The Separation of Normal Human Leukocytes by Density and Classification by Size

By Robert M. Zucker and Benedict Cassen

The separation of morphologically distinct cells by differences in their specific gravity has been demonstrated using density gradient centrifugation in several support media. From bovine serum albumin density gradient centrifugation experiments, morphologically similar lymphocytes have been shown to consist of several closely spaced, discontinuous, discrete populations. In addition, erythrocytes have been shown to become smaller and denser while aging in circulating blood.

Physical differences between morphologically similar cells (i.e., density and size) may indicate different functional properties of the cells. A Coulter counter, in conjunction with an electronic multichannel pulse-height analyzer system, can detect small differences in cell volumes which may be overlooked microscopically.

The electronic volume and density of leukocytes in bovine serum albumin density gradients is presented in this communication.

Materials and Methods

Preparation of Bovine Serum Albumin

The bovine serum albumin (BSA, fraction V, Armour and Co., Chicago), was prepared by a modified Leif and Vinograd technic. Four hundred thirty-two Gm. of BSA powder was layered on 800 Gm. deionized water containing 200 Gm. Amberlite MB-3 resin in a slowly rotating (1 r.p.m.) polyethylene container, inclined at a 30° angle. The duration of rotation was approximately 24 hrs. at 6 C. To permit further deionization, the solution was decanted into a smaller vessel containing 100 Gm. Amberlite MB-3 resin and slowly rotated for 2 hrs. The BSA liquid was then decanted into a 47 mm. Gelman pressure filtration funnel. This system contained a 5 and 1.2 μ filter to remove resin particles. Thereafter, the BSA was refiltered in the Gelman filtration funnel with a .22 μ filter. The BSA was diluted with deionized, distilled water to 33 per cent by weight (Refractive Index 1.3968, density, ρ = 1.1 Gm./cm.3 at 4C). The following salts were added per 100 Gm. of 33 per cent BSA solution: 0.3350 Gm. Na₂CO₃, 0.02668 Gm. KCl, 0.01334 Gm. MgCl₂, 0.0093 Gm. Na₂HPO₄, 0.067 Gm. glucose, 0.1874 Gm. NaCl, and 0.67 ml. antibiotic-antimycotic mixture (streptomycin 10,000 μg./ml., penicillin 10,000 units/ml., fungizone 25 μg./ml. Grand Island Biological Co.). To prepare isotonic BSA solutions the Na+ ion concentration was regulated to 144 meq units. The pH was determined to be between 6.5 and 7.0 using a pH meter with a sleeve junction reference electrode. To form light BSA (ρ = 1.05) the 33 per cent heavy BSA was diluted with Eagle's balanced salt solution with 1.53 Gm. NaCl.

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substituted for 2.2 Gm. NaHCO₃ (Na⁺ ion equivalent). All BSA solutions were stored at −70 C.

Preparation of the Gradient

Linear gradients of BSA were prepared with a system similar to that previously described by Leif. Into a mixing chamber containing a teflon stirrer, 4.25 ml. of light BSA, \( \rho = 1.05 \) was pipetted. Heavy BSA, \( \rho = 1.1 \), was then pumped into the mixing chamber containing light BSA at a rate of 0.15 ml./min. The BSA was continuously stirred at 150 r.p.m. and pumped out of the chamber at a rate of 0.30 ml./min. via a glass tee connected on one side to the chamber and on the other side to two identical pumping tubes. Therefore, the peristaltic pump regulates the flow out of the mixing chamber to be twice that of the flow of heavy BSA into the mixing chamber. Between \( 10^7 \) and \( 10^8 \) blood cells in .1 cc. to .5 cc. Hank’s balanced salt solution (HBSS) were layered with a micropipet on top of the gradients. All work was done at 6 C.

Specially designed polycarbonate centrifuge tubes were constructed to fit the HB-4 Sorvall rotor (Fig. 1). These tubes contained two holes which were 0.50 inch and 0.040 inches in diameter. These holes were parallel to the longitudinal axis of the tube and were approximately ¼ its length. The two holes were connected by a small .040 inch channel parallel to the horizontal axis. This .040 inch aperture permitted the formation and fractionation of the density gradient. A small 1/32 inch lengthwise groove on the outside of the tube facilitated removal of the tube from the centrifuge bucket. Five to 10 drops of water were placed in the centrifuge bucket prior to centrifugation to prevent breakage of the polycarbonate tube.

Fig. 1.—Polycarbonate centrifuge tube specially made to fit into the Sorvall HB-4 rotor. The centrifuge cap enabled fractions to be withdrawn from top of centrifuge tube.
Centrifugation
An RC-2B Sorvall centrifuge with an HIB-4 Sorvall swinging bucket rotor was used. The centrifuge was accelerated and decelerated slowly with a specially made acceleration trimmer (Sorvall, Norwalk, Conn.). Centrifugation continued for one-half hour at 5000 r.p.m. (4080 gm.)

Fractionation of the Gradient
Two methods of fractionation were used because of variables noted in this study (Fig. 2). Initially, downward fractionation was used. Equal volume fractions of BSA were removed from the bottom of the centrifuge tube with the peristaltic pump. The pump was automatically stopped by a calibrated system.

Secondarily, upward fractionation was used. BSA was withdrawn from the bottom of the centrifuge tube as in downward fractionation, until the pumping tube was filled. The pumping tube was then put into Fluorocarbon 75, \((\rho = 1.4 \text{ Gm./cm.}^3 3M \text{ Co., St. Paul, Minnesota})\) and the peristaltic pump was reversed in direction. Fluorocarbon 75 was pumped into the bottom of the centrifuge tube which pushed BSA out of the centrifuge tube through a specially adapted conical centrifuge cap (Fig. 1). An electronic size distribution, a refractive index measurement, and a differential count of 100 leukocytes was made on each fraction.

Electronic Multichannel Analyzer System
The equipment used in the electronic size distribution and counting measurements included: a Coulter transducer with a 90 \(\mu\) long and 90 \(\mu\) diameter jeweled orifice; a RIDL preamplifier model 31-25; a RIDL pulse shaper model 52-58; a RIDL amplifier model 30-30; a Packard multichannel pulse-height analyzer model 15; and a Monroe digital print-

![Diagram of fractionation procedures](image-url)
out model MC 10-40. The multichannel analyzer system was calibrated with 3.49 µ poly-

styrene spheres, erythrocytes of man and mouse and a pulse generator. All HBSS to be used

for counting was filtered with a .22 µ millipore filter.

The concentration of cells in each density fraction was determined. In addition, a size

distribution was made on 20,000 cells in the fraction.

**Density Measurements**

Refractive index measurements were made with a Zeiss-Abbe refractometer and re-

lated to the densities of BSA fractions by the equation \( \rho = 1.543 N_{25} - 1.0553 \). The presence

of \( 10^7 \) cells per cc. in BSA does not significantly alter the refractive index measurements.

**Cytology**

Cells were spun onto slides at 750 r.p.m. for 10 minutes with the Shannon cytocentri-

fuge. A small amount of BSA in HBSS was necessary to prevent excessive cell breakage
during centrifugation. The slides were stained with Giemsa stain. Differential counts of

100 leukocytes were made on each slide.

**Preparation of Blood**

Ten ml. of human blood was obtained by venous puncture using sterile plastic disposable

syringes. Ten units of heparin per ml. of blood was used as an anticoagulant. The syringes

were placed in a vertical position for approximately 1½ hours. Supernate fluid (2-5 cc.)

was collected by attaching a butterfly-23 infusion set (Abbott, Chicago, Ill.), without the

needle, to the syringe and pushing the plunger up. This method collects almost all the super-
nate that is formed during sedimentation. The supernate was diluted with HBSS to the 10

cc. mark in a disposable plastic centrifuge tube. It was centrifuged for 10 minutes at 200 g.
The resulting supernate fluid was removed, the pellet was dispersed, and 5 cc. of HBSS was
added for further washing. The tube was next spun for 10 minutes at 200 g., and the

supernate was removed from the pellet. The pellet was then diluted and dispersed with

1/2 cc. of HBSS for layering on the gradient.

**Erythrocyte Density Check**

In order to check the BSA lot and BSA tonicity, two drops of blood from a finger punch

were put in .3 ml. Alsevers anticoagulant solution (Delco Labs, Inglewood, Calif.). The

mixture was then diluted with .3 ml HBSS and an approximate concentration of \( 5 \times 10^7 \)
cells, determined by the Coulter counter, was layered on top of the gradient. The ery-

throcyte mean density was then calculated and compared to the average value reported

by Leif and Vinograd.

**Determination of the Mean Density of Cell Populations**

The electronic size distribution spectrums were divided into three regions corresponding
to the three major peaks. These regions correlated with the three major cell populations:
erthrocytes, lymphocytes, and granulocytes. The percentage of a cell population was cal-
culated by the summation of counts from the digital print-out tape in the corresponding
region. To find the quantity of a specific cell type in each fraction, these percentages
were multiplied by the total quantity of cells determined from the Coulter counter. Fra-
ctions containing a quantity of specific type cells less than 10 per cent of the modal quantity
were considered insignificant and excluded. In addition, fractions were excluded if differ-
ential counts did not correlate with electronic size data. To determine the mean popula-
density, the number of cells in each fraction was multiplied by the fraction’s density. The
results were added and then divided by the total number of specific type cells in the
fractions.

\[
\rho_{\text{avg}} = \frac{\sum N_i \rho_i}{\sum N_i},
\]

where \( i = \text{fraction number}, N = \text{specific type cells in fraction}, \) and \( \rho = \text{density}. \)
RESULTS

The size distribution of cells layered on top of the gradient and the size distribution of the resulting fractions after centrifugation are represented in Figure 3. The erythrocyte, lymphocyte and granulocyte population were correlated to the three distinct peaks in Figure 3 by microscopic differential counts. The first size peak, observed in Figure 3A, 3G and 3H, coincides with size dis-
tributions of erythrocytes from whole blood. Differential counts have shown the second size peak, Figure 3B-3E, to be lymphocytes and the third size peak, Figure 3E-3H, to be granulocytes. Since it is known that erythrocytes have a mean equivalent volume of $90 \mu^3$, they were used as a standard for the calibration of the electronic sizing equipment. Small and medium lymphocytes have a modal equivalent volume varying between $142 \mu^3$ and $265 \mu^3$, while granulocytes have a modal equivalent volume between $35 \mu^3$ and $500 \mu^3$. Monocytes have a modal equivalent volume of approximately $600 \mu^3$. The electronic volumes of all leukocyte populations were smaller than those determined microscopically. Our reported volumes of lymphocytes, granulocytes, and monocytes were different from the results reported by Van Dilla, Fulwyler and Boone. These investigators used an electronic cell separator, and saponin which can alter leukocyte volume distributions. We believe calibration methods, cell preparations, and electronic sizing equipment may explain this discrepancy.

The observed distribution of a population of cells in a gradient is partially dependent on the direction of fractionation. The skewness of the distributions is to the right in downward fractionation, and to the left in upward fractionation. In addition, the mode of a population is less dense than the mean in upward fractionation, while the mode is more dense than the mean in downward fractionation. The order of cell populations appearing in downward fractionation is erythrocytes, granulocytes, lymphocytes, and monocytes, while upward fractionation reverses the order. End fractions in both methods may be contaminated by partial mixing with cells adhering to the centrifuge tube walls. Some mixing between neighboring fractions may occur due to the parabolic flow of a liquid through a small orifice during fractionation. The first population removed from the gradient is the least contaminated, and in each successive population the resolution is diminished. Fractionation procedures produce tails of the population distributions, i.e., erythrocytes and granulocytes are seen in the lymphocyte range in downward fractionation but not in significant quantities in upward fractionation. Upward fractionation should, therefore, be used to study lymphocytes or monocytes while downward fractionation is useful in studying erythrocytes and granulocytes. In most experiments the modes of the different populations are similar. However, variations in distributions cause the means to vary. The densities of blood cells combining the results of upward and downward fractionation are reported in Table 1.

The distribution of cells in a representative upward fractionation experiment is indicated in Figure 4. The populations of erythrocytes and granulocytes have been observed to overlap as much as 90 per cent and as little as 15 per cent. In the granulocyte population, basophils are seen in the least dense region, eosinophils are seen in the denser region, and neutrophils are seen throughout the entire granulocyte distribution. In some experiments, the light density regions of the granulocyte population consists predominately of band neutrophils, while the dense region consists of segmented neutrophils. The modal volume of the granulocyte population was observed to decrease between the lightest and densest granulocyte regions. This correlation of size versus density was seen in the lymphocyte, granulocyte and erythrocyte populations.
Fig. 4.—Population distribution of blood cells in an upward fractionated density gradient.

Table 2 represents a typical experiment where the lymphocyte modal volume decreases as the density of the gradient fractions increases. The modal cell volume varies from $264 \mu^3$ to $184 \mu^3$ as the density varies from 1.0547 Gm./cm.$^3$ to 1.0644 Gm./cm.$^3$. This inverse relationship between lymphocyte size and density is observed both microscopically and electronically. Differential counts reveal that monocytes, which appear in the same region as the large and medium lymphocytes, are the least dense population.

Leif and Vinograd have demonstrated that the erythrocyte density is dependent upon BSA tonicity. The proper salt concentration in the BSA lot is evaluated by layering approximately $5 \times 10^7$ erythrocytes on a BSA gradient ($\rho = 1.1- = 1.06$). The mean density of erythrocytes of one individual in 19 experiments was $1.0811 \pm .0011$.

Table 1.—Density of Blood Cells

<table>
<thead>
<tr>
<th></th>
<th>Erythrocytes</th>
<th>Granulocytes</th>
<th>Lymphocytes</th>
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<tr>
<td>Density of Hematologically Normal Blood</td>
<td>(13 trials—3 Upward Fractionation)</td>
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<td></td>
</tr>
<tr>
<td>Mean of Density</td>
<td>1.0800</td>
<td>1.0753</td>
<td>1.0618</td>
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<tr>
<td>Standard Deviation</td>
<td>.0025</td>
<td>.0047</td>
<td>.0023</td>
</tr>
<tr>
<td>Density of Hematologically Normal Blood</td>
<td>(16 trials—8 Upward Fractionation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean of Density</td>
<td>1.0793</td>
<td>1.0747</td>
<td>1.0632</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>.0031</td>
<td>.0054</td>
<td>.0025</td>
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Table 2.—Lymphocyte Volume vs. Density

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Density Gm./cm.³</th>
<th>Volume µ³</th>
<th>% of Lymphocyte Population</th>
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<tr>
<td>2</td>
<td>1.0547</td>
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<td>3</td>
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<td>0.18</td>
</tr>
<tr>
<td>5</td>
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<td>0.15</td>
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<tr>
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<td>191</td>
<td>0.13</td>
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<tr>
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</tr>
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<td>9</td>
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<td>0.06</td>
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<tr>
<td>10</td>
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<td>191</td>
<td>0.04</td>
</tr>
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</table>

Discussion

Shortman et al.⁴ reported that rat lymphocytes form discrete bands in BSA gradients. In our experiments done on the supernatant of human blood, a continuous distribution (Fig. 4) of erythrocytes, lymphocytes, and granulocytes was observed. However, each cell population consists of different sized cells which can be correlated to different densities. The smaller cells are usually more dense.

Pertoft et al.³ reported distributions similar to ours, using colloidal silica-polyvinylpyrrolidone (Ludox-PVP) gradients and differential counts to determine the distributions of various cells. The following densities were reported using Ludox-PVP: erythrocytes 1.09–1.1 Gm./cm.³; neutrophils 1.082 Gm./cm.³; eosinophils 1.07 Gm./cm.³; small lymphocyte 1.063 Gm./cm.³; medium and large lymphocytes 1.058 Gm./cm.³; and monocytes 1.055 Gm./cm.³. In contrast, our experiments used BSA, electronic counting and sizing, and differential counts. The mean densities of erythrocytes and neutrophils were found to be 1.080 Gm./cm.³ and 1.075 Gm./cm., respectively. The overlap between granulocyte and erythrocyte populations was greater in BSA. We found eosinophils were located throughout the entire granulocyte population and have a higher mean density than the granulocyte mean density. Medium and small lymphocytes form a continuous size distribution in BSA with a mean occurring at 1.062 Gm./cm.³. The monocytes are denser in BSA, overlapping the medium lymphocyte range.

Noble et al.¹ ² reported distributions similar to ours, using human and chicken blood with Ficoll, an inert, high molecular weight polysucrose, as the gradient medium. Ficoll agglutinates cells, obscuring an accurate size distribution of the cell populations. These size distributions were essential to our experiments to correlate cell types, size and density. Ficoll also has a high viscosity at a density of 1.1, making it difficult to use in our peristaltic pump. The five distinct bands described by Noble were, in order of increasing density: platelets; lymphocytes; light erythrocytes; monocytes; granulocytes and erythrocytes. The monocyte band in Ficoll is denser than that found in Ludox-PVP or in BSA. The erythrocyte band exists as a continuous distribution in BSA and no evidence of a distinct light erythrocyte band was found. It is
suggested that the type of media, tonicity, and pH are all important variables in cell separation.

With the normal blood cell distributions known, an investigation is now in progress to determine the density and size of different lymphocytes in chronic lymphocytic leukemia.

**Summary**

Leukocytes of hematologically normal human blood have been studied by separating them in bovine serum albumin gradients. The blood cells of the density fractions have been characterized by electronic volume sizing and microscope differential counts. The electronic volumes of different leukocyte populations have been determined from density gradient experiments. Lymphocytes of various sizes can be separated by differences in density. The small lymphocyte population is the most dense while the large population is the least dense. The density of blood cell populations in BSA in order of increasing density were: monocytes, lymphocytes, granulocytes and erythrocytes.

**SUMMARIO IN INTERLINGUA**

Leucocytos e hematologicamente normal sanguine human esseva studiate per le procedimento de lor separation in gradientes de albumina de sero bovin. Le cellulas sanguine del fractiones de densitate esseva characterisate per determinaciones electronic de volumine e microscopic contation differential. Le volumes electronic del differente populations leucocytic esseva determinate a base de experimentos a gradiente de densitate. Lymphocytes de differente dimensiones pote esser separate a base de differentias in densitate. Le population de micre lymphocytos es le plus dense, durante que le population grande es le minus dense. Le densitates del populationes de cellulas sanguine in albumina de sero bovin esseva in ordine ascendente illos de monocytes, lymphocytos, granulocytos, e erythrocytos.

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**REFERENCES**

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