

## Separation and Purification of Leukocytes and Platelets

By JAMES L. TULLIS, M.D.

SEVERAL TECHNIQUES exist for the isolation of white blood cells and platelets from each other, and from the erythrocytes of human blood. Some of the methods are applicable to small aliquots of blood and employ only simple laboratory equipment. Others are designed for the rapid processing of large quantities of blood and generally employ extensive mechanical equipment. The former have the advantage of low cost and simplicity, but usually yield leukocytes which are not as normal biologically as the leukocytes separated with more complex, refrigerated equipment.

All methods (except one)\* utilize the difference in density of the three formed elements: red cells 1.092, leukocytes 1.065, platelets 1.030. Gravitational force is employed either at 1 *G*, as in sedimentation, or at *x G* as in mechanical centrifuges. Efforts to utilize characteristics other than density have met with limited or no success: i.e., utilization of surface charge by migration in an electrophoretic cell; phagocytosis of iron particles followed by exposure to a magnetic field; specific antisera (antiplatelet antibodies, antileukocyte antibodies); and specific cell toxins such as saponin to lyse erythrocytes. All these methods are predicated on purification of a single formed element through destruction of the other formed elements and thus are manifestly wasteful of blood. The constantly increasing use of blood and blood derivatives demands economy and specificity in the utilization of its component parts.

### SEDIMENTATION

Erythrocytes are generally the first and easiest cells to remove from whole blood. With no treatment at all, erythrocytes will settle out of suspension on standing and eventually pack to a uniform hematocrit. Fåhræus<sup>1</sup> in 1921 showed that erythrocyte sedimentation was primarily a function of rouleaux formation and that this in turn was a factor of plasma fibrinogen concentration, or to a less extent, globulin content. Later, Grey and Mitchell<sup>2</sup> added Plasma Fraction I to whole blood to obtain a more rapid sedimentation. Oncley, Cohn and others<sup>3</sup> showed that the rouleaux-augmenting property of fibrinogen was its physical shape and that any long asymmetric molecule would be equally effective. Minor and Burnett<sup>4</sup> first used sedimentation as a means of obtaining plasma for later concentration of leukocytes. Many of the plasma volume expanders serve as excellent sedimenting agents. Dextran, dextrin, glutamyl peptide, periston and gamma globulin have all been used in this laboratory with success. Data are presented in table 1 to show representative results with each of these substances.

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\* The sequestration and elution of platelets from resin columns.

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The optimal time for erythrocyte packing, without white cell sedimentation, averages 40 minutes. Glucose is generally added in a final concentration of 0.3 Gm. per cent to support metabolism during the period of sedimentation.

It should be noted that satisfactory sedimentation is seldom achieved at temperatures below 12 C. At lower temperatures rouleaux formation is inhibited and the red cell aggregates do not settle properly once formed. This is due to increased viscosity of the plasma. Thus, room temperature is generally best for sedimentation. One should bear in mind, however, the resultant decrease in survival that accrues from this added period of metabolic activity. It is not possible to obtain purity of either white cells or red cells by rouleaux technics. A single sedimentation of the type reported above will reduce the customary 1000:1 ratio of red cells over white cells to a ratio of about 1:1. When the red cells become too widely dispersed to rouleaux properly, no amount of a sedimenting agent will cause further aggregation. Thus technics for improving purity by

TABLE 1.—*Erythrocyte Sedimentation*

Agent	Final Concentration	Time	Hematocrit Obtained
Dextran (intrinsic Viscosity 0.46).....	0.8%	35 min.	47.5
Dextran (intrinsic Viscosity 0.44).....	0.8%	35 min.	37.0
Dextrin (α-amylase pectin)*.....	0.8%	35 min.	45.0
Glutamyl Peptide†.....	0.6%	35 min.	32.0
Glutamyl Peptide‡.....	0.6%	35 min.	35.0
Periston.....	2.5%	40 min.	50.0
Gamma Globulin, Human.....	7.0%	60 min.	56.5

All sedimentations carried out by standard technics using a Wintrobe hematocrit tube. Temp. 21 C.

\* Lot #3245: 77B Northern Regional Laboratories.

† Mol. wt. 8160; prepared by Dr. Max Bovarnik, Veterans Administration Hospital, Brooklyn 9, N. Y.

‡ Mol. wt. 23,000; prepared by Dr. Max Bovarnik, Veterans Administration Hospital, Brooklyn 9, N. Y.

secondary rouleauxing have met with little success. Certain substances, such as jack-bean extract as recently developed by Osgood,<sup>5</sup> cause aggregation of the erythrocytes rather than rouleaux formation, and yield a high purity of leukocytes in a minimum of time.

One can also sediment white cells by extending the exposure time. After the red cells have settled and have been removed from the system, a later sedimentation of leukocytes and platelets will occur. This generally requires approximately 90 minutes at 37 C. and yields moderately good concentration of cells, if done in narrow tubes to facilitate removal of the supernatant. The leukocytes are clumped in small aggregates by this process. Although they can later be disengaged partially by agitation in fresh dextrose, saline, or buffer media, irreversible damage has occurred as can be shown by metabolic studies or subsequent preservation. This technic has the further disadvantage of yielding a mixed population due to trapping of platelets. It serves, however, as a simple method for obtaining leukocytes with minimal laboratory equipment, and yields cells which are entirely adequate for short term chemical experiments. Greater

freedom from entrapped red cells can be obtained by transferring the resultant cell mass to a protein-free media and allowing a secondary settling to occur. In this instance, the distance between red cells is of no importance as rouleaux formation does not occur and sedimentation is simply a factor of individual cell density, magnified by the decreased viscosity of the suspension media. With such a final differential sedimentation one can often obtain a white cell to red cell ratio of 50:1 and occasionally as high as 100:1.

#### MECHANICAL SEPARATIONS

Any type of centrifuge with sufficient force to overcome the resistance of plasma, will separate the formed elements into three layers: the red cell mass with a superimposed buffy coat consisting of white cells covered by platelets. Improvements in mechanical separation are of two general types: those which vary the vessel in which the blood is placed, so as to increase the efficiency of the recovery; and those which vary the actual principles of centrifugation. A typical example of the former is the Cushman tube.\* This vessel, of both 10 and 25 ml. capacity, has a bulbous bottom calibrated to varying hematocrits and a thin capillary tube in the middle with a second bulbous reservoir at the top. On centrifugation, red cells pack in the bottom, a buffy coat in the thin central portion and plasma on top. Due to the capillary nature of the central compartment, the white cells cover a long enough area to permit physical removal with a syringe and needle. Still another variation of the centrifuge vessel has been developed in this laboratory by Dr. Maurice D'hont.\* It consists of a pair of stoppered vessels connected end-to-end through a needle or glass tube. The top vessel is filled with plasma from which the red cells have been removed previously by sedimentation. The two vessels are then connected and placed in a standard centrifuge cup, one on top of the other. By means of a one minute centrifugation, at any given  $G$ , the plasma is forced from the upper to the lower vessel. The centrifuge is then abruptly stopped. The relative vacuum which has been created by passage of the plasma from the upper vessel to the lower vessel then siphons the plasma back into the upper compartment leaving the leukocytes behind. By employing different centrifugal speeds (150  $G$  for white cells 400  $G$  for platelets) one can effect a partial purification of white cells from platelets with a minimum of effort, time and expense.

The recent "slicer" developed by Randolph and Ryan<sup>6</sup> represents still another modification of recipient vessel. With it, one can remove an intact plastic segment containing the desired cell type. This method has been further developed and modified by Wall and Doan.<sup>7</sup>

During the past two years, this laboratory has been engaged in extensive research on the development of newer types of mechanical equipment for the more rapid and atraumatic separation of blood into all of its component parts: both formed elements and proteins. Under the stimulus of Dr. Edwin J. Cohn, a group of engineers, cytologists and chemists have collaborated to produce a series of devices, each more simple than the first, and each designed to fractionate blood rapidly and effectively. The first<sup>8</sup> was a falling-film centrifuge for the dif-

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\* Available through MacAlaster Bicknell Company, Cambridge 48, Mass.

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ferential separation of platelets and leukocytes, following a preliminary erythrocyte sedimentation. It has had extensive use during the past eighteen months and the bulk of the leukocyte studies reported from this laboratory<sup>9</sup> has been with cells obtained in this manner. It was engineered for continuous operation, however, and subsequent study has shown that it is inadvisable to mix the white cells from various donors even for in vitro experiments. The second development was a long-traverse centrifuge for the washing of red cells and the simultaneous partial separation of white cells from platelets by the use of high density medium as suggested earlier by Buckley, Gibson and co-workers.<sup>10</sup> More recently,

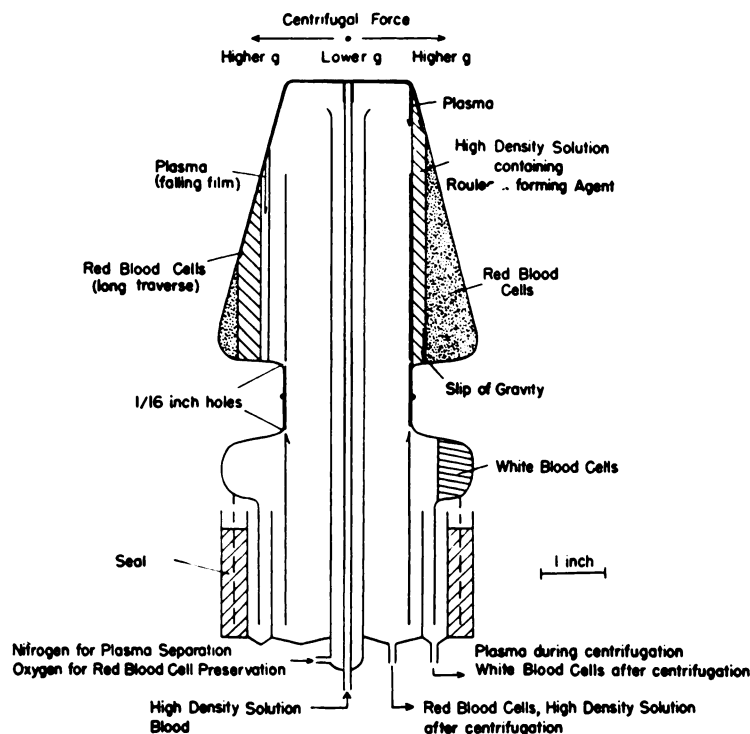


FIG. 1—Principles of the new blood centrifuge.

a simplified apparatus for the rapid separation of blood at a low gravitational force in a continuous closed-system has been developed by Dr. Cohn. The new process eliminates a blood collecting bottle. The blood flows from the arm of the donor through a vessel containing an exchange resin to effect removal of calcium and platelets, thence, through a heat exchange unit for reduction of blood temperature, and into a light, inverted centrifuge bowl of siliconed glass (fig. 1). This bowl contains a high density solution selected to be such that the red cells, with a specific gravity of 1.092, will pass through it, while the plasma, and leukocytes with a lower specific gravity will not. At the proper flow rate, the high density solution becomes, in effect, a cutting edge. The red blood cells, passing down the sloping walls of the centrifuge bowl, therefore simulate a long-traverse centrifuge, while the plasma, passing in laminar flow down the inside wall of the high density solution, simulates a falling-film centrifuge. Addition of

a rouleaux agent to the high density solution makes the separation more effective as the weight of the aggregated red cells, according to Stokes's law, allows a more ready passage through the high density solution.

Inside the centrifuge vessel, the outward force at the central weir is 60 times gravity. The force at the outer surface of the bowl is 200 times gravity. The white blood cells and plasma pass along the inner surface of the high density solution and overflow into a lower bowl where they are concentrated by the falling-film principle. After the plasma has been separated for further processing in the closed, sterile system, the red blood cells and white blood cells are washed from the respective centrifuge bowls into separate containers containing the desired preservative solutions. The platelets are eluted from the exchange resin column into a separate collecting vessel. Thus, almost simultaneously with the completion of a standard 500 ml. bleeding, one has a fairly high yield of sterile formed elements in what is believed to be a close approximation of their state in nature.

In the new blood collecting and processing apparatus, the details of which are outlined more fully elsewhere,<sup>11, 12</sup> the separation of plasma from cells is a continuous process. The volume in the centrifuge vessel at any one time is, therefore, always less than the volume of blood processed. As a result, all parts of the apparatus are of very light construction. Processing in the compact, portable machine is carried out in a closed system from the vein of the donor to the final plastic containers for the blood components. All parts of the apparatus which come in contact with the blood are non-wettable and sterilized as a single replaceable unit.

#### CHEMICAL SEPARATION

The sequestration of platelets onto resin beads provides the single chemical method for isolating one cell type from another. Efforts to improve the percentage sequestration of platelets onto cation columns became important when Freeman<sup>13</sup> showed that platelets could be recovered from such resin complexes. On the suggestion of Dr. Edwin J. Cohn, methods have been developed by Drs. William Batchelor and Douglas Surgenor to remove platelets quantitatively from blood.\* The concentration of platelets in the subsequent elution has not improved proportionately, but the addition of sodium acetate and metal-complexing agents (citrate or ethylenediaminetetraacetate) has increased the final yields to around 30 per cent. The failure to recover the platelets completely suggests the future use of resins with a lower affinity for calcium and platelets.

#### SUMMARY

Methods and equipment for the separation of the formed elements of the blood are outlined and discussed. Selection of any given method should be predicated on the purposes for which the isolated cells are to be used. The technics vary from simple fractional sedimentation requiring no equipment (when viable cells are

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\* Recycling commercial Dowex-50 onto the acid cycle followed by treatment with NaOH. Before use, a few of the sodium sites are replaced by calcium. (Insufficient calcium to interfere with the decalcification of whole blood but sufficient to provide an excess of available calcium sites for platelet interaction.)

not necessary) to complex, refrigerated, centrifuge equipment when cells in their true state in nature are desired. An apparatus for the rapid separation of blood in a continuous, closed-system is described and discussed.

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