Quantitative Studies of the Influence of Plasma Proteins and Hematocrit on the Erythrocyte Sedimentation Rate

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Since the publication of Fahreus' work in 1918 and in 1921 there have been thousands of articles in medical literature dealing with the sedimentation rate of erythrocytes. In general, these can be divided into three categories: those proposing a new method or a revision of an existing one; those discussing the clinical application of the test; and those attempting to discover what physical and chemical factors may be responsible for the different rates sometimes found in health and in illness.

No attempt will be made to review the literature. Excellent reviews can be found in articles by Katz and Leffkowitz, Nichols, and Thygesen. A more complete review may also be found in the doctorate thesis upon which the present article is based.

From the welter of disagreement regarding the erythrocyte sedimentation rate, two factors are obviously significant: the size and character of the rouleaux formed by the erythrocytes, and some measure of the amount of cell mass present. Furthermore, the factors most generally agreed upon as affecting the size of rouleaux are the plasma proteins, chiefly the fibrinogen and globulin. As shown by Gordon and Wardley, the sedimentation rate of a given sample of blood is not dependent upon any one factor, but is the result of a complex interplay among various factors. Little work has been done along this line of investigation.

In the Child Research Council, simultaneous studies were made of the sedimentation rate, cell volume, and plasma protein levels of some ninety-five children. On forty-three of these, plasma protein analyses were done both by a modified Howe method and by electrophoretic separation. Thus an opportunity existed to make a study of the combined effects of several factors upon the sedimentation rate. We first attempted to devise a "correction chart" for cell volume, hoping to eliminate this one variable.

The Problem of a "Correction Chart" for Cell Volume

Correction charts have been published for the Wintrobe and Westergren sedimentation methods, by means of which the sedimentation rate at a given cell volume level can be corrected to the value it would have at a standard cell volume. It was our intention to devise such a chart for our modified Smith method.

Three techniques for manipulating cell volumes were employed:

1. Experiments 1 to 26. Five Smith tubes were filled with well-mixed, heparinized blood. Tube no. 1 was filled to the 50 mm. mark, nos. 2 and 3 to different levels above this mark, and nos. 4 and 5 to different levels below this 50 mm. mark. After the tubes had been centrifuged for 5 minutes, plasma was removed from tubes no. 2 and 3 and added to no. 4.

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and 5 to make the height of the column in each tube 50 mm. Then the samples were re-mixed for 3 minutes by gentle agitation with a capillary pipet. Readings were taken of the upper level of the red blood cell column at 5 minute intervals for an hour, the maximum fall during a 5 minute period being designated as the erythrocyte sedimentation rate (E.S.R.). Then the tubes were centrifuged to obtain their respective cell volumes.

2. Experiments 27 to 30. The entire sample of heparinized blood was centrifuged for 10 minutes. All the plasma was removed and 1.0 cc. added to each of five vials. Then 1.0, 0.8, 0.7, 0.6, and 0.5 cc. of red blood cells were added to the vials, respectively. After the vials had been shaken for 3 minutes, two Smith tubes were filled from each, and the sedimentation rate and cell volume determined as above.

3. Experiments 31 to 40. This group differs from the first (experiments 1 to 26) in that the manipulations were carried out in small test tubes and vials. These vials were allowed to stand for 15 minutes, then shaken for 5 minutes, after which the Smith tubes were filled.

In all, forty individual curves were obtained showing the relationship in a given case between the sedimentation rate and the cell volume. Ten such curves are depicted in figure 1. They intersect each other and show little common pattern, except that in all cases the sedimentation rate decreases as the cell volume increases. The thirty curves not drawn showed the same type and degree of inconsistency. No difference was discernible in the types of curves obtained by use of the various technics discussed above.

Despite criticisms levelled at correction charts in general, other workers have devised such charts for use with their methods. Three possible explanations might be suggested for our failure to obtain a satisfactory chart for this method:

![Diagram of ESR (mm/5 min) vs Hematocrit (%) for different diagnoses and cell volume values.](image-url)
1. Technics used in the present study for manipulating cell volume were faulty.

2. The Smith tube method of determining sedimentation rates is not as accurate as methods using a taller column of blood.

3. Correction charts which had been published were so constructed as to give an exaggerated impression of orderliness.

The first two explanations do not seem to be the answer. Our technics are similar to those used by other workers; furthermore, there was no apparent difference between the results obtained with the three different technics used. Also, in eleven of the experiments discussed above, simultaneous sedimentation rates were determined using the Wintrobe tube (height of blood column, 100 mm.). These showed no greater consistency than when the Smith tube was used.

However, a close scrutiny of the original data upon which the published charts were based shows the third possible explanation to be true. Wintrobe and Landsberg obtained a series of curves whose essential pattern was similar to figure 1. To obtain smoothed curves for the final chart, they calculated an average line from the individual curves, and then drew lines at regular intervals, parallel to this median line. Such a group of lines hardly seems to fit their original data. Wintrobe later termed correction charts "crude and artificial." Rourke and Ernstene have as their correction chart a series of curves which are convergent at the higher cell volume levels. This seems much more in line with the type of data on which these smoothed curves were based. However, their original individual curves show much of the irregularity which led us to abandon the attempt to devise correction charts for our method. Individual curves obtained by Westergren and by Gram for the Westergren method show more consistency than the ones just mentioned. Nevertheless, Westergren later found better correlations between the sedimentation rate and plasma protein fractions if he used uncorrected rates than if he used rates corrected according to his chart.

Two possibilities may be suggested for the inconsistent types of curves obtained.

First, the relationship between the sedimentation rate and cell volume may not be independent of the levels of the plasma proteins, as has been assumed. As shown in figure 1, more similarity exists between the curves of healthy and of pregnant individuals than between either of these groups and the curves of those with pathologic processes. Attempts were made without success to interpret these curves statistically, assuming their difference in shape to be due to differences in the plasma protein levels.

Secondly, in manipulating cell volumes in vitro, a new and unknown factor may be introduced into the process. This was suggested by Cutler, Park, and Herr who state that "the dilution experiment involves large-scale manipulations on a relatively small quantity of blood, a phenomenon which does not take place during life."

Despite our failure to devise a satisfactory correction chart for cell volume, it obviously plays a significant role in determining the sedimentation rate. The treatment of this factor in the statistical analyses will be discussed later.

**Quantitative Relationships between the Sedimentation Rate, Cell Volume, and Plasma Proteins**

*Materials and Methods*

*Subjects.* The subjects from whom blood was drawn for these experiments can be divided into two categories:

1. Forty-three of the children being studied in the "birth series" of the Child Research Council. Blood was taken at approximately six month intervals. The total of seventy-eight bloods in this group includes one determination on each of fourteen children, two on each of twenty-three children, and three on each of the remaining six children. These subjects range in age from 3 to 16 years. In all but five instances the children were considered healthy by a staff pediatrician.

2. Twelve ward patients at Colorado General Hospital, whose ages range from 10 to 71 years.

* Original data are available from the authors.
Obtaining blood sample. Approximately 8 cc. of venous blood were drawn with a minimum of stasis. The following apportionments were made:

1. One cc. was placed in a small shell vial containing 0.3 mg. of heparin (110 units/nl). The vial was stoppered and shaken for about 15 seconds. This portion was used for determinations of the hemoglobin and hematocrit values and of the erythrocyte sedimentation rate.

2. Another cc. was placed in a small test tube containing 0.25 mg. of heparin. This tube was stoppered and shaken. Plasma protein determinations by the salting-out method were done on this sample.

3. The remainder of the sample was put into a plain test tube, allowed to clot, and the serum used for electrophoretic analysis of serum proteins.

Methods of analyzing samples. 1. Sedimentation rate. In order to obtain reproducible results it was necessary to adhere very closely to the following routine. After the initial mixing of blood and heparin, the vial was set aside for 15 minutes; it was then shaken for 5 minutes, using a vigorous and irregular motion and avoiding the production of foam. Immediately the blood was drawn up into a capillary pipet; from this, the sedimentation tube was filled and placed vertically in a rack. The tube used was the one described by Smith; it has an internal diameter of about 2.5 mm. and a height of 50 mm., with constant bore throughout the length of the tube. Readings in mm. of the descent of the red blood cells were made at 5 minute intervals and the maximum fall in any one 5 minute interval during 1 hour designated as the sedimentation rate. Determinations were performed in duplicate on most samples and the average of the two values used. Tests were performed at ordinary room temperatures (22 to 27 C.). Our own previous experiments had shown no significant variation in the sedimentation rate within this temperature range.

2. Cell volume (hematocrit value). After the sedimentation rate had been determined, the Smith tube was centrifuged for 1 hour at 2500 rpm in an International Centrifuge, S.B., No. 1. The height in mm. of the column of packed red blood cells was divided by the height in mm. of the entire sample to obtain the packed red cell volume. Plasma volume was obtained by subtracting the cell volume as a per cent from 100.

3. Hemoglobin content. This was determined by the standard alkaline hematin method, using a Benciner-Mass photocolorimeter.

4. Mean Corpuscular Hemoglobin Concentration. This constant, proposed by Wintrobe, is obtained by the following formula:

\[
M.C.H.C. = \frac{Hgb. (Gm./100 cc. blood)}{C.V. (cc./100 cc. blood)} \times 100
\]

5. Plasma proteins (salting-out method). The modified Howe method described by Hill and Trevorrow was used. Total protein, albumin, globulin, and fibrinogen concentrations were determined on each sample.

6. Serum proteins (electrophoretic analysis). These analyses were done with the table-model apparatus described by Moore and White. Barbiturate buffer with a pH of 8.6 was used. The following fractions were determined: albumin (A); alpha-1 globulin (a1); alpha-2 globulin (a2); beta globulin (b); and gamma globulin (y). Edsall, Perry, and Armstrong and Armstrong, Budka, and Morrison pointed out that the so-called fibrinogen as determined in plasma by this or any of the usual electrophoretic methods actually consists of a mixture of fibrinogen with portions of the beta and gamma globulins. Since the particular interest in the present study is each separate fraction, serum was used for the electrophoretic analyses and the fibrinogen content of plasma determined by the salting-out method.

After the electrophoretic separation of the protein constituents of serum, photographs were taken and the area representing each fraction measured. The per cent of each protein in the sample was calculated on the basis of the total protein area photographed; these percentages were in turn multiplied by the total protein concentration as determined by Kjeldahl analysis to obtain absolute values in Gm./100 cc. of plasma.
Results

a. Correction of sedimentation rates for variations in specific gravity of cells and plasma. According to Stokes' law the speed of fall of a spherical solid body through a liquid medium varies directly with the difference in specific gravity between the body and the liquid. In blood, the solid body consists of aggregates of red blood cells and the liquid medium is the plasma. However, the solid bodies are not spherical, but irregular in outline; therefore, Stokes' law cannot be applied directly to sedimentation rates.

In the present study an indirect method for estimating relative values for the specific gravity of cells and of plasma was adopted. Total plasma protein concentration (Gm./100 cc. plasma) was used for the relative value for specific gravity of the plasma. Mean corpuscular hemoglobin concentration (Gm./100 cc. cells) was used as an index of cell specific gravity. For convenience the arithmetic difference between these two values (M.C.H.C.—T.P.) has been labelled as the factor “H”. Obviously the values have meaning only as a basis for differentiating the various blood samples in respect to relative specific gravities.

Inasmuch as the relationship between “H” and the sedimentation rate should be independent of other factors, each rate was corrected to the value it would have been with an “H” factor of 25. This corrected figure was used in the mathematical derivations now to be described.

Derivation of prediction formulae. Table 1 lists the simple coefficients of correlation between these variables: corrected E.S.R., cell volume, plasma/cell ratio, and plasma proteins as determined by the salting-out method. Combination of the data by the multiple correlation method of Doolittle leads to a prediction of E.S.R. from the cell volume value and the protein levels. This prediction formula is:

$$\text{E.S.R.} = \frac{H}{25} (12.368 \text{ fib.} + 1.361 \text{ glob.} + .090 \text{ alb.} - .179 \text{ C.V.} + 3.377)$$

(Formula K-1)

The values of the constants preceding each factor represent the relative importance of that factor. Albumin was omitted from the later formulae since it was without influence except as it enters into the factor “H”. Although the coefficient of correlation between observed and predicted sedimentation rates was \(+ .960 \pm .008\), the results indicated an error probably in the underlying assump-
tion of a linear relation between cell volume and sedimentation rate. Those bloods with a high cell volume gave a low predicted sedimentation rate (twenty-nine of forty-six cases) while those with low cell volumes predicted high (thirty of forty-four cases). The difference was significant by the Chi square test. We theorized that the influence of the plasma proteins on the size of rouleaux might be better measured in terms of the concentration of protein per unit of cell surface or volume. In the prediction formula which follows, each of the protein fractions was computed in Gm./100 cc. of cells by multiplying its original value by the plasma/cell ratio:

E.S.R. = \[P/C(7.546 \text{ fib.} + .722 \text{ glob.} - .603) - 1.809\] \[\frac{H}{25}\]  
(Formula K-2)

The coefficient of correlation between observed sedimentation rates and those predicted by the formula is +.971 ± .006. Tabulation of the values predicted by this formula for bloods in the high and low cell volume groups mentioned above gives an even distribution. This may indicate that the data have been more properly handled by this method of including the cell volume factor, even though the difference between +.960 and +.971 is not significant.

In those cases where the globulin fraction was greatly elevated, agreement between observed and predicted values was not as good as when only the fibrinogen value was high or when all the factors were in the normal range. It is known that globulin as determined by the salting-out method is not a single substance, but consists of a mixture of several globulin fractions. It seems probable that these various components may have different influences upon the rouleaux formation of red blood cells and therefore upon the sedimentation rate. If so, higher coefficients of correlation should be obtained if prediction formulae based upon protein values found by electrophoretic analysis are used.

Computation of prediction formulae using such data was done in the same manner as above. Simple coefficients of correlation are given in table 2. As before, better results were obtained by using the concentration of protein fractions as

### Table 2.—Coefficients of Correlation between Various Factors which Influence the Sedimentation Rate

(Serum Proteins Determined by Electrophoretic Analysis)

<table>
<thead>
<tr>
<th></th>
<th>Fib.</th>
<th>γ glob.</th>
<th>β glob.</th>
<th>α1 glob.</th>
<th>α2 glob.</th>
<th>Alb.</th>
<th>P/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected E.S.R.</td>
<td>+.901</td>
<td>+.442</td>
<td>+.195</td>
<td>+.730</td>
<td>+.744</td>
<td>−.734</td>
<td>+.524</td>
</tr>
<tr>
<td>Fibrinogen*</td>
<td></td>
<td>+.291</td>
<td>+.114</td>
<td>+.673</td>
<td>+.711</td>
<td>−.656</td>
<td>+.255</td>
</tr>
<tr>
<td>γ globulin</td>
<td></td>
<td>+.390</td>
<td>+.146</td>
<td>+.067</td>
<td>−.355</td>
<td>+.114</td>
<td></td>
</tr>
<tr>
<td>β globulin</td>
<td></td>
<td></td>
<td>+.079</td>
<td>+.066</td>
<td>−.196</td>
<td>+.067</td>
<td></td>
</tr>
<tr>
<td>α1 globulin</td>
<td></td>
<td></td>
<td></td>
<td>+.730</td>
<td>−.543</td>
<td>+.294</td>
<td></td>
</tr>
<tr>
<td>α2 globulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−.650</td>
<td>+.384</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−.469</td>
<td></td>
</tr>
</tbody>
</table>

* Fibrinogen values are the same as used in table 1. No values for this fraction were obtained electrophoretically, as explained in the text.
Gm./100 cc. of cells; and, as before, the albumin fraction was found to have very little influence and was therefore omitted from the final formula, which is:

\[
E.S.R. = \frac{P}{C}(6.060 \text{ fib.} + .866\alpha + .326\beta + 2.091\alpha_2 \\
+ .183\alpha_1 - 1.958) - 1.327 \frac{H}{25}
\]

(Formula T-1)

The coefficient of correlation using these data is \(+ .982 \pm .004\). Although this value is not significantly higher than the corresponding one using total globulin (formula K-2), it does indicate a great difference between the various globulin fractions with respect to their influence upon the sedimentation rate, as shown again by the constants preceding each factor. The three most influential fractions are fibrinogen, alpha-2 globulin, and gamma globulin, with alpha-1 globulin and beta globulin exerting but little influence.

In figure 2, observed sedimentation rates are plotted against the rates determined by prediction formula T-1.

**Discussion of Results**

It should first be pointed out that the protein fractions as determined by the two methods used are not comparable because of the fact that in the first they are separated on the basis of solubility, and in the second on the basis of electrical properties. In general, however, albumin as determined by the salting-out
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method equals, in amount, the albumin, alpha-1 and alpha-2 globulins as determined electrophoretically; and "total globulin" equals beta plus gamma globulins.

The three fractions found to be most influential upon the sedimentation rate are fractions which are most likely to change with certain illnesses. Fibrinogen and alpha-2 globulin have been associated with tissue repair; gamma globulin is the antibody-containing fraction of the proteins. From the simple correlations shown in table 2, however, it is obvious that an increase in any one of these fractions is not necessarily accompanied by an increase in either or both of the other.

An elevated sedimentation rate gives no indication as to which of the fractions may be raised. For example, experiment no. 83 can be compared with experiment no. 87:

<table>
<thead>
<tr>
<th>No.</th>
<th>Sc</th>
<th>P/C</th>
<th>Fib.</th>
<th>Globulins</th>
<th>Total glob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
<td>9.8</td>
<td>1.74</td>
<td>.525</td>
<td>2.49</td>
<td>1.15</td>
</tr>
<tr>
<td>87</td>
<td>9.9</td>
<td>1.72</td>
<td>.825</td>
<td>0.65</td>
<td>0.88</td>
</tr>
</tbody>
</table>

The sedimentation rates and plasma cell ratios are identical, within limits of error. On the other hand, the plasma protein fractions are extremely different. No. 83 shows a high gamma globulin, with a somewhat elevated fibrinogen. No. 87 shows a normal gamma globulin but a greatly elevated fibrinogen.

Moreover, a high cell volume (low plasma/cell ratio) can produce a slow sedimentation rate despite elevated plasma proteins. This can be shown by a comparison of experiments no. 86 and 89:

<table>
<thead>
<tr>
<th>No.</th>
<th>Sc</th>
<th>P/C</th>
<th>Fib.</th>
<th>Globulins</th>
<th>Total glob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>6.8</td>
<td>1.86</td>
<td>.424</td>
<td>0.90</td>
<td>1.02</td>
</tr>
<tr>
<td>89</td>
<td>0.5</td>
<td>0.74</td>
<td>.427</td>
<td>1.13</td>
<td>1.26</td>
</tr>
</tbody>
</table>

This confirms the clinical observations of Numbers, Bannick and his co-workers, and others who emphasize that a normal sedimentation rate does not rule out an infectious process.

The high degree of correlation obtained does not preclude the possible existence of other factors not studied in this work. An example is a blood from a patient under acacia therapy for nephritis, whose predicted sedimentation rate (formula K-2) was 3.9 mm., but whose observed rate was 20.8 mm. Such effects of acacia have previously been reported in the literature.

Comparisons of the simple correlations obtained in this study with those found in the literature are extremely difficult because of the different technics employed, both for sedimentation rate determinations and for plasma protein analysis. Our results agree qualitatively with those of other workers in that positive correlations are found between the sedimentation rate and fibrinogen and globulin levels, and negative correlations between the rate and
albumin and cell volume (or erythrocyte count) values. The same qualitative agreement exists between our prediction formulae and those of previous workers.3,38

**ADDIION OF PURIFIED PROTEINS TO BLOOD**

The statistical results described above do not prove that an increase in one or more of the protein fractions is necessarily the cause of an increase in the sedimentation rate. To study this point, purified protein fractions obtained from the laboratory of Dr. E. J. Cohn* were added to samples of heparinized blood.

Eight to 10 cc. of blood were drawn and placed in a test tube containing heparin; this tube was centrifuged and the plasma removed and divided into two portions. In one of these the protein fraction was dissolved. Varying proportions of these two plasmas were pipeted into each of five vials, with the final plasma volume always 1.0 cc. Then the cell mixture left in the original test tube was mixed and 0.6 cc. pipeted into each vial. Sedimentation rates and cell volumes were determined in duplicate on each sample. From each vial, portions of plasma were removed and the proteins determined by the salting-out method. This type of experiment was performed using three different fractions—albumin, fibrinogen, and gamma globulin.

Results with these proteins followed the same trend as that suggested by the statistical analysis. Addition of albumin causes some slowing of the rate. Increasing amounts of fibrinogen and of gamma globulin, on the other hand, cause increases in the sedimentation rate. Furthermore, these increases are of

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* We wish to thank Dr. Cohn and the Harvard Laboratory for so graciously supplying these materials.
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the same magnitude as the increases which would be predicted on the basis of formula K-2. This is shown in figure 3, where observed and predicted results from these addition experiments are plotted against the background of the regression line calculated from prediction formula K-2.

These experiments demonstrate a causal relationship between the proteins and the sedimentation rate in vitro, and confirm the work of many previous investigators who performed similar experiments. Only two of these studies can be compared quantitatively with our addition experiments and prediction formulae. Gray and Mitchell used protein fractions similar to those used by us. Good agreement exists between their results and ours. They found that the addition of fibrinogen caused 6 times as much increase in the sedimentation rate as did gamma globulin. According to formula T-1, this ratio is 7:1. In their experiments addition of albumin caused considerably greater decrease in the sedimentation rate than was found in the present study. Kylins used proteins similar to those of our salting-out technic and found that the addition of fibrinogen caused 8 times as much increase in the sedimentation rate as did addition of an equal amount of globulin. According to formula K-2, this ratio is 10:1. These ratios indicate good agreement between the statistical results of the present study, which are in a sense in vivo experiments and those of in vitro experiments.

These studies suggest that the influence of the proteins on rouleaux formations, and therefore the sedimentation rate, is determined by the concentration of that protein per red blood cell.

SUMMARY AND CONCLUSIONS

The failure to devise a satisfactory means of adjusting all sedimentation rates to a standard cell volume, together with a critical analysis of the results of other investigators, casts serious doubt on the validity of any such correction charts.

Two formulae are presented by means of which the sedimentation rate of a given sample of blood can be predicted on the basis of the plasma/cell ratio and plasma protein concentrations. One formula utilizes the results of salting-out analysis of plasma proteins, the other those of electrophoretic analysis of serum proteins. The most influential factors in determining sedimentation rate are plasma/cell ratio, fibrinogen, alpha-2 globulin, and gamma globulin. The results suggest that the effective concentrations of the plasma proteins are most adequately expressed as concentrations per unit of cell volume.

Addition of purified protein fractions (fibrinogen, gamma globulin, and albumin) to the blood in vitro confirm the findings from the statistical studies and suggest a direct cause and effect relationship between the concentration of these proteins and the rate of erythrocyte sedimentation.

Determination of the erythrocyte sedimentation rate cannot give, per se, any clue as to the level of any one of the responsible factors. An appreciation of the multiple factors involved is essential for an adequate interpretation of the clinical significance of a sedimentation rate determination.

REFERENCES


Quantitative Studies of the Influence of Plasma Proteins and Hematocrit on the Erythrocyte Sedimentation Rate

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