EFFECT OF PROTEINS ON ELECTROPHORETIC MOBILITY AND SEDIMENTATION VELOCITY OF RED CELLS

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Since the increased sinking velocity of red cells seen in pregnancy and in various diseases is due to an aggregation of the cells, the question of the mechanism of the increased rate of sinking becomes that of the factors promoting aggregation; the influence of changes in cell or plasma specific gravity and in plasma viscosity is usually negligible.

The problem which presented itself may be stated as follows. The aggregation and therefore the sinking velocity of red cells are increased in human plasmas high in fibrinogen, as in pregnancy and in various febrile and neoplastic disorders. Aggregation and sinking velocity are increased by pure fibrinogen solutions and by various other agents, as gelatin, gum acacia, and casein; serum globulin is much less effective than fibrinogen and albumin almost without effect. Sinking velocity is proportional to the degree of aggregation of the cells as determined microscopically. The aggregation brought about by fibrinogen or gelatin is a rouleau formation and not, as in specific agglutination, a sticking at the first point of contact. The principal differences between slowly and rapidly settling human bloods are in the plasma, since the cells of a non-pregnant woman sink almost as fast in "pregnant plasma" as do those of a pregnant woman, while pregnant and non-pregnant cells both sink slowly in non-pregnant plasma. The possibility of a difference in the cells of slowly and rapidly settling human bloods has not been excluded; we have demonstrated such differences in the cells of different species. The cells of horse blood settle many times as fast as those of beef blood, al-

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though horse plasma contains no more fibrinogen than does cow plasma.[1] Since the cell concentration is a very important factor in determining settling rate, the sinking velocity increasing rapidly as cell concentration decreases, any quantitative work must be done with a constant ratio of cell to medium volume. Most of the above facts are reported by Fåhraeus (1921).

The problem has been clearly stated by Fåhraeus (1929), who says,[2] "The problem seems to hinge upon the manner in which the globulin increase changes the interface between the corpuscles and the plasma or, let us say, the surface of the red cells. Is it by increasing the surface tension, or by reducing the negative electric charge of the corpuscles or finally by depriving their surface colloids of adsorbed water?"

Most of the previously reported views on the mechanism of increased sedimentation velocity demand the formation of an adsorption coat of protein on the red cell surfaces, which should act by lowering surface charge or increasing surface tension. The first question to be answered, therefore, is whether such an adsorption coat is formed. Earlier evidence purporting to demonstrate this is unsatisfactory. Thus, an important line of evidence advanced has been that the isoelectric point of red cells is that of some plasma protein. Netter (1925) concludes that beef red cells carry an adsorption layer of albumin, horse cells of globulin. For a discussion of the sources of error in isoelectric point determinations see Abramson (1930), who concludes that the isoelectric point of normal red cells cannot be measured, that the isoelectric points observed are merely those of protein coats adsorbed on *damaged* cells, and that normal red cells do not adsorb any of the proteins investigated on the alkaline side of the isoelectric point and do not adsorb gelatin even when the gelatin is positively charged. We fully confirm Abramson on all of these points. These remarks do not apply, of course, to the reactions between specific antibodies and cell antigens, which are adsorption processes.

Another line of evidence is that of Wöhlisch (1924), which is used

[1] We have not seen any report, previous to our work, of the settling of horse cells in beef plasma and *vice versa*.

[2] P. 259.

by Bendien, Neuberg, and Snapper (1932) in an attempt to explain the action of fibrinogen on sedimentation velocity. Wöhlisch and Wöhlisch and Bohnen (1924) report microscopic evidence of elastic fibers passing between red cells, which they interpret as evidence of a layer of "denatured fibrinogen" or fibrin on the cells, causing the cells to stick together. They have no evidence that these fibers are fibrin or that a fibrin coat exists but merely assume its existence in order to retain the Höber-Mond (1922) theory.[3] The fact that rouleau formation consists in a sliding of cells into such position that the minimum surface is exposed proves, as Fåhraeus has pointed out, that a mere stickiness is not involved (in contrast to specific agglutination). This fact does not appear to have been adequately appreciated (Ponder, 1926; Abramson, 1934[4]).

Rothe (1924) emphasizes increase of interfacial tension as the principal factor in promoting aggregation and sinking velocity, assuming a fibrinogen adsorption coat. He gives no experiments but argues by exclusion of other factors.

Abramson first presented evidence against the presence of an adsorption coat. He showed (1929) that repeated washing of red cells does not change their electrophoretic mobility; this means either that there is no adsorption coat or that it cannot be washed off. Abramson (1930) further showed (his Fig. 2) that the mobility of washed red cells in diluted serum is at no pH (above 3.5) the same as that of quartz particles in the same medium; this means either that there is no protein adsorption coat on the cells, or that the red cells selectively adsorb a protein having a different mobility from that adsorbed on the glass. He further showed (1934) that the mobility of horse red cells in oxalated horse plasma is about the same as in horse serum; this means either that the red cells do not adsorb fibrinogen or that they already had a fibrinogen coat before being transferred to the serum. More convincing evidence is his finding (1930) that while the isoelectric point of normal red cells cannot be measured, it is certainly lower than that of any plasma protein; while this does

[3] The evidence quoted from Ley (1922) that the maximum agglutination of red cells in fibrinogen solutions is at pH 5.86 is not convincing, since Ley's cells were undoubtedly damaged by washing.

[4] P. 260.

not exclude the adsorption of small amounts of protein, it proves that a complete coat is not formed. Again, Abramson (1929) found that the mobility of pregnant human cells in their own plasma is normal, which demonstrates that the increased sinking velocity of the pregnant blood is not due to an adsorption coat of fibrinogen. Abramson's suggestion that the increased sinking velocity may be due to the adsorption of amounts of fibrinogen too small to be detected electrophoretically, but sufficient to make the cell surface more sticky is incompatible with the finding referred to above, that agglutination by rouleau formation is a surface tension phenomenon and not explicable by an increased stickiness.

EXPERIMENTAL

We may first consider our experiments designed to answer the question of a protein adsorption coat on the cells. These fall into two groups, one based on a comparison of the isoelectric point of the cells in protein solutions and the other based on determinations of the effect of added protein on the electrophoretic mobility of the cells. Electrophoretic determinations were made in a cylindrical cell of the Mattson (1928) type, the readings being taken 0.147 diameter from the top of the cell. The applied voltage was 9.1 volts per cm., the 232 volt power line being used. Each mobility figure is the average of five observations with each direction of current, with reversal between consecutive observations. Change in velocity, due to drift, on reversal of current, was significant only with low mobilities. When a difference does occur the average velocity for the two directions must be taken, rather than calculating velocity from the average time in the two directions. Temperature was noted and correction for viscosity made; in all cases the temperature was close to 25°C.

Viscosity determinations were made with the Bingham (1922) viscometer in a water bath at 25° ±0.02, using pressures from 60 to 300 cm. of water, with transfer times from 90 to 440 seconds. The gelatin stock sols showed a slight component of plasticity since the observed viscosities at pressures of about 60 cm. of water were a few per cent higher than with pressures of 300 cm. of water. The values obtained with the high pressures were used for correcting the mobilities. We confirm Abramson (1928) in finding that the plasticity

does not hinder mobility at the voltages employed, since the same mobility was observed with 4.55 as with 9.1 volts per cm. The viscosity determinations were easily reproducible in our hands to ±0.2 per cent.

In the first group of experiments we found that within 2 minutes after adding fresh, unwashed human cells to solutions of gelatin or plasma dilutions in M/50 phthalate buffer of pH 4.0 + 3 per cent glucose + 0.3 per cent NaCl, the cells were still negatively charged although the proteins were positively charged.[5] However, the cells gradually reverse their sign of charge upon standing in the acid solution. Cells washed in M/50 buffer + 5 per cent glucose reverse their

TABLE I

Electrophoretic mobility of gelatin-coated glass particles and of *washed* cow and dog red cells in 0.04 per cent gelatin as a function of pH. The cells were washed 5 times in M/50 buffer pH 7.4 + 5 per cent glucose. The gelatin was dissolved in M/50 phthalate buffer + 5 per cent glucose. Readings made within 2 minutes after addition of cells to buffer mixtures.

pH	Glass	Cow	Dog
	μ/sec./v./cm.	*μ/sec./v./cm.*	*μ/sec./v./cm.*
7.4	0.92	1.90	2.30
6.0	0.88	1.86	2.35
5.0	0.48	1.65	1.72
4.6	0.21	1.25	0.91
4.0	+0.27	+0.26	+0.27

charge immediately when placed in protein solutions on the acid side of the protein isoelectric point. Thus, dog cells washed repeatedly in M/50 phosphate buffer, pH 7.4 + 5 per cent glucose, were immediately reversed when transferred to a solution of cow fibrinogen in M/10 buffer pH 5.0 (isoelectric point of fibrinogen at pH 5.6, determined electrophoretically); dog, cow, horse, and human cells similarly washed were immediately reversed in gelatin solutions or plasma dilutions at pH 4.0. The results shown in Table I are typical of the behavior of cells washed in such glucose-dilute buffer solutions. We interpret these results as meaning that normal red cells do not

[5] This test of adsorption could not be applied to casein, since at pH 4.0 insufficient casein dissolves in the buffer to coat completely even glass particles.

adsorb proteins even when the latter are positively charged. Washing with 5 per cent glucose, however, or allowing the cells to stand in acid solution damages the cell surface; such damaged cells may then adsorb protein. Cells washed in 0.9 per cent NaCl are less likely to be damaged than those washed in sugar-buffer, but cells allowed to stand in any solution, even their own plasma, sometimes show abnormalities both in electrophoresis and in sedimentation velocity. For this reason we have confined our later experiments to unwashed cells from oxalated blood not more than 2 hours old.

TABLE II

Corrected electrophoretic mobility of protein-coated glass particles as compared with that of fresh, unwashed horse red cells. Egg albumin, casein, and gelatin were dissolved in M/50 phosphate buffer pH 7.4 + 3 per cent glucose + 0.3 per cent NaCl, horse fibrinogen in M/10 phosphate buffer pH 7.4. Albumin was dialyzed.

Protein	Concentration	Relative viscosity	Mobility		Corrected mobility (mobility × viscosity)		Ratio Horse cells / Glass
			Glass	Horse cells	Glass	Horse cells	
	per cent		*μ/sec./v./cm.*	*μ/sec./v./cm.*			
Egg albumin	0.01	1.07	0.75	1.50	0.80	1.61	2.01
	3	1.24	0.69	1.50	0.85	1.86	2.19
Casein	0.01	1.07	1.54	1.56	1.72	1.67	0.97
	3	1.99	1.24	1.62	2.47	3.08	1.25
Gelatin	0.01	1.07	0.71	1.62	0.76	1.73	2.28
	2	6.38	0.45	1.27	2.87	7.72	2.69
Horse fibrinogen	0.01	1.01	0.82	1.47	0.83	1.49	1.80
	1.6	1.44	0.64	1.49	0.92	2.15	2.34

In the second group of experiments, we find that at pH 7.4 the mobility of fresh, unwashed horse, beef, human, and dog cells in M/50 phosphate buffer + 3 per cent glucose + 0.3 per cent NaCl is unchanged on addition of several hundredths per cent of gelatin, casein, egg albumin, or homologous fibrinogen, more than sufficient to coat glass particles completely. Since in no case was the mobility of the cells changed by the protein it seems unnecessary to present tables of data. While this indicates that an adsorption coat is not formed at these low concentrations of protein, the possibility re-

mained that at the higher concentrations required to increase the sinking velocity of the cells, an adsorption coat is formed. Therefore the mobilities of glass particles and of fresh, unwashed horse, beef, human, and dog cells were compared also in 3 per cent casein, 3 per cent egg albumin, 2 per cent gelatin, and 1.6 per cent fibrinogen, all brought to pH 7.4, with a final electrolyte concentration of M/50. The ratio of the mobility of the cells to that of the protein-coated glass particles was always at least as high in the concentrated protein solutions as in the dilute. The results of a typical experiment are shown in Table II. These results demonstrate that the proteins investigated are not adsorbed by the red cells even in concentrated solutions. All theories of increased sinking velocity based on changes in surface tension or charge brought about by adsorbed protein must therefore be discarded; the proteins act without being adsorbed.

We may now consider the third possibility mentioned by Fåhraeus, namely, that the proteins may act "by depriving their (the cells') surface colloids of adsorbed water." Such surface dehydration should lower the suspension stability of the cells. Actual evidence for such surface hydration has not heretofore been presented. In investigating this point on protein sols we find that observed mobility of gelatin and casein decreases with increasing protein concentration less than bulk viscosity increases. This must mean that double layer viscosity increases less than does bulk viscosity; since the electrolyte medium remains constant, charge density and double layer thickness are unchanged. The changes in mobility and viscosity with egg albumin are too small to permit a definite conclusion as to whether or not double layer viscosity remains the same as bulk viscosity.

It is difficult to visualize a protein molecule inside a layer only a few mμ thick, as is the case with M/50 to M/15 solutions, the concentrations employed in this protein work. Furthermore, electrical forces should prevent adjacent protein particles from approaching each other closely enough for either to be within the limits of the other's double layer. Nevertheless, it is seen that added protein does have some influence on double layer viscosity, since observed mobility decreases somewhat. The suggestion is therefore made that the decrease in observed mobility, which means increase in double layer viscosity, observed on increasing the protein concentra-

tion, takes place in some way without the added protein actually entering the double layer. An ability of a protein particle to orient water molecules within a zone extending beyond the limits of its double layer could account for this effect. This means that a given protein particle possesses a layer of oriented water molecules extending beyond this double layer and is thus able to influence the viscosity at the surfaces of its neighbors. This argument applies to the protein of the medium; with the protein-covered glass particles the surface of the particle under observation is therefore also hydrated, since its surface is the same as the added protein.

In the blood work, however, the particle under observation is a red cell, having a surface different from the added protein. A decrease in its observed mobility less than the increase in bulk viscosity of medium with increasing protein concentration cannot on the basis of the above argument be taken as evidence that the original surface of the cell was hydrated.

But if the cell surface were not hydrated, added gelatin or casein should effect an orientation of water molecules about the added protein particles, the effect extending to the originally unoriented water molecules in the cell's double layer. The double layer viscosity of the cell would increase even more than is the case with protein particles (since the double layer viscosity with the latter was already above normal before the additional protein was added) and the observed mobility of the red cell would decrease even more with increasing protein concentration than is the case with the protein particles themselves.

If, on the other hand, the observed mobility of the cells is but little decreased on increasing protein concentration, this may be taken to indicate that the viscosity of the cell's double layer was already high (due to orientation of water molecules) and is therefore but little increased by the action of the protein. Such a finding would therefore indicate that the red cell is normally surrounded by a layer of oriented water molecules. It therefore becomes desirable to determine how the electrophoretic mobility of red cells is influenced by added protein. Ponder (1926) found corrected mobility of human red cells greater in plasma than in 0.85 per cent NaCl. He ascribed this to a greater charge on the cells in plasma than in saline, without

considering the possibility that double layer viscosity differed from bulk viscosity. Since he does not give his viscosity data one cannot tell whether or not observed mobility decreased.

We have measured the electrophoretic mobility of fresh, unwashed horse, beef, human, and dog red cells in various protein solutions and have found that their observed mobility is unchanged or only slightly decreased from that in the protein-free medium. We interpret this as meaning that viscosity of the cell double layer does not increase, or only slightly, with greatly increasing bulk viscosity. This is compatible with the view that the cell surface is hydrated. Since our protein solutions differed from the protein-free medium only in that protein was present, there is no reason to believe that cell charge was altered in the various solutions.

The sedimentation experiments were carried out in glass tubes of 3.5 mm. bore and 35 cm. long. Fresh, unwashed cells were used, a constant ratio of 1 volume of cells to 2 volumes of protein solution being maintained in all experiments. After considerable experimentation to find a medium suitable for both sedimentation and electrophoresis observations the protein solutions were finally made up in M/50 phosphate buffer + 3 per cent glucose + 0.3 per cent NaCl at pH 7.4. The thoroughly mixed cell suspensions were sucked up into the glass tubes to form columns 30 cm. long. The tubes bore short segments of rubber tubing on their lower ends, which, after filling, were closed with spring clips.

The ash content of the undried gelatin was 1.0 per cent, of the casein 1.1, and of the egg albumin 5.9 per cent. Any correction for the salt effect on mobility here must be very rough. If one assumes that the ash represents an equal weight of NaCl we have, in terms of molar electrolyte carried into the solutions by the proteins in Table III, 0.03 M with albumin, 0.006 M with casein, 0.003 M with 2 per cent gelatin, and 0.0015 M with 1 per cent gelatin. The molar concentration of electrolyte due to the M/50 buffer and 0.3 per cent NaCl constantly present is about 0.07 M. The concentration of electrolyte due to salt content of the protein is thus increased by about 50 per cent with the albumin and about 10 per cent with the casein; the change in salt concentration with the gelatin is negligible. Since κ varies as the square root of concentration (Müller, 1933), κ is increased, and mobility decreased, by about 22 per cent with the albumin and 5 per cent with the casein. The column headed "mobility corrected for salt effect" designates what the observed mobility would have been if the proteins had been salt-free. This figure multiplied by bulk viscosity gives the "corrected mobility" of the 8th column.

The findings on fresh unwashed cells are shown in Table III. It is evident that with the cells, such as horse, human, and dog, which

TABLE III

Mobility and sinking velocity of fresh, unwashed (except where otherwise specified) red cells. All solutions made up in M/50 buffer + 3 per cent glucose + 0.3 per cent NaCl, pH 7.4.

Medium	Relative viscosity	Mobility	Mobility corrected for salt effect	Sinking for min. indicated			Corrected mobility (mobility × viscosity)	Corrected sinking (sinking in 30 min. × viscosity)
				10	20	30		
		μ/sec./v./cm.	μ/sec./v./cm.	mm.	mm.	mm.		
Dog 1								
Buffer	1.07	1.60	1.60	0	0	0	1.71	0
3 per cent albumin	1.24	1.27	1.55	0	0	0	1.92	0
3 per cent casein	1.99	1.39	1.46	18	47	77	2.90	153
2 per cent gelatin	6.38	1.32	1.32	40	102	139	8.43	887
Dog 2								
Buffer	1.07	1.59	1.59	0	0	0	1.70	0
3 per cent albumin	1.24	1.32	1.61	0	0	0	2.00	0
3 per cent casein	1.78	1.40	1.47	27	85	134	2.62	238
1 per cent gelatin	3.58	1.43	1.43	10	53	115	5.12	412
Human 1								
Buffer	1.07	1.44	1.44	0	0	0	1.54	0
3 per cent albumin	1.24	1.13	1.38	0	0	0	1.71	0
3 per cent casein	1.99	1.19	1.25	6	23	39	2.49	78
1 per cent gelatin	3.58	1.29	1.29	12	40	73	4.62	261
Human 2								
Buffer	1.07	1.36	1.36			0	1.46	0
3 per cent albumin	1.24	1.09	1.33			0	1.65	0
3 per cent casein	1.99	1.13	1.19			10	2.37	20
1 per cent gelatin	3.58	1.13	1.13			21	4.05	75
Beef								
Buffer	1.07	1.04	1.04			0	1.11	0
3 per cent albumin	1.24	0.88	1.07			0	1.38	0
3 per cent casein	1.78	0.92	0.97			0	1.73	0
1 per cent gelatin	3.58	0.83	0.83			0	2.97	0
Horse 1								
Buffer	1.07	1.44	1.44	0	0	0	1.54	0
3 per cent albumin	1.24	1.18	1.44	0	0	0	1.79	0
3 per cent casein	1.78	1.50	1.57	51	118	135	2.80	239
1 per cent gelatin	3.58	1.43	1.43	22	64	97	5.12	347
Horse 1 washed in M/50 buffer pH 7.4 + 5 per cent glucose								
Buffer	1.07	1.33	1.33			0	1.45	0
3 per cent casein	1.78	1.24	1.30			1	2.32	2
1 per cent gelatin	3.58	1.29	1.29			1	4.70	4

can be aggregated and made to settle rapidly by added protein, gelatin is the most effective in increasing sinking velocity, with casein next, and albumin practically without effect. Beef cells do not settle at all in the 30 minute period in any of the proteins investigated. We have also found that beef cells will not sink more than 1 mm. in an hour in either beef or horse plasma while horse cells sink 100 mm. or more in 20 minutes in either horse or beef plasma.

DISCUSSION

In this paper data have been presented which are interpreted as indicating that red cell surfaces are hydrated. The establishment of a surface hydration for the red cells is a necessary but not a sufficient condition for the acceptance of the third possibility of Fåhraeus. The remaining step is to show that certain proteins, as gelatin, casein, and fibrinogen have a capacity to dehydrate the surfaces, not possessed by albumin. We have not been able to devise any positive independent test of such dehydrating action. The only evidence that they do act in this way is the fact that the red cells are aggregated by gelatin and casein and not by albumin. The viewpoint may be briefly recapitulated as follows. All types of red cells investigated possess a layer of oriented water molecules extending beyond the limits of the double layer. Added gelatin, casein, or fibrinogen abstracts the water from the outer shell in the case of those red cells which are aggregated by these proteins but is unable to do so in the case of the red cells, as beef, which are not aggregated, presumably because the latter cells hold the water more tenaciously. This disorientation, *i.e.* with respect to the cell, does not, however, extend into the double layer since double layer viscosity, as indicated by the electrophoretic findings, is but little changed by added protein. The surface energy at the interface of the cell and its medium is greater the thinner the oriented water layer, the increased surface tension holding the less hydrated cells together.

SUMMARY

The isoelectric point of normal red cells cannot be measured but is certainly lower than that of any plasma protein. Red cells are easily damaged so that they will adsorb proteins from low concentra-

tions. Normal red cells do not adsorb protein even from concentrated solutions, as is evidenced by the finding that the ratio of the mobility of the cells to that of the proteins themselves is at least as high in concentrated casein, albumin, gelatin, or fibrinogen solutions as in dilute.

The finding that the observed mobility of red cells is unchanged or only slightly decreased when bulk viscosity is increased by added protein is interpreted as indicating that the red cell surfaces are hydrated. The aggregating effect of certain proteins has been determined and is assumed to be due to their dehydrating effect on the cells. Some types of cells, as beef, are not aggregated, presumably because they are resistant to this dehydrating effect. The difference in the behavior of different types of red cells demonstrates the importance of the nature of the cell as well as of the medium in determining the rate of aggregation and therefore of sedimentation.

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