The Zeta Sedimentation Ratio

By Brian S. Bull and J. Douglas Brailsford

The zeta sedimentation ratio (ZSR) is a measurement similar to the determination of the erythrocyte sedimentation rate (ESR) but possesses several advantages. It is unaffected by anemia and responds in a linear manner to increase in fibrinogen and/or gamma globulin. The normal range is identical for males and females. A blood sample, contained within a vertically oriented capillary tube, is subjected to four cycles of dispersion and compaction. At the conclusion of this process, the hematocrit of the blood in the red cell-containing portion of the capillary tube is measured. This hematocrit, a measure of the closeness with which red cells will approach each other under a standardized stress, is the ZSR.

In 1924, ALF WESTERGREN published a paper entitled "Die Senkungssreaktion"—The Sedimentation Reaction. The technique of performing an erythrocyte sedimentation rate determination (ESR) has remained unchanged ever since. In 1970, the International Committee for Standardization in Hematology proposed adoption of the Westergren Technique as the standard method. In 1971, the American National Committee for Clinical Laboratory Standards followed suit. The ESR is, thus, the same test, requiring the same amount of time, and subject to the same uncertainties of interpretation in anemic patients as it was 47 yr ago.

The ESR has not persisted unchanged because investigators have ignored it. On the average, more than one paper a year has been written on various improvements in sedimentation rate methodology since it was first described. These improvements have been concentrated in three main areas: (1) reducing the amount of blood and/or the time required to perform the test; (2) corrections for the effect of anemia; (3) making the test respond in a linear fashion to changes in asymmetrical macromolecules, such as fibrinogen and gamma globulin.

Attempts to reduce the time required for the ESR and to speed up the determination have, in general, followed the approach of shortening the tube and decreasing the bore. None of the microtechniques has met with wide acceptance because the tubes become more difficult to read as they get shorter, and as the bore goes below 2.5 mm the sedimentation process becomes unstable and blockage of the tube occurs. Another commonly used short cut is the elimination of the dilution step, even though a Westergren...
tube is used. The hazards of this approach and the inaccuracies that result have been emphasized by Dawson.4

Charts for anemia correction were proposed by Wintrobe and Landsberg in 1935.5 Hynes and Whitby, in 1938,6 pointed out that the curves drawn for normal blood were inaccurate when applied to pathologic blood and that no single set of curves could ever be expected to accomplish the desired result. As a result, correction charts have fallen into disrepute, although surprisingly 67% of laboratories in the United States still use them.7 A much superior approach to correction for the effects of anemia was proposed by Rourke and Ernstene in 1930.8 They showed that hematocrit corrections were possible if the sedimentation rate was determined continuously over a 1-hr period and the maximum rate of fall recorded, rather than the distance traversed by the red cells in the first hour. The complexity of making multiple readings on each sample over a 1-hr period, plotting the results, and finding the maximum rate of fall militated against the widespread acceptance of this approach.

In 1952,9 Hardwicke and Squire reexamined the question and plotted the maximum sedimentation velocity (determined by the method of Rourke and Ernstene) at a standard hematocrit of 30% against increasing concentrations of macromolecules. They found that if they corrected the plasma sample to a standard viscosity, the results were linear when plotted on log paper. They recognized that the complexity of the procedure precluded its clinical use and suggested that the measurement of plasma viscosity be substituted for the ESR. The clinical measurement of plasma viscosity has been facilitated by the introduction of a clinical viscometer by Harkness,10 but the equipment is bulky and relatively expensive, and the results are expressed in units foreign to most clinicians. As a result, the Harkness technique has not been widely accepted in this country.

Despite these well-recognized weaknesses, the ESR continues to be used. It is simple to perform and does respond to an increase in plasma content of asymmetrical macromolecules. These proteins, particularly fibrinogen and gamma globulin, are good indicators of the presence of inflammatory disease.

As fibrinogen and gamma globulin increase in plasma, the zeta potential of the suspended red cells falls. The zeta potential results from negatively charged sialic acid groups on the red cell membrane. The repulsive effect of this negative charge on adjacent red cells is attenuated by the presence of ions and modified by the dielectric effect of proteins in the surrounding medium. All proteins affect the dielectric coefficient of plasma, but asymmetrical macromolecules are oriented by the field and hence exert a disproportionately large effect.11 Therefore, as fibrinogen and gamma globulin in plasma increase, they decrease the zeta potential of suspended red cells, permitting increased rouleaux formation and a more rapid sedimentation rate. Such a mechanism is the most likely explanation for elevations in the sedimentation rate in disease.

The primary effect, the increase in fibrinogen and gamma globulin, can be quantitated by electrophoresis, but the technique is too complex to serve as a screening test. The decreased zeta potential of the red cells can also be
Fig. 1. Diagrammatic illustration of Zetafuge principle and the way in which zeta sedimentation ratio (ZSR) is determined.

measured directly but not at the cellular concentrations found in whole blood. Fortunately, the effect of this decreased zeta potential on adjacent red cells can be measured simply and quickly by determining the extent to which red cells will pack under a standardized stress. The technique to be described measures the closeness with which red cells approach one another under such a standardized stress. Unlike measurements of rate of fall such as the ESR, the ZSR is unaffected by anemia and responds in an approximately linear fashion to an increase in asymmetrical macromolecules throughout the range of clinical significance.

The ZSR has two drawbacks. The results are expressed in new units, %, rather than mm/hr. As a consequence, there is a new normal range, 40%–51%. This range is, however, identical for both males and females. The second disadvantage is the need to purchase special capillary tubes and a specific piece of new equipment, the Zetafuge. However, there are compensations: the capillary tubes are easier to fill, require less sample, and are cheaper than standard ESR tubes.

MATERIALS AND METHODS

The forces of alternating compaction and dispersion are produced by the Zetafuge, a centrifugal device shown in Fig. 2. A blood sample of approximately 100 µl contained within a vertical capillary tube is spun at 400 rpm. The resultant 7–8 g force causes the red cells in the sample to travel outward until they approach the outer edge of the containing tube. After 45 sec of centrifugation the centrifuge head is stopped by a timer. The small capillary tubes are rotated 180°, and the centrifuge is restarted automatically. As the centrifuge head regains its operating speed the clumped red cells now travel from the inner wall of the tube. They are partially dispersed during the initial phases of this
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Fig. 2. Photograph of Zetafuge, the centrifugal device that produces the repetitive cycles of compaction and dispersion.

journey before being deposited in rouleaux once more on the outer wall. In the process of traversing the tube, they are exposed to the normal downward force of gravity and, thus, come to rest upon the outer edge of the tube at a point somewhat lower than that from which they started at the beginning of the cycle. This process is repeated four times, causing the red cells to follow a zigzag path down the capillary tube and become more densely packed all the while. Four 45-sec cycles of compaction, dispersion, and recompaction are employed (Fig. 1). At the conclusion of this 3-min period the degree of compaction achieved by the red cells is measured. This measurement, the zeta sedimentation ratio (ZSR), is the hematocrit of the red cell-containing portion of the capillary tube at the conclusion of the compaction-dispersion cycles. It is a measurement of the ease with which red cells will pack under a standardized compaction-dispersion stress and is, therefore, presumably inversely related to the zeta potential of these cells when suspended in the particular plasma under consideration. Blood samples anticoagulated with tripotassium EDTA were obtained from 100 male blood donors and an equal number of females. The degree of compaction-dispersion stress applied by the zetafuge was then adjusted so that 95% of the resultant ZSR’s were less than 51%. Increasing quantities of various asymmetrical macromolecules were then added to additional samples so as to reproduce quantitatively blood with any selected sedimentation rate or any desired ZSR. (The macromolecules studied were obtained from the following sources: gelatin, (nutrient) Difco Lab., Figs. 3 and 4; fibrinogen (human); and gamma globulin (human), Cutter Lab.) The results on 100 consecutive patients, on whom an ESR had been requested, were analyzed in depth. Duplicate Wintrobe and Westergren determinations were run using standard methodology. ZSR’s were also run in duplicate in the same samples. From these duplicates the coefficient of variation for each method was derived. The hematocrit of each sample was then adjusted using autologous plasma or red cells, females to 45% and males to 47%, and both the Westergren and the Wintrobe ESR’s were repeated. All of the Westergren and Wintrobe results were then analyzed independently by three internists who were provided with randomized lists giving the patients’ sex, hematocrit, and Wintrobe (or Westergren) value. The team of internists assigned each ESR to one of five categories: normal (N), probably normal (PN), and elevated (+, 2+, 3+). The categories assigned by each referee to a patient before and after hematocrit adjustment were compared to evaluate the facility with which a practicing clinician can mentally make hematocrit adjustments. The data were then compiled to form two “standards.”
Fig. 3. ZSR of three saline suspensions of washed red cells to which increasing quantities of gelatin have been added. Curves superimpose except in range below ZSR of 40, where each describes a separate "tail" that intersects Y-axis at ZSR value identical to its hematocrit value (28, 20, 12). 99% of patient whole blood samples will have ZSR's in region where curve is still approximately linear.

The first standard was the average of the eight independent assignments made on each patient. These were the categories assigned on the Westergren determination by the three internists, the value assigned by the same group on the Wintrobe results, plus two ZSR determinations. As a result of combining this data, each of the 100 patients was given a value ranging from N to 3+. This value was assumed to be the true value, and this list composed standard No. 1. A second list of values was prepared for the same 100 patients by use of the Hynes and Whitby curves for the Wintrobe results. This list of values constituted standard No. 2. Each of the three internists and the ZSR were then evaluated against these two standards, and the frequency with which their individual assignment matched the standard value was calculated.

To measure the ZSR, a capillary tube is read after the compaction-dispersion cycles as if it were a standard hematocrit tube. This value is referred to as the zetacrit, to avoid confusion with the true hematocrit. The same capillary can then be spun in a microhematocrit centrifuge or the hematocrit determined by using the Coulter S on another aliquot of the same sample. (The outside diameters of the zetacuff capillaries require that the rubber gasket in the outer rim of the International Centrifuge be raised slightly and cemented in place. The Clay Adams microhematocrit centrifuge head will accept the tubes if a heated tube is paused to enlarge slightly the plastic grooves.) The true hematocrit is divided by the zetacrit; the resultant figure, expressed as a percentage, is the ZSR.

Several variations of the above method are possible and may be more convenient, depending on the methods employed in a given laboratory to determine the hematocrit. For instance, the level of the zetacrit may simply be marked on the capillary tube with a glass marker. After spinning the tube at high speed to determine the centrifuged hematocrit, the tube is placed on a hematocrit reader with the zetacrit mark aligned with 100%. The ZSR now corresponds with the upper meniscus of the red cell column. The meniscus
Figs. 4 and 5. Comparison between Westergren and Wintrobe methods of sedimentation rate determination and ZSR. To the same blood sample was added increasing quantities of gelatin or fibrinogen, following which the ZSR and the two sedimentation rates were performed. At each level of gelatin or fibrinogen, sample was diluted with its own plasma so as to halve the hematocrit, and determinations were repeated. Note large range of uncertainty induced by this hematocrit change in the Westergren and Wintrobe methods.
Table 1. Frequency With Which Three Internists Were Able to Place a Patient in the Same Category (Normal, Probably Normal, +, 2+, 3+ Elevated) Before and After Adjustment of the Hematocrit to Normal

<table>
<thead>
<tr>
<th>Referee</th>
<th>Wintrobe ESR (%)</th>
<th>Westergren ESR (%)</th>
<th>ZSR</th>
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<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>74</td>
<td>Unaffected by anemia</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>54</td>
<td></td>
</tr>
</tbody>
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of the zetacrit is not completely horizontal, for a tail of red cells rises above it on one side and a small wedge of plasma depresses it on the other. The convention followed in reading the meniscus is to turn the tube sideways, as shown in Fig. 1, and to mark the zetacrit at the "knee" of the curve between the red cell tail and the meniscus.

RESULTS AND DISCUSSION

In normal individuals the ZSR is 51% or less. Values between 51% and 54% are considered probably normal; the ranges 55%-59%, 60%-64%, and 65% or greater are, respectively, mild, moderate, and markedly elevated. Under experimental conditions, gelatin is a more convenient macromolecule to use than fibrinogen for in vitro elevation of the ESR; fibrinogen has a high content of sodium citrate and hence, osmolar effects. The effect on the ZSR of adding increasing quantities of gelatin to washed, protein-free, red cell suspensions in saline is shown in Fig. 3. The curve that results can be conveniently treated in three sections. The portion of the ZSR curve from 40% to 70% is virtually a straight line. Above 70% the red cells are approaching their maximum packing ratio under 1 g (80% for most samples), and the curve, thus, approaches this upper limit asymptotically. In the region below a ZSR of 40%, the curve described depends on the hematocrit of the red cell suspension. Since the ZSR can never be lower than the cell content of the sample, each curve must intersect the Y axis at a ZSR that corresponds to the hematocrit. The curves described for progressively more dilute red cell suspensions indicate that the line is straight and does pass through the origin.

More than 99% of ZSR's determined on whole blood samples from patients fall within the range 40%-70% and, thus, will lie in the approximately linear portion of the curve. None will fall below a ZSR of 40% unless the hematocrit is also quite low, thus lengthening the linear portion of the curve. Very

Table 2. Per Cent of Interpreted ESR's That Agreed With Each of Two ESR Standards

<table>
<thead>
<tr>
<th>Wintrobe ESR</th>
<th>Westergren ESR</th>
<th>ZSR</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard 1</td>
<td>78</td>
<td>68</td>
</tr>
<tr>
<td>(3 Westergren results, 3 Wintrobe results, 2 ZSR results)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard 2</td>
<td>76</td>
<td>60</td>
</tr>
<tr>
<td>(Hynes-Whitby)</td>
<td>60</td>
<td>63</td>
</tr>
</tbody>
</table>


rarely, a patient with a markedly elevated sedimentation rate by usual methods will show a ZSR approaching 80%, and it should be appreciated that the curve has become nonlinear in this region.

The particular selection of length of cycle, number of cycles, and centrifuge speed is not critical and could be varied within certain limits. Shorter cycles will compensate for increased centrifuge speed, etc. Such absolute limits as do exist arise from the fact already noted that red cells under 1 g will not pack to a hematocrit greater than 80% no matter how long or how intensely the compaction-dispersion stress is applied. To gain the greatest sensitivity, the upper limit of the normal range must be kept as far from 80% as possible yet still higher than a normal hematocrit, since the ZSR can never be less than the starting hematocrit of the particular blood sample under test. (If a ZSR is needed on a patient whose hematocrit exceeds 54%, it can be obtained by allowing an aliquot of blood to sediment in a test tube. A portion of the red cell layer is pipetted off, the sample remixed, and placed in a ZSR tube. Since the hematocrit will have been lowered by this procedure, it must be determined in the same altered blood sample.)

The marked effects of hematocrit variations on sedimentation rates determined by the Westergren and Wintrobe techniques are shown in Figs. 4 and 5. The particular type of gelatin employed in this experiment had an effect that, milligram for milligram, was very similar to that of fibrinogen. The standard deviation of each method is also shown diagrammatically in Fig. 5. These deviations were calculated from the 100 duplicate determinations further analyzed in Tables 1 and 2. The ZSR and the Wintrobe have a standard deviation of 0.49. The Westergren has a standard deviation of 1.2 mm/hr. Even though the Westergren has a higher standard deviation, the addition of 1 mg of fibrinogen elevates the Westergren ESR from 8 to 12 mm/hr depending on the portion of the curve being analyzed. Comparable figures for the ZSR are 5–6 mm/hr and for the Wintrobe, due to early onset of the packing phase, the range is from 2 to 14 mm/hr. As a result, the relative sensitivities (units of elevation per milligram fibrinogen/standard deviation) are virtually identical for all three methods.

The ZSR is slightly less sensitive to storage at room temperature than is the Westergren or the Wintrobe. Periods of 12 hr on the laboratory bench produced no changes, whereas 24-hr storage at room temperature caused marked erythrocyte swelling and decreases in the ZSR of most samples. The effect of sickle cells and marked poikilocytosis is likewise similar for the three methods.

The parameter measured by the ZSR is the closeness with which the red cells will approach each other if permitted to do so under controlled alternating compaction and dispersion. As a result, if the hematocrit is low at the inception of the determination, the red cells continue to sediment until the hematocrit stabilizes at a ZSR appropriate for that blood sample making the ZSR independent of anemia. The effectiveness with which highly skilled internists were able to correct mentally for the effects of anemia on the Westergren and Wintrobe ESR’s is shown in Table 1. Three internists who
held faculty appointments with a university medical school and evaluated more than five ESR's a day in the course of their clinical practice evaluated each of the 100 patients. Since there are no generally accepted "normal ranges" each internist was asked to place each patient in one of five categories before and after hematocrit normalization; thus, each was scored against himself. The lists were randomized so that each ESR had to be categorized without knowledge of the category previously assigned to that particular patient. Although one of the group (a hematologist) was able to correct somewhat better than the others, the over-all results are not reassuring, and it is reasonable to suppose that the average user of ESR data would not do as well.

The ZSR is faster, requires less sample, is unaffected by anemia, and has equivalent sensitivity to the Westergren and Wintrobe methods of measuring the ESR. Is it a better method clinically? Phrased this way the question is unanswerable. The Westergren and the Wintrobe have been compared for decades by many investigators without either emerging as clearly the best method. Based on frequency of usage in the United States, the Wintrobe is superior. Yet the National Committee for Clinical Laboratory Standards recommends the Westergren method for perfectly valid reasons. Comparison of the ZSR with either or both of these methods is not likely to clarify the situation. The problem is that no absolute standard exists against which all three methods could be compared. Relative standards can, however, be formulated, and the comparison of the various approaches with these relative standards should reveal any gross differences between the methods.

Each of the three internists and two ZSR determinations were graded, in turn, against two such standards. Standard No. 1 consisted of data to which all four methods under evaluation had contributed, in contradistinction to standard No. 2 where there was no contribution from the internists or the ZSR. The faults and deficiencies of each of these relative standards were hopefully compensated for by the other. It seems reasonable to conclude that a single ZSR value is as useful as the considered evaluation of a Wintrobe or a Westergren ESR by a skilled internist (Table 2).

The ZSR should serve well in those situations where the ESR has already established itself. The additional advantages it possesses, not the least of which is ease of interpretation, should extend its usefulness to yet other areas. Anemic patients, multiphasic health screening centers, and blood bank donors are among the more obvious applications.

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