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Biological and Medical Research Group (H-4) of the Health Division -- Annual Report July 1965 Through June 1966

Group Leader, W. H. Langham
Division Leader, T. L. Shipman

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CHAPTER 1

INTRODUCTION

The increased emphasis over the past 5 years on attempts to contribute to the understanding of radiation effects at the cellular- and molecular-levels has been primarily responsible for expansion of the biological and medical research program at Los Alamos. These investigations have largely taken the direction of studying some of the detailed processes of information transfer in in vitro biological systems and the sequential biochemistry of phased mammalian cells grown in culture medium. The eventual objective of these investigations is to determine the influence of radiation, radioactive materials, and drugs of known biological effect on the previously established cellular and molecular processes. The purpose of studying drug effects is to observe whether there is parallelism between radiation effects and the known action of the drug. As an example, is there similarity between cycloheximide inhibition of synthesis of essential protein for cell division and inhibition of cell division by irradiation?

Many of those new projects are now yielding sufficient results to justify beginning of radiation studies which will be reflected in future program direction. Expansion of the more fundamental aspects of the program has essentially leveled off insofar as staff-level personnel are concerned. At the present time, however, the staff-to-technician plus research assistant personnel ratio is about 0.9. Some expansion in the latter category is needed and contemplated to increase the efficiency of utilization of staff-level scientists.

Past emphasis of the cellular radiobiology aspect of the program has resulted in increased emphasis on biophysics and instrumentation, largely along the lines of developing electronic means of quantitatively monitoring the rate of growth of cells in culture medium and on means of actually separating living cells electronically on the basis of their volume. Other means of studying the properties of

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cells and cell populations and separating living cells electronically (e.g., fluorescence and light absorption and scattering) are being investigated.

Although over 50 percent of the present effort is devoted to more fundamental studies at the cellular- and molecular-levels, the applied aspects of the program are being re-emphasized, particularly along the lines of potential risks of radiation exposure and contamination from space applications of nuclear energy in rocket propulsion systems and auxiliary power supplies. The nuclear weapons incident in Spain in January 1966 has focused programmatic attention on the need for development of methods of diagnosing plutonium exposure to replace or to supplement the standard urine analysis method, which appears inadequate for inhalation exposures to insoluble radioactive particulates. Sophisticated proportional counters and solid-state detector systems are being considered for this purpose.

A summary of the past year's research effort can be seen in the Table of Contents in the preceding pages.

The present group organization and personnel are shown in the Table of Organization. New personnel are as follows:

Staff Members

C. H. Blomquist, Ph.D., Cellular Radiobiology Section
L. M. Holland, D.V.M., Mammalian Radiobiology Section
P. F. Mullaney, Ph.D., Biophysics Section
K. D. Munkres, Ph.D., Cellular Radiobiology Section
B. R. Burchill, Ph.D., LASL Postdoctoral Appointment, Cellular Radiobiology Section

Research Assistant

E. L. Martinez, Jr., B.S., Molecular Radiobiology Section

Clerk-Typist

J. H. Montague

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D. G. Ott, Ph.D., Alternate Group Leader
O. S. Johnson, B.S., Administrative Deputy
E. M. Sullivan, Secretary
J. M. Montague, Clerk-Typist

CELLULAR RADIOBIOLOGY SECTION

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Section Leader

Staff Members

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C. M. Blomquist, Ph.D.
I. U. Boone, M.D.
M. D. Enger, Ph.D.
C. T. Gregg, Ph.D.
K. A. Hyatt, Ph.D.
P. M. Kraemer, Ph.D.
K. D. Munkres, Ph.D.
A. G. Saponara, Ph.D.
R. A. Tobey, Ph.D.

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S. G. Carpenter, B.A.
S. B. Cox, B.A.
P. W. LaBauve, B.A.
P. C. Sanders, M.S.

Technicians

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E. Mueller
E. C. Wilmoth

Postdoctoral Appointees

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W. D. Currie, Ph.D.

AEMU Doctoral Candidate

B. A. Walters

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A. Murray, M.S.
R. L. Ratliff, Ph.D.
G. S. Shepherd, Ph.D.
D. A. Smith, Ph.D.
T. T. Trujillo, B.S.
D. L. Williams, M.S.

Research Assistants

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E. Hansbury, M.A.
E. B. Lilly, B.S.
A. M. Martinez, B.S.
E. L. Martinez, Jr., B.S.
B. J. Noland, B.A.
C. M. Roberts, B.A.

Technician

V. E. Mitchell

Postdoctoral Appointee

A. W. Schwartz, Ph.D.

MAMMALIAN RADIOBIOLOGY SECTION

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Section Leader

Staff Member

L. M. Holland, D.V.M.

Research Assistants

C. F. Bidwell, M.S.
M. R. Brooks, B.Ch.E.

Technicians

R. F. Archuleta
M. J. Bassmann

Animal Caretakers

F. Archuleta
J. E. Atencio
S. Cordova
L. Ortiz
A. Trujillo
F. Valdez
J. G. Valdez
E. A. Vigil

MAMMALIAN METABOLISM SECTION

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Section Leader

Staff Member

J. E. Furchner, Ph.D.

Research Assistants

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J. E. London, B.S.
J. S. Wilson, B.S.

Animal Caretakers

F. Benavidez
B. Martinez

BIOPHYSICS SECTION

M. A. Van Dilla, Ph.D.,
Section Leader

Staff Members

P. N. Dean, M.A.
M. J. Fulwyler, B.S.
J. M. Larkins, B.S.*
P. F. Mullaney, Ph.D.
J. D. Perrings

Research Assistant

J. M. Hardin, M.S.

Electronics Technicians

M. T. Butler
L. J. Carr

*Casual employee.

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AUTOMATIC CELL COUNTER FOR MAMMALIAN CELLS IN SUSPENSION CULTURE (E. C. Anderson, D. L. Carlson, R. B. Glascock,* J. H. Larkins, J. D. Perrings, and R. A. Walters**)

INTRODUCTION

The objectives of this development were: (a) the automatic measurement of concentrations of mammalian cells at frequent intervals (5 to 15 minutes) from several (up to 4) parallel suspension cultures in spinner flasks; (b) a precision of measurement of $\pm$ 0.5 percent or better for each determination; (c) rapid response to transients; and (d) the potential for expansion to include automatic cell spectrometry and chemostat operation.

A Coulter probe was chosen as the sensing element because of the precise volume spectrometry possible with this transducer. The automatic system of James and Anderson (1) was considered but was rejected because it was feared that the continuous-flow rate-meter method would not yield the desired precision and because the thin aperture required would not permit cell volume spectrometry. In addition, direct digital output was desired for computer processing.

METHODS AND RESULTS

In preference to continuous-flow systems using in-line apertures, an intermittent batch-sampling method with adjustable sample dilution was chosen. This choice offered considerable flexibility, with ease of changing the dilution factor as well as sampling frequency and volume. Cell concentration in the counting solution could be regulated to avoid coincidence losses over a large dynamic range, and long apertures (e.g., 90 x 300 microns) could be used for spectrometry. Volumetric syringes were readily available with a precision of better than 0.1 percent, and counts could be taken on 10^6 cells, reducing the statistical error to 0.1 percent.

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*Group P-1 of the Physics Division, Los Alamos Scientific Laboratory.

**Associated Rocky Mountain Universities, Inc., Predoctoral Fellow, Colorado State University.

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The basic unit of the electronic control circuitry was a "logics control chassis," which initiated performance of each operation, received the "operation complete" signal from the mechanical system, and initiated the following step. A relay system originally, the logics control was redesigned with transistorized plug-in logical units. The steps in the logics cycle were: (a) "ready" -- cycle completed and waiting for an elapsed time signal to initiate the next round; (b) "select" -- a rotary selector valve advances to sample the next culture in sequence; (c) "empty" -- previous sample removed from the counting beaker by opening a solenoid valve to vacuum drain, followed after 2 seconds by a saline rinse; (d) "drop Hg" -- solenoid valve opens, applying vacuum to the Coulter probe to lower the mercury in the manometer below the volumetric contacts; (e) "set" -- solenoid valve closes as mercury reaches "set" position, and scaler is reset; (f) "count" -- rising mercury reaches lower volumetric contact, beginning the count; and (g) "readout" -- mercury reaches upper volumetric contact, stops count, and initiates printing of flask number, count number, time of day, and total cell count. Completion of printing returns cycle to either position "5" for a replicate count (1 to 4) or to position "1" to await next time signal.

Figure 1 shows the front panel of the "logics" chassis of the control system. The status of the system at any time is indicated by lights which show the conditions of the various valves and syringes, as well as of the current stage of the logical cycle and subcycles. The "start" and "stop" controls in the upper right permit the independent setting of times at which the "rinse," "flush," "set," and "empty" valves are opened or closed during the "empty" cycle. These times are interpreted as positions of the "Y register," which advance at 2-second intervals.

The selector valve ("flask number") is a rotary stopcock (stainless Kel F) driven by a 1-RPM Bodine motor with microswitch positioning. The diluent syringe is a commercial pneumatic dispenser (Research Specialties Company), modified to be driven by laboratory vacuum and pressure supplies. The sample syringe is a glass hypodermic type (5-ml capacity) driven by a 3-RPM motor through a "walking beam" mechanical coupling. Volume is continuously variable by setting a micrometer stop to limit the intake stroke.

Each culture flask is fitted with a special sampling valve designed to minimize hold-up in the system by permitting the saline diluent to be injected at the sampling point. The

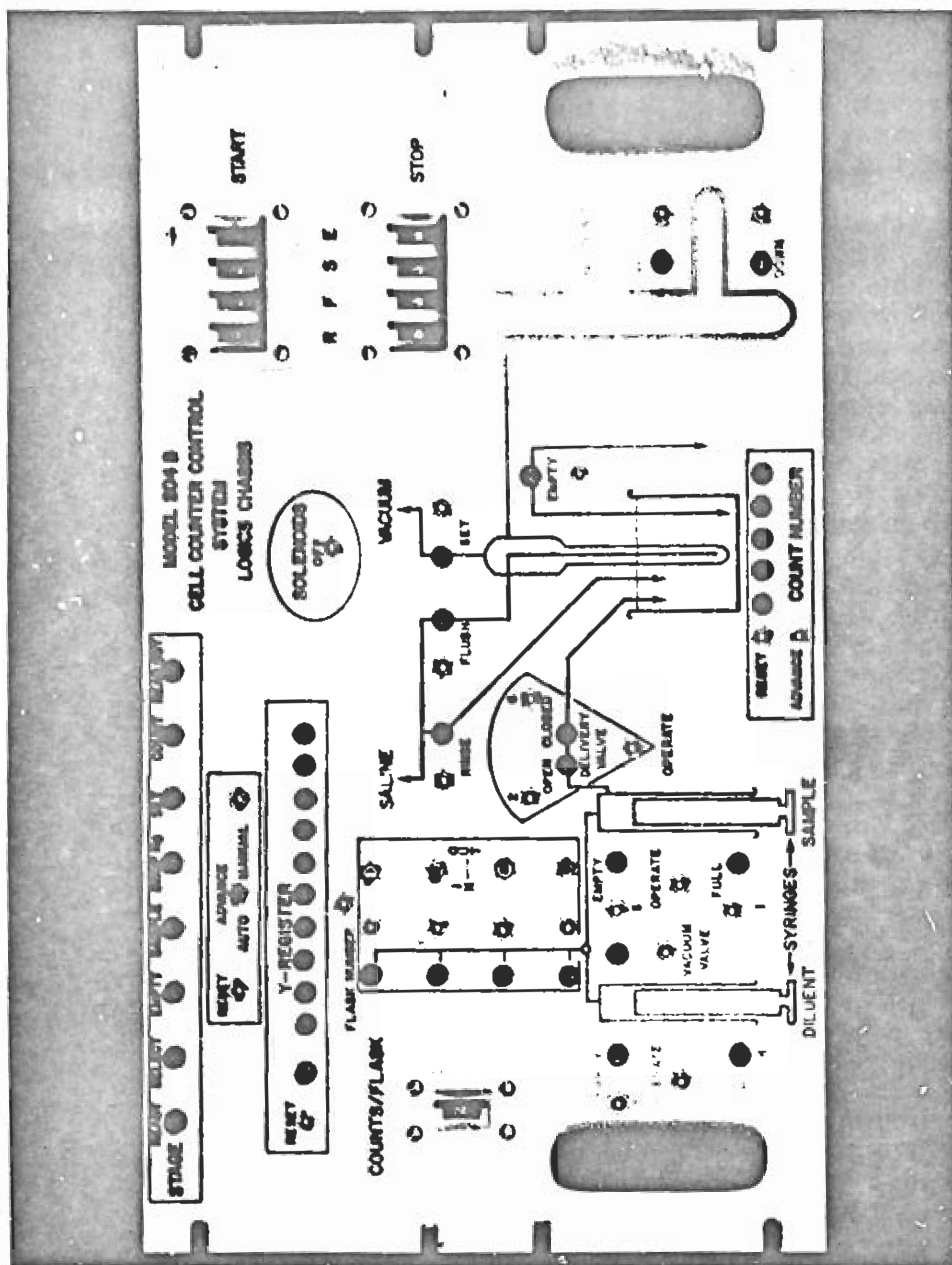

Fig. 1. Logics control chassis.

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entire sampling line is thus swept out by the saline, and there is minimal "memory" from sample to sample. Tubing diameter does not have to be kept extremely small, and thus pumping pressures are not excessive. A cross section of a sampling valve is shown in Fig. 2. It consists of a central tube of 1/8-in. diameter stainless steel, which is moved up and down through a pair of O-ring seals by application of a vacuum or pressure to the piston at the top. In the "down" position the filling port in the side is exposed to the culture medium, and a sample can be drawn into the sample syringe. In the "up" position the filling port is above the lower O-ring, and the sample is delivered into the counting beaker from the sample syringe. The saline diluent then passes into the outer tube of the sampling valve, enters the filling port, and flushes all the plumbing (including the empty sample syringe) into the counting beaker. "Memory" of the preceding sample was found to be less than 3 percent.

Immediately before entering the counting beaker, the solutions are passed through a Swinney filter unit containing only the 100-micron mesh plate. The mesh alone was of proper porosity to trap large foreign objects which could clog the aperture. No interference with passage of cells was noted, and very few instances of plugging were noted after addition of the filter. Occasional difficulties were due to such things as airborne lint falling directly into the beaker, and frequency of occlusions was greatly reduced by keeping the beaker covered at all times.

Cell suspensions were grown in jacketed spinner flasks of 250- to 1000-ml capacity with access tubes in the lid rather than in the sides to reduce int .ference between sampling probe and spinner. Temperature was regulated by a thermostated circulating water bath.

The sampling probes were sterilized by immersion in 70 percent alcohol at all times when not in use. Successful runs extended over a period of several days without bacterial contamination.

The Coulter probe was of conventional design except for replacement of manual stopcocks with solenoid valves to set the mercury manometer and to flush the interior. "Long" apertures (90 x 100 to 90 x 300 microns) were used.

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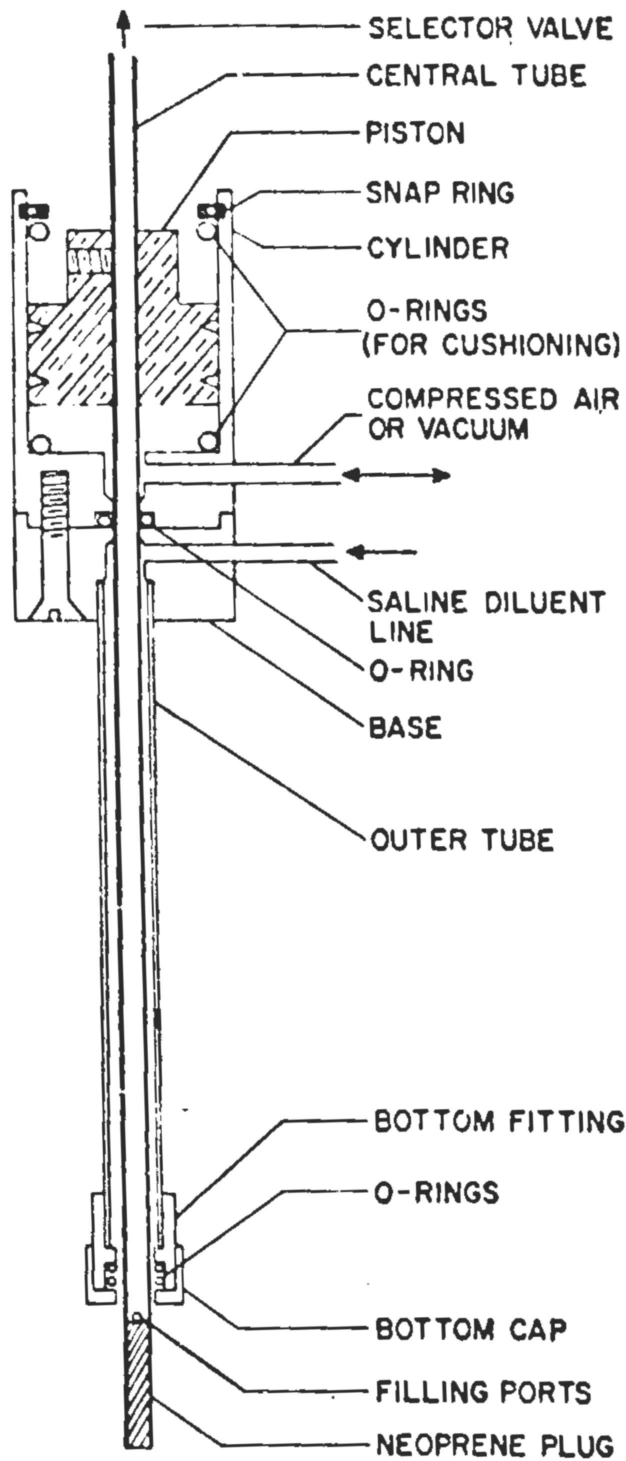

Fig. 2. Sampling valve.

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DISCUSSION

The instrument has operated to design specifications for extended periods of 24 hr/day without failures, but also without complete freedom from servicing the somewhat complicated plumbing and valving. The difficulties were primarily leaking stopcocks and sticking valves and were largely eliminated by preventive maintenance and a few design changes.

The precision of the system was estimated from scatter of the data about a least-squares regression line for cultures in exponential growth and for cultures with constant cell number (as a result of thymidine blockade). Under the best conditions, standard deviations of 0.4 to 0.6 percent were observed, compared with counting statistical errors of 0.3 percent. Unexpectedly, the growth rate of a supposedly "random" culture often varied, sometimes by as much as a factor of 2, over periods of a number of hours. Manual counting indicated this to be a true variation and not an error in the automatic system.

This device has been in use for a number of months in experiments to determine the effects of radiation and drugs on random and synchronized mammalian cell cultures. Its principal shortcomings are inherent complexities of the electronic control and plumbing system and the finite sample size requirement. A sample of 2 ml (typical) taken every 4 minutes removes 30 ml/hr. If this depletion rate is not to exceed the growth rate of the culture, then a 500-ml initial volume is required for a culture with a generation time of 12 hours and proportionately more for slower growing cultures. Work is currently underway on a simplified model which counts the sample without dilution and returns it to culture after measurement, thereby significantly reducing culture volume requirements. Coincidence losses are significant at high cell concentrations, and volume spectrometry is not possible; however, the resulting system should be far simpler and more economical to build and to operate.

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(1) T. W. James and N. G. Anderson, Science 142, 1183 (1963).

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FURTHER DEVELOPMENT OF THE CELL SEPARATOR (M. J. Fulwyler)

INTRODUCTION

Refinement of the cell separator, the operation of which has been described earlier (1,2), is expected to result in an instrument suitable for routine application to a variety of biological and nonbiological problems. Preliminary application has been made to separation of human leucocytes (3), mouse bone marrow cells (4), and several other cell types.

RESULTS AND DISCUSSION

As shown in Fig. 1, recent improvements in the device permit simultaneous separation of 4 discrete volume fractions of a distribution. The dotted line indicates the volume distribution of polystyrene spheres (diameter 7 to 14 microns) prior to separation. The remaining 4 curves represent the distribution of each of the 4 separated fractions. The separator has been used to isolate volume fractions of normal human leucocytes (5) and leucocytes from some abnormal states (6).

The entire separator mechanism may be sterilized by autoclaving. The pressure reservoir may be heated or cooled as needed to maintain the cell suspension at a desired temperature. These improvements will permit experiments in which it is desired to recover and to culture sterile, separated volume-fractions of living cells.

Improved electronics incorporating an anticoincidence circuit have been designed and built and are being tested. The anticoincidence circuit will sense the time between passage of particles through the Coulter aperture. This circuit will prevent separation, should two particles pass through the aperture so close in time that they might be collected in the same group of deflected droplets. This and other improvements are expected to increase the analysis rate of the separator from 10^5 to perhaps 2 to 4 x 10^5 particles/min, which will greatly enhance the usefulness of the device for certain biological experiments.

Described elsewhere in this report (7) are efforts to develop a sensor of cell optical properties. Such a sensor may have great value in electronically resolving cells of differing morphological character.

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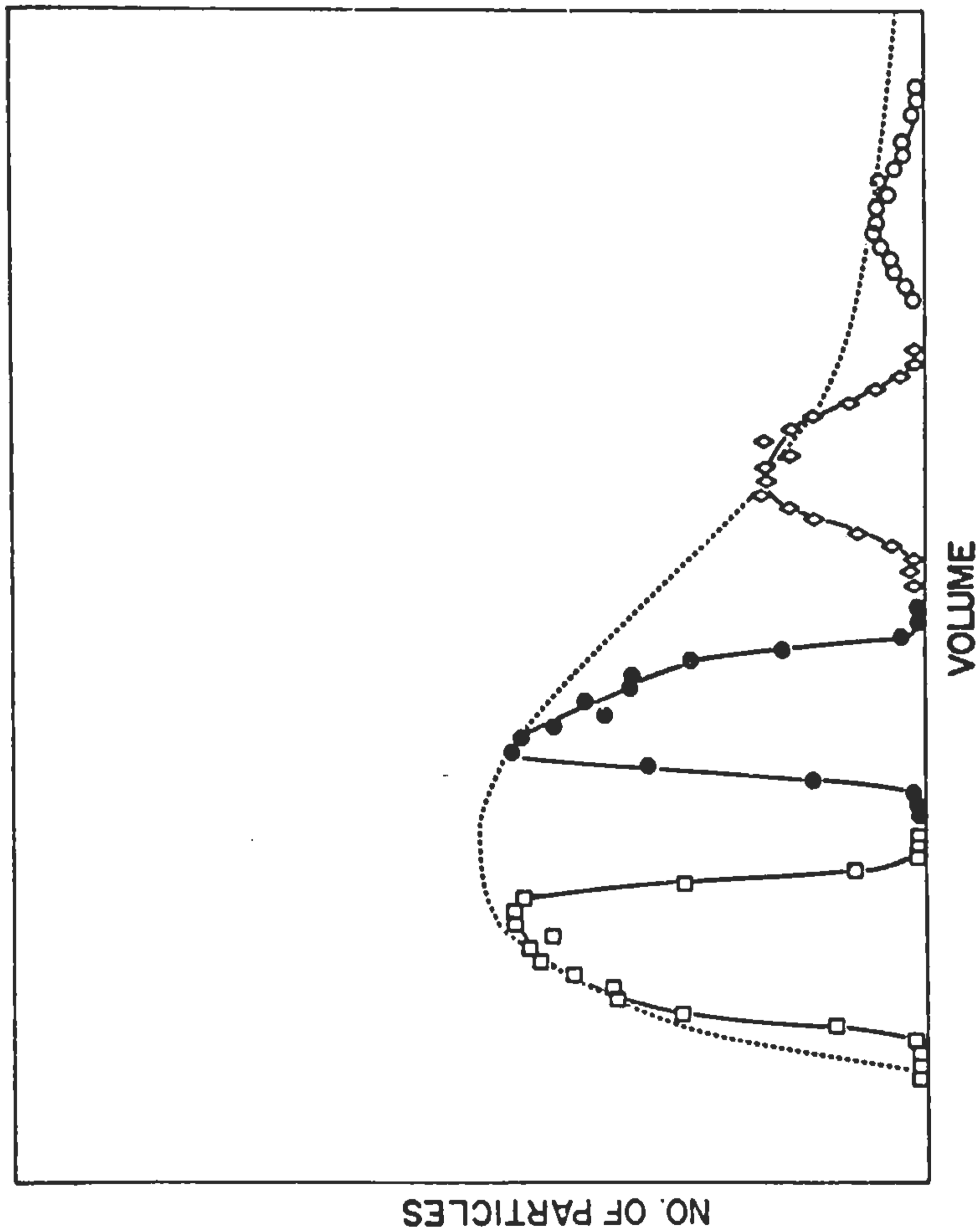

Fig. 1. Simultaneous separation of 4 discrete volume fractions from a distribution of polystyrene spheres ranging in diameter from 7 to 14 microns.

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(2) M. J. Fulwyler, Los Alamos Scientific Laboratory Report LA-3432-MS (1965), p. 114.

(3) M. A. Van Dilla and J. M. Hardin, this report, p. 234.

(4) M. A. Van Dilla, J. M. Hardin, and C. F. Bidwell, this report, p. 239.

(5) M. A. Van Dilla, M. J. Fulwyler, and I. U. Boone, submitted to Science.

(6) I. U. Boone, M. J. Fulwyler, and M. W. Stewart, In Proceedings of the American Association for Cancer Research 7, 8 (1966), 57th Annual Meeting, Denver, Colorado. Abstract No. 28.

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DETECTION OF BIOLOGICAL CELLS BY LIGHT-SCATTERING (M. A. Van Dilla)

INTRODUCTION

The phenomena of light-scattering and absorption by biological cells and their fluorescence when stained suitably are of great value in studying the properties of both normal and pathological cells. A study of light-scattering by single cells has been started; future adaptation to the electronic cell separator developed by Fulwyler (1) and dual-sensor systems may be feasible. The optical components of two commercial optical blood cell counters have been modified to yield pulse-height distributions for several types of inert particles and biological cells and these compared with volume distributions from a Coulter spectrometer. These data have enough interesting features to make it worthwhile to proceed with the design of a more flexible and suitable light-scatter sensor. This development, which uses a laser light source and attempts at optimal stray light control, is currently in progress.

METHODS

The most straightforward experimental approach to the problem of cell detection by light-scattering was to take advantage of existing commercial optical blood cell counters. The photomultiplier circuit of a Sanborn-Frommer blood cell counter was modified to allow pulse-height analysis of the output. The pulses generated by cells traversing the illuminated region of the scattering chamber were amplified and analyzed by the same electronic equipment used with the Coulter sensor (2). The resulting pulse-height distributions for leucocytes obtained by saponin lysis of diluted whole blood and erythrocytes are shown in Fig. 1. These distributions are quite different from those obtained with a Coulter spectrometer (see below), and hence something other than volume is being sensed.

Inherent disadvantages in the Sanborn-Frommer scattering chamber made it worthwhile to look for a design better suited to our needs. A new unit recently marketed by Vickers Instruments Ltd. seemed promising in this respect, since it generated a cell suspension stream of small diameter (about 100 microns), each cell of which traversed the illuminated region before exiting

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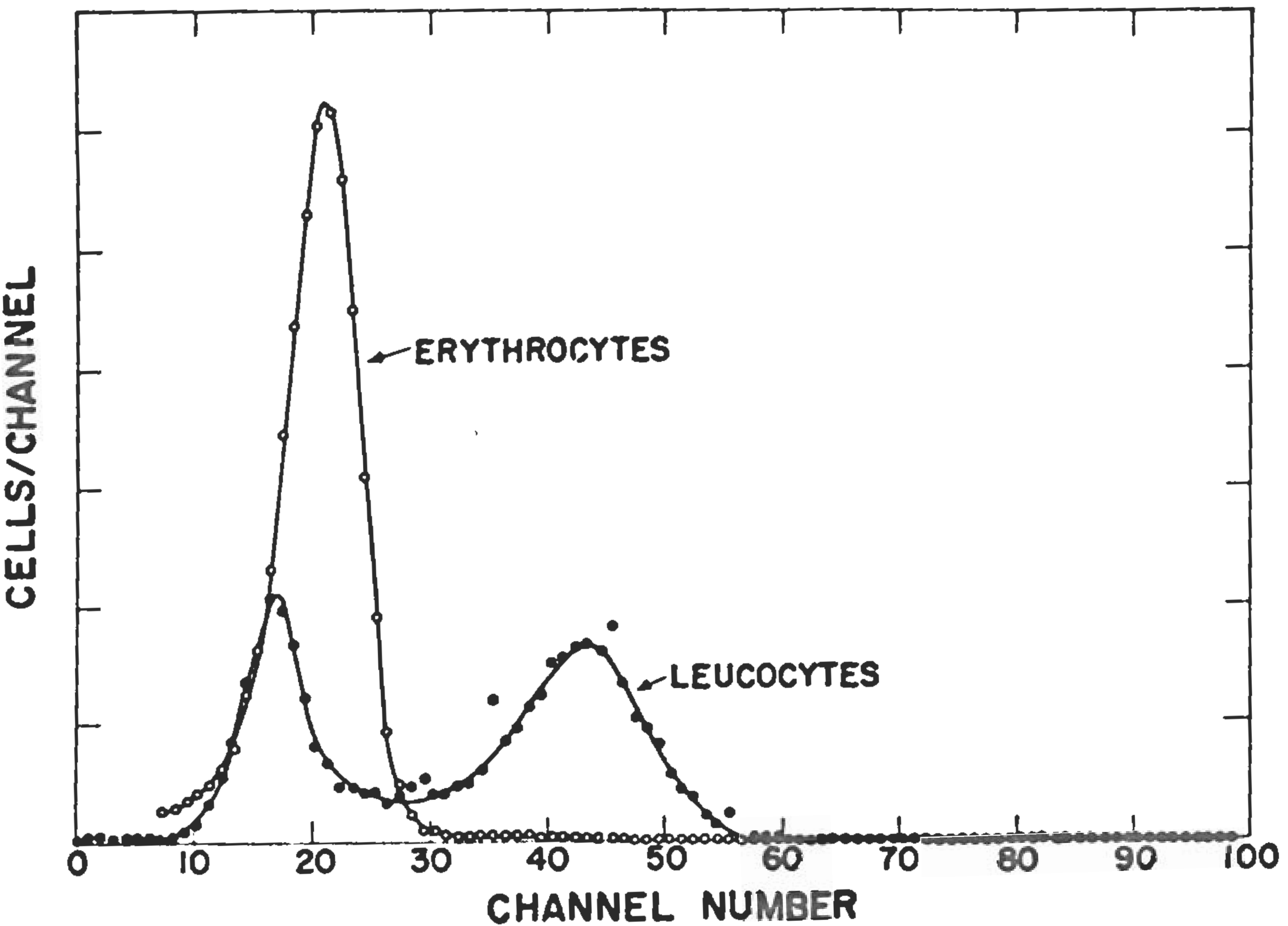

Fig. 1. Pulse-height distribution of normal erythrocytes and leucocytes obtained with a modified Sanborn-Frommer counter.

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through a small aperture (about 500 microns diameter). With the photomultiplier circuit modified as in the case of the Sanborn-Frommer counter and the same amplifier and analyzer, pulse-height distributions generated by leucocytes, erythrocytes, ragweed pollen, and paper mulberry pollen were compared with those from a Coulter spectrometer (Figs. 2, 3, 4, and 5). The interesting features of these data, combined with the limitations of the Vickers system for our purposes, led to the design of a system using a helium-neon laser light source, the Vickers scattering chamber, and arrangement to allow variation of scattering angle. This experiment is currently in progress.

RESULTS AND DISCUSSION

First results with the Sanborn-Frommer detector are shown in Fig. 1. The erythrocyte peak shape resembles that obtained with the Coulter spectrometer. Both are approximately Gaussian with a standard deviation of about 15 percent. However, the leucocyte peaks obtained with the optical detector are spread further apart than in the volume spectrum. In addition, the lymphocyte and erythrocyte peaks in the optical spectrum have similar mean values, whereas the lymphocyte mean volume is known to be about triple the erythrocyte mean volume from the Coulter spectra (see below). These results indicate that the light-scatter sensor is detecting different cellular properties than the Coulter sensor and hence would seem worthy of further study. However, design of the Frommer scattering chamber, although suitable for red cell enumeration, was not suitable for our purposes. Its limitations are that only a small fraction of the cells are detected, that stray light due to dirt on the windows is hard to control, and that future adaptation to the cell separator or dual-sensor arrangements seems very difficult.

The Vickers instrument, based on Crosland-Taylor's design (3), seemed to offer a way to circumvent many of these difficulties. Here a sheath flow of distilled water carries a cell suspension stream across the scattering chamber and out through a small aperture (about 500 microns diameter). The cell stream exits axially, while the sheath flow exits peripherally through the remainder of the aperture. Just before exiting, the cell stream diameter is about 100 microns; the dark-field image of a slit illuminated by a tungsten filament lamp is focused on the stream at this point. Thus every cell is counted, stray light generated by dirt on windows is reduced, and the possibility exists of converting the exit aperture into a Coulter

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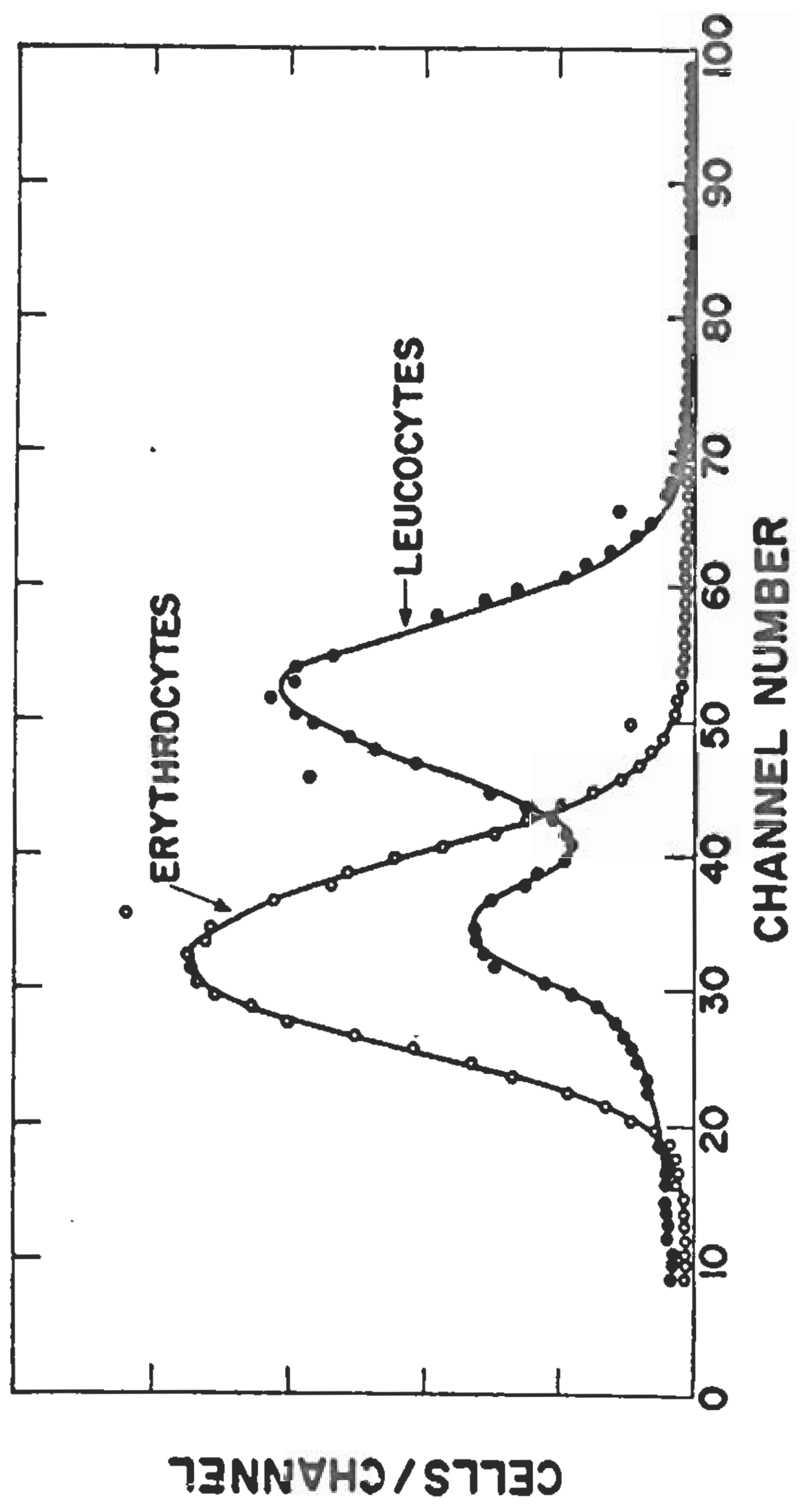

Fig. 2. Pulse-height distribution of normal erythrocytes and leucocytes obtained with a modified Vickers counter.

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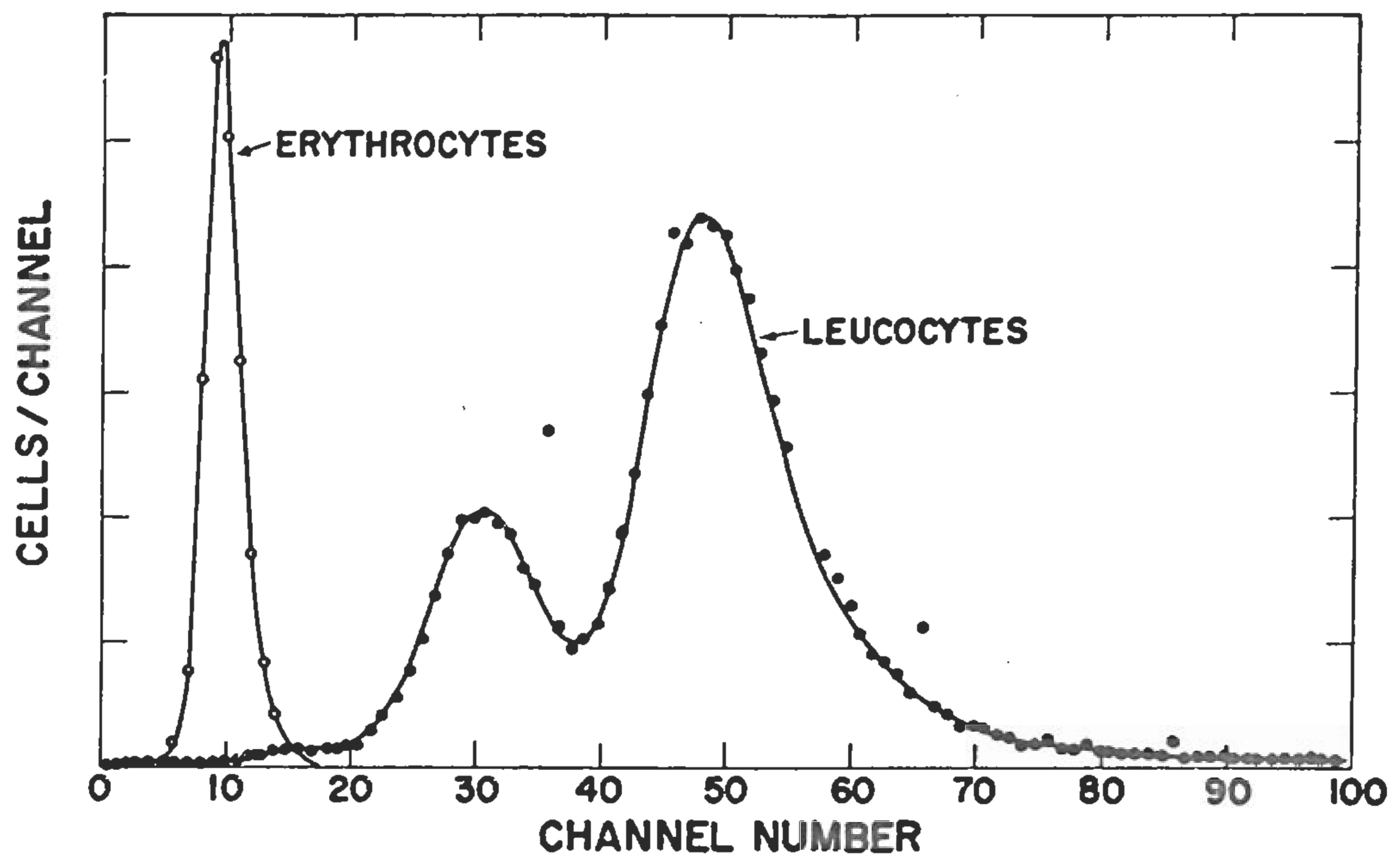

Fig. 3. Pulse-height (volume) distribution of normal erythrocytes and leucocytes obtained with a Coulter spectrometer.

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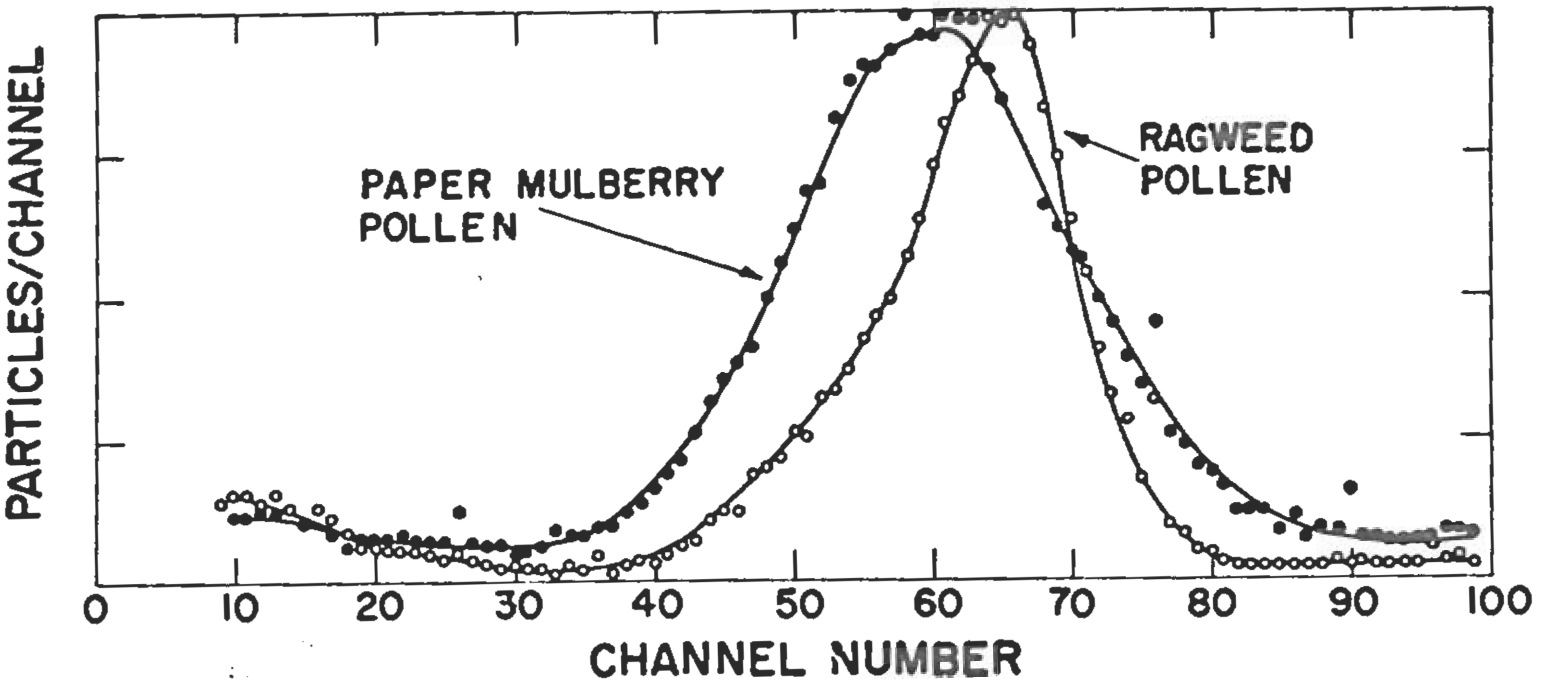

Fig. 4. Pulse-height distribution of ragweed and paper mulberry pollens obtained with a modified Vickers counter.

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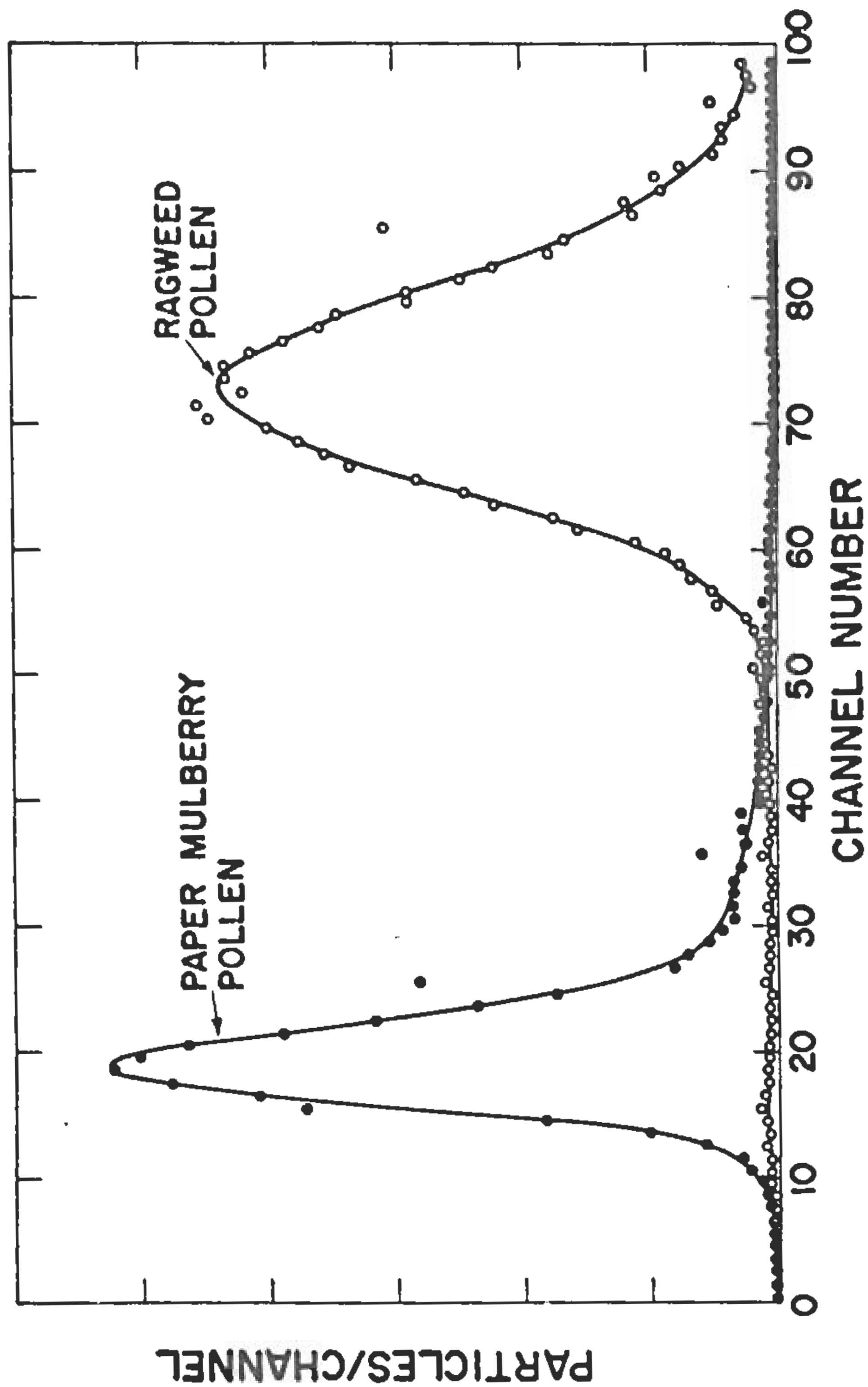

Fig. 5. Pulse-height (volume) distribution of ragweed and paper mulberry pollens obtained with a Coulter spectrometer.

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sensor. With this sensor the pulse-height distributions shown in Figs. 2, 3, 4, and 5 were obtained. Now the shape of the leucocyte distribution agrees quite well with the volume distribution, but again the optical sensor shows little difference between lymphocytes and erythrocytes. In addition, the erythrocyte standard deviation is now larger (about 21 percent) than that observed with either the Coulter or Sanborn-Frommer sensor. This may be a cell orientation effect due to the disc shape of the red cell. In all three sensors the fluid flow pattern would be expected to cause the red cell axis to line up perpendicular to the flow direction. In the Sanborn-Frommer sensor this would result in fixed orientation to the light beam, and in the Coulter sensor independence of orientation. However, in the Vickers sensor red cells would be expected to orient with respect to the beam edgewise, broadside, or an angle in between, thus producing a wider pulse-height distribution. The difference between the leucocyte spectra from the two optical sensors was quite unexpected but may have to do with the angle of acceptance of scattered light. This question will be investigated with the more flexible experimental arrangement discussed below.

In Figs. 4 and 5 we see again a striking difference between the volume spectrum (Coulter sensor) and light-scatter spectrum (Vickers sensor) of two different particles, this time ragweed and paper mulberry pollens. This emphasizes the point that different particle properties are being detected. The theory of light-scattering by particles large compared to the wavelength of the incident light (4,5) applies to all particles and cells discussed above; the ratio of particle diameter to wavelength is about 20 to 1. In this case the concepts of physical optics apply, and incident light interacts with the particle by ordinary processes of reflection, refraction, and diffraction. All of the light incident on the particle is removed from the beam by reflection from the surface, refraction, traversal of the interior (with some absorption), and exiting through the opposite surface. These processes cause large-angle deviations from the incident beam direction. An equal amount of light is diffracted, but this light is confined to small angles (about 3 to 4°). Thus the angle subtended by the photomultiplier is probably quite important in determining what cellular properties are being sensed. This point is under investigation with an experimental system which uses a laser light source (Spectra-Physics, Model 130 B) and the Vickers scattering chamber arranged to allow variation of scattering angle.

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VOLUME DISTRIBUTION OF NORMAL HUMAN ERYTHROCYTES AND LEUCOCYTES (M. A. Van Dilla and J. M. Hardin)

INTRODUCTION

Leucocytes have been concentrated from the whole blood of normal individuals, their volume measured, and the resulting data subjected to computer analysis. Each peak of the bimodal volume distribution can be well fitted by a skewed normal distribution -- the same function that gives a good fit to erythrocyte volume distribution data. The coefficient of variation of each leucocyte peak and erythrocyte peak is close to 15 percent; this is about what has been observed for several types of mammalian cells in tissue culture at division or just after division. The leucocyte peak at larger volume has a tail that cannot be fitted, suggesting a different population. Subsequent studies showed this tail highly enriched in monocytes.

METHODS AND RESULTS

Erythrocyte distributions were obtained from whole blood diluted in physiological saline buffered at pH 7.3 in the way previously described (1). An aperture 40 microns in diameter and 190 microns long and an aperture current of 100 μA were used. The leucocytes were concentrated from whole blood by the method of Herbeuval et al. (2) and were measured under the same conditions. A computer program described by Dean (3) was used to fit the experimental data. Normal, log-normal, and skewed-normal functions were tried; the latter function gave the best fit for both erythrocyte and leucocyte data. The program reads-in the data, fits the peaks, prints out the parameters of the function, and plots the data points and computed function. Typical graphical computer outputs are shown in Fig. 1 (erythrocyte volume distribution) and in Fig. 2 (leucocyte volume distribution). In these plots, channel number is proportional to cell volume; the constant of proportionality for Fig. 1 was made one-fourth that for Fig. 2 so that both distributions would fall in mid-scale. Parameters of the computed functions are listed in Table 1, where σ/m is the coefficient of variation (i.e., ratio of standard deviation to mean) and γ is the coefficient of skewness (4).

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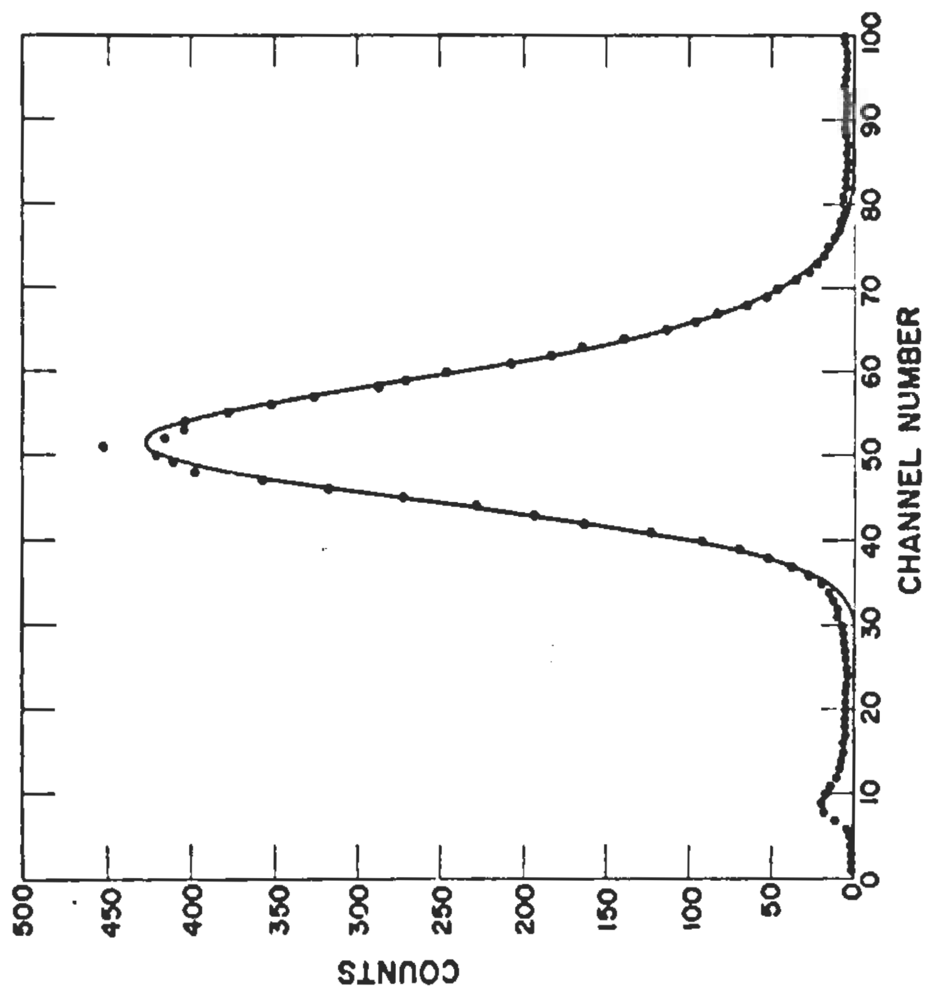

Fig. 1. Volume distribution of erythrocytes from a normal adult.

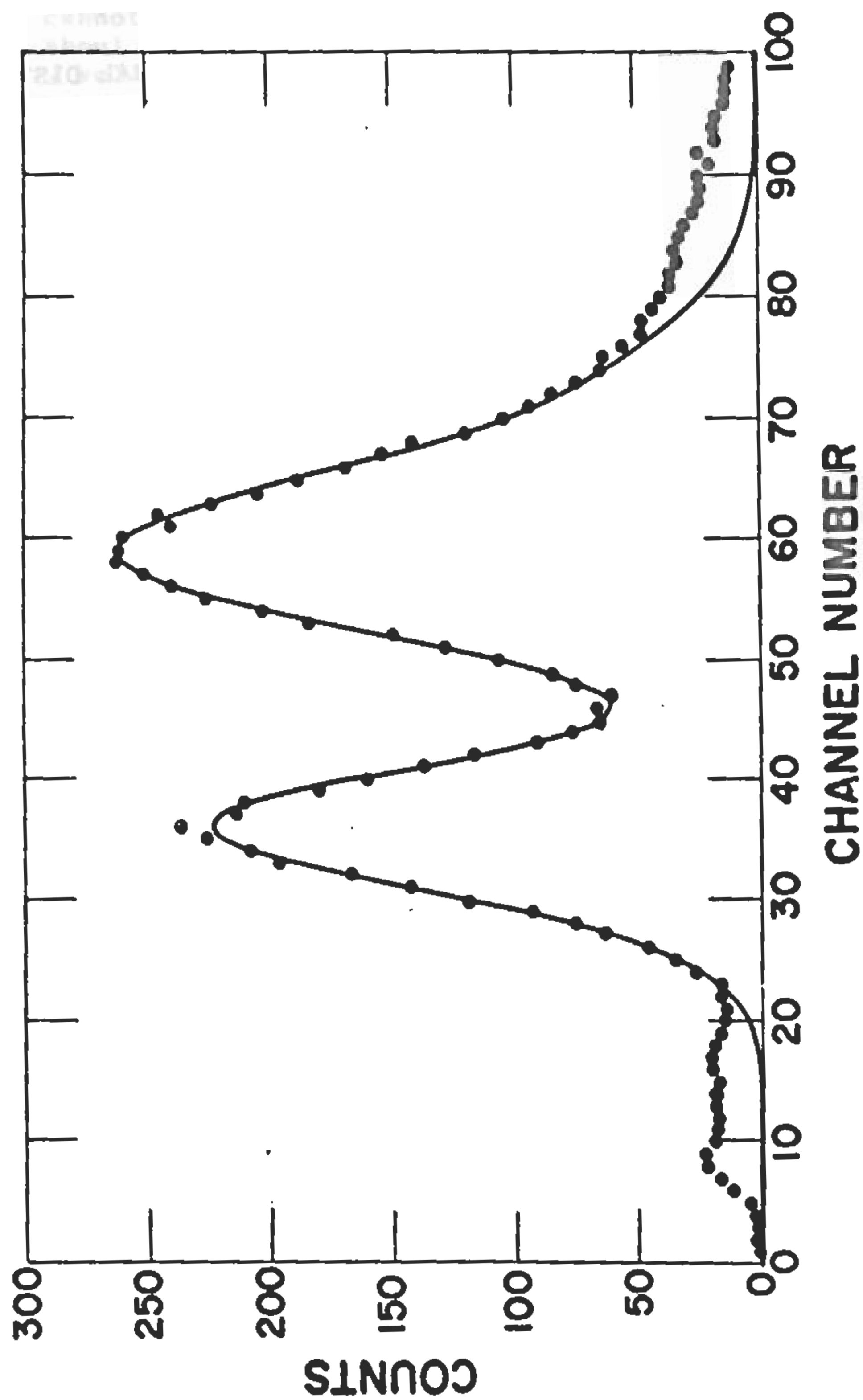

Fig. 2. Volume distribution of leucocytes from a normal adult.

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TABLE 1. PARAMETERS OF COMPUTED SKEWED NORMAL DISTRIBUTION FUNCTION WHICH BEST FITS BLOOD CELL DATA FOR A NORMAL ADULT

Cell Type	σ/m (percent)	γ	Mean Volume Relative to Erythrocytes
Erythrocyte	14	0.40	1.0
Leucocyte (peak 1)	13	0.23	2.6
Leucocyte (peak 2)	15	0.67	4.6

DISCUSSION

Experiments with the cell separator (5) have shown that the leucocyte peak with the smaller mean volume is due mostly to lymphocytes and that the other peak is due mostly to granulocytes with the large-volume tail (not fitted) highly enriched in monocytes. Thus, we see that volume distributions of normal erythrocytes, lymphocytes, and granulocytes are quite similar in shape, with similar coefficients of variation and asymmetry. Lymphocytes are larger than erythrocytes by a factor of about 2.5 and granulocytes by a factor of about 4.5. Data on several other normal adults are very similar, so that the results presented above are typical.

It was a surprise to find similar distributions for diverse cell types of quite different morphology and function and, in the case of lymphocytes, site of origin. Data of E. C. Anderson of this Laboratory on CHO, HeLa, and mouse L cells indicate that the volume distributions of these mammalian cells at division and when in the steady state have coefficients of variation of about 15 percent. Studies by Kubitschek (6) on cell generation rates show a coefficient of variation of 10 to 20 percent for several widely different kinds of cells. It is conceivable, therefore, that there exists a generalization which states that cells of a given

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type cannot be too dissimilar in size (coefficient of variation about 15 percent) when growing in the steady state (i.e., volume distribution constant). The mechanism is unknown.

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VOLUME DISTRIBUTION OF MOUSE BONE MARROW CELLS (M. A. Van Dilla, J. M. Hardin, and C. F. Bidwell)

INTRODUCTION

Bone marrow plays a vital role in the biological effects of radiation and recovery from radiation injury. Much can be learned from study of the circulating blood cells (1,2), but study of the source of these cells in the bone marrow is of primary importance whenever possible. A recent paper by Lee and Richards (3) describes bone marrow cell volume distributions for normal and irradiated mice which contrast with the results obtained at this Laboratory, as described below.

METHODS AND RESULTS

An amount of blood equal to 2 percent of body weight was taken by orbital bleeding from 15 RF female mice, and volume distributions of the circulating blood cells and femoral bone marrow cells were measured at daily intervals thereafter for 5 days. The animals were 3 to 4 months old; average weight was 25 g. Bone marrow cells were obtained from the femur by cutting the ends off and flushing physiological saline buffered at pH 7.3 through each shaft with a No. 23 needle on a 2-ml syringe to yield 5 ml of cell suspension. Passage through the needle several times was sufficient to break up clumps with no apparent damage to the cells. Dilution by a factor of 25,000 was followed by a 1-minute measurement of volume distribution of cells from each femur in a Coulter spectrometer (4). Whole blood was diluted by a factor of 10^5 and measured in the same way. Blood smears were examined for reticulocytes using standard techniques.

Circulating cell volume distributions were fit by a skewed Gaussian function using computer methods (5). The data for the initial 2 percent bleeding of the 15 animals are shown in Table 1. The parameters of the fitted functions are area (proportional to cell count), mean channel number (proportional to mean cell volume), standard deviation, and skewness coefficient (upon which the asymmetry of the distribution depends). Accurate calibration of channel number in terms of volume in cubic microns was not undertaken. An approximate calibration with Dow monodisperse polyvinyltoluene latex spheres yielded a conversion factor of 0.91 μ^3/channel,

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TABLE 1. PARAMETERS OF ERYTHROCYTE VOLUME DISTRIBUTION OF 15 NORMAL MICE

Mouse No.	RBC (in 10^6 cells/mm^3)	Mean Channel Number	Standard Deviation (channels)	Skewness Coefficient
1	10.30	42.4	6.32	0.316
2	11.90	43.4	6.84	0.237
3	9.58	41.9	6.19	0.256
4	10.70	43.2	6.48	0.246
5	9.70	42.5	6.31	0.292
6	9.71	42.2	6.15	0.240
7	10.50	43.5	6.41	0.250
8	10.60	42.6	6.15	0.293
9	9.42	42.9	6.39	0.304
10	10.50	42.2	6.14	0.295
11	11.00	42.2	6.11	0.284
12	9.81	43.4	6.25	0.272
13	9.28	43.2	6.23	0.271
14	10.40	43.1	6.24	0.250
15	9.92	42.3	6.15	0.280
Mean	10.20	42.8	6.29	0.269
1 (2 hours)	8.67	52.9	7.93	0.180

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so that channel number 42.8 = 39 μ^3. Table 1 also shows the deleterious effect on erythrocytes of standing in the saline counting solution for 2 hours, emphasizing the point that the counting suspension should be measured quickly after preparation.

Anemia caused by bleeding stimulated a macrocytosis illustrated in Figs. 1 and 2. Figure 1 shows the circulating erythrocytes 3 days after bleeding compared with a control. Figure 2 shows the variation of erythrocyte volume distribution parameters with time after bleeding and also the reticulocyte count as percent of circulating cells determined from the stained slides.

The volume distribution of normal mouse bone marrow cells is shown in Fig. 3, along with the normal erythrocyte volume distribution. Bleeding stimulated the marked changes shown in Fig. 4. All bone marrow cell distributions represent a standard dilution of cells from a single femur measured under standard conditions so that the vertical scales are identical.

DISCUSSION

The control erythrocyte volume distribution data are notably homogeneous. Thus, mean channel number averaged over the 15 mice is 42.8 $\pm$ 0.52, or a coefficient of variation (i.e., percent standard deviation) of about 1 percent. Hence, mean erythrocyte volumes are very tightly distributed for this group of animals. Inspection of Table 1 shows further that the other parameters of the cell distribution function also show little variance, with the exception of area (i.e., red cell count). This exception may be due to the fact that red cell count is directly proportional to fluid content of the blood; total red cell mass would be a more meaningful quantity but much more difficult to measure accurately.

Figure 1 shows that the effect of bleeding is a macrocytosis, as reported by Brecher and Stohlman (6). A similar effect has been noted following radiation-induced bone marrow arrest (1,2). Figure 2 shows that the skewness coefficient is the parameter most correlated with degree of reticulocytosis; variation of this parameter with time after bleeding closely parallels variation of the reticulocyte fraction seen in stained preparations. The standard deviation is also correlated with reticulocyte count; mean channel number (not plotted) shows a less striking correlation.

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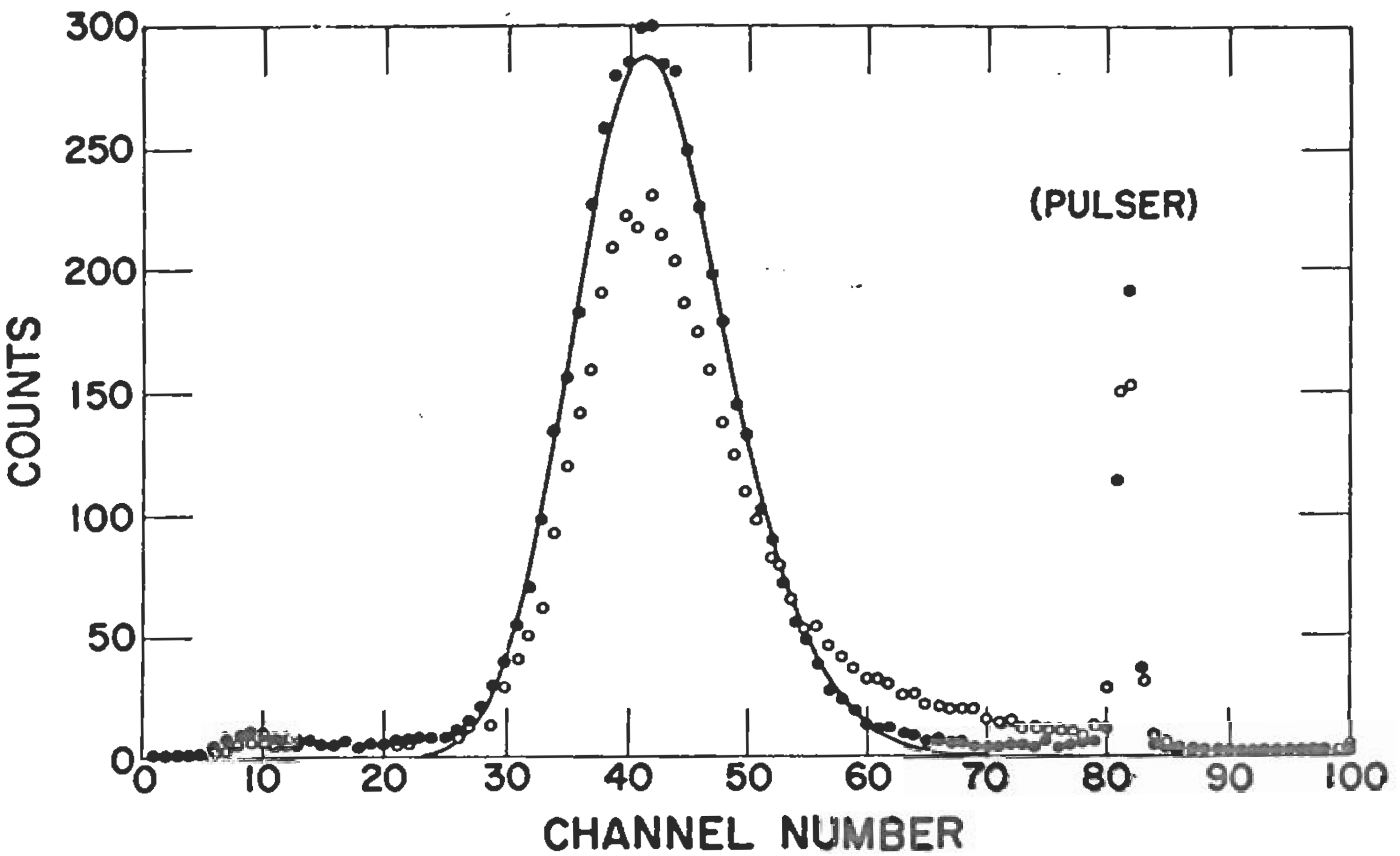

Fig. 1. Erythrocyte volume distribution of a mouse 3 days after bleeding (●) compared with control (O); solid line is computer fit of control data.

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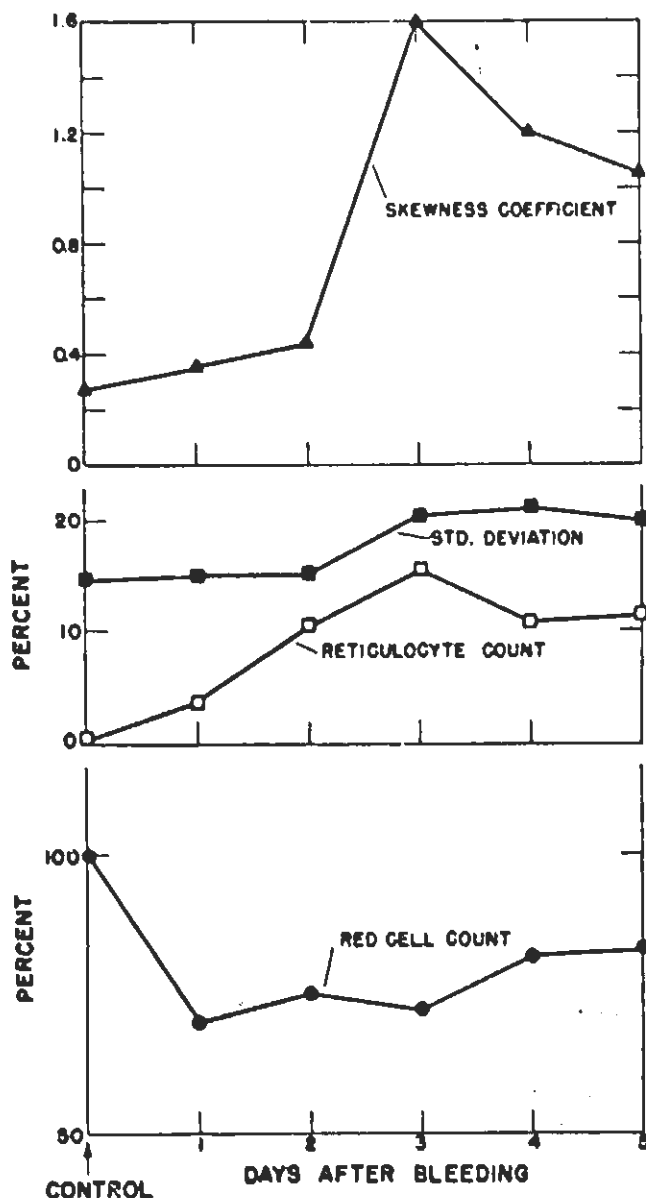

Fig. 2. Dependence of parameters of erythrocyte volume distributions on time after bleeding. Skewness coefficient is dimensionless. Standard deviation is expressed as percent of mean red cell volume. Reticulocyte count is expressed as percent of total red cells. Red cell count is expressed as percent of control value.

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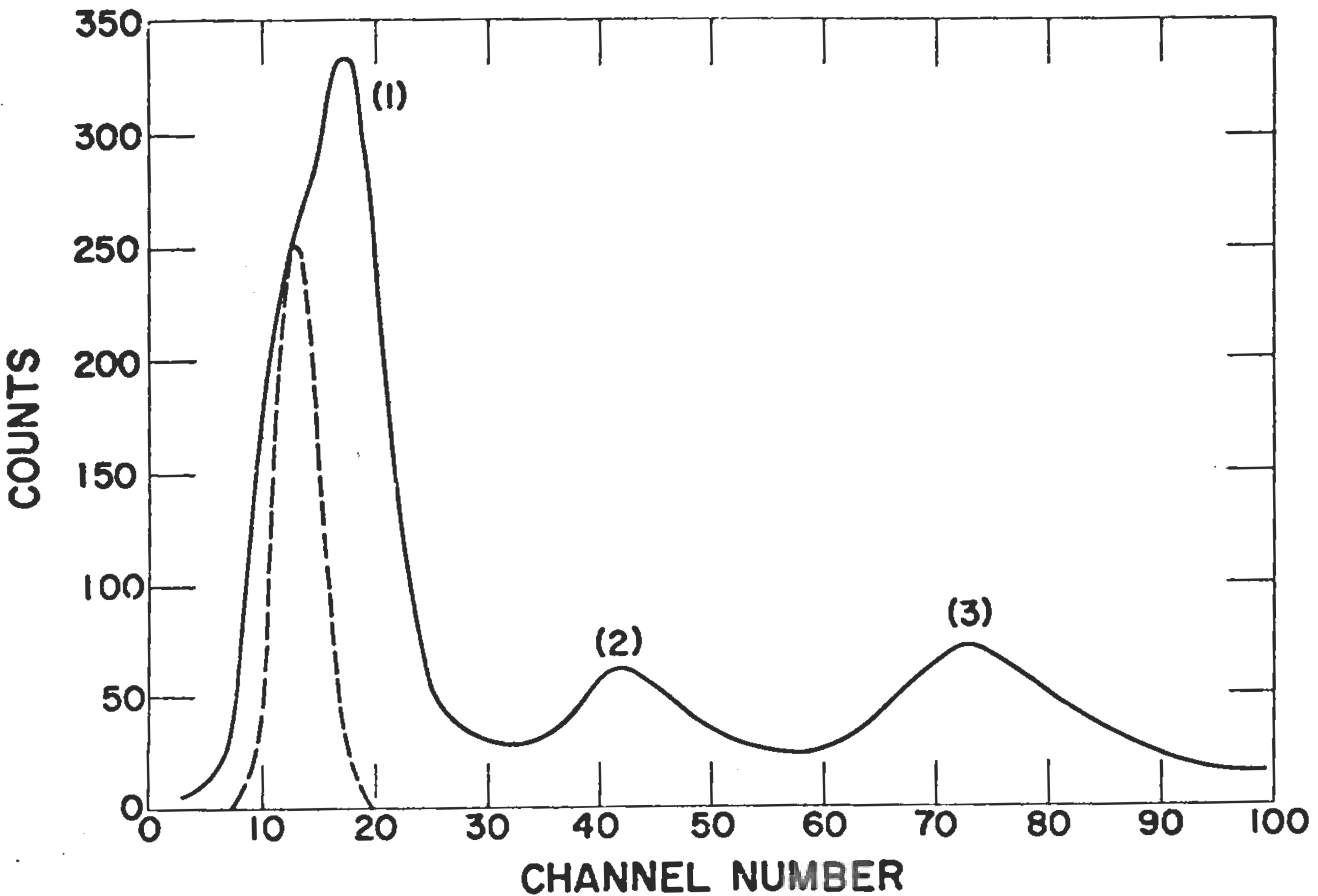

Fig. 3. Normal bone marrow cell volume distribution (———) compared with circulating erythrocyte distribution (-----).

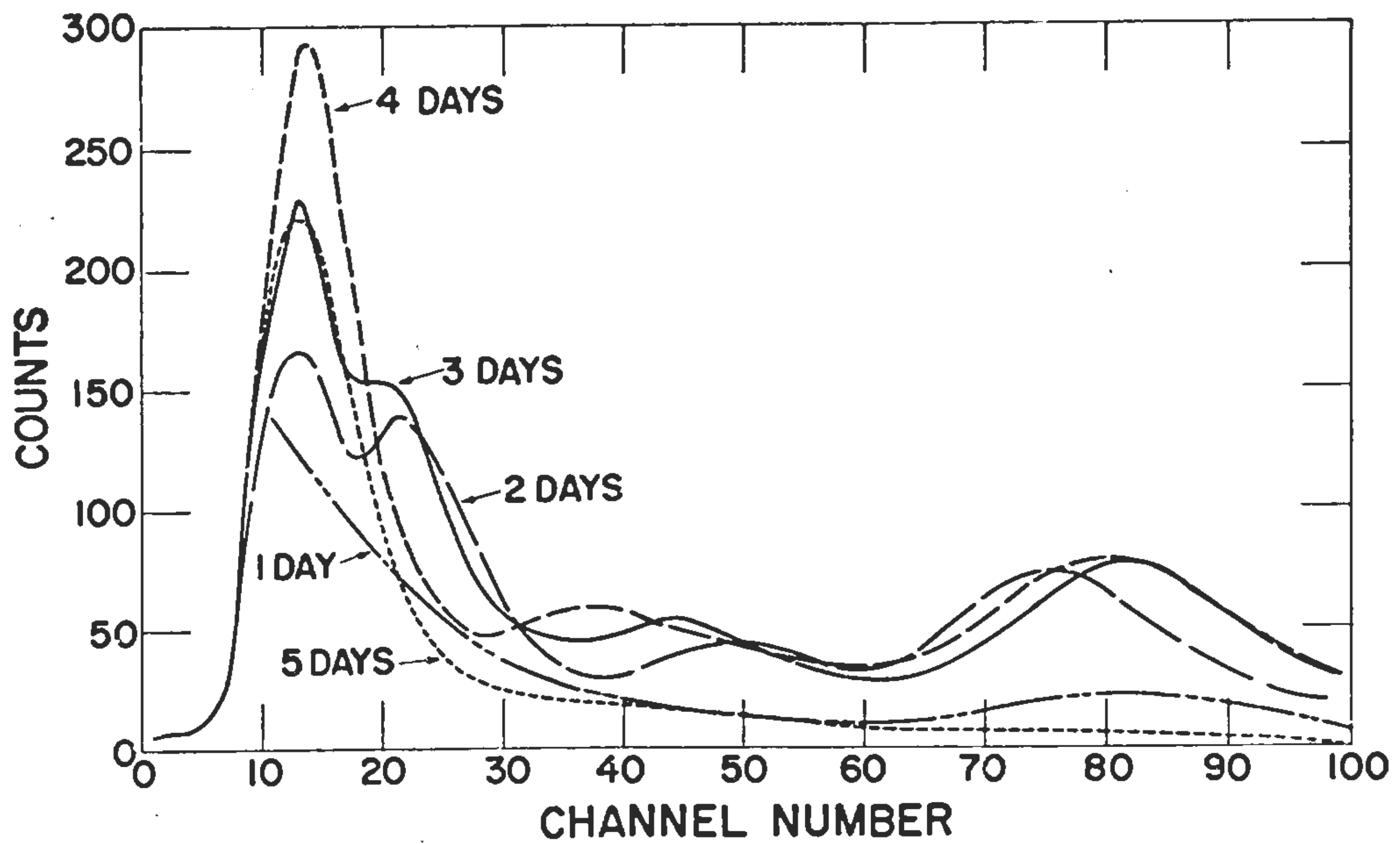

Fig. 4. Changes with time in volume distribution of bone marrow cells induced by bleeding (0.5 ml).

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Normal bone marrow cell distribution (Fig. 3) has 3 peaks. The first (at minimum volume) is very broad and appears composite, perhaps a mixture of circulating erythrocytes and late marrow cells of the erythroid series. The cell types comprising the other 2 peaks are not obvious, but it seems likely that they are more immature precursors of the erythroid and granulocytic series. A comparison between Fig. 3 and the comparable data of Lee and Richards (3) shows marked differences. Their control distribution does not show a peak corresponding to our peak 1; they say these cells are excluded, since they were mostly erythrocytes mixed with a great deal of debris. Nor is there a peak corresponding to our peak 2. Their only peak (at 40 threshold units) corresponds to our peak 3.

The reason for these differences is not clear. Lee and Richards also report that their only peak disappears with whole-body X-ray doses of 100 r and over, in contradiction of their data on "cellular dry mass and the percent of radiation-induced giant cells." They attribute this to "changes in conductivity of the cell caused by cell membrane injury from irradiation." We have some, although certainly inconclusive, evidence to the contrary in our mouse bone marrow arrest experiments (1,2); after doses of 1600 rads spread over a 2-week period, no such effects could be observed on macrocytic reticulocytes or erythrocytes.

Bone marrow cell distributions undergo considerable change after bleeding (Fig. 4). Peak 1 disappears at 1 day and then shows a bimodal structure which suggests a mixture of circulating erythrocytes (channel 13) and marrow reticulocytes or late normoblasts (channel 21). Two cell groups of similar volume were observed in the circulating blood of mice in the bone marrow arrest experiments (1,2) and were identified as erythrocytes and macrocytic reticulocytes by microscopic examination. If the morphology of the erythrocytic and granulocytic series and the myeloid-to-erythroid ratio in mice are similar to humans, then perhaps peak 2 is predominantly erythrocytic and peak 3 predominantly granulocytic. Experiments with the cell separator (7) are planned to clarify this situation.

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BIOPHYSICS SECTION

PUBLICATIONS AND ABSTRACTS OF MANUSCRIPTS SUBMITTED

SOME BIOLOGICAL ASPECTS OF RADIOACTIVE MICROSPHERES, W. H. Langham, C. R. Richmond, J. C. Hensley, P. N. Dean, and M. A. Van Dilla. Los Alamos Scientific Laboratory Report LA-3365-MS (August 23, 1965).

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COMPUTER REDUCTION OF METABOLIC DATA OBTAINED FROM SCINTILLATION COUNTERS, P. N. Dean. Los Alamos Scientific Laboratory Report LA-3298 (November 15, 1965).

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ELECTRONIC SEPARATION OF BIOLOGICAL CELLS BY VOLUME, M. J. Fulwyler. Science 150(3698), 910-911 (1965).

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AUTOMATIC DATA ACQUISITION, REDUCTION AND ANALYSIS, P. N. Dean and C. R. Richmond. In Radioisotope Sample Measurement Techniques in Medicine and Biology, International Atomic Energy Agency, Vienna (1965). pp. 139-168.

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COMPUTER ANALYSIS OF CELL VOLUME DISTRIBUTIONS, P. N. Dean. Los Alamos Scientific Laboratory Report LA-3440 (February 7, 1966).

Two computer programs have been written to analyze cell volume distributions measured with a Coulter type of cell spectrometer. One of the programs, called AVØL, is used to calculate the mean cell volume of any type of distribution and to plot the data in various formats. The other program, called CELVØL, uses an iterative least-squares technique to fit either normal, log-normal, or skewed-normal distributions to the data. Either single- or double-peak distributions can be fitted. The program also converts the results of the fit to cell concentration in each peak in cells per cubic millimeter, standard deviation in cubic microns, and mean cell volume in cubic microns. For red blood cells the mean cell volume is also calculated from the hematocrit and included in the data output listing.

ISOLATION OF NORMAL AND ABNORMAL CIRCULATING CELLS BY AN ELECTRONIC PARTICLE SEPARATOR, I. U. Boone, M. J. Fulwyler, and M. W. Stewart. Proceedings of the American Association for Cancer Research 7, 8 (April 1966), 57th Annual Meeting, Denver, Colorado. Abstract No. 28.

Separation of discrete cellular fractions of normal and abnormal WBC and abnormal circulating cells on the basis of cell volume has been undertaken using an electronic particle separator (Fulwyler, Science 150:910, 1965). Cell volume distribution is first obtained using a modified Coulter aperture, and cells are subsequently isolated in droplets of suspending medium which are charged and deflected according to sensed volume. Circulating normal WBC were separated into lymphocyte-, granulocyte- and monocyte-rich fractions. Mononuclear cells from patients with infectious mononucleosis were separated into four distinct fractions and subsequently lymphocytes from at least two fractions cultured in artificial medium. Viability of separated cells was proven by obtaining chromosomes with usual techniques used in cultured cells. Tne technique has been further extended to isolation and concentration of large abnormal hematopoietic elements and cancer cells from peripheral blood of a patient with terminal neuroblastoma.

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THE CELL SEPARATOR -- DESIGN AND USAGE, M. J. Fulwyler. Radiation Res. 27, 501 (1966). Abstract No. Ca-4.

An electronic device has been developed that is capable of separating particles (suspended in a conducting liquid) on the basis of volume. Particle volume is sensed by a Coulter aperture. The suspension is ejected from a nozzle as a fluid jet and is uniformly broken into droplets by high frequency vibrations produced by a piezoelectric crystal. It takes 250 μsec for a particle to travel from the volume-sensing point to the jet separation point, where it is caught in a forming droplet. At the time of formation droplets are given an electric charge predetermined from the measured volume of the contained particle. The charged droplets pass through an electrostatic field and are deflected in proportion to their charge. The deflected droplets are collected in a series of vessels.

Volume fractions of mouse lymphoma cells randomly growing in suspension culture have been separated, and mixtures of chicken and human red blood cells have been quantitatively separated. Volume fractions have been isolated from normal human white blood cells and from white blood cells found in disease states such as leukemia and infectious mononucleosis. Application is being made to bone marrow cytology using mice. Tests with Chinese hamster ovary cells showed 98 percent viability and normal growth rate after passing through the device.

The separation principle may be extensible to the separation of particles on the basis of any electronically measurable property such as ultraviolet absorption or fluorescence. We are investigating such sensors.

ERYTHROCYTE VOLUME DISTRIBUTION DURING RECOVERY FROM RADIATION-INDUCED BONE MARROW ARREST, M. A. Van Dilla and J. F. Spalding. Nature (in press).

It has been shown that macrocytosis follows erythropoietic stimulation by phenylhydrazine anemia, bleeding, or administration of erythropoietin. Here we report that bone marrow arrest induced by whole-body radiation also produces a similar macrocytosis in mice. It was observed in the course of the experiment that the volume of mouse red cells decreases as they age.

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VOLUME DISTRIBUTION AND SEPARATION OF NORMAL HUMAN LEUCOCYTES, M. A. Van Dilla, M. J. Fulwyler, and I. U. Boone. Science (submitted).

The volume distribution of human leucocytes has been obtained using a modification of the Coulter counter. The position of lymphocytes, granulocytes, and monocytes within this distribution has been determined by morphological examination following isolation of cell groups of desired volume with an electronic cell separator.