The Measurement of Lymphocyte Volume: Importance of Reference Particle Deformability and Counting Solution Tonicity

By George B. Segel, Giles R. Cokelet, and Marshall A. Lichtman

We have determined the influence of reference particle deformability and suspending buffer tonicity on the measurement of lymphocyte volume by an electronic particle volume analyzer. When the volume analyzer was standardized with latex spherules having a shape factor \( f \) of 1.5, red cell volume was 96 cu \( \mu m \) and lymphocyte volume was 289 cu \( \mu m \). The red cell volume corresponded closely to the true red cell volume; the true lymphocyte volume, however, was 218 cu \( \mu m \) when measured by the lymphocytocrit/lymphocyte count and 203 cu \( \mu m \) by wet lymphocyte weight and density (mean \( \sim 210 \) cu \( \mu m \)). The difference between the electronic volume \( V_e \) of 289 cu \( \mu m \) and true lymphocyte volume of 210 cu \( \mu m \) was due to the influence of lymphocyte deformability (shape factor) as it traverses the sizing aperture. Since the true volume equals the \( V_e / f \), the red cells with a shape factor near 1.0 were sized appropriately by this method. In contrast, the lymphocyte shape factor was 1.38; thus, the true lymphocyte volume was 289 cu \( \mu m / 1.38 \) or 210 cu \( \mu m \). The tonicity of the suspending solution also influenced the measurement of particle volume when osmotically inactive standard particles (e.g., latex spherules) were used as a reference. Whereas the true lymphocyte volume was 210 cu \( \mu m \) at 286 mosmole/liter, it was 194 cu \( \mu m \) at 330 and 229 cu \( \mu m \) at 250 mosmole/liter. The standard counting solution, isoton, is hyperosmolar (330 mosmole/liter) and causes an 8% shrinkage of osmotically active cells.

THE ELECTRONIC particle volume analyzer has become a standard tool in many biomedical research laboratories. Many of these instruments were designed principally for use in a clinical laboratory setting. Therefore, standardization has been established for counting blood red cells, white cells, and platelets, but for sizing only human red cells. When such instruments have been used in research laboratories, there has been a marked variation in the volume of the same nucleated cells. For example, the reported lymphocyte volume varies from 180 to 280 cu \( \mu m \).1-9 We have explored the reasons for the marked variation by assessing the effect of standard particle deformability and suspending buffer tonicity on the electronic measurement of lymphocyte volume. Further, we have measured lymphocyte volume by two independent methods to allow calculation of a shape or deformability factor, \( f \), for lymphocytes. Knowledge of this shape factor allows determination of the true lymphocyte volume from measurements on an electronic particle volume analyzer.

MATERIALS AND METHODS

Preparation of Lymphocytes

Lymphocytes were prepared from the mononuclear cell-rich platelethpheresis residues of healthy donors as previously described.8 To ensure a low proportion of monocytes, the white cell concentrates were suspended in tissue culture medium 199 (TC-199), GIBCO, Grand Island, N.Y.) at a concentration of 2 \( \times 10^6 \) cells/ml and incubated for 30 min at 37\( ^{\circ} \)C with an equal volume of twice concentrated lymphocyte reagent, a balanced salt solution containing iron filings (Technicon Inst. Corp., Tarrytown, N.Y.). The mononuclear cells were then separated on a step gradient,10 and the mononuclear cell layer was harvested, washed, and resuspended at a concentration of 10\( ^7 \) lymphocytes/ml. This cell suspension was passed through a nylon fiber column prepared from a leukopheresis filter (no. 4C2450; Fenwal, Inc., Ashland, Mass.). Forty milliliters of filter material were tightly packed in a 50-ml syringe. The lymphocytes were eluted from the column with 20 ml portions of TC-199. Lymphocytes prepared in this way contained fewer than 1.5% monocytes.

Measurement of Lymphocyte Volume by Lymphocytocrit and Lymphocyte Count

Lymphocytes were suspended in TC-199 plus 20% AB plasma at a concentration of 6 \( \times 10^6 \) cells/ml. Three microcuries of \( ^{14} \)C-sucrose containing 1.66 mmole unlabeled sucrose was added per ml of the cell suspension. Precisely 60 \( \mu l \) of this suspension was pipetted into microhematocrit tubes to provide 8 replicate measurements. These cell suspensions were sedimented at 8000 \( g \) for 6 min in a microhematocrit centrifuge and the packed cell volume measured as a percentage of the total column height. The tubes were then severed at the juncture of the supernatant and pellet. Twenty microliters of the supernatant was added to Instagel (Packard Instruments, Downers Grove, Ill.) scintillation fluid with 80 \( \mu l \) of 0.625 N KOH.

The excess supernate was blotted from the end of the cell pellet, and the pellet was pushed from the severed tube into a tube containing 0.4 ml distilled water. The pellet was thoroughly dispersed and allowed to freeze at \(-20^{\circ} \)C for 45 min. It was then thawed and digested with 100 \( \mu l \) of 2.5 N KOH. After 30-min incubation at 37\( ^{\circ} \)C, the digest was thoroughly mixed and 100 \( \mu l \)
added to Instagel in scintillation vials. The beta radioactivity was determined in a Packard beta spectrophotometer. The quenching of photon emission was equivalent in the supernatant and pellet samples. The lymphocyte volume was calculated from the cytocrit volume minus the volume of trapped supernate measured by 14C-
sucrose as follows:

\[
\text{Lymphocyte volume} = \frac{\text{Lymphocytocrit volume} - \text{Trapped volume}}{\text{Cell count} \times 10^8 \text{cu } \mu\text{m} / \mu\text{l}}
\]

Measurement of Lymphocyte Volume From Lymphocyte Wet Weight and Density

A lymphocyte suspension was prepared in TC-199 with 20% AB plasma at a concentration of 6 x 10^9 cells/ml. Three microcuries of 14C-sucrose containing 1.66 mmole unlabeled sucrose was added per ml of the cell suspension. One hundred and eighty microliters of this cell suspension was added to a 2.5-ml conical tube that was weighed on an analytical balance. The cells were sedimented at 900 g for 10 min and 50 \mu l of the supernate was added with 500 \mu l of 0.5 N KOH and 50 \mu l TC-199 to Instagel scintillation fluid for measurement of beta radioactivity. The insides of the tubes were dried with filter paper and the cell surfaces blotted. The tubes with the wet pellet were reweighed on the analytical balance. The cell pellet was homogenized with 0.4 ml of 2.5 \% KOH added. This digest was incubated at 37°C for 30 min and thoroughly dispersed. A 500-\mu l sample was added to Instagel for measurement of the beta radioactivity. The quenching of photon emission was identical in the supernate and pellet samples. Blank samples were prepared by adding a 14C-sucrose containing buffer to the cell pellet after sedimentation. This supernate was removed and the pellet treated as before. This blank served to assess the radioactivity contributed by medium not trapped within the pellet but on the pellet surface and on the tube walls. Further studies were performed to measure the proportion of the suprapellet medium that evaporated during the weighing process, since this evaporation resulted in radioactivity that did not represent water weight. The radioactivity that did not represent water weight was subtracted from the total 14C-sucrose radioactivity and accounted for about 19% of the total radioactivity. The lymphocyte volume was calculated from the weight of lymphocytes (total weight - supernate wet weight) and lymphocyte cell density of 1.06^{11} by the formula:

\[
\text{Lymphocyte volume} = \frac{\text{Cell pellet wet weight} \times \text{Cell density}}{\text{Cell count} \times 10^8 \text{cu } \mu\text{m} / \mu\text{l}}
\]

Measurement of Lymphocyte Volume By Electronic Particle Counting

Particle volumes were determined with a Coulter Model ZBI particle volume analyzer and Channelizer attachment. Particle volumes were derived from the particle size distribution generated on the Channelizer oscilloscope. The number (n) of accumulated particles was integrated under the curve, and the median channel calculated from this integration: median channel = channel encompassing n/2 particles + base channel threshold. This provided the best and most reproducible perspective of particle volume when compared to the mean or modal measurements. The Coulter Model ZBI was equipped with a 100 by 150 \mu m aperture and particles were aspirated at a flow rate of 2.5 ml/min. The base channel threshold was set at 6 and the upper threshold at 100. The current was set at 2 mA and the amplification at 1.

The Coulter particle volume analyzer operates with a constant electric current through the aperture. When a nonconducting particle enters the aperture, the voltage drop across the aperture must increase in order to maintain the constant current. The voltage pulse height, \Delta v, is related to the particle volume, V, by the equation:12

\[
\Delta v = \frac{\rho_2}{A^2} f_s V
\]

where \rho_2 is the specific resistivity of the electrolyte (suspending) medium, I is the electric current, A is the aperture cross-sectional area, and \( f_s \) is a shape and conductivity factor. For a given suspending medium and the instrument parameters \rho_2, A, and I,

\[
\Delta v = K f_s V
\]

where \( K \) is a constant. The shape and conductivity factor, \( f_s \), is a function of the particle shape and orientation in the aperture, the ratio of the resistivities of the suspending medium to the particle, and the particle to orifice diameters ratio. Under the usual operating conditions with blood cell suspensions, \( f_s \) is only a function of particle shape and orientation in the orifice; these factors are in turn dependent on the particle deformability. Since \( \Delta v \) is the signal used to measure cell volume, these equations show that this signal reflects the product \( f_s V \), which is called the "effective" or "electronic" volume of the particle, \( V_e \). In order to correctly evaluate \( K \) in equation 2 (calibrate the instrument) and to determine \( V \), the correct value of \( f_s \) must be determined.12 (Reference 12 is a good recent review that describes the principals of operation of electronic particle volume analyzers.)

The value of \( f_s \) for various shape particles has been calculated from theory and confirmed experimentally for some shapes. For rigid spheres, \( f_s \) has a theoretical and experimental value of 1.50.15,16 A deformable spherical particle will have a smaller \( f_s \) value, as it deforms into a prolate ellipsoid. A prolate ellipsoid with a large axial ratio, oriented in the aperture with its longer axis parallel to the direction of flow, will have \( f_s \approx 1.0.15,16 \) It has been observed that normal human erythrocytes deform as they flow into analyzer apertures so that they are approximately prolate ellipsoids with long axis orientation parallel to the flow.15 An experimental determination of \( f_s \) resulted in a value of 1.04 for erythrocytes. These \( f_s \) values illustrate the range of values encountered (1.1-1.5) when calibrating and using electronic particle volume analyzers.

RESULTS

Calibration of the Instrument for Measurement of Latex Spherules and Red Cell Volume

Calibration studies with the Coulter Model ZBI electronic counter and "Channelizer" particle volume analyzer were performed with 3 particles: (1) latex spherules (source with a true, mean volume of 113 cu \mu m—Particle Information Services, Los Altos, Calif.), (2) Coulter 4C Standard (Coulter Electronics, Hialeah, Fla.), and (3) normal human red cells. Each particle was studied in 3 media: (1) Coulter’s Isoton (330 mosmole/liter), (2) isotonic phosphate-buffered saline (PBS) (286 mosmole/liter), and (3) hypotonic PBS (250 mosmole/liter).
Latex spherules. Since latex spherules do not change their volume when placed in media of different tonicity and they are rigid, their effective or electronic volume \( V_e \) is represented by \( V_e = f_e V \). In this case, \( f_e \) is 1.5, \( V \) is 113 cu \( \mu \)m and thus, \( V_e \) is 170 cu \( \mu \)m. When these spherules were sized electrically, the data in Table 1 were obtained. Since the instrument does not record \( \Delta V \) values but rather sorts the individual \( \Delta V \) values into "channels," the channel number, \( n \), is proportional to \( \Delta V \), and equation 2 becomes:

\[
 n = K_2 f_e V = K_1 V_e
\]  
(3)

The last row in Table 1 shows the cu \( \mu \)m/channel (1/\( K_2 \)) calculated from the data and equation 3.

Coulter 4C Standard. The red cell standard in Coulter 4C Standard is said to have a volume of 83 cu \( \mu \)m, which may be \( V \), the true particle volume, or \( V_e \), the electronic volume. We used the calibration factors determined with the latex spherules to obtain \( V_e \) values in the three different suspending media (Table 2). A volume ratio can be calculated as \( V_e / 83 \) cu \( \mu \)m. The 4C Standard is designed for use with Coulter’s Isoton solution as the suspending medium (330 mosmole/liter). In this medium, the volume ratio of 0.97 is essentially unity, indicating that the reported volume of 83 cu \( \mu \)m is the electronic volume (with unknown \( f_e \) value) or else the particle has a true volume of 83 cu \( \mu \)m and a shape factor \( (f_e) \) of 1.0. The change in electronic volume as the suspending medium tonicity is changed reflects swelling of the 4C red cells.

Normal human red cells. The red cells from eight different healthy donors were added directly from a finger stick to the three suspending media and were sized individually with the electronic particle volume analyzer. The median channel and electronic volume, \( V_e \), (calculated with the \( K_2 \) values obtained with the latex spherules) are tabulated in Table 3. Since the true volume of a human red cell varies with suspending medium tonicity, the red cell volume in the three suspending media was calculated using the ideal osmotic swelling law.\(^4\) The law states that red cell volume is a linear function of the reciprocal of the suspending medium tonicity. The experimental red cell volume data of Evans and Fung\(^5\) were plotted according to this law and a linear relationship in the tonicity range of our suspending media was obtained. Red cell volumes were also calculated from the ideal osmotic swelling law using the same slope but a value of 87 cu \( \mu \)m for the isotonic cell volume.\(^4\) These calculated volumes are included in Table 3.

The experimental \( f_e \) values were calculated from \( V_e / V \), where \( V \), the true volume, was taken either as 87 cu \( \mu \)m at 286 mosmole/liter\(^6\) or from Evans and Fung's data.\(^5\) From these data, it is apparent that the shape factor for normal human red cells is between 0.99 and 1.1, and that its precise value is sensitive to what we take as the true erythrocyte volume. A value of \( f_e \) equal to 1.04 for normal red cells appears to be reasonable.\(^6\)

### Table 1. Sizing of Latex Spherules

<table>
<thead>
<tr>
<th>Suspending Medium Osmolarity</th>
<th>330</th>
<th>286</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median channel (10)</td>
<td>23.8±0.1</td>
<td>23.7±0.1</td>
<td>24.3±0.2</td>
</tr>
<tr>
<td>cu ( \mu )m/channel</td>
<td>7.12±0.05</td>
<td>7.15±0.05</td>
<td>6.98±0.05</td>
</tr>
</tbody>
</table>

The median channel (mean ± SE) and cu \( \mu \)m/channel are indicated for 1/amp = 1 on the Coulter electronic particle volume analyzer. The number of measurements is shown in parenthesis. The buffer osmolarity is expressed as mosmole/liter.

### Table 2. Sizing of 4C Standard

<table>
<thead>
<tr>
<th>Suspending Medium Osmolarity</th>
<th>330</th>
<th>286</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median channel (6)</td>
<td>11.3±0.4</td>
<td>12.2±0.4</td>
<td>13.4±0.4</td>
</tr>
<tr>
<td>Electronic volume, cu ( \mu )m</td>
<td>80.5±2.8</td>
<td>87.2±2.8</td>
<td>93.5±2.8</td>
</tr>
<tr>
<td>Volume ratio ( V_e / 83 ) cu ( \mu )m</td>
<td>0.97</td>
<td>1.05</td>
<td>1.13</td>
</tr>
</tbody>
</table>

The median channel and electronic (effective) volume (mean ± SE) are indicated for 1/amp = 1 on the Coulter electronic particle volume analyzer. The number of measurements is shown in parenthesis. The buffer osmolarity is expressed as mosmole/liter.

### Table 3. Sizing of Human Red Blood Cells

<table>
<thead>
<tr>
<th>Suspending Medium Osmolarity</th>
<th>330</th>
<th>286</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median channel (8)</td>
<td>12.3±0.09</td>
<td>13.5±0.03</td>
<td>14.8±0.2</td>
</tr>
<tr>
<td>Electronic red cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume cu ( \mu )m</td>
<td>87.6±0.6</td>
<td>96.5±0.2</td>
<td>103.3±1.4</td>
</tr>
<tr>
<td>True volumes, cu ( \mu )m, based on</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evans and Fung data</td>
<td>89.3</td>
<td>97.4</td>
<td>105.4</td>
</tr>
<tr>
<td>87 cu ( \mu )m at 286</td>
<td>79</td>
<td>87</td>
<td>95</td>
</tr>
<tr>
<td>Shape factors, ( f_e ), based on true volumes from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evans and Fung data</td>
<td>0.98</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>87 cu ( \mu )m at 286</td>
<td>1.11</td>
<td>1.11</td>
<td>1.09</td>
</tr>
</tbody>
</table>

The median channel and electronic (effective) volume (mean ± SE) are indicated for 1/amp = 1 on the Coulter electronic particle volume analyzer. The number of red cell populations studied is shown in parenthesis. The buffer osmolarity is expressed as mosmole/liter.
Measurement of Lymphocyte Volume

The data presented above indicate that a particle’s true volume, $V$, can be calculated from its electronic or effective volume, $V_e$, only if the shape factor, $f_e$, is known. The volume of human blood lymphocytes can be determined only if the particle volume analyzer is calibrated with a particle having the same shape factor or if the shape factors for the calibrating particle and the lymphocyte are known. Except for a few special cases (such as latex spherules), the shape factor, $f_e$, must be determined from experimental measurements of $V$ and $V_e$.

We have measured the true lymphocyte volume, $V$, by two independent methods at 286 mosmole/liter. These included measurement of the packed cell volume by a lymphocytocrit method and measurement of the net lymphocyte wet weight. The lymphocyte volume was determined from the lymphocytocrit in 5 cell populations (Table 4). The cell volume was calculated from the lymphocytocrit volume minus the trapped volume (see Materials and Methods). The percentage of trapped volume in these studies averaged $19 \pm 0.4$. The mean lymphocyte volume in these studies was $218 \pm 4 \text{ cu } \mu\text{m}$. Alternatively, lymphocyte volume was determined from the cell pellet weight and lymphocyte density (Table 5). The cell pellet weight was calculated from the total weight minus the supernate wet weight. The percentage of supernate wet weight was $17 \pm 2.6$, and the mean lymphocyte volume by weight/density was $203 \pm 3 \text{ cu } \mu\text{m}$. The mean volume from these two methods, $210 \text{ cu } \mu\text{m}$, is compared to the electrical volume, $289 \pm 5 \text{ cu } \mu\text{m}$ (286 mosmole/liter) measured in the same lymphocyte populations using latex spherules as the standard particle. These measurements are significantly different ($p < 0.001$). Since the shape factor, $f_e$, equals $V_e/V$, $f_e$ for lymphocytes is the ratio $289/210$ or 1.38.

In practical terms, the knowledge of $f_e$ permits the simplest method for the electronic measurement of lymphocyte volume; that is, the particle volume analyzer is calibrated with latex spherules in isotonic buffer (286 mosmole/liter) using their electronic volume, $V_e$. The $V_e$ for lymphocytes is determined directly in the same buffer. The true lymphocyte volume, $V$, is calculated from $V_e/f_e$. The instrument can be calibrated also by using the electronic volume of Coulter 4C Standard or normal human red cells with the same results. The median channels of lymphocytes sized in Isoton (330 mosmole/liter) PBS (286 mosmole/liter) and PBS (250 mosmole/liter) are shown in Table 6. When the change in median channel is taken as a change in lymphocyte volume and the shape factor of 1.38 is used to determine the true lymphocyte volume at each osmolarity, an 8% reduction from 210 to 194 cu \mu m is observed in Isoton. This may not be an exact calculation, since the shape factor was determined at physiologic osmolarity, 286.
mosmole/liter, and $f_e$ may be slightly altered in Isoton (330 mosmole/liter). The reduction results from the use of osmotically inactive particles, latex spherules, to standardize the instrument for measurement of osmotically responsive lymphocytes.

**DISCUSSION**

The accuracy of volume determinations on an electronic particle volume analyzer is dependent on several factors. These include electronic features of the instrument, the area and length of the aperture, and the flow characteristics and deformability of the particle used as a standard and the particle to be sized. We have evaluated the effects of (1) the deformability of particles by calculating the shape factor, $f_e$, and (2) the buffer osmolarity on the measurement of human blood lymphocyte volume. These findings apply, however, to the measurement of any cell’s volume.

The precise determination of cell volume is of particular importance in transport and metabolic experiments in which cell volume contributes to the estimation of the distribution space of internal contents. Cell volume is a rapid, easy, accurate, and reproducible basis for calculating the cell water when compared to more tedious methods. Cell water or volume can serve as the denominator for calculation of the concentration or content of internal substance. The influence of cell volume on the apparent concentration of internal solutes is exemplified by its effect on monovalent cation concentration. The cell potassium concentration has been reported between ~110 and 180 mmole/liter cell water in mammalian cells. Although some variation may be due to difficulties in measuring the cations themselves, the influence of cell volume on cation concentration may be marked, as shown in Fig. 1. In this analysis, the lymphocyte K content of 25 fmole/cell and Na content of 3 fmole/cell have been used to calculate the lymphocyte monovalent cation concentration at lymphocyte volumes from 175 to 300 cu µm. At a lymphocyte volume of 175 cu µm, the Na and K concentrations are 22 and 183 mmole/liter cell water, respectively. In contrast, at a volume of 300 cu µm, the Na and K concentrations are 12 and 108 mmole/liter cell water. Thus, differences in the apparent cell volume resulting from measurement errors can account for a difference in cell concentration of a constituent of up to 80%.

The reported values for lymphocyte volume range from 180 to 280 cu µm. Such variation would be predicted if the volume measurements were made without regard for the deformability characteristics of the cell to be measured and the standard particle used to calibrate the instrument. For example, if the shape factor is ignored and the instrument is calibrated with latex spherules, the lymphocyte electronic volume in isoosmotic medium is 193 cu µm. In contrast, the lymphocyte electronic volume is 276 cu µm if the instrument is calibrated with the Coulter 4C Standard. This range of volumes corresponds to the reported values for lymphocyte volumes; moreover, the lowest values were measured when latex spherules were used to calibrate the electronic particle size and the highest values when red blood cells were used for calibration. In these studies, shape factor was ignored and true volume was not measured.

Two groups of investigators have considered the shape factor in the sizing of human lymphocytes. One group concluded that the lymphocyte behaved as a rigid sphere on the basis of direct microscopic observations of cells flowing through the sizing aperture and concluded that lymphocyte volume was 180 cu µm.19
They did not provide an independent measurement of lymphocyte volume, however, to verify an $f_c$ of 1.5. The other group used data calculated from measurements of cell diameter as the independent volume determination. They considered the lymphocyte shape factor to be that of a rigid sphere ($f_c = 1.5$) and calculated a lymphocyte volume of 195 cu μm.

By using two independent methods to measure lymphocyte volume, we have arrived at a shape factor value of 1.38. This results in a lymphocyte volume slightly greater than that reported by Ben-Sasson and coworkers. The difference between a shape factor of 1.38 and 1.5 is small, and the important insight is that lymphocyte volume is about 200 cu μm rather than the higher values of 250–290 cu μm.

An additional but less critical problem is introduced in the measurement of cell volume if the commercial diluting solution, Isoton, is used to suspend the particles for measurement and an osmotically inactive particle is used as a standard. Isoton, despite its name, has an osmolarity of 330 mosmole/liter. The hyperosmolarity of Isoton results in about an 8% decrease of lymphocyte volume measured in isotonic medium with latex spherules as the standard particle.

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