which had been washed with saline. This observation is compared with the results carried out by many investigators in the presence of plasma. These polymers are classified for the convenience sake into several groups and their agglomerating effects are shown in Table 1. It can be seen in this table that plasma does not play any important role in this phenomenon, except the case of globulin. Moreover it may be deduced from this table that among the negative colloids, fibrous ones generally show this effect, while spherical ones exert but little action at best. It has been observed that the agglomeration brought about by negative colloids is reversible and the agglomerate forms rouleaux structure, while the positive polymers which exert far stronger agglomerating action than the negative ones, cause an irreversible agglomeration and the agglomerate forms random clumping, but not rouleaux structure. In order to clarify, first of all, the mechanism of the agglomeration by

(1) This work was reported at the meeting for general discussion of colloid chemistry under the auspices of Chemical Society of Japan, Kyôto, Nov., 1950. The earlier part of this work was carried out at the Faculty of Science of the University of Kyûshû.

(2) L. Pauling, *J. Amer. Med. Assoc.*, **62**, 2643 (1940).

(3) R. Fahraeus, *Acta Med. Scand.*, **3**, 55 (1921).

(4) K. Meyer, E. Hahnel and R. R. Feiner, *Proc. Soc. Exptl. Biol. Med.*, **58**, 36 (1945).

Table 1

Water soluble polymers	Agglomeration effect on red cells: in whole blood	Agglomeration effect on red cells: washed with saline
Negative colloid		
(1) Fibrous colloid		
(a) Electrolytic colloid		
Na-alginate	+(5)	+
Na-glycolcellulose	+	+
Na-desoxyribonucleate	+(4)	+
Pectin	+(6)	+
Gelatin	+(7)	+
Potassium salt of polyvinyl alcohol sulfuric ester	+	+
Gum arabic	+(3)	
Fibrinogen	+(5)	
Na-hyaluronate	+(4)	
Type-III pneumococcus polysaccharide	+(9)	
(b) Non-electrolytic colloid		
Glycolcellulose	+	+
Mannan	+	+
Polyvinyl alcohol	+(10)	+
Dextran(10)	+(11)	+
(2) Spherical colloid		
(a) Electrolytic colloid (Globular protein)		
Ovalbumin	−	−
Serum albumin	−(8)	−
Serum globulin	+(8)	−
Hemoglobin	−	−
(b) Non-electrolytic colloid		
Glycogen	±(6)	±
Positive colloid		
Poly N-trimethyl glucosamine (Macramine)		+++
Clupeine sulfate		+++

these substances, sodium alginate was mainly used as a representative of these polymers.

Decrease in Concentration of Alginate in Bulk.—The decrease in the concentration of alginate was measured, which was expected to occur by the addition of erythrocytes if the adsorption of alginate by erythrocytes would take place. The decrease in concentration, however, was so small that it barely exceeds the limit of experimental error.

Electric Charge of Erythrocytes by the Addition of Alginate.—The change of the electric charge on the erythrocytes by the addition of alginate was also measured using a flat microelectrophoretic cell. As shown in Fig. 1, the electric charge on erythrocytes increases

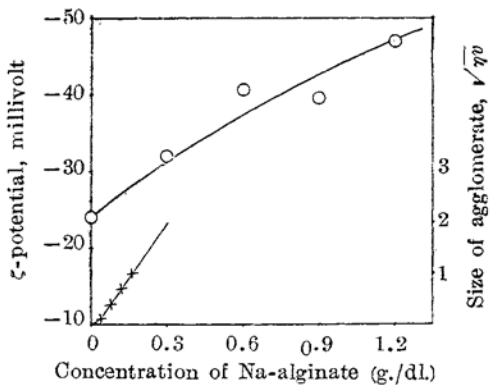

Fig. 1.—Charge of red cell and size of red cell agglomerate *versus* alginate concentration: circles show ζ-potential and crosses, the size of agglomerate, temp., 16°C; *p*H, 7.3.

proportionally with the amount of alginate. This result clearly shows some change have taken place on erythrocyte surface.

Adsorbability of Erythrocytes on Alginate Film.—Thin film of alginic acid made on the glass by treating Na-alginate film with hydrochloric acid, was immersed in an isotonic saline suspension of erythrocyte for a few minutes and then washed with isotonic saline solution. Some number of erythrocytes thus were found remaining on the film without having removed by washing. The adsorbabilities of cells on the films of several metallic alginates which are insoluble in water were observed in the same manner. As shown in Table 2, it was found that the adhering abilities of erythrocyte on alginate films are different according to the kind of metal ion of alginate,

Table 2

Numbers of erythrocytes adhered to the various metallic alginate films (Relative number)

H-alginate	340, 320	Zn-alginate	58, 89, 48
Ca- 〃	10, 5, 12	Ba- 〃	35, 37, 43
Cu- 〃	84, 87, 78	Mn- 〃	43, 32, 36

These metalic alginate films were prepared by treating the Na-alginate films in various metallic solution.

(5) Maynard B. Chenoweth, *Ann. Surg.*, **127**, 1173 (1948).
(6) Ch. Wunderly, *Experimentia*, **1**, 332 (1945).
(7) Koop, *Surgical Clinics of North America*, Philadelphia number, 1313 (1944).
(8) J. Gray and E. B. Mitchell, *Proc. Soc. Exptl. Biol. Med.*, **51**, 403 (1942).
(9) W. J. Nungester and Klein, *Proc. Soc. Exptl Biol. Med.*, **36**, 315 (1937).
(10) cf. F. P. Turner *et al.*, *Surg. Gyn. Obst.*, **5**, 673 (1949).
(11) A. Grönwall and B. Ingelman, *Acta physiol. Scand.*, **9**, 188 (1945).
(16) Ch. Wunderly, Viertel jahrschr, Naturforsch. Ges. Zürich, **89**, 170 (1944).

namely Ca-alginate film almost failed to show the ability to adhere erythrocytes on it, while they adsorb considerably on the film of alginic acid.

The Effect of Some Change Occurred on Erythrocyte Surface.—With the purpose of finding the effect of some change occurred on the erythrocyte surface, the amino radical, considered to be the positive center of erythrocyte, was attempted to be blocked up. Fraenkel[12] reported that the amino radical of serum albumin was attacked by formaldehyde. The effect of alginate upon erythrocytes, treated with formaldehyde, was thus observed. The results are shown in Fig. 2. It shows that the

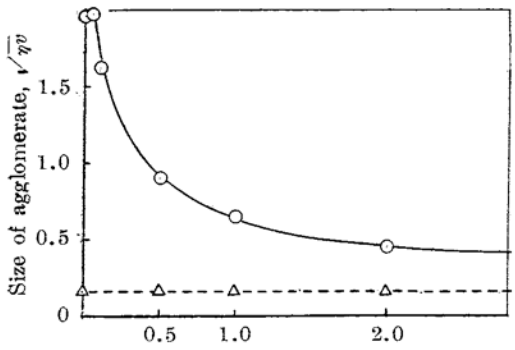

Fig. 2.—Effect of formaldehyde upon the red cell agglomeration by alginate (circles). Triangles show the only effect of formaldehyde on red cells in the absence of alginate, temp., 16.5°C.; Na-alginate, 0.1%; *p*H, 6.6.

agglomerating action of alginate decreases with the concentration of formaldehyde used. Moreover, Klotz[13] has shown that some organic anisons, *i.e.*, methyl orange and dinitrophenol, picric, and cinnamic ions combine with the amino radicals of serum albumin. The effect of these anions upon erythrocyte agglomeration by alginate was observed. It was shown that the coexistence of 0.1 mol/dl. of these four anions prevents completely the agglomeration by alginate. Salicylic, benzoic, and sulfanilic ions, which have been considered to give similar action to the afore-stated substances, also show the inhibiting effect on erythrocyte agglomeration by alginate.

Effect of Degree of Polymerisation of Alginate[14] on the Agglomeration.—As

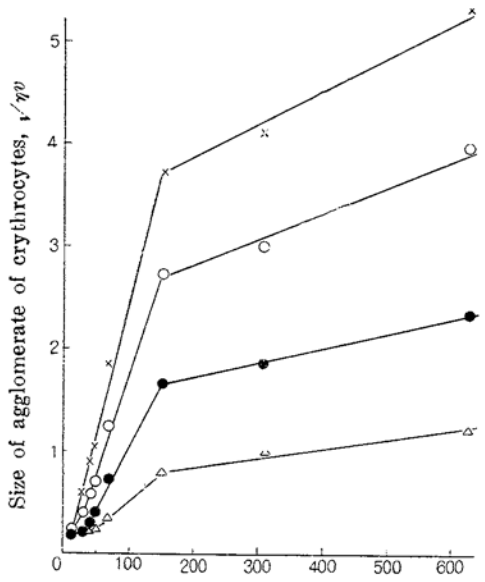

Fig. 3.—Size of agglomerate of red cell *versus* degree of polymerisation of alginate added, × 0.16%, ○ 0.12%, ● 0.08%, △ 0.04%.

shown in Fig. 3, it has been observed that the agglomerating action of alginate increases remarkably with increase in the degree of its polymerisation as well as its concentration. The effect of the former factor is the greatest in certain range of the degree of polymerisation.

Discussion

Possible mechanism of Agglomeration of Erythrocytes by Na-alginate.—From the afore-stated results that the electric charge of cells increases with the increase in concentration of alginate added, and that erythrocytes are capable to adsorb on the alginic acid film, it may be considered that alginate molecule is adsorbed on erythrocyte surface.

It was observed by the author that diacetyl alginate, prepared by the acetylation of OH radicals of Na-alginate, aggregetes to practically the same extent as the original alginate. Furthermore as shown in Table 2, it was found that the adhering abilities of erythrocyte on alginate films are different according to the kind of metal ion of alginate. These facts show that the dissociated radical of alginate, *i.e.*, carboxyl one, is the centre of its adsorption on erythrocytes. On the other hand, the attempts to block up the amino groups on cell surface have brought the preventing effect of agglomeration of cells. This shows that the centre

(12) Fraenkel-Conrat and Olcott, *J. Biol. Chem.*, **174**, 827 (1948).
(13) Klotz and Curme, *J. Amer. Chem. Soc.*, **70**, 937 (1948).
(14) Polymer homologues of alginate was prepared by the method reported by the author in *Memoirs of the Faculty of Science, Kyushu University*, Ser. C, **1**, 115 (1950).

of adsorption on erythrocyte are considered to be the amino groups on its surface, presumably that of lysine, arginine, histidine, etc. From these facts, it may be concluded that alginate molecule is adsorbed on erythrocyte surface by the electrostatic force. Although alginate is a macromolecule having carboxyl radicals on each unit, simulatneous-fitting of all the carboxyl radicals of alginate in the positive centres on the cell probably cannot occur due to the steric hinderance. It is more likely that alginate molecule combines with red cell with a small portion of the radicals and that the remaining portion of them is left unreacted. It is probable that the unreacted radicals then combine with the second cell, thus alginate molecule holding the cells together. Such a view is supported by the experimental fact, as mentioned above, that the agglomerate of erythrocytes increases in size with the increase in the degree of polymerisation of alginate added. The increase in the degree of polymerisation causes the increased molecular extentsion in solution, which in turn facilitates the molecule to act as link joining cells together.

Other substances shown in the group of negative electrolytic fibrous colloid in Table 1 are generally considered to provide the similar properties as alginate with respect to this effect. Besides, it was also reported that with pectin,[6] sodium desoxyribonucleate,[4] and sodium hyaluronate[4] their agglomerating effects increase with the polymerisation degree as seen in sodium alginate. The above indicated mechanism is, therefore, the common one for negative fibrous electrolytes.

Non-electrolytic Fibrous Colloids.—As shown in Table 1, glycolcellulose, mannan, dextran, and polyvinyl alcohol have been known to possess the action to agglutinate red cell. These substances are similar in structure to the fibrous electrolytes, except only that the hydrophilic radicals are OH group which is not dissociated. The dependence of agglomeration upon the polymerisation degree was also reported for this series of substances.[4][6] Moreover, it has been found by the present author that these substances show their agglomerating action neither in the presence of formaldehyde, nor of some organic anions such as cinnamic, salicylic, etc. These facts also support the view that the same mechansim as that of alginate is also available for these substances. However, the force contributing to the adsorption of the colloids upon cells probably is a nature of hydrogen bond, acting between OH groups of colloids and the amino radicals on red cell surface.

Reason for the Lack of Agglomeration Effect of Globular Protein.—As shown in Table 1, the native globular proteins, for instance, ovalbumin, serum albumin, serum globulin, hemoglobin, cause no agglomeration of red cell. The question arises why these proteins lack this action in spite of the presence of dissociated radicals in their molecules. As to this problem, at least two possible reasons may be considered, *i.e.*, the lack of adsorption of these proteins on cell surface or too small molecular extension of these proteins. Pauling[15] reported that erythrocytes previously treated with bisdiazoarsanilic benzidine forms agglomerate and this is due to the formation of –NN-⟨⟩-⟨⟩-NN- bridge between the cells. Obviously, the length of the bridge between the protein molecules is much larger than that of bisdiazobenzidine. Moreover, according to the present author's experiment, the partially degraded alginate, which is estimated to have smaller order of molecular extension in comparision with that of serum albumin, shows the agglomerating action. In view of these experimental facts, the lack of agglutination effect cannot be considered to be due to their too small molecular extension. Thus, we prefer another possibility that this negative result is due to the lack of the adsorption of the protein on cells. Now we face to the problem why globular protein cannot attach to the cell surface, while fibrous colloids can do. Red cells, in this case, possess negative charge as a whole, which causes the repulsive force against negative colloids. Therefore we must consider a necessary condition for the combination of these colloids with cell surface in spite of the repulsion between them. The author proposes the following view concerning this point. In order that negative colloid can couple with the weakly positive radicals situated among strongly charged negative groups on cell surface, it is necessary that the negative group in the polymer molecule has a mobility in solution strong enough to be capable of attaching themselves to the positive centres surrounded by negative ones on the surface of cells. This view is easily understood by the fact that the low molecular organic anions are capable of combining with the amino group of negatively charged protein. A fibrous molecule is considered to have a favorable property to satisfy the necessary condition mentioned above, since each part of the molecule seems to possess a sufficient flexibility in solution. In addition to this property, fibrous molecule easily holds cells together on account of the large molecular extension in solution.

(15) D. Presman, D. H. Campbell and L. Pauling, *J. Immunol.*, **44**, 101 (1942).

Globular protein, however, does not satisfy this necessary condition, because each part of the protein molecule is closely packed in space by virture of hydrogen bonds. The negative radicals of the protein, which are fixed each other in space and accordingly unfavorable to change their mutual positions in solution, thus cannot appreciably attach to the positive centres on cells surrounded by strongly charged negative ones due to steric hinderance. Owing to such properties of the molecular shape of the polymers, a fibrous molecule exerts agglomeration effect on erythrocytes, while a spherical one does not show it.

It is very interesting to note that a considerable agglomeration occurs when globulin is added to whole blood, which is in accord with the results obtained by Gray and others, while it does not occur in the absence of plasma as shown in Table 1. Many interesting problems concerning globulin remains unexplained, waiting for further research.

Agglomerating action of Glycogen. — Generally speaking, the agglomerating effect of this substance upon cells is very small, but in higher concentration the agglomeration occurs to some extent. The molecular extension of glycogen in solution is considerably small as seen from the knowledge of viscosity. Moreover, the mobility of each part of the molecule in solution is considered to be neither so poor as that of globular protein, nor so rich as of fibrous colloid. In view of these properties of glycogen, the experimental result obtained can be explained.

Some Suggestions to Clinical Problems. —In general, the rate of erythrocytes sedimentation in pathological state has been considered to be dependent on the nature and concentration of the protein components of the plasma. However, some experimental evidences, suggesting that erythrocytes sedimentation rate may likewise be profoundly influenced by non-protein substances, were reported by several workers.[4] If it might be assumed that certain polymers of fibrous shape appear in blood in the course of diseases and they were responsible for the increased sedimentation rate in the pathological state, the mechanism of this clinical phenomenon may also be explained by the same mode proposed above.

Summary

The mechanisms of agglomeration of erythrocytes caused by hydrophilic negative polymers were investigated and the following views were proposed.

(1) Among the negative colloids, fibrous colloids show the erythrocyte agglomeration, while spherical ones do not cause it.

(2) Agglomeration by the negative colloids is caused by two actions, *i.e.*, adsorption of these polymers upon cells and their action to join cells together. Those substances which lack either or both of these actions fail to cause the agglomeration.

(3) Fibrous structure of macromolecule favors these actions on account of the large extension of molecule and adequate flexibility of each part of the molecule in solution.

(4) The dissociated or dipol radicals of the negative polymers and amino groups of the protein of cell surface are considered to be the centres of adsorption respectively.

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