thorax; presumably a disproportionate loss of fat occurred in these animals.

The chests of the operated and sham-operated animals at autopsy were relatively free of adhesions. It seems feasible to us to remove the bands from living animals; therefore our method may be useful for various studies before, during, and after vascular obstruction.

In view of the choice of experimental animal the method may prove especially useful for studies of endocrine function, cardiac hypertrophy, metabolic balance, etc., in which the expense of a larger animal might be prohibitive.

Summary. We have demonstrated the feasibility of banding intrathoracic vessels in the weanling rat, with survival of an acceptable proportion of the animals for 9 weeks. The vascular obstruction resulted in reduced food intake, growth failure, cardiac hypertrophy, and other changes indicative of cardiac failure.

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Volume Distribution and Separation of Normal Human Leucocytes.* (32093)

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The volume distribution of leucocytes of normal adults has been measured with a modified Coulter counter described below. This distribution curve has been investigated with the electronic cell separator described by Fulwyler(1). Cells from the separated fractions were identified and counted using leucocyte differential staining procedures. These methods can be extended to separation of different classes of white blood cells for metabolic and other studies in both health and disease.

Materials and methods. The electronic equipment for determining cell volume distribution uses a modified Coulter counter sample stand,† a specially designed aperture 75 μ diameter and 250 μ long, an aperture current supply set normally at 100 μamp, a transistorized low-input impedance amplifier‡ (integrating time 5 μsec, differentiating time 50 μsec, maximum gain 10^4), and an RIDL Model 34-12 400-channel analyzer§ with input modified to accept the relatively slow pulses from the Coulter aperture. The purpose of the low-input impedance is to make the pulse amplitude insensitive to diluent conductivity. The analyzer modification consists of increasing the input RC coupling time constants and duration of the linear gate which admits the pulse to the analog-digital converter, to accommodate the longer pulses (25-

* This work was performed under the auspices of U. S. Atomic Energy Commission.

† Coulter Electronic Sales Co., Hialeah, Fla.

‡ Designed at Los Alamos Scientific Laboratory; details available on request.

§ Radiation Instrument Development Laboratory, Melrose Park, Ill.

40 μsec). A constant pressure difference of 0.4 atmosphere is maintained across the aperture by a Conoflow vacuum regulator,‖ resulting in flat-topped pulses of about 25 μsec duration. The live timer in the 400-channel analyzer is used to time a measurement (typically 1 minute, about 50,000 cells).

With this apparatus, we were able to observe a leucocyte peak of mean volume about 5 times the erythrocyte mean volume in a 10-20 minute measurement of heparinized whole blood diluted by a factor of 50,000 with buffered physiological saline. However, smaller leucocytes were obscured by the much larger number of erythrocytes, pointing up the necessity of concentrating the leucocytes.

None of the concentration methods tried was completely satisfactory for quantitative cell recovery. These methods included hemolysis, accelerated sedimentation, and centrifugation at an interface(2). Best results were achieved with a modification of the procedure described by Herbeuval *el al*(3) using saponin to lyse the erythrocytes. The starting material was 5 ml of heparinized whole blood. We modified Herbeuval's procedure by first spinning the cells down and then washing them with phosphate-buffered saline, a step shown by Ericksson(4) to reduce interference of plasma lipids with saponin hemolysis. After lysis, we layered the cell suspension over 1 ml of the donor's own plasma and centrifuged for 10 minutes at 160 g to transfer the leucocytes from the saponin to a medium known to minimize cellular disruption (*i.e.*, plasma). Finally, the pellet was resuspended, and the resulting suspension was diluted with phosphate-buffered saline for size distribution measurements and separation. This method produced a cell suspension which was stable for about 1 hour. Lymphocyte recovery was quantitative, but there was some loss of other cell types.

A different approach was also investigated. We attempted to increase the leucocyte concentration of diluted whole blood relative to the erythrocyte concentration by electronic cell separation. Enrichments of the order of 10^3 were obtained, and the granulocyte peak which was observed had the same shape and modal volume as the one obtained by saponin lysis. However, this method encountered difficulty because of the very large erythrocyte-to-leucocyte ratio in whole blood; the number of leucocytes recovered was small, and the lymphocyte peak was obscured by unidentified cells of similar volume. These cells may have been large immature erythrocytes or two or more attached cells. Whatever their character, they prevented resolution of the lymphocyte peak.

The volume distribution of the leucocyte suspension at a concentration of 50,000-100,000 cells/ml was measured, and cells from selected volume bands were separated. Because of limitations of cell stability and separation speed, the number of cells ordinarily removed from a mixture was 10,000-50,000, requiring special methods to prepare the slides for microscopic examination.¶ A specially designed cylindrical centrifuge tube was used to deposit the cells on a circular coverslip (diameter 18 mm) prior to staining with Wright's stain.

Results. The volume distribution of leucocytes from a normal male subject obtained using the modified Herbeuval procedure is shown in Fig. 1. The erythrocyte distribution

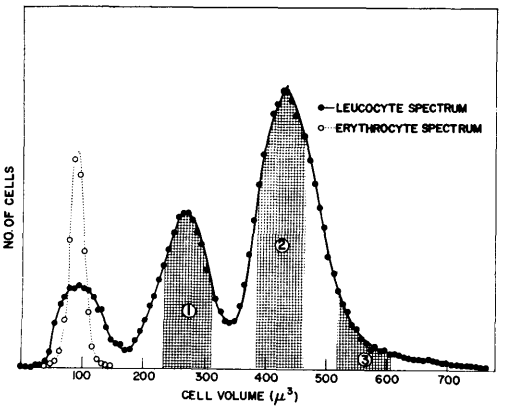

FIG. 1. Volume distribution of erythrocytes (open circles) and leucocytes (closed circles) for a normal male subject. The shaded areas show the separated cell fractions.

‖ Conoflow Corp., Philadelphia, Pa.

¶ The electronics of the cell separator have recently been improved to the extent that the current speed of operation is several times greater than reported here. In addition, it is now possible to separate simultaneously as many as four discrete volume fractions from a distribution.

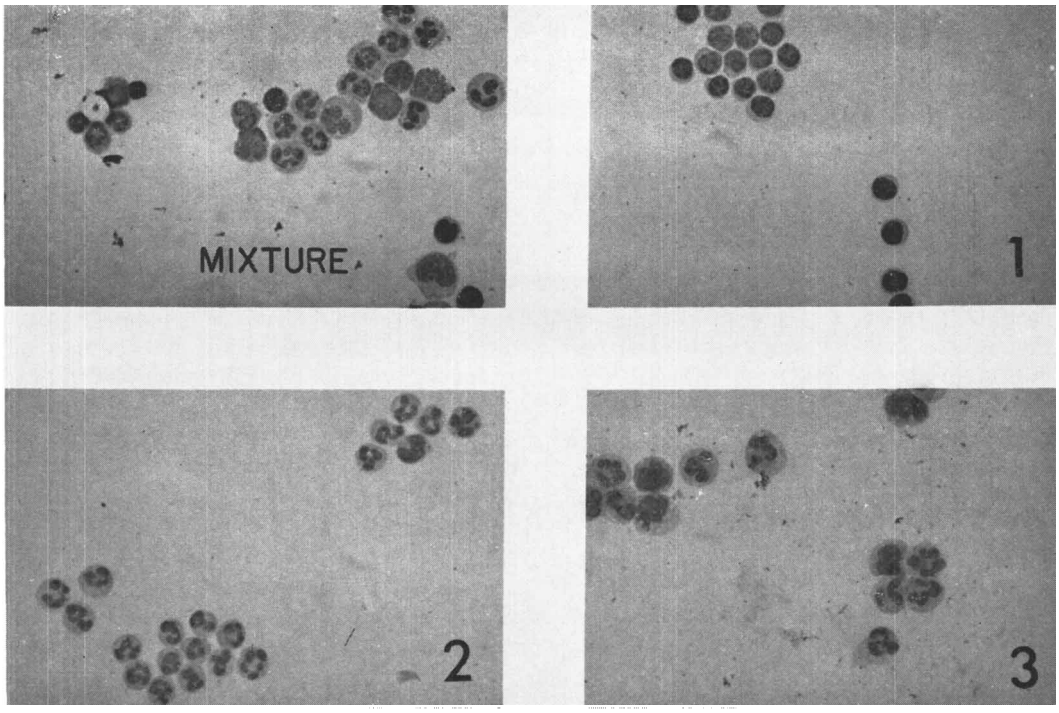

FIG. 2. Photomicrograph of separated cell fractions compared with the original mixture. The numerals 1, 2 and 3 refer to Fig. 1.

(open circles) was obtained directly from whole blood diluted in buffered physiological saline. The cell concentrations used to obtain the two distributions of Fig. 1 were unrelated (*i.e.*, the vertical scales of the erythrocyte and leucocyte distributions were quite different).

The volume distribution of the erythrocytes can be well described by a slightly skewed normal distribution function with a mean value of 90 μ^3 and a standard deviation of 13.5 μ^3(5). The peak of similar modal volume (closed circles) is due to incomplete erythrocyte lysis. The leucocytes are larger and show a bimodal distribution. Cells from the indicated areas 1, 2 and 3 were electronically separated, examined for morphology and photographed (Fig. 2). These photographs were taken at the same magnification ($\times$ 680) and show the size progression among the separated fractions. In Table I the cells of the separated fractions are divided into 3 classes and compared with the unseparated mixture. The smaller volume leucocyte peak (fraction 1) represents almost pure lymphocytes. The central part of the larger peak (fraction 2) is mostly polymorphonuclear granulocytes, and the tail (fraction 3) shows a 15-fold enrichment of monocytes.

TABLE I. Human Leucocyte Separation: Cellular Types Present in Three Areas of Volume Distribution.

Volume Separation	Distribution (%)		
	Lympho-cytes	Granulo-cytes	Mono-cytes
Unseparated	40	58	2
Fraction 1	99	1	0
" 2	5	93	2
" 3	3	67	30

Table II shows a quantitative correlation between electronic sizing and standard hematological analysis made by subjecting 15 blood samples from 8 normal male subjects to both differential leucocyte counts and electronic sizing. The lymphocyte peak was the more stable of the two. Its area yielded a count in good agreement with the small lymphocyte count obtained from the differential. The larger peak (mostly granulocytes) was more sensitive to slight variations of the

TABLE II. Leucocyte Counts in Peaks of Volume Spectrum Relative to Standard Differential Count.

	First peak (small lymphocytes) (%)	Second peak (large cells) Saponin lysis (%)	Second peak (large cells) Whole blood (%)
Average of 15 measurements ± S.D.*	102 ± 23	69 ± 20	107 ± 13

* Standard deviation.

saponin lysis method, was less stable, and generally yielded a count lower than the differential. This is consistent with the experience of others(6), where it was observed that lymphocytes almost always displayed a more normal morphological appearance and swelled less readily than polymorphonuclear granulocytes. However, the large peak obtained with whole blood that had merely been diluted yielded a count in good agreement with that obtained by standard hematological procedures. Thus, it appears that some quantitative information on the distribution of cell types can be obtained from the volume spectrum; however, this is no substitute for a standard differential. In order to improve this technique, a more satisfactory leucocyte concentration method is needed; work on this problem is in progress.

Summary. Leucocytes from normal adults have been studied by electronic sizing and separating methods. The volume distribution was found to be bimodal. Three cell groups of small volume dispersion were isolated and subsequently identified by hematological methods. The smaller-volume leucocyte peak was found to contain small lymphocytes and little else. The large-volume peak contained granulocytes in its central portion and monocytes in its large-volume tail. Using these methods, the relative abundance of a specific type of leucocyte can be greatly increased. These methods may also be applied to other cell suspensions.

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An *in vitro* Assay of Erythropoietin.* (32094)

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The ability of erythropoietin to stimulate heme synthesis in cultures of bone marrow has been demonstrated by many workers (1-3). Recently, Krantz and coworkers(3,4) described a method of bone marrow culture in which heme synthesis, for periods of incubation longer than 12 hours, was dependent upon the presence of erythropoietin in the culture media. They suggested that the ability of erythropoietin to prolong heme synthesis *in vitro* might be used as an assay for erythropoietin.

We(5) recently used the Krantz method of

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