Improved Measurement of Erythrocyte Volume Distribution by Aperture-Counter Signal Analysis

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In aperture counters, particles in fluid suspension flow through a small orifice or aperture, causing a change in the electrical resistance of the aperture. This change is sensed by an external electronic circuit and translated into a voltage pulse, the signal height of which is proportional to the volume of the particle in the aperture. These signal pulses are collated into a spectrum of pulse heights by a multichannel pulse-height analyzer. The channel number (voltage increment) spectrum is proportional to the volume distribution of the particles sensed. A problem is that pulse height not only depends on cell volume, but also on the orientation and shape of the particle sensed and the current density along the path taken by the particle through the aperture. Uneven current density exists, primarily at the aperture entrance and exit, close to the wall. Orientation and shape of particles are altered near the wall by the unbalanced shear forces there. Toward the center of the aperture, the shear forces act so as not to induce continuous change in the orientation of the particles sensed. Thus introduction into the pulse-height spectrum of pulses that do not show a good proportionality to volume is primarily caused by particles that are traveling near the aperture wall. Residence time in the aperture for a particle traveling near the wall will be longer than that for a particle traveling down the center of the aperture, because of the smaller fluid velocity near the wall. Duration of the signal pulse created by a particle traveling near the wall will be correspondingly greater. We discuss an electronic filter to remove from the pulse-height spectrum those pulses that appear to result from particles traveling near the wall and the effect of the filter on the measured signal height and hence the volume distribution of erythrocytes. Use of this technique to characterize erythrocytes by volume distribution is described.

Additional Keyphrases: fluid mechanics • electrical field effects • effect of particle shape and orientation • mean cell volume

Measurement of size and number of human erythrocytes has long been important in diagnostic medicine. Manual methods for these evaluations are time consuming and susceptible to technical error. Counting of erythrocytes is now commonly mechanized, by use of instruments that will be referred to in this discussion as “aperture-counter devices.” No generally accepted generic name for these instruments has been established, but they are often referred to as “electronic cell counters” or by the trade name of the specific manufacturer. The term “aperture counter” is proposed because the feature that is unique to that instrument is a small aperture where the cells can be sensed and measured individually. Furthermore, the work to be discussed is based on evidence that the major phenomena affecting use of these instruments take place in the counter aperture. Description of commercial instruments may be found in various manufacturers’ literature (1) and (2). The aperture-counter principle has been used in several research studies (3, 4).

In the aperture-counter technique, a suspension of the erythrocytes to be measured flows through a small aperture under a pressure gradient (Figure 1). The suspension is dilute enough so that only one cell at a time passes through the aperture. An electric current of about 1 mA is maintained through the aperture. The change in the electrical resistance in the aperture caused by the presence of a particle can be measured, and this is the basis of electronic counting of cells.

The amplitude of the output signal of the counter is a function of the measured particle's volume, shape, and orientation with respect to the electric field within the aperture. The counts may be arranged into a distribution function according to the magnitudes of the voltage of the output signals by feeding the signal from the counter into a multichannel pulse-height analyzer. This distribution is normally assumed to be proportional to the volume dis-
distribution of the measured particles, and a linear calibration for cell volume in terms of channel number is applied. In the typical distribution for erythrocytes shown in Figure 2, the population of voltage pulses is strongly skewed to the right, resulting in a bimodal appearance that has led some investigators to believe that aperture counters do not give a true measure of the erythrocyte volume distribution.

Aperture counters are now widely used to determine the mean cell volume of erythrocytes and, in combination with the count, the hematocrit. The skewness problem is negated by using chemically fixed blood cells of known volume for calibration. Both such fixed and native erythrocytes exhibit skewed volume distributions when aperture-counters are used. This calibration scheme yields reproducible results of reasonable accuracy as compared with those for manual techniques (5). However, the use of fixed cells may not be valid if the erythrocytes to be analyzed have an unusual volume distribution, as could occur in certain disease states in which cell volume is affected. Moreover, the mean cell measurement is an averaging technique that does not necessarily denote a unique distribution around the mean.

For studying the effect of many biochemical and physiological stresses on the erythrocyte, the erythrocyte volume distribution must be accurately measured. To study cell volume distributions with aperture counters, the counter response for each fraction of cell sizes must be understood.

Authors dealing with the existence of skewness in measured erythrocyte volume distributions are divided into those who propose that the skewness reflects the true volume distribution of erythrocytes (6, 7) and those who consider the skewness to be an artifact of the method of measurement (8, 9, 10, 11). Most recent writers on the subject hold the latter view.

1 Each channel in the multichannel analyzer represents a voltage increment. For example, if the total voltage range was 1 V and the analyzer contained 100 channels, the voltage increment would be 0.01 volt, and the fiftieth channel would contain the count of all signals whose peak voltage was between 0.50 and 0.51 V. Hence, the channel number can be calibrated in terms of peak signal voltage and in turn in terms of particle volume.

The following discussion analyzes the evidence that the artifact is a result of a complex interaction among the inhomogeneity in the electric field across the aperture, measured particle shape and orientation, and the hydrodynamic field of the fluid traversing the aperture. We describe a signal-analysis technique that can remove most of this artifact.

**Phenomena in Counter Aperture**

The output signal is governed by three phenomena in the counter aperture: electrical field effects, particle shape and orientation, and fluid mechanics. These interact to produce the erythrocyte volume measurement artifact as discussed below.
Electrical Field Effects

The change in the electrical resistance of the aperture produced by the passage of a particle will be a function of the strength of the electric field through the aperture. Theory and experiment show that the magnitude of the resistance change, and hence the pulse height of the signal output produced by a particle of given volume, is directly proportional to the current density along the line of travel taken by the particle. If the electric field is of uniform current density, a particle will produce the same maximum pulse height along different paths through the aperture. However, the aperture geometry is that of an extended conductor narrowing into a conductor of small cross-section. The apertures used in erythrocyte measurements are usually 50 to 100 μm in diameter with axial length to diameter ratio (X/D)^2 of approximately 1. Lines of electrical current in the fluid conductor converge into the aperture and pinch together at the edges of the aperture. Thus high current densities result near the wall of the aperture at the entrance and exit, and the electrical field at these two points is not radially homogeneous. At an X/D of about 0.5, the field approaches radial homogeneity. The high current density at the edges causes a particle traversing this region to produce a higher signal pulse than a particle of the same volume traveling closer to the axis of the aperture. Large voltage responses for some particles out of the total population of particles would cause the measured volume distribution to skew to the right.

Grover et al. (9) performed a theoretical analysis of the potential gradient within the aperture. The potential gradient at a point is directly proportional to the current density. Their results agree with the experimental data gathered by Thom et al. (8) for the case of a long aperture. The mathematical derivation holds true only for apertures with an X/D of a least one. In Figure 3, Grover’s calculations are shown in terms of the output signal response of a particle of given volume relative to the response of the same particle if the electric field were radially homogeneous. The relative response is a function of the fractional radial distance from the axis (FRD) and the ratio of the distance from the aperture mouth to the aperture radius (X/R). Figure 3 shows that the electric field approaches radial homogeneity at an X/R = 1.0 (equivalent to X/D = 0.5). The electric field will extend into the reservoir at the entrance and exit of the aperture. This is reflected in Figure 3, because the relative response is not zero at X/R = 0. A particle passing through the aperture at a given FRD will produce a relative response denoted by the appropriate FRD curve.

Particles traveling down the center (FRD less than about 0.7) produce output signal pulses that slowly rise to a maximum value and then descend to form symmetrical shapes. Within 0.7 FRD from the axis for a long aperture (X/D greater than one), the maximum values of pulse height achieved by a particle are roughly equal. For a shorter aperture, they are not. Particles passing near the wall (FRD greater than 0.7) give an output signal that rises in a short X/R to a maximum and then falls back to a minimum at an X/R of one. Because of the slower fluid flow near the wall these signals are also longer in duration. This effect will be discussed in more detail in the discussion of fluid mechanics which follows. Near the wall the high current densities at entrance and exit cause a very high response producing a characteristic “M” shaped curve. 3

The pulse height analyzers used to process these signals record the maximum pulse height produced. Hence, for the same actual volume, a particle near the wall will have a larger measured volume than one near the aperture axis. Kachel et al. (11) have correlated the signal shape with erythrocyte radial position by using a capillary director. This analysis is consistent with their results.

Typical output signals produced by an aperture-counter system are shown in Figure 4 for spherical latex particles and erythrocytes. The traces marked

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2 Nonstandard abbreviations used: X/D, ratio of axial length to diameter; FRD, fractional radial distance (from the axis); RE, Reynolds Number; LPR, long-pulse rejector; and MCA, multi-channel analyzer.

3 Note that Figure 3 only shows the entrance region of the aperture. The exit region would show a mirror image of the response with respect to the X/R coordinate.
with a "B" display the "M" shaped pulses of particles traveling near the walls. Those marked "A" exhibit the shape characterized by axial passage through the aperture.

Particle Shape and Orientation

The pulse height of the signal from the counter is proportional to a particle's volume but is affected by the particle's shape and orientation with respect to the electric field in the aperture. Orientation refers to the spatial relationship between the axis of a particle and the lines of current. A spherical particle would not be subject to orientation-dependent artifact. The shape and orientation of a particle determines the extent to which the particle disrupts the electric field within the aperture. Shear forces in the hydrodynamic field can cause rotation and deformation, causing the orientation and shape of the particle to be altered. The following discussion will more closely examine shape and then orientation.

Several researchers (4, 12) have investigated shape relationships by using models in scaled-up aperture systems. The results are shown in Figure 5 for some basic geometric shapes. The number associated with each shape is called the "shape factor," which is the ratio of the signal pulse height produced by a given particle to the minimum pulse height that can be produced by a particle of the same volume. A long, slender body has a shape factor approaching 1.0. If the resistance change caused by a particle in the aperture were proportional only to the volume displaced, then all shapes would have a shape factor of 1.0. When the electric field does not conform to the particle shape an "electrical shadow" is formed, which makes the particle appear to displace a larger "volume" than its actual volume. It can be shown from a theoretical analysis (9, 13) as well as by model building that the shape factor of a sphere is 1.5. Thus the measured volume of a sphere is 50% larger than the measured volume of a slender, tapered body of the same volume.

Figure 6 shows a native biconcave erythrocyte and the possible shape factors it could have as a result of its orientation with respect to the electrical current lines in the aperture. It is well documented that fluid shear will cause rotation of asymmetrical particles (14, 15) such as erythrocytes. Thus the fluid flow in the aperture will affect the erythrocyte orientation and hence its effective shape factor. In addition, the erythrocyte is extremely flexible. For example, Guest et al. (16) have shown that even small fluid shear can have a pronounced effect on the erythrocyte shape.

Fluid Mechanics

In analyzing the fluid mechanics of the aperture flow it is convenient to examine two regions: the region just ahead of the aperture entrance and the region inside the aperture. The aperture exit can be treated as a viscous jet and does not affect the analysis.

**Fig. 5.** Shape factors for some common geometric shapes with the same volumes

In the region ahead of the entrance, the flow must fall between the limiting cases of potential flow (negligible viscous forces) and creeping flow (negligible inertial forces). Potential flow would cause a uniform velocity distribution at the aperture entrance; creeping flow would cause a velocity distribution that was partially developed.

Within the aperture, the flow can be treated as a boundary-layer flow. Viscous forces slow the flow near the wall, and a viscous boundary-layer grows to fill the tube. As the flow slows near the wall, the central core accelerates. Assuming the flow is laminar (Reynolds Number 4 (RE) less than 2000) and the aperture is long enough, the velocity profile develops down the aperture to produce the classical Poiseuille velocity profile. The central accelerating flow exhibits zero radial shear (the velocity gradient is zero).

One of us has extensively investigated the aperture flow with the aid of computer solutions to the partial differential equations that describe the fluid mechanics. Based on pressure-drop measurements, it was determined that potential flow ahead of the aperture

\[
\text{Re} = \left( \frac{\text{aperture diameter}}{\text{average velocity}} \right) \left( \frac{\text{fluid density}}{\text{fluid viscosity}} \right)
\]

where

\[
\text{average velocity} = \frac{\text{volumetric flow rate}}{\text{aperture cross-sectional area}}
\]

4 Denoted in this work by RE =

erture was a good assumption for a Reynolds Number in the range of 200 or higher. Most commercial instruments operate in this range. For example, in this work the average Reynolds Number was 233 [aperture diameter, 50 μm; length to diameter, 1; linear average velocity, 466 cm/s; pressure drop, 26.6 kPa (20 cm Hg)].

Figure 7 gives the results of fluid velocity profile calculations within the aperture, assuming a uniform entrance profile. This figure gives the ratio of the axial velocity to the average velocity as a function of radial position, the distance from the entry of the aperture, and the Reynolds Number. The three-dimensional function can be displayed in a two-dimensional graph because analysis of the fluid mechanics shows that X/D and RE can be combined into a single variable. Thus if the volumetric flow rate, the aperture diameter, and the fluid properties of viscosity and density are known, the fluid velocity at any point in the aperture can be determined from Figure 7. For a 50-μm diameter orifice and conventional flow rates, the exit velocity profile will fall between profiles 3 and 4 in Figure 7. Profile 5 is fully developed. Note that the exit flow for most apertures is not fully developed. Therefore there is a central core of uniform velocity in the aperture. Particles traveling in this central core experience no radial shear stress. Acceleration is also an important aspect of the flow field. At distances greater than one diameter from the aperture entrance, the fluid is essentially at rest. Hence the flow accelerates from rest to its average velocity at the aperture entrance over a space of one aperture diameter. A linear average velocity of 450 cm/s is typical. Within the aperture, viscous forces slow the flow near the wall, and the velocity down the central core must increase in order to maintain a mass balance. The flow in the core is therefore accelerated continuously from entrance to exit.

Calculations based on the velocity profiles show that at an FRD of about 0.8 (radial distance from axis/aperture diameter of 0.4 in Figure 7) there is a sharp break in the residence times of fluid particles in the aperture. Particles within the viscous boundary-layer traverse the aperture much more slowly than do particles in the accelerating central core.

The radial shear in the viscous boundary-layer is quite large, ranging from about 3 × 10^4 μN (3000 dyn/cm²) at the boundary-layer edge up to about three times that value at the aperture wall. Neglecting acceleration effects, calculations show that rotations of 25% or greater are possible throughout the boundary-layer. This would represent a rotation between extreme shape factors shown in Figure 5 for a rigid erythrocyte. Because Nevairt et al. (17) have shown that at continuous shear forces > 3 × 10^4 μN/cm², hemolysis is greatly increased, the assumption of a rigid shape probably is not justified.

Kachel et al. (11), in a remarkable piece of work, photographed both native and rigid erythrocytes passing through a 100-μm diameter aperture at an average linear velocity of 320 cm/s (RE = 160). Native erythrocytes near the wall were distorted, and rotation was apparent with both native and fixed cells. In the central core, the native erythrocytes appeared to be stretched into long, slender bodies along the axis of the aperture. No rotation was apparent. The rigid erythrocytes appeared to be in a longitudinal orientation with respect to the aperture axis.

The cells in their pictures were directed with a microcapillary close to the entrance of the aperture and the cells were not introduced singly, which may disrupt the normal aperture flow. However, this would not change the conclusion that native erythrocytes distort and rotate in the viscous boundary-layer and that they stretch because of the acceleration in the central aperture core and align with the axis of the aperture.

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For example, if X = 50 μm, D = 50 μm, then

\[ \frac{X}{D \cdot RE} = 4.3 \times 10^{-3} \]  

(having the dimension one)
Signal-Analysis Technique

The signal-analysis technique is based on a number of points that were developed in the preceding discussion. These points are summarized below:

1. Erythrocytes passing near the aperture in the viscous boundary-layer distort and rotate, making it impossible to calibrate the output signals produced by these cells to their volume.

2. The nonhomogeneity in the electric field that causes large "M" shaped output signals falls within the viscous boundary-layer. (Compare Figures 3 and 7.)

3. Erythrocytes that traverse the central aperture core (which comprises about 65% of the cross-section) are stretched into slender bodies aligned with the aperture axis.

4. The erythrocyte residence time in the central core of the flow is constant as a result of the flat velocity profile. At the boundary-layer edge the residence time sharply increases. The duration of the output signal of the aperture counter is proportional to the erythrocyte residence time in the aperture.

The conclusion we draw from these points is that by rejecting output signals of long duration it should be possible to analyze only signals caused by flow of erythrocytes in the aperture core. Because these cells are aligned and involve only a single shape factor, it should be possible to calibrate the output signals of these cells to cell volume by using spherical particles that have a known volume and shape factor.

The electronic module that was used to eliminate the signals of long duration from the aperture-counter output will be referred to as the long-pulse rejector (LPR). The technique used in the LPR is to compare the duration of a signal above a set threshold voltage to the duration of a preset signal. If the duration of the input signal is less than the preset signal, a linear gate is opened and the LPR outputs a signal with the same pulse height as the input signal to the LPR. If the input signal duration is equal to or longer than the preset signal, the linear gate is not opened and no output signal is produced by the LPR. Figure 8 illustrates the relationship of the LPR to the aperture-counter system. The control on the LPR which determines the duration of the signals to be rejected is denoted as the LPR delay setting. A large delay setting indicates a long pulse duration.

Instrumentation and Materials

We used two separate aperture-counter/multichannel analyzer systems in this work:

**System 1:** Aperture-counter, Coulter Model F
- 50-μm diameter aperture
- 0.57 ml/min flow rate
- Multichannel analyzer, Packard Model 115

**System 2:** Aperture-counter, Particle Data Celloscope
- Model 111
- 48-μm diameter aperture
- 0.5 ml/min flow rate
- Multichannel analyzer, Nuclear Data Series 2200

Typical settings are shown below:

**Erythrocytes:**

<table>
<thead>
<tr>
<th>System 1</th>
<th>System 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aperture current</td>
<td>1</td>
</tr>
<tr>
<td>Aperture gain (Attenuation)</td>
<td>64</td>
</tr>
<tr>
<td>MCA sensitivity</td>
<td>2 V/100 channels</td>
</tr>
<tr>
<td>MCA attenuation</td>
<td>16 (650)</td>
</tr>
<tr>
<td>2.02-μm diameter</td>
<td></td>
</tr>
</tbody>
</table>

**Latex spheres:**

<table>
<thead>
<tr>
<th>System 1</th>
<th>System 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aperture current</td>
<td>8</td>
</tr>
<tr>
<td>Aperture gain</td>
<td>64</td>
</tr>
<tr>
<td>MCA sensitivity</td>
<td>2 V/100 channels</td>
</tr>
<tr>
<td>MCA attenuation</td>
<td>16 (650)</td>
</tr>
</tbody>
</table>

The data output for System 1 was perforated paper tape, which could be read into the Scientific Date System's Sigma 5 computer used for data analysis. The output for System 2 was typewriter hard copy.

The LPR was manufactured by Applied Data Laboratory, P. O. Box 57822, Webster, Tex. 77598. Its description follows:

**Input:** Positive 0, 10 V
- Duration 5, 100 μs

**Delay adjustment (rejection):** Adjustable from 5–50 μs. Inputs of greater duration than this setting and greater than the threshold will not appear at the output. Adjustable by 10-turn-pot.

**Threshold:** Adjustable to accept pulses greater than 0.1 to 5 V. Adjustable by 10 turn-pot.

**Output:** Positive 0.1 to 10 V if input width is less than the delay duration setting. Duration approximately 5 μs.

**Packaging:** One U. S. AEC Nuclear Instrument Module

The delay setting calibration is shown in Figure 9. The threshold setting used was approximately 0.15 V. Identity of signal input and output pulse heights was verified by calibrated signal generator.

The materials used in this work are listed below:
Measured Particle Source:
Erythrocytes: Whole blood, with heparin or ethylenediaminetetraacetate as anticoagulant
Latex spheres: Dow Chemical
Diagnostic Products Division, Indianapolis, Ind. 46268
Fixed erythrocytes: Dade Normal Hematology Reference
Suspension Media:
Eagle’s solution, as marketed by Coulter Electronics, Hialeah, Fla. 33010, under the name of “Isoton.”

Results
Correlation with Fluid Mechanics
The residence time of a particle in an aperture depends on the radial distance from the axis at which it enters and is determined by the fluid mechanics describing the path and velocity of the particle in the aperture. Thus the equation solutions that allow the calculations shown in Figure 7 can be used to calculate the residence time as a function of radial entry position. In general, the residence times will be longest near the wall and will increase to a minimum in the core of the aperture.

Now consider two other points. First, the particles in suspension are randomly distributed throughout the fluid, making the chance of a particle entering the aperture at any radial position equally probable. Second, the suspension of particles is very dilute, making negligible the probability that two or more particles will simultaneously pass through the aperture. Thus, each passage through the particle aperture can be treated as a separate event. These two points mean that within a cumulative count, the fraction of particles that passed through the aperture and entered the aperture in a cross-section between the wall and any given radial position is equal to this cross-section divided by the total aperture cross-section. For example, 35% of the particles will enter the aperture in the cross-section between the wall and a distance from the axis equal to or greater than 0.8 of the aperture radius.

If the total aperture cross-section is considered, the fraction of total counts will be 1.0 and the longest residence time will approach infinity (if the particles occupied no volume) owing to zero flow at the wall. Then the cumulative distribution of particle aperture passages having residence times less than infinity will be 1.0. If the longest residence time in the cross-section around the axis which represents 0.8 of the total cross-section is 12 μs, then 0.8 of the total counts will have residence times of 12 μs or less. By using the calculated residence times it is possible to construct such a residence time/fractional count distribution curve.

This same cumulative distribution can be measured indirectly by using the LPR. As the delay width is gradually decreased, the number of pulses determined by the LPR as being of acceptable width is decreased. By counting the pulses allowed through the LPR, the number of particles having a pulse width less than a reference width can be determined. This distribution is an indirect measure of the distribution of residence times, because the particles are sensed before they arrive at and after they leave the aperture. The pulse width is therefore greater than the residence time within the aperture. The length along which the particle is “sensed” is approximately equal to the sum of the aperture length plus 84% of the aperture diameter (19). For a length/diameter ratio of 1, the sensing zone is considerably longer than the aperture length. This factor will offset, by a constant factor, the cumulative distribution of pulse widths from the distribution of residence times. Figure 10 presents the results of this comparison by
using erythrocytes. We assumed a flat entrance velocity in calculating the residence times. The curves are offset by a constant factor but both have a sharp break characteristic of undeveloped flow. The sharp break occurs when about 35% of the particles traveling through the aperture have been removed from the spectrum. If the assumption of an equal distribution of particles through the aperture is correct, 35% of the particles entering the aperture will enter at a distance equal to or greater than about 0.8 of the aperture radius from the axis.

The purpose of the LPR is to filter out the pulses originating from particles traveling in this region. These pulses will have artifacts in them as a result of the unusually high current densities near the walls at the aperture entrance and exit and as a result of shear forces that can induce tumbling. The 65% of the total particles traveling closer to the axis will largely be free of these artifacts. Because some electric field inhomogeneity exists down to a fractional radial distance of 0.8 from the axis, the twin sources of artifact disappear at about the same point.

**Erythrocyte Volume-Distribution Measurements**

Figure 11 shows the decrease in the number of erythrocytes counted into the volume distribution as the delay-width setting is decreased. Again, the delay-width setting determines the maximum signal width that is used in collating the distribution of peak heights. Figure 12 shows the spectra found at different points in Figure 11. The feature to notice is the gradual disappearance of the skew. The spectra corresponding to a 35% decrease in counts is nearly gaussian. Again this is the point at which theory and experimental evidence indicate that the artifact should be removed. Figure 13 shows a least-squares gaussian fit to the spectra corresponding to this point. The coefficient of variation of the best-fit line is 17.4%.

In theory a further decrease in LPR delay width setting beyond the setting that gave a 35% decrease in total aperture counts should diminish the count to zero, and in fact the decrease is quite sharp, as shown in Figure 11. This decrease in counts is not instantaneous because the velocity profile in the core is not perfectly flat throughout the aperture and because of the resolution of the LPR used in this work. The resolution of the LPR will be discussed in the following, under The Effect of Signal Height on Signal Width. However, a spectrum at 75% count reduction is also shown in Figure 12, illustrating the effect of decreasing the delay width beyond the optimum.

**Measurement of Erythrocyte Mean Cell Volume**

One significant aspect of removing the artifact with the LPR is that it makes it possible to measure erythrocyte mean cell volume based on an absolute calibration rather than on a relative calibration, by using a fixed-cell standard having some "typical" volume distribution curve. Until the artifact is removed no single calibration will apply to the volume distribution curve. In this section we present results of the measurement of erythrocyte mean cell volume by using microspheres, whose diameters were accurately determined by electron microscopy, as a calibration standard. The shape factor for spheres is known to be 1.5. The shape factor for erythrocytes, based on pictures of stretched aligned cells in the aperture core (11), should approach 1.0.

The results are reported based on an erythrocyte shape factor of 1.0 and are compared to the conven-
tional fixed-cell standard and a microhematocrit method.

Calibration requires the determination of the relationship between volume and the channels of the multichannel analyzer used to construct the distribution of signal pulse heights. The peak value of the distribution of spheres of 2.02-μm diameter was assumed to have that volume corresponding to a sphere with the average diameter of the distribution. This procedure is accurate, because the manufacturer's reported standard deviation of diameters is only 0.0135 μm. This gives an average volume of 4.32 μm³ with a standard deviation of 0.086 μm³.

The shape factor of the spheres must be taken into account when making the calibration. Again, the shape factor is the ratio of the maximum voltage response caused by a particle of given shape and volume to the response generated by an elongated, tapered body of the same volume. Relative to an elongated body of the same volume, a sphere will produce a voltage pulse 1.5 times as large. Thus the channel at which the peak of the distribution of spheres occurs would be at a volume of 1.5 × 4.32 = 6.48 μm³, and the multichannel analyzer would be calibrated on the basis of actual volume. If a particle is measured on this scale it is only necessary to divide by its shape factor to get its actual volume.

For example, if for an instrument gain of 16, the microspheres peak fell in channel 121, the calibration would be given by

$$\frac{6.48}{121} (16) = 0.86 \mu m^3 \text{ per channel}$$

If a population of erythrocytes is counted in channel 100 with an instrument gain of 1, and if a shape factor of 1.0 is assumed, the volume of these cells would be

$$\frac{(100)(0.86)}{(1.0)} = 86 \mu m^3$$

The mean cell volume of the measured erythrocyte volume distribution can be obtained by numerically integrating the distribution function.

$$\text{Mean cell volume} = \frac{\sum \Delta V_i N_i}{\Delta V_i}$$

where

- $N_i$ = number of counts in the $i^{th}$ channel
- $V_i$ = volume in the $i^{th}$ channel (Corrected for shape factor)
- $\Delta V_i$ = volume increment per channel in the $i^{th}$ channel.

We determined the mean cell volume for four different blood samples by using the aperture counter technique. The blood was drawn from astronauts as part of the National Aeronautics and Space Administration (NASA) Project Skylab. NASA also supplied mean cell volume measurements based on the microhematocrit technique. Table 1 gives a comparison between the volumes as measured by the microhematocrit technique and by the aperture counter technique. For comparison, two different volumes are given for the results of the aperture counter technique. One of the volumes corresponds to the value measured without the LPR (zero reduction in counts), with use of fixed human erythrocytes as the calibration standard. (Fixed cells are the current commercially employed calibration standard.) The second volume is that volume measured with use of spheres as a calibration standard, assuming a shape factor of 1.0 for stretched erythrocytes, and eliminating the artifact with the LPR. The point of measurement was taken as the mean volume corresponding to 65% of the total aperture counts. Although four samples cannot be considered a statistically valid population, the volumes as determined by both aperture calibration schemes are reasonably close to the volumes determined by manual methods. Recall that the shape factor for the erythrocytes, in the case where the spheres were used for calibration, was assumed to be 1.0. Nothing was assumed about the shape factor of the fixed erythrocytes used in calibrating the measuring system. The calibration volume of the fixed cells was supplied by the distributor.

Table 1 also gives the shape factors for the native cells, calculated by taking the ratio of the volumes measured by the aperture counter to the volume measured by the microhematocrit determinations. If, for the case of the calibration with spheres, it is as-
summed that all of the artifact has been eliminated, then the ratio just defined could be considered to be the shape factor. The shape factors based on sphere calibration closely approach, but exceed, one. Three of the values are almost equal. This is encouraging, because a constant shape factor from sample to sample would allow accurate determination of mean cell volume.

Again, the significance of these results is that an absolute calibration standard can be used in measuring mean cell volume with the LPR. Based on Figure 12, the use of microspheres with the 100% curve would result in an error in mean cell volume of greater than 25%. With the artifact removed and a shape factor of 1.0, the error is about 3 to 6% and is as good as the fixed cell standard. If the shape factor for erythrocytes can be more accurately determined, the accuracy of the LPR technique can be improved.

Effect of Signal Height on Signal Width

The signal pulse width as measured by the LPR was found to be sensitive to signal pulse height. For example, increasing the amplification of the aperture-counter output increased the number of pulses rejected by the LPR. In addition, about 10% of the 2.02-μm spheres stick together to form pairs. The LPR provides good resolution showing two distinct peaks, but at 35% reduction in total counts the pairs, which have at least a 60% greater response, seem to show a disproportionate decrease in counts. Oscilloscope tracings indicate that the LPR may not be distinguishing sufficiently between the small, wide flat pulses near the wall and the relatively large pulses caused by pairs in the center of the aperture.

To test this effect on the erythrocyte measurement, we measured a sample of large (3–7 μm diameter) latex spheres under the same conditions as for the erythrocytes. Figure 14 shows the results. At a 40% decrease in count, which is extreme, signal pulses 30% larger than the erythrocyte are being counted with the LPR. This gives confidence that in this range the erythrocyte volume distribution measurement is not altered by the influence of pulse height on the ability of the LPR to measure pulse width.

Discussion

This work adds to the evidence that the artifact in measuring erythrocyte volume distribution by use of aperture counters is primarily attributable to effects of electric field and of fluid flow dynamics near the wall. The LPR technique will effectively remove this artifact and allows the accurate measurement of erythrocyte volume distributions and the absolute calibration of the volumes by using microspheres.

In comparison to the LPR, several investigators, including Bull (18) and Grover et al. (19), attempted to eliminate the inhomogeneity of the electric field by installing an electronic circuit that delayed the measurement of the signal pulse height until the particle reached the homogeneous field deep within the aperture. This technique will not handle artifact because of the viscous boundary-layer, and it does not allow for different particle residence times.

On the other hand, the capillary director technique has been used by a number of investigators, notably Thom et al. (8), Shank et al. (10), and Kachel et al. (11). These investigators used a capillary tube to direct the flow of the cell suspension into the core region of the aperture. Aside from the possible effect of the capillary on the flow field in the aperture, this technique is very effective in eliminating the electric field and viscous boundary-layer artifact. Although we had no capillary director for direct comparison, our results and the results of other investigations are in general agreement. For example, Thom et al. (8) arrived at a shape factor of 1.0 for erythrocytes in the aperture core as compared to 1.03 to 1.06 measured in this work. Our determination of a shape factor of 1.03–1.06 compared to 1.2–2.9 for a rigid erythrocyte

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Microhematocrit technique</th>
<th>Fixed cell calibration without signal analysis</th>
<th>Sphere calibration with signal analysis</th>
<th>Calculated shape factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87.0</td>
<td>84.5</td>
<td>89.6</td>
<td>1.03</td>
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<tr>
<td>2</td>
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<td>90.2</td>
<td>97.0</td>
<td>1.06</td>
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<tr>
<td>3</td>
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<td>86.0</td>
<td>89.3</td>
<td>1.03</td>
</tr>
<tr>
<td>4</td>
<td>90.0</td>
<td>85.5</td>
<td>92.5</td>
<td>1.03</td>
</tr>
</tbody>
</table>

*a* Shape factor taken as 1.0.
*b* The shape factor was calculated by dividing the sphere calibration value for mean cell volume by the microhematocrit value. For example, in sample number 1: Shape factor = 89.6/87.0 = 1.03
supports the evidence that acceleration in the core flow of the aperture aligns and stretches the native erythrocyte. In addition, the coefficient of variation for the gaussian fit to the measured erythrocyte volume distribution reported by Shank et al. (10) was 20% and by Kachel et al. (11) was 17.5%. This compares well with the 17.4% we found in this work, and indicates that the same artifact is being removed from the cell volume distribution. Although the capillary director eliminates artifact, it requires a complicated mechanical system and will restrict count rate considerably; on the other hand, the LPR is simple to install on existing aperture-counter systems and decreases count rate by only 35%.

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References