Centrifugal Subfractionation of Polymorphonuclear Leukocytes, Lymphocytes and Erythrocytes

By Bernard W. Agranoff, Bert L. Vallee and David F. Waugh

A method has been described by which leukocytes may be separated from erythrocytes. The technic takes advantage of the difference in density between the two cell classes. Fractionation is accomplished by sedimenting the population of cells through two media, plasma and albumin, having a differential of density intermediate between the cell classes at their juncture. The principle has been applied to the separation of neoplastic cells from other cell types. Since the technic described used a single density interface, the separation could not be extended to the subfractionation of leukocytes simultaneously with their separation from erythrocytes. The procedure to be presented here is a step in this direction since it permits the separation of the formed blood elements into polymorphonuclear leukocytes, lymphocytes, and erythrocytes.

Methods and Results

A sample of blood is layered over two isosmotic albumin solutions of different density (see Fig. 1a), which are in turn layered over each other.

Armour fraction V lot 678B2, 35 per cent bovine albumin reworked to lower the salt content, was found to have a density of 1.092 Gm./cu.cm., a pH of 6.9, and to be hypotonic to blood. The addition of 0.907 mg. per ml. sodium bicarbonate and 2.57 mg. per ml. sodium chloride produced an albumin solution having a density of 1.094 and a stable pH of 7.4 after standing two weeks. The solution was isosmotic with blood. Since a pH of 7.4 was desirable, isosmotic sodium chloride was used for all further adjustments of density. Each adjusted albumin was kept in a screw-cap bottle under refrigeration, the pH remaining constant two months.

The quantities of salts necessary to produce an isosmotic albumin stock solution were determined by measuring changes in red cell volume when various amounts of salts were added to the original albumin. The volume of red cells was determined by hematocrit at 2000g for 30 minutes, first on whole blood and then after resuspending the same cells twice in albumin, the erythrocytes thereby serving as an osmometer. The Barger gaseous membrane technic was found not to be applicable for this purpose.

Different densities were produced by diluting the stock albumin of density 1.094 with isosmotic physiologic saline of measured density. The density of the particular dilution was calculated from

\[ D = \frac{D_a V_a + D_s V_b}{V_a + V_b} \]

de where \( D \) = the density desired, \( D_a \) = the density of the albumin stock solution, \( D_s \) = the density of the physiologic saline, \( V_a \) = volume of stock solution added, and \( V_b \) = volume of saline which must be added. Possible small differences between the total volume and \( V_a + V_b \) were neglected.

Wintrobe tubes (3.0 mm. I.D.) are filled to the 20 mm. mark (blue scale) with albumin solution II (density = 1.094) using dry polyethylene tubing attached to a syringe. Then
The initial distribution in the Wintrobe tube. The blood sample is indicated as solid black. The interfaces are blood (plasma) and albumin I designated A and albumin I and albumin II designated B. After centrifugation the red blood cells (RBC) have passed through all layers. Interfaces A and B are thus displaced centripetally. For details see text.

Albumin solution I (density = 1.065 to 1.085) is carefully layered over solution II to the 40 mm. mark. That little mixing has occurred is indicated by the sharp interface observed. This interface, designated B, is made visible by the difference in refractive indices of the two solutions. Oxalated capillary blood* is layered over solution I to the 50 mm. mark. The interface between blood and solution I is designated A in figure 1. The volume of whole blood is \( \pi (1.5 \text{ mm.})^2 10 = 71 \text{ cu. mm.} \). The filled tube is placed in an 8BV-1 International centrifuge with the base of the tube 17.1 cm. from the center of rotation. The tube is spun at 500 rpm for 10 minutes at 24°C., then 3000 rpm for an additional 20 minutes. The initial slow centrifugation is necessary for best results.

After centrifugation the tube appears as illustrated in figure 1b with the leukocytes and platelets forming distinct layers at the interfaces between plasma and albumin solution I (A) and between albumin solution I and albumin solution II (B).

The interface regions A and B are withdrawn through a pipet constructed by attaching a length of polyethylene tubing to a syringe. The polyethylene tube is sealed at the end and perforated with two small lateral openings through which the leukocytes are aspirated. Following aspiration of the layer near A, the albumin solution is withdrawn to just above the next interface and discarded. The interface region B thus revealed is then aspirated. The interface specimens are immediately smeared on cover slips and stained with Wright's stain.

Five normal bloods were examined with albumin solution I adjusted to a density of 1.072. In each case 90 to 96 per cent lymphocytes were found at interface A (fig. 2a) and 93 to 95 per cent polymorphonuclear leukocytes at interface B (fig. 2b). Platelets and occasional erythrocytes appeared in both layers, erythrocytes being more common near inter-

* A potassium-ammonium oxalate mixture was used to minimize cell volume changes.
After centrifugation, 90 to 96 per cent lymphocytes are found at interface A (a) as indicated above while 93 to 95 per cent polymorphonuclear leukocytes are found at interface B (b).

Monocytes appeared with the lymphocytes. When found, eosinophils appeared in either layer but not both.

When albumin solution 1 was less dense, e.g., 1.065, fractions containing as high as 98 per cent lymphocytes were obtained at A, or, when albumin solution 1 was 1.083, over 95 per cent polymorphonuclear leukocytes were found at B; in each instance at the sacrifice of purity in the other layer. For example in the first case, some of the lymphocytes appear at B. Aggregates of cells within a layer were common but the aggregates consisted of one type of cell only and were characteristic of the layer.
In several technics the densities of visible particles or droplets, inert with respect to the materials of the suspension medium, are determined by allowing them to seek, under the influence of gravity, their appropriate levels in a gradually changing density gradient. The density gradient is established either by diffusion or by layering solutions of different density over each other, mixing slightly, and allowing gravity and diffusion to smooth out the boundaries thus produced.

The materials used in the present technic cannot be suspended in the inert media (bromobenzene-xylene mixtures) often used, nor can they be observed easily in situ in the tube since fixation and staining are required to reveal type difference. Thus, the fractionated cells must be removed and stained smears examined microscopically. Since small amounts of blood are employed in each run, it seemed desirable that the technic should concentrate as well as fractionate; the former being accomplished most easily by using a series of sharp density interfaces. As detailed under methods this is accomplished by carefully inserting serially small volumes of albumin solution of different density into a capillary tube. In theory, one should be able to fractionate a cell population into subgroups having small density differences, the limit being set by the number of interfaces which can be introduced in a given tube. In practice, however, the nature of the starting material (blood) introduces an element of mixing due to the mass transport of plasma with groups of red cells. This factor and others to be discussed below, have limited the present method to the use of only two density interfaces.

The fractionation of cells is attended by two serious complications: first, the gravitational field producing separations must be greater than unity and, therefore, established by centrifugation; and second, water exchange between cells and medium must be minimized by maintaining all layers isosmotic with blood. The technic for accomplishing the latter has been described under Methods. Maximum efficiency of separation is accomplished when the axis of the tube is in the direction of the gravitational field (i.e. an angle centrifuge cannot be used). Since laboratory centrifuges generally rotate around a vertical axis, one has the choice of loading either with the tube axis permanently horizontal or with the tube axis initially vertical, the centrifugal field causing it to swing to a horizontal position. In the former case density differences will produce flow and therefore mixing in the tubes while the rotor is stationary; while in the latter case, when the tubes swing out (and in) during acceleration (and deceleration), swirling will tend to mix the contents. Of the two alternatives, the latter has been chosen.

According to Poiseuille's equation the rate of flow of a liquid through a capillary varies directly with the fourth power of the radius and inversely with the capillary length other factors being equal. Stabilization against mixing due to swirling should therefore be obtained when the radius of the tube is made as small as possible while the length of each column of a given density is made as long as possible.

If each cell sediments as an independent unit, the full centrifugal field could theoretically be applied at the start of fractionation. Experimentally it has been
found necessary to apply an initial centrifugation of low gravitational field (varying from 34 to 48g through the fluid in the Wintrobe tube) for 10 minutes before applying the final field (varying from 1215 to 1719g) for 20 minutes. If the final field is applied at the start, relatively large groups of red cells with their accompanying plasma are pulled through albumin solutions I (mainly) and II. Not only does considerable mixing take place but the leukocytes also are carried along with the red cells to the bottom of the tube from whence they will not ascend. The slow initial centrifugation evidently produces a preliminary separation of red cells, leukocytes, and plasma which (1) allows the formed elements to sediment either as units or as the “species pure” types noted previously, and (2) prevents excessive mixing by holding the separated plasma above layer I.

As each cell type reaches an interface of equal or higher density, the effective mass is reduced to zero, and the cell is arrested. Actually, if the maximal centrifugal field is allowed to continue for over an hour instead of 20 minutes, all of the white cells will be found resting over the red cell mass below albumin layer II (density 1.094), despite their initially lower density. This may be due to an interaction between cells and albumin. However, it seems more likely that the pressure produced by forcing a cell against a layer of higher density causes water to leave the cell with a concomitant increase in density and subsequent sedimentation. It has been found that lower centrifugal fields (i.e. 135 to 191g produced by centrifuging at 1000 rpm) will not produce the same effect in 110 minutes. Thus no simple reciprocity exists between gravitational field and time of centrifugation since changes in the composition of the cells may occur.

A number of combinations of tube diameters, differentials of density, lengths of albumin columns, and blood volumes have been examined. Tubes of less than 3 mm. internal diameter do not give adequate yields of samples on aspiration, a finding which led to the adoption of the Wintrobe tube. Density differences less than 0.010 were not adequate for good separation. The introduction of a third albumin column into the Wintrobe tube is possible. The third column was not added since a subfractionation of leukocytes into two types was the immediate objective. Samples of blood larger than those used (71 cu. mm.) disturbed the interfaces in passing through them.

If one were first to separate leukocytes from blood in large quantities, resuspend them in saline, and centrifuge them against an isosmotic solution of density 1.072, large scale subfractionation would be possible, since there is only the saline-albumin solution interface to maintain, with a density difference of .070.

It is noteworthy that in this limited series the monocytes were found characteristically with the lymphocytes while the eosinophils appeared in any given centrifugation either with the lymphocytes or with the polymorphonuclear leukocytes. This suggests that the eosinophils have a characteristic density intermediate between the other leukocyte cell types.

It has previously been held that the polymorphonuclear leukocyte has a lesser density than the lymphocyte. Under the stated conditions, the opposite was found to be the case.

It would appear that lymphocytes have been thought to have a higher density than polymorphonuclear leukocytes because of their intensely basophilic nuclei
which give the visual impression of high density when stained with Wright's stain. However, data obtained by x-ray absorption and with methods described here and elsewhere indicate that a preponderance of nuclear over cytoplasmic mass results in a lowering of the overall density of the cell due, presumably, to the lower density of the nuclear material.

**SUMMARY**

The separation of polymorphonuclear leukocytes from lymphocytes and erythrocytes has been accomplished by employing a method which takes advantage of their density differentials. During a single centrifugation the lymphocytes accumulate at a plasma-albumin interface (albumin I, density = 1.065 to 1.085), the polymorphonuclear leukocytes accumulate at an albumin I-albumin II interface (albumin II, density = 1.094) and the erythrocytes pass to the base of the Wintrobe tube. The yields are over 90 per cent pure cell types. All layers are isosmotic with blood.

**SUMMARIO IN INTERLINGUA**

Le separation simultanee de cellulas sanguinee in leucocytos polymorphonuclear, lymphocytos, e erythrocytos ha essite complite per medio de un methodo que utilisa le differentias de lor densitates. In le curso de un sol centrifugation le lymphocytos se accumula al interfacie del plasma e del prime del duo albuminas usate (densitate del albumina I: ab 1,065 a 1,085) durante que le leucocytos polymorphonuclear se accumula al interfacie del duo albuminas (densitate del albumina II: 1,094). Le erythrocytos passa al base del tubo Wintrobe. Le typos cellular assi segregate es plus que 90 pro cento pur. Tote le stratos es isosmotic con sanguine.

**REFERENCES**

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