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LOS ALAMOS SCIENTIFIC LABORATORY
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BIOLOGICAL AND MEDICAL RESEARCH GROUP (H-4) OF THE HEALTH DIVISION -- ANNUAL REPORT JULY 1962 THROUGH JUNE 1963

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

Contract W-7405-ENG. 36 with the U. S. Atomic Energy Commission

CHAPTER 1

INTRODUCTION

During this report period (FY 1963), reorientation of the biomedical research program toward molecular and cellular level studies continued at an accelerated rate. As a result, there have been a large number of changes in organization involving new hires, terminations, transfers of personnel within the group, and redefining and reallocating of responsibilities.

(a) Program Orientation

Continuing to increase emphasis on more fundamental research at the cellular and molecular levels and the termination of Dr. Lushbaugh, Section Leader of the Clinical Investigations Section, are resulting in a less diversified biomedical research program than in past years. During the next report period (FY 1964), projects involving clinical applications of radiation and radioactive isotopes will be brought to a reasonable conclusion, the section discontinued,

and the remaining personnel transferred to other sections. Some of the effort of the Low-Level Counting Section will be diverted also to other sections because of decreased interest in fallout as a result of the nuclear test ban treaty. This will result in the FY 1964 research activities being confined largely to the following program categories:

06-01-01	General Radiation Effects (Mammalian Radiobiology Section)
06-01-02	Toxicology of Radioelements (Mammalian Metabolism Section)
06-02-02	Radiation Genetics (Mammalian Radiobiology Section)
06-04	Molecular and Cellular Level Studies (Molecular and Cellular Radiobiology Sections)
06-06	Radiological and Health Physics and Instrumentation (Low-Level Counting Section)

The Low-Level Counting Section will continue a curtailed effort in environmental radiation studies (06-05), consisting of projects of potential interest to civil defense.

(b) Terminations

The following terminations occurred during or shortly after the end of the present report period:

Dr. C. C. Lushbaugh (Section Leader, Clinical Investigations Section).

GROUP H-4

BIOMEDICAL RESEARCH

W. H. Langham, Ph.D., Leader
D. G. Ott, Ph.D., Alt. Group Leader
O. S. Johnson, B.S., Asst. Ldr. for Administration
E. M. Sullivan, Group Secretary

MAMMALIAN METABOLISM

C. R. Richmond, Ph.D., Leader

Staff Member

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J. E. London, B.S.
G. A. Trafton, B.S.

Technical Staff

S. Cordova
R. Martinez

*Casual employee.

MAMMALIAN RADIOBIOLOGY

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Research Assistants

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G. L. Oakley, B.A.

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D. G. Valdez
F. Valdez
E. A. Vigil

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M. J. Fulwyler, B.S.
J. H. Larkins, B.S.*
J. D. Perrings

Technical Staff

R. L. DePriest, B.S.

Electronics Technicians

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A. R. Vigil

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S. H. Cox, B.A.
P. M. LaBauve, B.A.
P. C. Sanders, M.S.
F. Sapir, M.S.

Technical Staff

N. J. Basmann
V. M. Gibbs

Leave of Absence

I. U. Boone, M.D.

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F. N. Hayes, Ph.D.,
Leader

Staff Members

E. C. Anderson, Ph.D.
C. T. Gregg, Ph.D.
D. E. Hoard, Ph.D.
V. N. Kerr, M.A.
A. Murray, M.S.
R. L. Ratliff, Ph.D.
G. R. Shepherd, Ph.D.
T. T. Trujillo, B.S.
D. L. Williams, M.S.

Research Assistants

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P. A. Goldman, B.S.
E. Hansbury, M.A.
R. E. Hine, B.S.
E. H. Lilly, B.S.
B. J. Noland, B.A.

Technical Staff

V. E. Mitchell

Electronic Measurement of Cellular Volumes. IV. Determination of Scaling and Correction Factors for Conversion of Voltage to Cubic Microns

(C. C. Lushbaugh, D. B. Hale, and N. J. Basmann)

INTRODUCTION

Electronic measurement of cellular volumes has numerous potential applications in experimental and clinical hematology (1-4). The usefulness of these applications is presently limited by difficulties involved in accurate calibration of the electronic apparatus and in the mathematical interpretations of the results. The translation of frequency distribution profiles of pulse height voltages into populations of erythrocytes of varying volumes presents semantic difficulties to hematologic microscopists. A particle volume analyzer system assembled around an electronic particle counter* was recently described which seemed to resolve many of these difficulties and to produce hematologically useful results (1,2). Since then, this apparatus has been improved by several commercially available electronic units. In addition, a more accurate system of calibration has been developed which allows correction for changes not only in aperture currents as

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*Coulter Electronics, Miami, Florida.

previously described (1) but also for changes in aperture diameter, amplifier gains, and pH of the conducting solution used to suspend the cells. These determinations and the resulting scaling factors show that a much wider range of particles or cells can be sized with this device than previously envisioned. They also reveal that such an analyzer system and its set of apertures must be individually calibrated if actual, rather than relative, erythrocyte volumes are desired. The present paper reports a method for the comprehensive calibration of such equipment and the development of the various necessary scaling factors which are needed to convert pulse height voltages into cubic microns so that measurement and distribution of cellular volumes varying in size from bacteria to the largest HeLa cells can be determined.

DESCRIPTION OF THE APPARATUS

The particle volume analyzer system is composed of 9 pieces of equipment, as shown in Fig. 1. These are interrelated electronically as previously diagrammed (1). The basic units (Fig. 1, C and E) are the aperture tube and mercury manometer chassis (C), where the pulses are generated as the particles pass through the aperture (ApD) in the aperture tube and are enumerated in the particle counter (E).

This particle counter (Coulter Model A) was modified so that the internal coarse control for amplifying pulses after they were formed could be changed at will by turning an external dial knob (g) through 6 positions. The internal fine gain control was left fixed at 2.3 on its dial, since with this setting changes in the coarse gain (g) were constant. The Coulter threshold knob (t) was kept at 10 in order to delete counts due to small debris during the RBC counts used with Hmct to determine MCV. Since the pulses from the Coulter apparatus were obtained for pulse height analysis from the cathode follower circuit (1), the position of the Coulter threshold setting (t) does not limit the size of the pulses (PHv) going to the pulse height analyzer unit (A). Dial a of the Coulter counter enables the choice of 8 different currents (ApC) through the sizing aperture (ApD). The 400-channel analyzer (A) is divisible into four 100-channel units with separate memories so that 4 analyses can be made sequentially before printing out results. Correction for analyzer dead time is made automatically so that variable concentrations of particles are counted with constant statistical errors. As this unit sorts and stores with voltage of a pulse, this event is recorded on a cathode display of the contents of the 100 channels and a "count" is subtracted by the store pulse scaler unit (I). This unit (I) counts down

Fig. 1. The pulse height-particle volume analyzer and its component parts: (A) 400-channel pulse height analyzer;* (B) X-Y plotter or automatic grapher;** (C) aperture tube-manometer chassis;+ (D) digital recorder or numerical readout;++ (E) electronic particle counter, pulse amplifier (g), threshold control (t), and aperture current (ApC) control unit (a);+ (F) X-Y plotter calibration control unit;‡ (G) preset count control unit;‡‡ (H) pulse height calibration control unit;° and (I) store pulse scaler unit.°°

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*Model 34-12, Radiation Instrument Development Laboratory, Inc., Melrose Park, Illinois.

**Model 2, F. L. Moseley Company, Pasadena, California.

+Model A, Coulter Electronics, Miami, Florida.

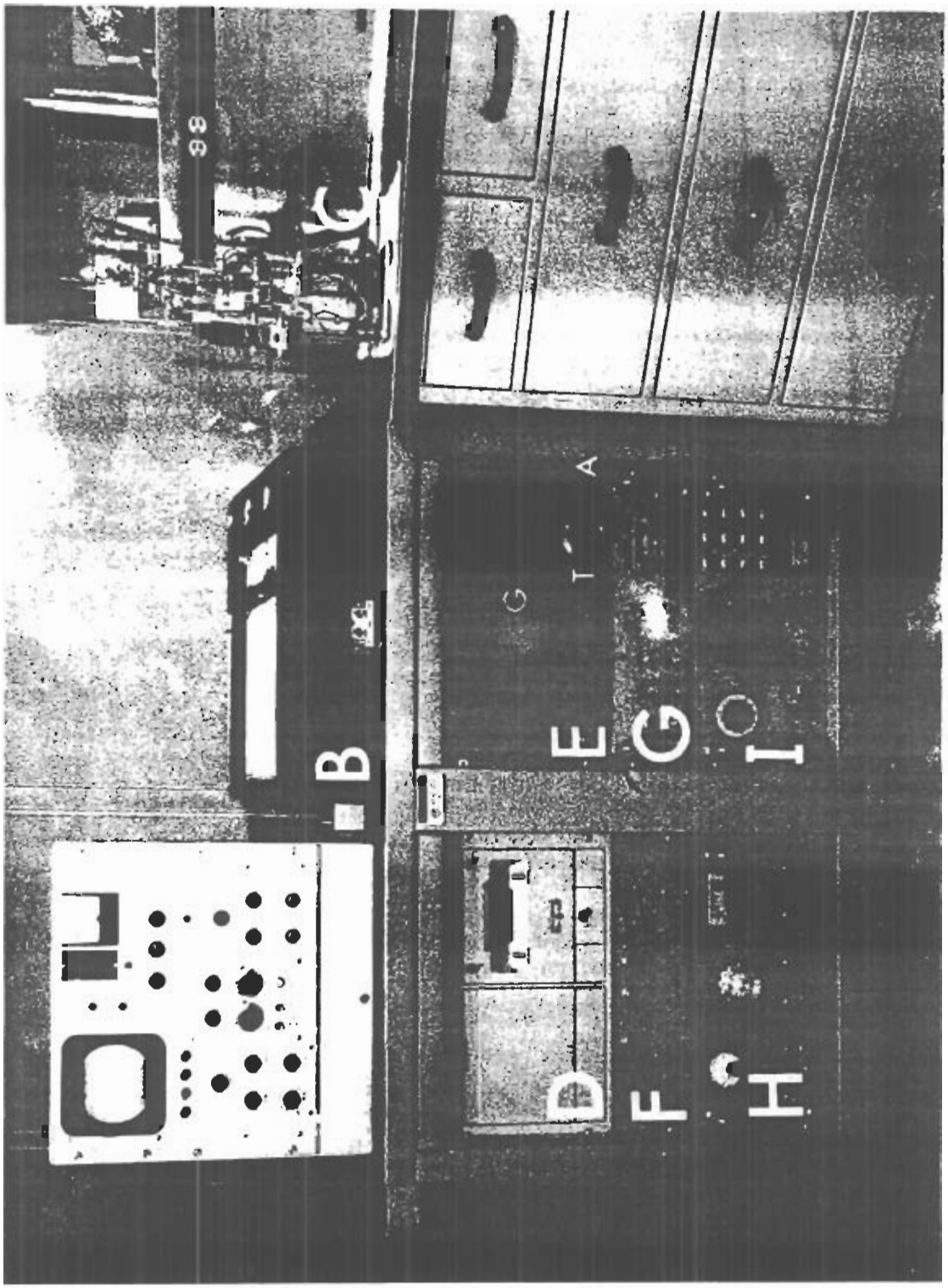
++Model 562-A Hewlett Packard Company, Palo Alto, California.

‡Model 34, Drawing 4Y-40796, Los Alamos Scientific Laboratory.

‡‡Model 7, Drawing 4Y-40806, Los Alamos Scientific Laboratory.

°Model 1560, Drawing 4Y-26828, Los Alamos Scientific Laboratory.

°°Model 750-A, Drawing 4Y-26065, Los Alamos Scientific Laboratory.


B
E
G
I
F
H

from any number preset on the preset count control unit (G) so that the number of analyses to be made for a given sample can be predetermined and controlled. For mathematical convenience, as well as for good statistics, the preset count in these determinations is kept at 100,000. RBC concentration in the solution being analyzed is kept at about 50,000/ml so that dead time loss is less than 10 per cent. Coincidence errors are not appreciable at this RBC concentration, and about 1 minute is required for each analysis. On completion of the analytical run, the data stored in the 100-channel analyzer memory are plotted out on the X-Y plotter (B) and printed out on tape by the digital recorder (D) on demand. The other 2 units shown in the apparatus (Fig. 1) are the plotter control unit (F), which determines that the X-Y axes of the graphic plotter correspond accurately with the analyzer, and a pulser (H), which enables control of the stability of the analyzer by direct measurement of pulse height voltage being stored in any particular channel from day to day.

In addition to the equipment shown in Fig. 1, 6 different aperture tubes with diameters (ApD) of 30, 50, 70, 100, 140, and 200 microns were used in this study. Another tube with an ApD of 10 microns was not used because it clogged too frequently and generated electronic noise with even low aperture currents.

CALIBRATION PROCEDURES

This pulse height analyzer system sorts PHv varying in height from 0.67 volt in channel 1 to 67 volts in channel 100, instead of 1 to 100 volts as in the previous analyzer (1), thus necessitating determination of a new scaling factor (F_1) for conversion of PHv to cellular volume (CV).

Red Blood Cell (RBC) Method

Red blood cells with different mean corpuscular volumes (MCV) were used as previously described (1,4), and an additional calibration was done with bacterial-sized latex particles.* The resulting line (Fig. 2) drawn by statistical analysis through the points correlating MCV with mean channel (MCh)** revealed that PHv from RBC 28.5 μ^3 in volume were being stored in channel 10 at ApD 100, ApC V, g 4. The number 2.85 is, then, the scaling factor (F_1) which enables conversion of any analyzer channel number into μ^3 at these electronic settings and physico-chemical conditions of the suspending solution (3,5-7).

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*Dow Chemical Company.

**Mean channel (MCh) corresponds semantically with mean threshold (MT) in terminology of the Coulter counter.

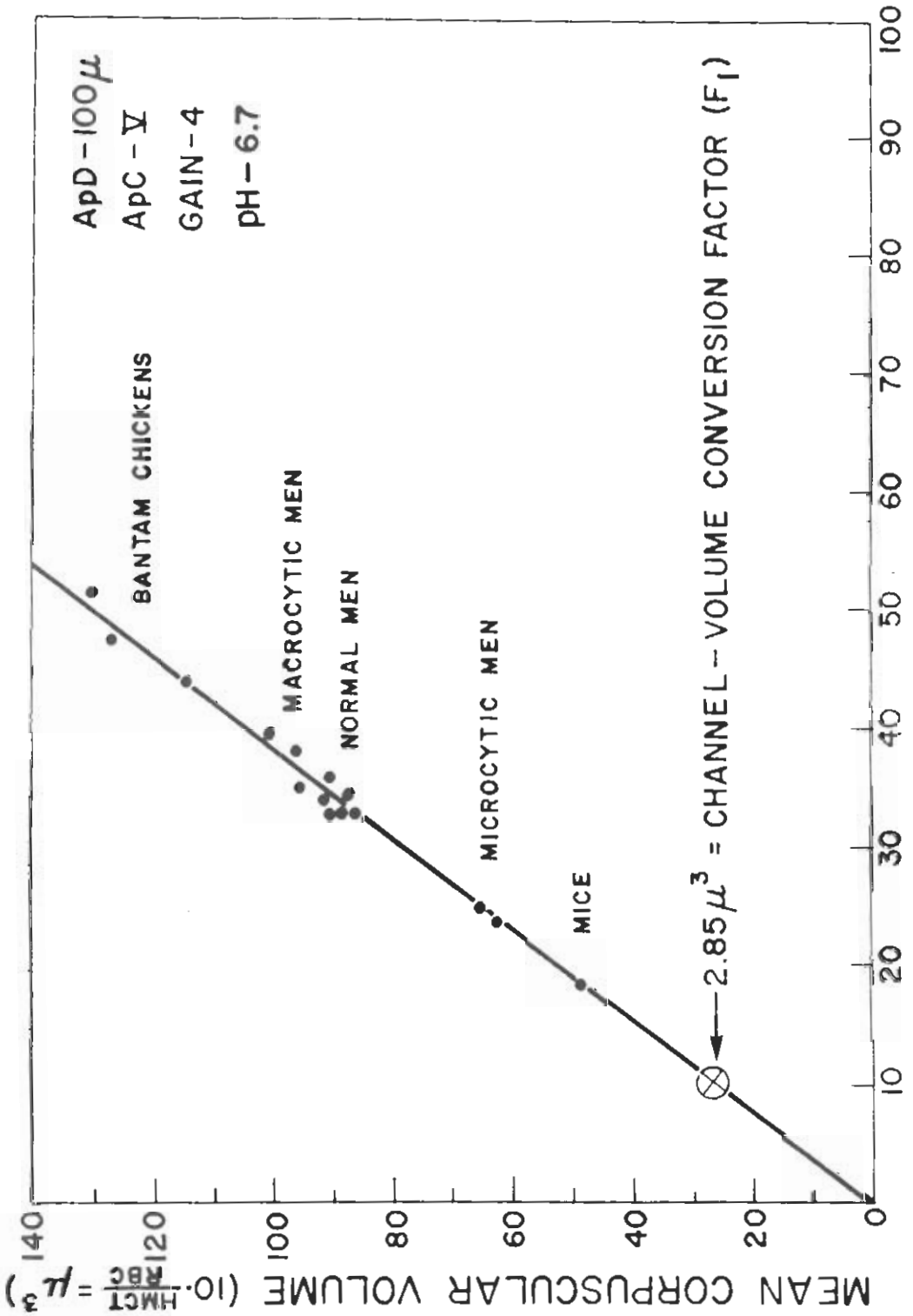

Fig. 2. The calibration line obtained for converting channel number (Ch) or pulse height volts (PHv) to cellular volume (CV) in cubic microns (μ^3) using RBC as "standard" particles.

Latex Particle Method

It is not necessary, however, to use RBC for determination of F_1 if other particles are available whose mean volume can be measured directly by some other means. Suspensions of latex particles of different mean diameters have recently been standardized and have become available commercially. The modal volumes of these particles are computed from their known mean diameter. Using three suspensions of latex particles of differing mean volume, frequency distribution profiles of their volumes were determined with appropriate ApD, ApC, and g settings. The best fit lines for these data were determined as shown in Fig. 3 (8). The volume of channel 10 is determined from these lines, and channel volume conversion factors (F_1) are then derived for the various ApD, ApC, and g. These values are tabulated in Table 1, along with F_1 for animal RBC.

Correction Factors

Only one F_1 needs to be determined directly with RBC or latex particles, since it is possible to determine further the correction factors which will compensate for use of other ApD, ApC, and g settings than those used in determining F_1. The definitions and purposes of these correction factors are outlined in Table 2. With these numerical factors, a

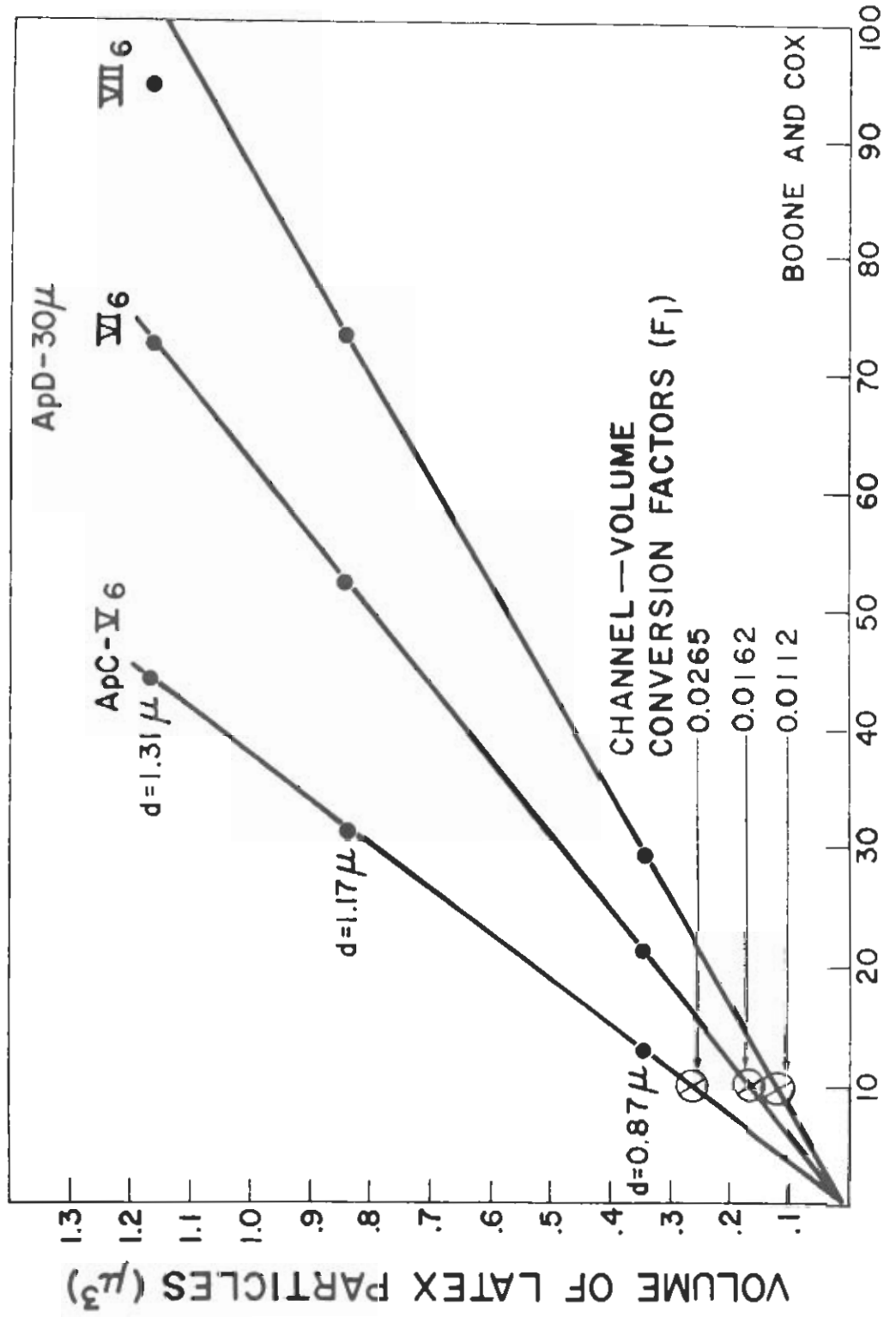

Fig. 3. Graph showing how 3 suspensions of latex particles of known mean diameter can be used to determine F_1 for a smaller ApD than that used with RBC.

TABLE 1. SCALING FACTORS (F_1) FOR CONVERTING CHANNEL NUMBER TO VOLUME

Latex Particles (5)

ApD = 30 μ, pH uncontrolled, 0.9 per cent saline

ApC V, g 6 = 0.0265 μ^3/Ch

ApC VI, g 6 = 0.0162 μ^3/Ch

ApC VII, g 6 = 0.0112 μ^3/Ch

Animal RBC

ApD = 100 μ, pH 6.7, 0.9 per cent saline

ApC I, g 1 = 111.6 μ^3/Ch*

ApC V, g 4 = 2.85 μ^3/Ch

ApC VII, g 1 = 3.04 μ^3/Ch*

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*By extrapolation using corrections $F_3 \times F_4$ for changes in ApC and g.

TABLE 2. PARTICLE VOLUME SCALING AND CORRECTION FACTORS

Factor	Converting	To
F_1	Analyzer channel number (pulse height)	Cubic microns (μ^3)
F_2	Aperture diameter of F_1	Any aperture diameter used
F_3	Aperture current of F_1	Any aperture current used
F_4	Amplifier gain of F_1	Any gain setting used
F_5	Physico-chemical conditions of F_1	Any pH, temperature, or osmolarity of suspending solution

pulse produced by a particle of unknown size can be corrected mathematically to standard conditions from those under which the pulse was produced.

PROCEDURE FOR DETERMINATION OF CORRECTION FACTORS

These correction factors (Table 2) were determined by measuring the shift in the channel number modes of populations of RBC caused by changing one variable while all other variables remained fixed. No attempt was made to measure the actual size of the cells or to determine mathematically their mean cell size or size of the cells at the mode. The assumption was made, based on the previously reported studies (1,2), that the cell size at the mode of frequency distribution curve of any one blood sample was constant. In the cases of pH (5) and osmolarity (6), the shift in modal peak is due to actual changes in CV because of the expansible RBC membrane.

As shown in Fig. 4, RBC from a patient were sized at pH 6.7, 0.9 per cent saline, 74° F, with ApD 140 microns and ApC VIII, while the amplifier gain settings were changed. Ratios were then made of the channel numbers of the modes at consecutive changes in one condition while other conditions were held constant, as shown in Table 3. The mean ratio for gain changes (g) determined in this manner was 0.707,

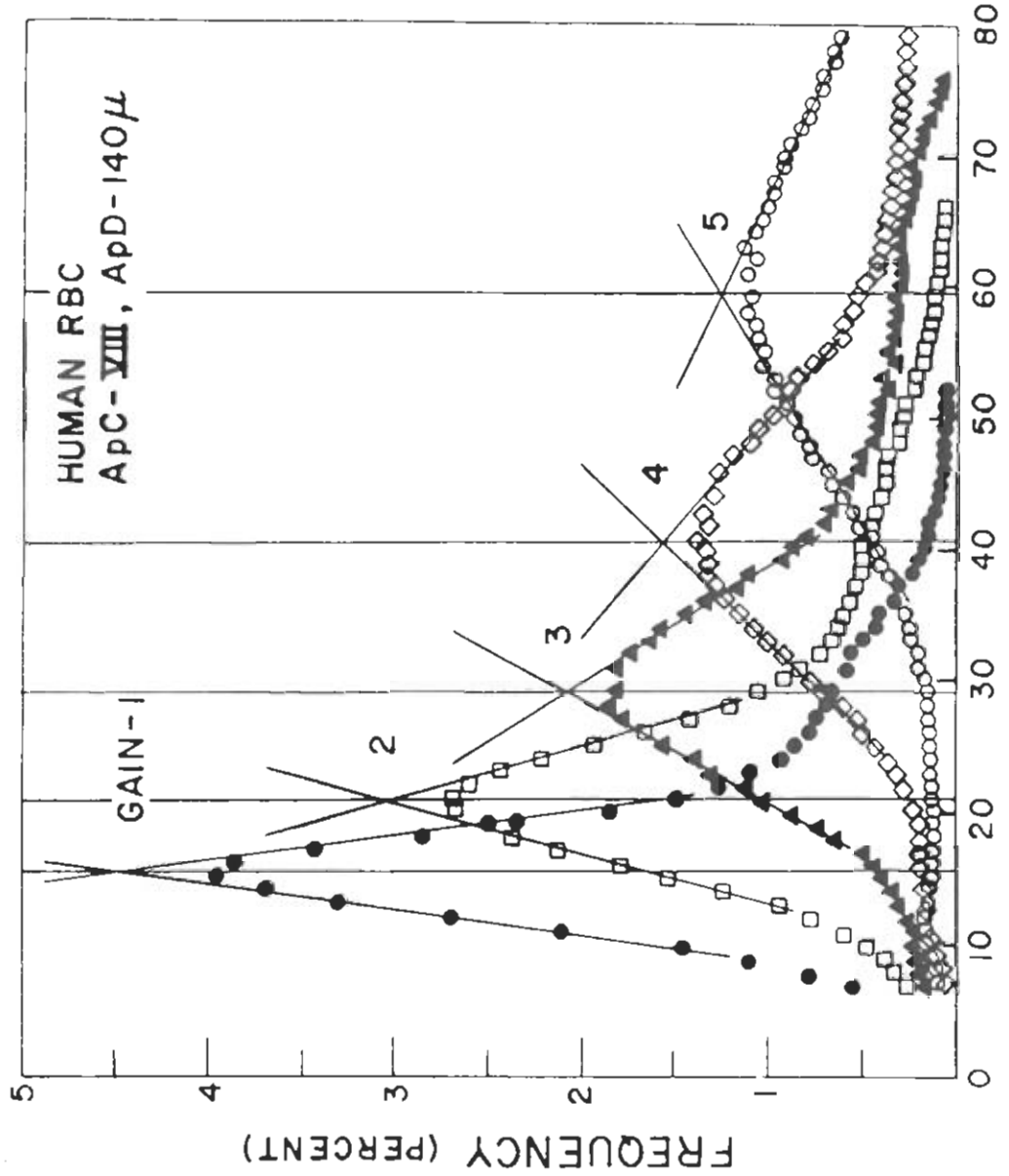

Fig. 4. Graph illustrating the "spectral peak" method of determining the effect of alterations in some condition of measurement upon the apparent mean diameter of erythrocytes.

TABLE 3. COMPARISON OF THE APPARENT VOLUME OF CHANNEL 10 AS MEASURED WITH MONODISPERSED LATEX PARTICLES (8) AND BY EXTRAPOLATION FROM ERYTHROCYTE CALIBRATION CURVES (2)

		Channel 10 Volume (μ^3)		
ApC	Gain	Latex Method*	RBC Method**	RBC/Latex
V	6	0.265	0.296	1.12
VI	6	0.16	0.17	1.02
VII	6	0.12	0.12	1.00

*At ApD 30.

**At ApD 100, ApC V, g 4, pH 6.7.

or $\sqrt{\frac{1}{2}}$. The ratios for ApD and ApC changes were not linear, and mean ratios could not be derived. Using these ratios, it is possible to derive the correction factors by which the experimentally determined conversion factor F_1 can be converted into scales for the other settings.

Use of Correction Factors

The accuracy of these correction factors was tested by using them to extrapolate from the F_1 determined with RBC at ApD 100, ApC V, g 4 (pH 6.7), to the F_1 values determined with latex particles at ApD 30, ApC V, VI, and VII, at g 6. The results of this test (shown in Table 3 and Fig. 5) confirm the validity of this method of calibration and correction. Figure 5 also shows the range of particle sizes that can be measured in channel 10 by appropriate changes in the three variables ApD, ApC, and g.

CONCLUSION

The inherent variability of electronic apparati requires a means of standardizing their measurements along with a method for compensating for changes in the conditions under which the measurements are made. RBC or latex particles can be used, as described here, as a nonelectronic means of such standardization that is facile and reliable.

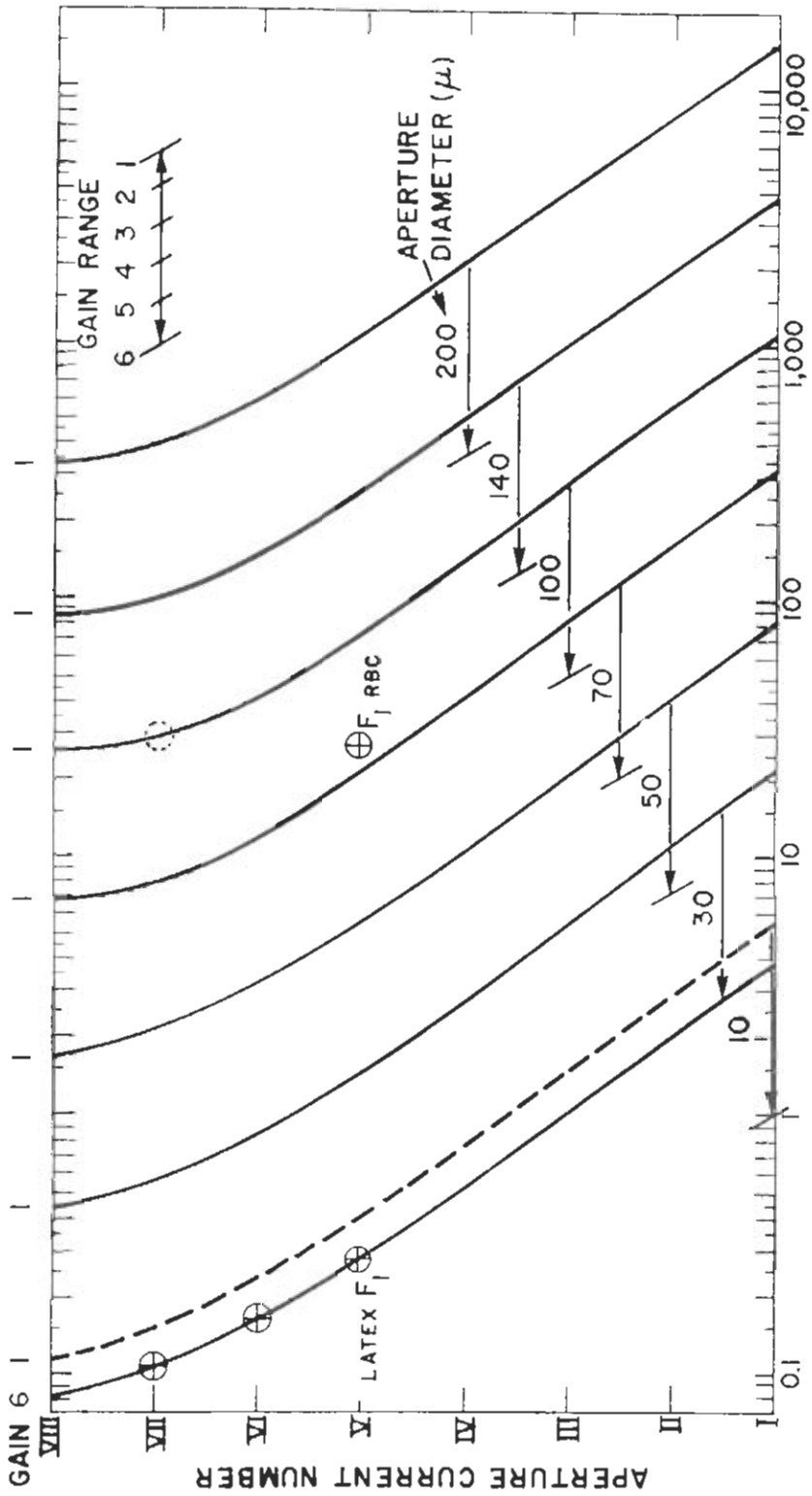

Fig. 5. Graph of the ranges of particle volumes that can be measured by various changes in aperture current, amplifier gain, and aperture diameter. The location of the latex and RBC conversion factors (F_1) that were determined experimentally are shown.

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Electronic Measurement of Cellular Volumes. V. Change in Red Blood Cells Resulting from Non-Physiologic pH (C. C. Lushbaugh, E. C. Anderson, H. I. Israel, D. B. Hale, and N. J. Basmann)

INTRODUCTION

Brecher et al. (1) have shown recently using the Coulter counter that 0.9 per cent saline causes an apparent increase in red cell volume when compared with measurements made in plasma or Eagle's solution. The unavailability of large inexpensive quantities of specific plasma and the difficulties involved in making solutions routinely with as many different salts as contained in Eagle's formula led us to look for a cause for this volume increase which might be controlled when using physiologic saline so that the "true size" of RBC could be calculated from electronic determinations of RBC volume distribution profiles (1,2). Since 1867, when Schmidt (3) and later Nasse (4) described increase in RBC volume in serum under increased CO_2 tension, a relationship between RBC size and pH of the suspending medium has been known to exist (5). Although no recent studies have been made of this phenomenon, it is generally accepted that RBC swelling can result from changes in osmotic pressure resulting from pH changes exterior to the cation impermeable membrane of the cell (6,7).

METHODS AND RESULTS

Commercially available saline is unbuffered and quite variable in pH, ranging usually from 5.8 to 6.0. Prolonged storage produces a pH as low as 4.0. Saline, however, can be buffered to a desired pH without changing its isotonicity or conductivity by appropriate amounts of phosphate buffer. A study of the effect of pH on RBC volumes measured electronically was made using this buffering system and commercially available saline. In this study, individual blood samples from 20 mice and 20 men were counted and sized electronically in 0.9 per cent saline solutions at 6.0, 6.5, 7.0, and 7.5 pH. The mean cell volume (MCV) at these pH's was determined as the mean analyzer channel (Mch) by integrating the volume distribution profiles of RBC volumes measured electronically (2). In order to determine whether aperture current (ApC) settings and amplification (g) of the pulses modify change in apparent volume, the mice were measured electronically at ApC VIII, g 2, and the men at both ApC VII, g 1, and ApC V, g 4. The resulting data are shown in Table 1. Figure 1 shows the best fit line for these points plotted as the logarithm of the mean channel number of the mean RBC volume versus the pH of the saline used in the determinations. The data show that, over the pH range investigated, change in RBC volume occurs as an exponential function of

TABLE 1. EFFECT OF pH OF PHOSPHATE-BUFFERED SALINE ON HUMAN AND MOUSE ERYTHROCYTE VOLUMES (SHOWN AS CHANGES IN MEAN PULSE HEIGHT ANALYZER CHANNEL NUMBER OF RBC FREQUENCY DISTRIBUTION PROFILES OF VOLUMES)

Blood Sample Source	Electronic Counter Settings	Mean Channel Number at pH								MCV (μ)
		6.0	6.3	6.45-6.5	6.65	6.70	7.0	7.45	7.5	
Mice (20)	ApC VIII, g 2	30.2	28.56	--	27.85	--	26.44	--	24.92	49.7
Men (12)	ApC VII, g 1	--	--	29.16	--	28.31	27.74	--	26.99	87.5
Men (8)	ApC V, g 4	--	30.71	--	29.76	--	28.01	26.55	--	86.0

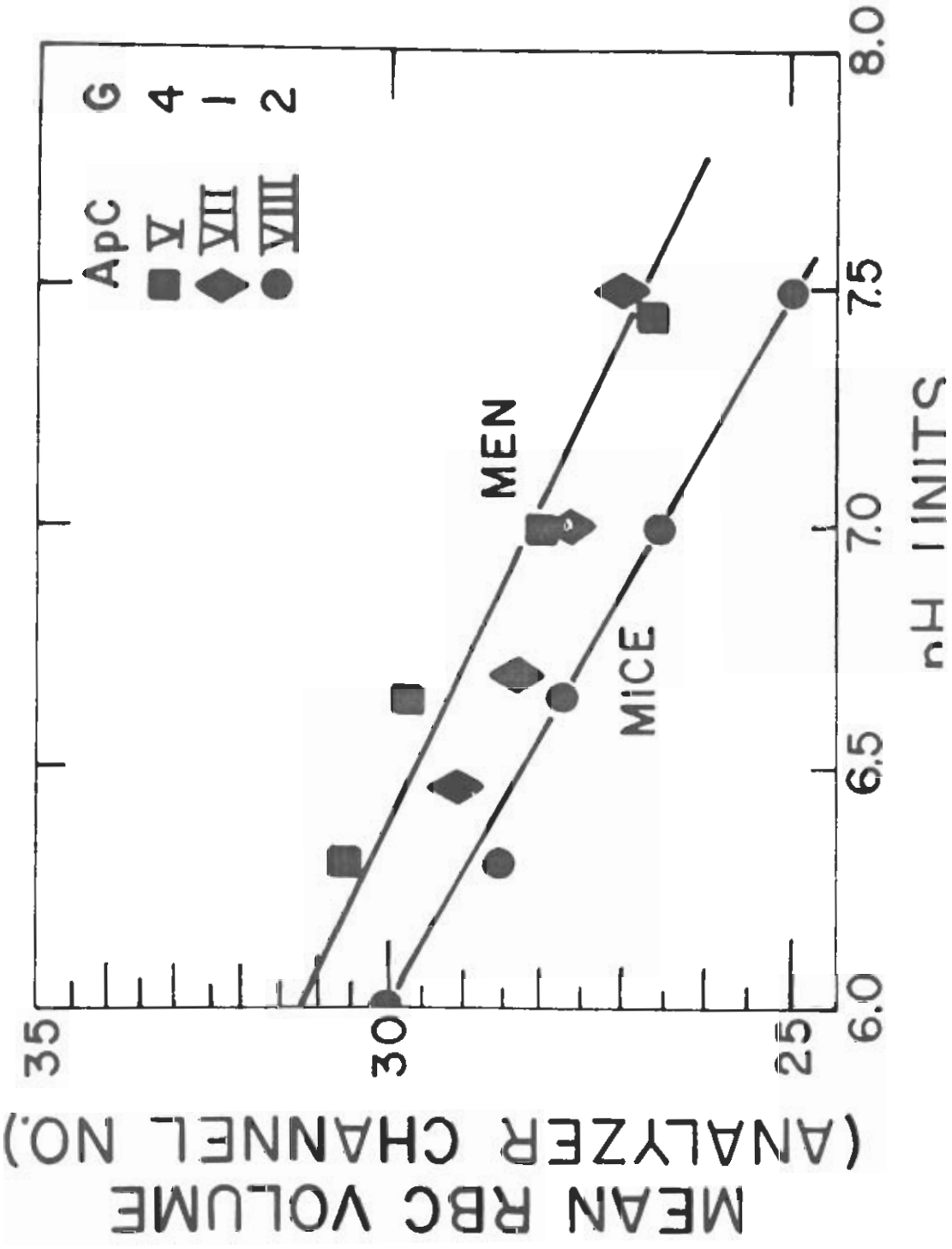

Fig. 1. Semilogarithmic plot of relationship of RBC volume to pH of the saline-phosphate buffer medium used in electronic sizing.

change in pH of the saline solution suspending them. This relationship between RBC and pH of the suspending medium implies that there is no single "true" size of RBC; human RBC in acidosis (< 7.35 pH) are larger than the same RBC in normal arterial blood (7.4 pH), and measurements of RBC volume must be referred to some standard set of conditions. The actual measurement, however, could be made under some condition of pH other than the "standard" if the appropriate mathematical correction for pH was known. Also, such a correction factor for counting solutions adequately buffered at the pH of human venous blood are needed, because blood samples open to the air change pH at the rate of 0.2 pH or more per hour and are, therefore, not measured in vitro at their in vivo pH. The figure shows that the fractional change in volume of RBC, as indicated by the channel number (Ch), is proportional to the change in pH; that is,

$$\frac{\Delta Ch}{Ch} = k \cdot \Delta pH.$$

(On a logarithmic scale, a constant fractional decrement, $\Delta Ch/Ch$, appears as a constant absolute change.) The constant (k) can be evaluated by substituting numerical values for the other parameters in this equation. [Note, however, that the equation is really only the differential form of the exact exponential function and can be used as an

approximation only when the fractional volume change is small (i.e., over the range to which 1 - x is a good approximation for e^{-x}). For the total range shown in Fig. 1, the volume change is 25/31 or 0.81, and the approximation is good to 2.5 per cent.]

The line of best fit drawn through the 5 mouse points shows volumes corresponding to Ch 30.0 at pH 6.0 and 26.5 at pH 7.0, giving a slope k = 0.124. A computer calculated least squares exponential fit to the same data gave k = 0.125. For man, the latter gave k = 0.106. This slope, indicating about a 10 per cent change in RBC volume per change in 1.0 pH, corresponds well with the fractional change observed by Warburg (6) with horse blood under different CO_2 tensions. This slope (k) can be used as a volume correction factor in the following way:

$$\text{True Size} = [1 + (k \cdot \Delta pH)] \cdot \text{Size Observed},$$

or for the electronic particle size analyzer:

$$Mch_{(true)} = [1 + (0.106 \cdot \Delta pH)] \cdot Mch_{(found)}.$$

Where scaling factors (F) have been determined for converting analyzer channel number to cubic microns (μ^3) at a certain ApC setting, amplification gain, and aperture diameter (ApD), it can be used in this formula to obtain true volume in cubic microns, as

$$V = F \left[1 + 0.106\ (\Delta pH)\right] \cdot \text{Ch No.},$$

where ΔpH is the difference between the pH used in the determination of the scaling factor and 7.4. Similarly, a scaling factor (F) determined at some pH can be corrected in this manner to that for any other pH.

Since it is still true, as Ponder (5) pointed out in 1948, that "no one knows what ought to be taken as the isoelectric point of the mixture of substances which enter into the architecture of the erythrocyte, nor is enough known about the swelling of anisotropic and elastic materials in the neighborhood of their isoelectric points," the choice of pH of the saline solution used in sizing RBC would seem to depend on the preferences and biases of the investigator. The factor for change in RBC volume due to pH, as determined here, would allow interconversion of the data of others to a standard or any other pH.

SUMMARY

The reported increased size of erythrocytes suspended in saline is due to an effect of the low pH of unbuffered saline solutions commonly used in electronic particle counters. This increase in size was found to occur exponentially with decrease in pH and to be 10.6 and 12.5 per cent per pH unit below 7.4 for man and mouse, respectively.

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Electronic Measurement of Cellular Volumes. VI. Electronic Improvement of Coulter Counter Resolution

(C. C. Lushbaugh, N. J. Basmann, and D. B. Hale)

INTRODUCTION

Resolution of energy spectra depends upon accurate measurement of pulse height voltage. As first pointed out by Kubitschek (1), resolution by the Coulter counter is not maximum since the circuitry and apparatus were designed primarily for rapid enumeration of pulses rather than voltage measurement. In order to minimize coincidence loss due to 2 or more particles occupying the sizing aperture simultaneously, the pore in the hollow glass sensing probe of the Coulter counter was made relatively shallow (e.g., the aperture of 100 μ in diameter is only 75 μ in depth). The length of time a particle remains in the electrical field of the aperture is shortened further by the rapidity with which the suspending solution is drawn through it. The rise time of pulses generated by particles is comparatively slow in relation to their velocity through the pore. Lengthening the gating interval of the pulse height analyzer does not correct the tendency for this system to report lower than actual height of the pulses, since the particle often passes through the sensitive volume of solution in the aperture before the pulse has time to reach its full height. To

complicate the analysis further, all particles do not pass through the aperture at the same velocity since, as is well known, fluid moves more slowly at the periphery of a stream than in its center. As a result of this phenomenon, the pulses of the slower moving particles are sized more accurately than those from the ones with greater velocity in the central core.

Improvement in resolution might be expected with this apparatus, therefore, by any mechanism that prolongs the sizing interval. Such prolongation could be accomplished by (a) slowing down the flow rate of the suspending solution; (b) increasing the sensing volume by physical elongation of the aperture; (c) forcing all particles into the slower moving peripheral areas by obstructing the central portion of the passage; or (d) increasing the critical volume of the electrical field around the aperture openings by the use of greater aperture currents than usually used.

Of these 4 possible means of improving resolution, the use of increased aperture currents (ApC) is technically the simplest and most easily varied. Some foundation for this approach to the problem is to be found in the recently reported work of Brecher et al. (2) that shows that frequency distribution curves of RBC volumes in a single blood sample show one mode at low ApC and two modes at high ones. Brecher

concluded that the appearance of the second modal frequency peak was due to a change in RBC size caused by the higher electrical currents.

Another interpretation of this finding may be that the higher currents enable better resolution of pulse heights so that large RBC are measured more accurately and resolved from the more numerous smaller RBC. The presence of such a large but unresolved subpopulation of large RBC in the size distribution curves of human RBC was suggested by Lushbaugh et al. (3) from a mathematical analysis of the curves based on the presence of 2 easily resolved subpopulations in the blood of birds. Since then, biological evidence for the presence of a volumetrically defined subpopulation of young RBC in mammals, as well as in birds, amphibia, reptiles, and fish, has been obtained (4,5), which makes improvement of the resolution of the Coulter counter pulses with human blood quite desirable.

METHODS AND RESULTS

Erroneous Sizing Caused by Coincidence

In the first series of experiments, the possibility was investigated that the second or larger volumed population ("B") of RBC was fictitious and due to doubling by coincidence. This possibility gained support from the fact that the mode

of the "B" population of birds was almost exactly twice that of population "A," although only 1.7 times as large in man. Figure 1 shows the result obtained when the blood of small birds was measured at the same electronic settings that failed to resolve two modal peaks in the blood of man. Although the concentration of RBC in both cases was the same, the two populations were easily defined in the bird at ApC V, g 4, ApD 100, pH 6.7 (the common settings) but not in man. Obviously, if the larger modal peak was due to coincidence, it should have been found with both blood samples. Figure 2 shows in a suspension of latex particles, which are known to consist almost entirely of monodispersed particles but also containing doublets, triplets, etc., due to agglutination, that appropriate ApC can resolve peaks with doubling modalities. This figure also shows how their apparent frequency can be increased by changing the readout scale of the pulse height analyzer memory bank. Attempts to change the frequency distribution curves of RBC by coincidence of particles by increasing the RBC/ml were unsuccessful because of the apparent inability of the Coulter counter amplifier circuitry to feed proper sized pulses to the analyzer at the counting rates used.

In the second series of experiments, RBC of different modal volumes obtained from different patients were used. In each experiment, the blood of two persons was sized separately

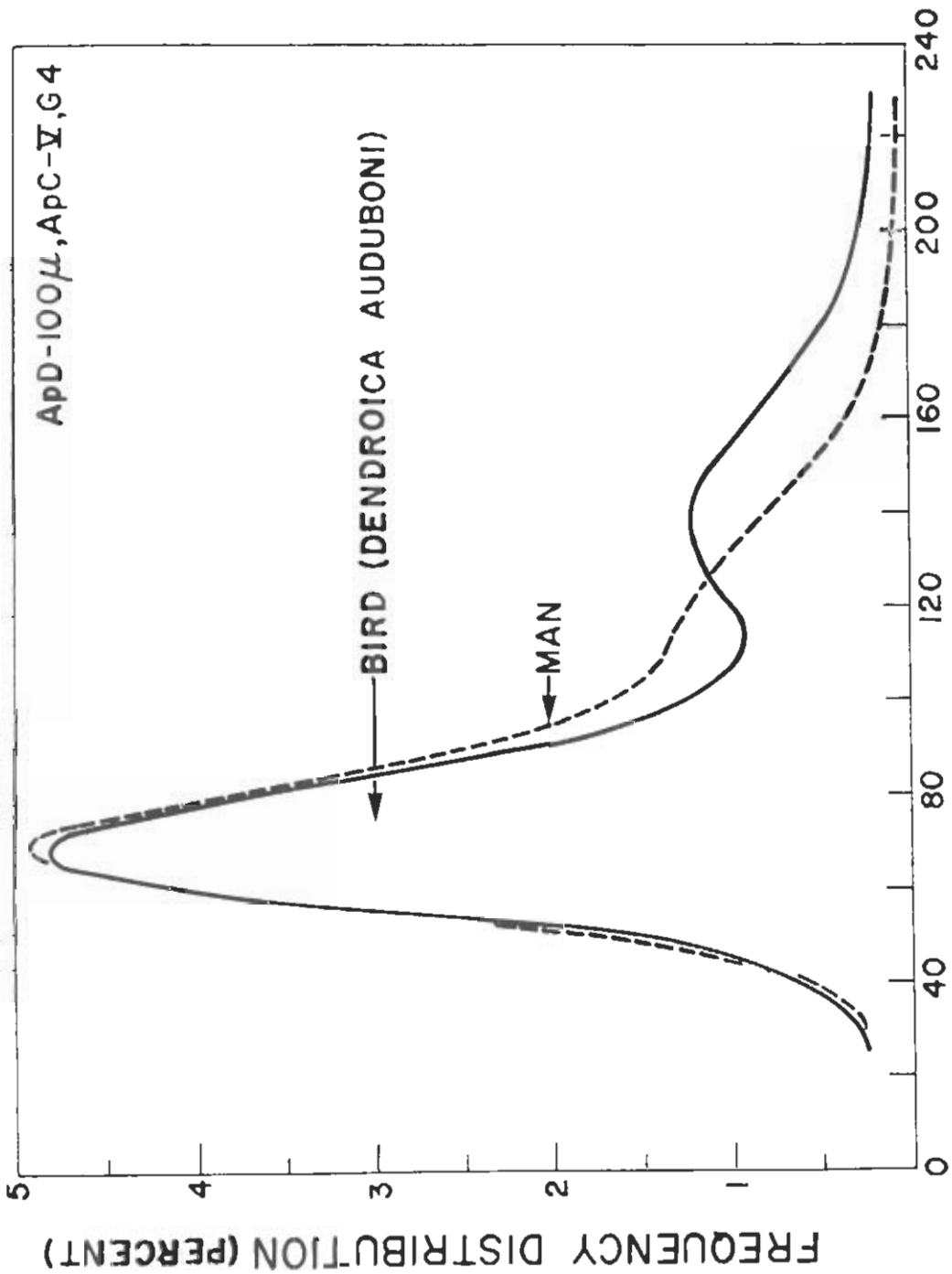

Fig. 1. Frequency distribution curves of RBC volumes of a bird and man determined under the same electronic and physical conditions, showing the resolution of two modes in the bird and only the suggestion of a second one in man.

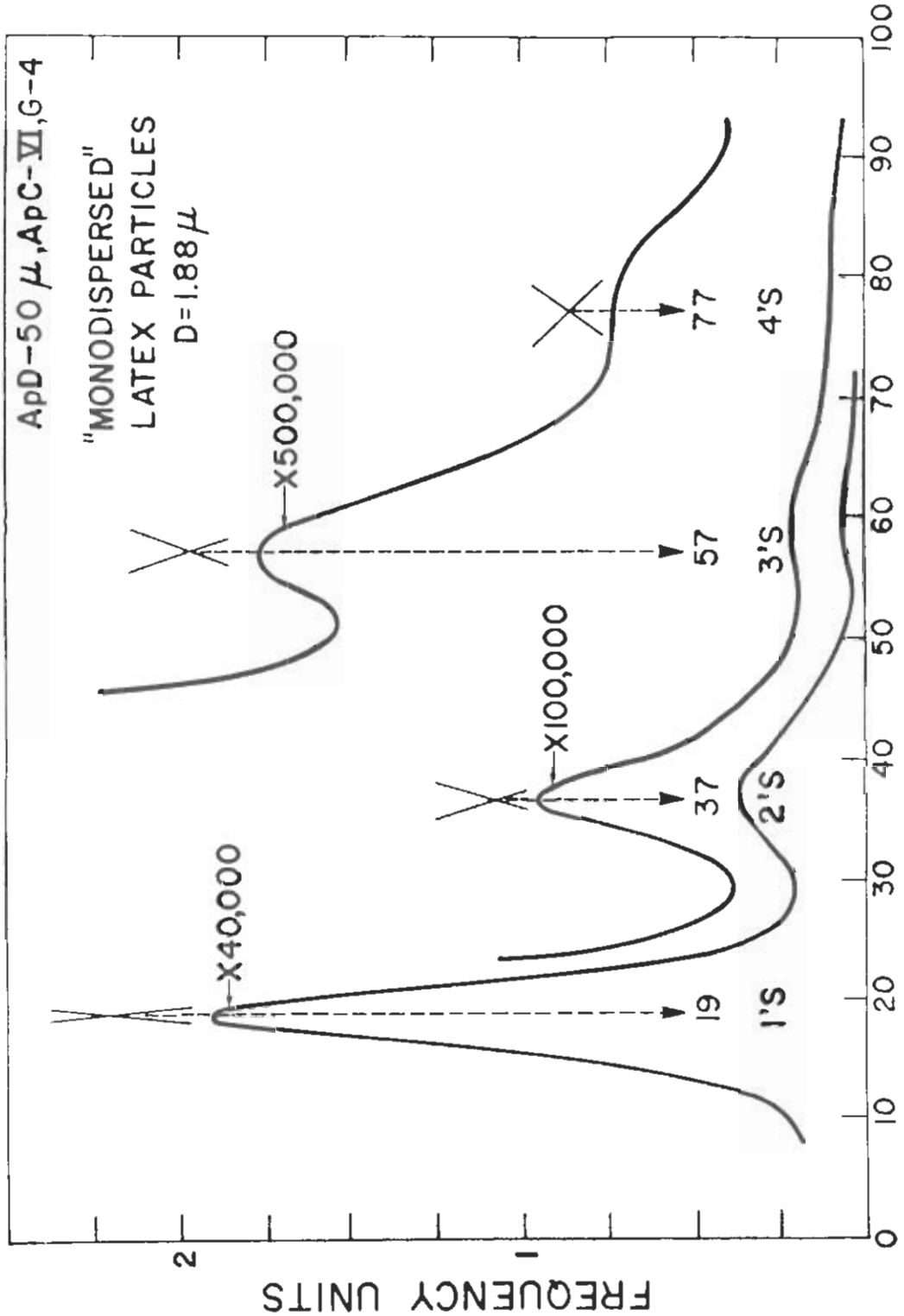

Fig. 2. Frequency distribution curves of monodispersed latex particles showing the coexistence of doublets, triplets, and quadruplets. The curves marked X 100,000 and X 500,000 are electronic magnifications of the X 40,000 curve.

at the "recommended" low ApC and g (2) and again at the highest ApC and g that seemed to resolve two subpopulations in human blood. The two frequency distribution curves that resulted were then summated, as with the shaded curves of Fig. 3. The summated curve was then transposed to another sheet of graph paper on the X-Y plotter, where it was designated the "predicted curve." An equal number of RBC from the blood of each person was then combined in vitro and suspended in the saline solution and analyzed. The resulting distribution of RBC volumes was then printed out mechanically upon the graph. The small open circles on the right-hand graphs in Fig. 3 show the excellent correspondence of the predicted and experimentally obtained curves. Comparison of the curves obtained with the different electronic settings shows quite well the better resolution of the modal peaks obtained with the higher ApC.

DISCUSSION AND CONCLUSIONS

These results seem to show that resolution of pulses from RBC passing through the sensing aperture of the Coulter counter is improved by increasing the ApC appropriately. They indicate that the appearance of double RBC populations does not result from enlargement of RBC due to excessive electrical current nor coincident pile-up. The conclusion would seem warranted that the presence of a second subpopulation

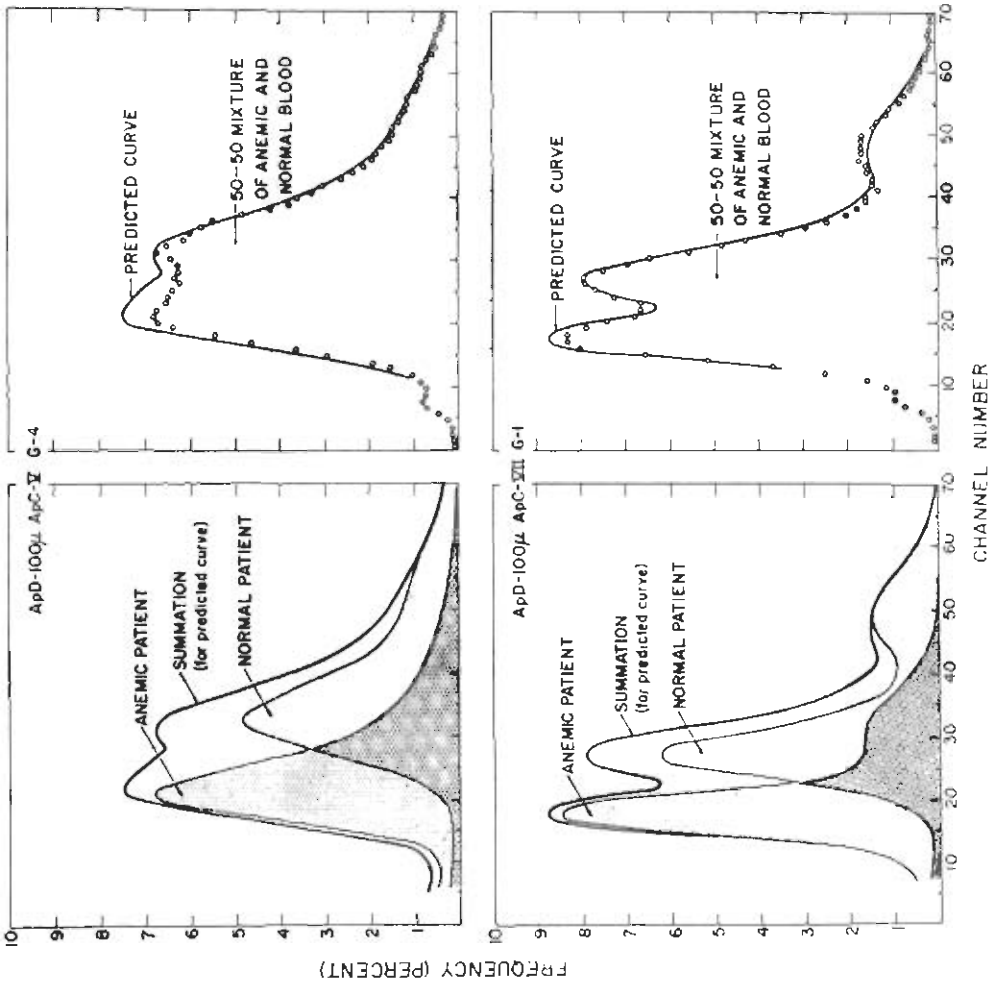

Fig. 3. Graphs showing improved resolution of the modal frequencies of distribution curves of RBC volumes by high ApC. Graphs on the left are superimposed curves of 2 blood samples analyzed separately; on the right, the curves of dots were obtained from analysis of a 50-50 mixture of the 2 blood samples.

of RBC, resolvable from another population of smaller modal size but more numerous individuals, is not due to an artifact of the techniques of measurement. This conclusion receives further support in the following study, which characterizes the "B" population as new and young RBC.

The increased resolution obtained here with increased aperture current would seem to encourage a search for other means of improving pulse generation so that RBC volume and pulse height have a more reliable constant relationship.

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Electronic Measurement of Cellular Volumes. VII. Biologic Evidence for Two Volumetrically Distinct Subpopulations of Red Blood Cells

(C. C. Lushbaugh and D. B. Hale)

INTRODUCTION

Some biologic support was reported previously (1) showing that it was possible to destroy red blood cells around the mode of population "A" and to leave a subpopulation of saponin-resistant red blood cells around the locus of the mathematically predicted mode of population "B." These results were obtained in the blood of man, as well as in birds where the "B" population does not require mathematical differentiation at aperture current V and gain setting 4.

The present study reports the refinement of these observations by the better resolution obtained by ApC VII, g 1 (2) and by the use of $Fe^{59}SO_4$ as a hemoglobin label in newly produced RBC.

METHODS

As in the previous study, increments of a 1:100 saponin solution were added to suspensions of human and rabbit RBC that were then sized electronically. In order to identify newly formed RBC, the rabbits were labeled with Fe^{59} (10.3 μc Fe^{59}/μg Fe) as $FeSO_4$ intravenously 3 days previously. The resulting changes in the distribution of cellular volumes were

then related graphically to saponin concentration so that the effect on 0.1 ml of blood of 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 ml of 1:100 saponin made up to 4.9 ml with saline could be determined. The hemolytic destruction was stopped after 1-1/2 minutes by (a) dilution of 0.05 ml aliquot to 100 ml for volumetric analysis, and (b) 3-minute centrifugation of the remainder. One-ml aliquots of the supernatant solution after centrifugation were then analyzed spectrophotometrically for hemoglobin content using cyanomethemaglobin, and another 1-ml aliquot was analyzed in a NaI (Tl) crystal-wall photospectrometer for Fe^{59} content.

RESULTS

The results of these experiments are shown graphically in Figs. 1, 2, 3, and 4. Figures 1 and 2 demonstrate that the hemolytic effect of saponin is dependent upon the saponin concentration and time of reaction. They also show that the volume distribution curves are shifted progressively to the right (large volumed RBC) as increasingly larger RBC are destroyed successively. As the result, the cells of population "A" are destroyed first and then those of population "B" are progressively hemolyzed. In Figs. 3 and 4, the frequency distribution curve of rabbit RBC has been related to the saponin concentration that was required to destroy all rabbit

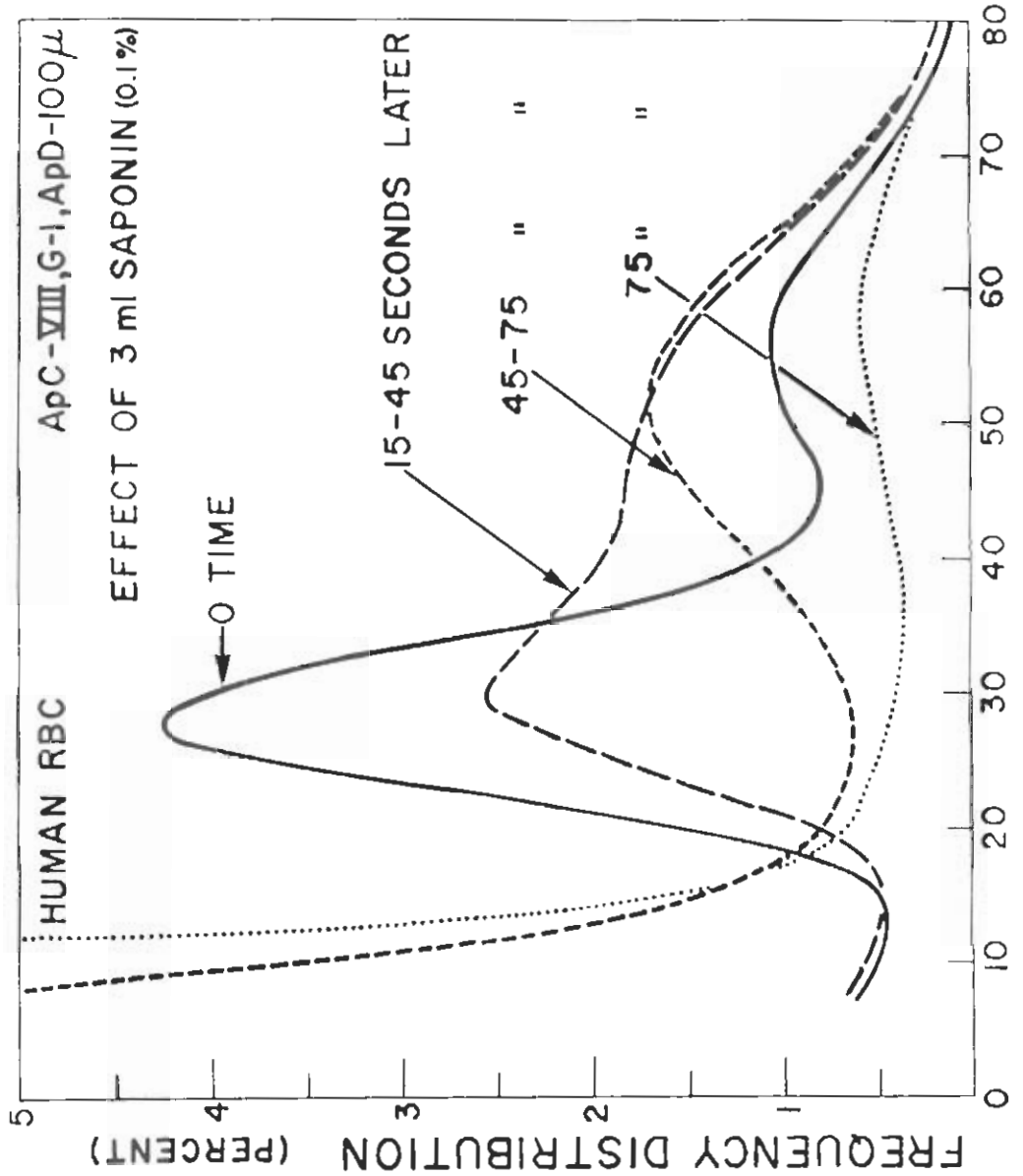

Fig. 1. Effect of saponin upon the frequency distribution of RBC volumes as a function of time after its addition.

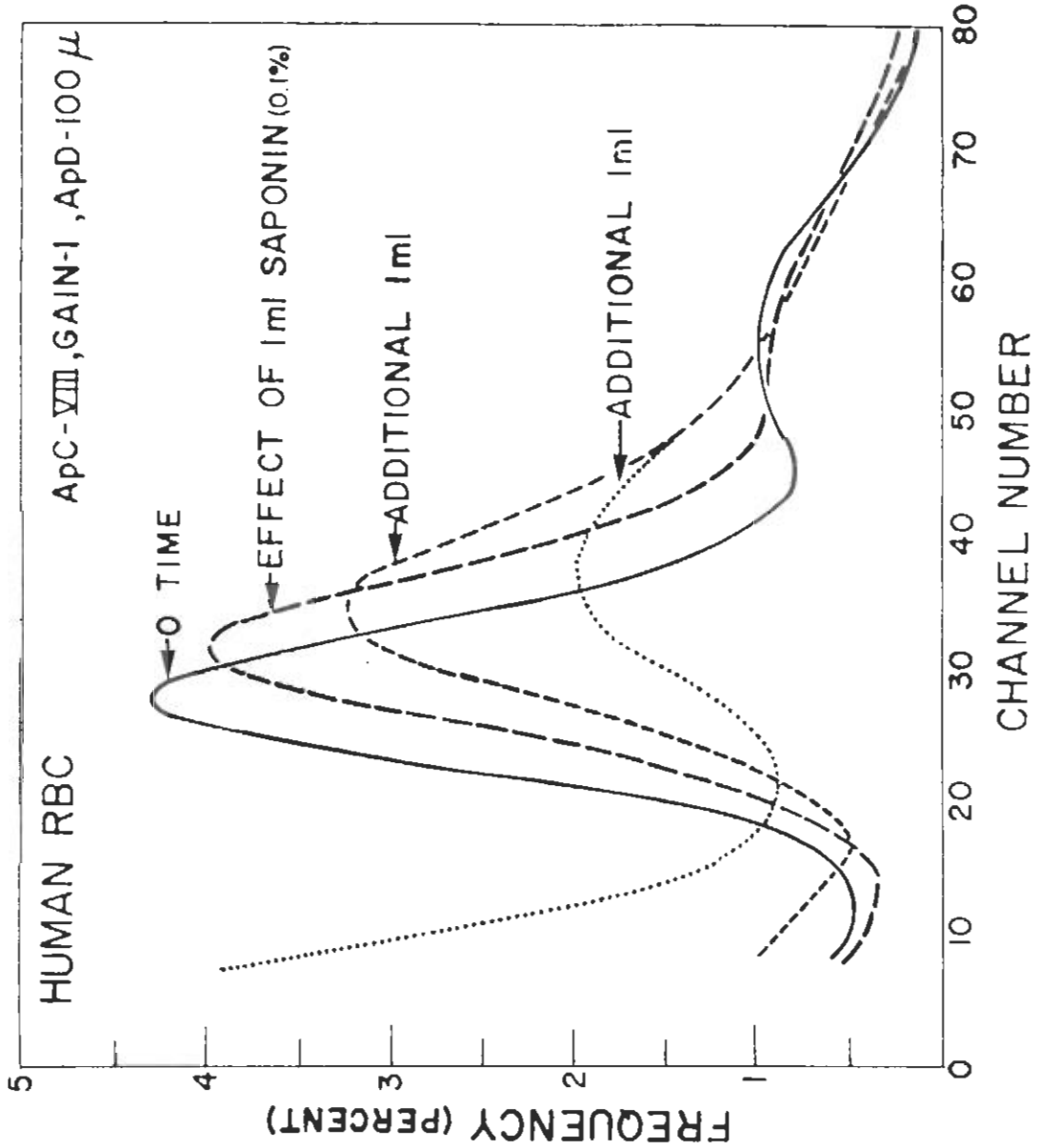

Fig. 2. Effect of increasing amounts of saponin upon the frequency distribution curves of RBC.

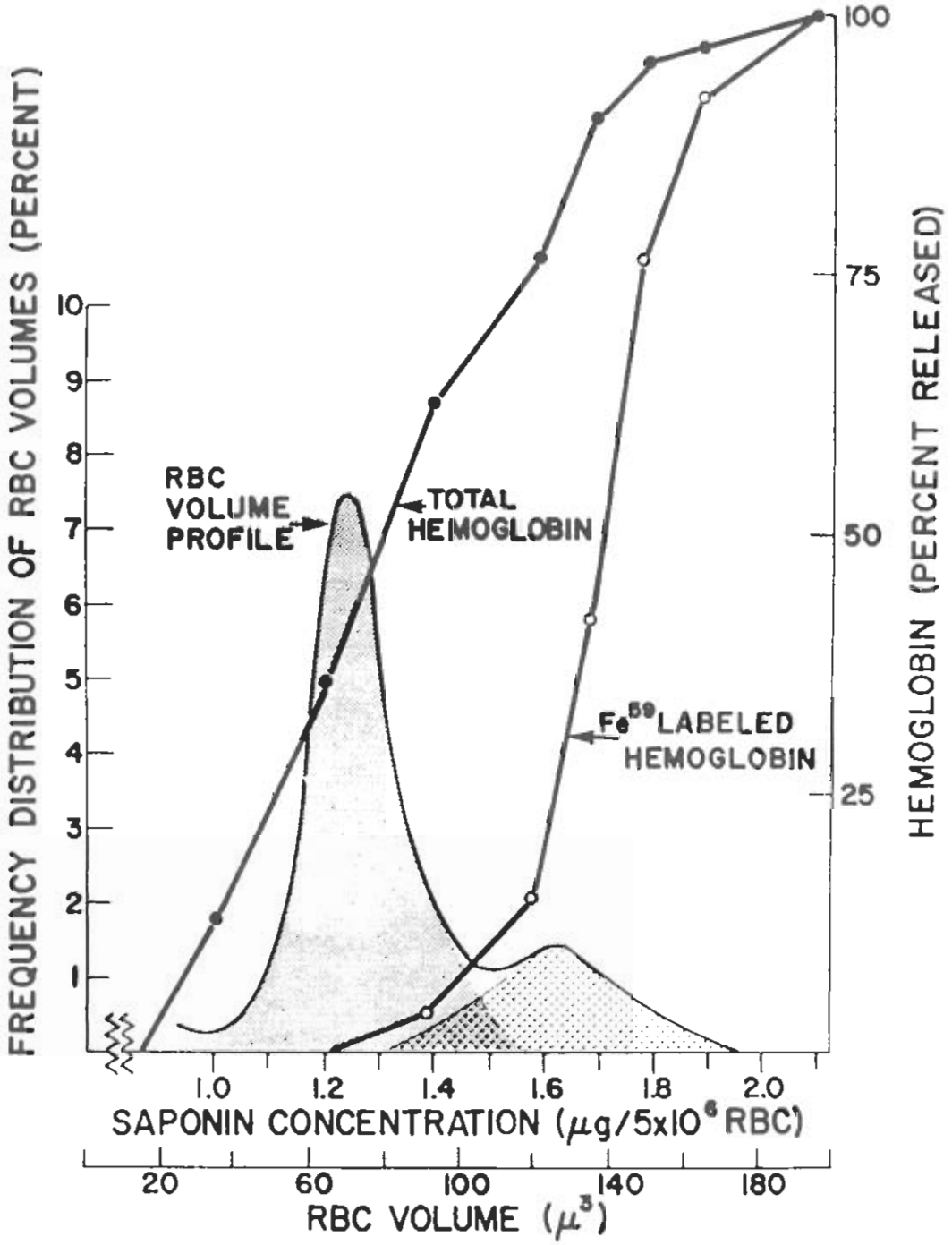

Fig. 3. Integrated curves of hemoglobin and Fe^{59}-labeled hemoglobin liberation by increasing saponin concentration shown in relation to the RBC volume frequency distribution curve of rabbits.

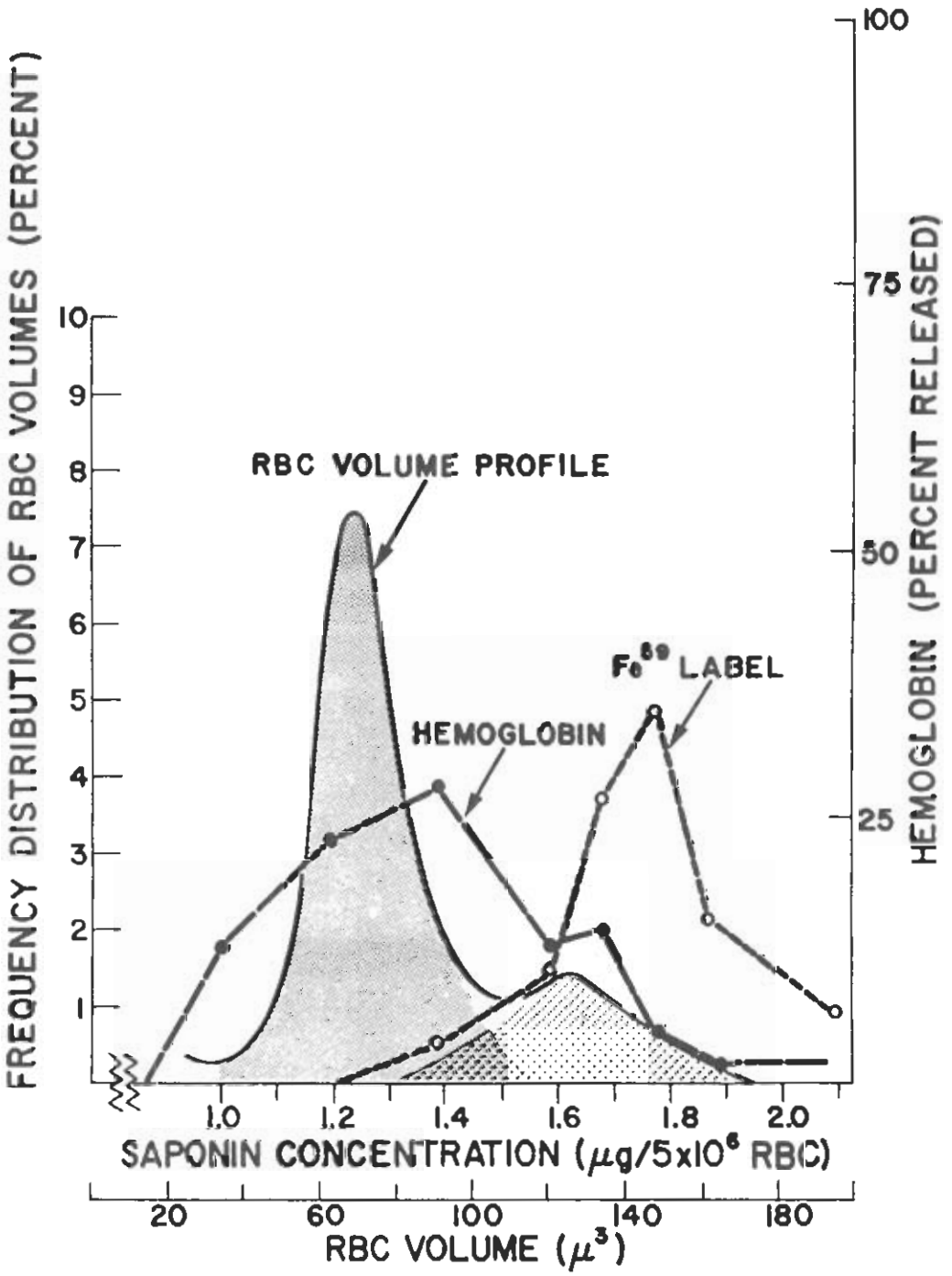

Fig. 4. Differentiated amounts of hemoglobin and Fe^{59}-labeled hemoglobin liberated by increasing saponin concentration in relation to the RBC volume frequency distribution curve of rabbits.

RBC smaller than that size. In Fig. 3, the total amounts of hemoglobin and Fe^{59} liberated by the destruction caused by the increments of saponin are shown. The same data are shown in Fig. 4 but are shown as the differential amount of hemoglobin and Fe^{59} liberated. The hemoglobin and Fe^{59} curves are dissimilar and disassociated; the largest amount of hemoglobin was unlabeled and released by destruction of population "A," and the largest amount of Fe^{59} labeled hemoglobin was obtained by destruction of the cells from the larger side of population "B."

DISCUSSION

These experiments demonstrate that the small RBC of population "A" are more sensitive to hemolysis by saponin than the large ones of population "B" and that the population of large RBC is composed of the youngest cells, since only this group of cells contained Fe^{59}-labeled hemoglobin 3 days after parenteral administration of radioactive iron. These conclusions would seem to justify alteration of the names of the bimodal peaks of RBC frequency distribution curves from "A" and "B" to "mature" and "immature," respectively. Although the names "old" and "young" might seem sufficiently descriptive and more adaptable to everyday usage, these terms do not embody the concept that RBC undergo maturation as they

age and decrease in size. Furthermore, the existence of two distinct modal frequencies in the distribution of the mature and immature RBC would seem to indicate that the process of RBC maturation is significantly shorter in duration than the mature life of the RBC. Experiments in progress with phenylhydrazine poisoned rabbits appear to show that the time involved in this volumetric change to mature size is about half of the rabbit RBC life span.

SUMMARY AND CONCLUSIONS

Taking advantage of the well-known facts that RBC sensitivity to hemolysis by saponin increases with RBC aging and that parenterally administered Fe^{59} labels RBC only as they are produced, experiments were done which showed that hemoglobin released by saponin destruction of the two subpopulations of RBC was unlabeled when only the smaller volumed RBC of population "A" were lysed. The Fe^{59} label was found in the larger volumed RBC of population "B." These findings are considered biologic evidence for the existence of two distinct subpopulations of RBC and for the belief that the bimodal frequency distribution curve of RBC volumes obtained with the LASL cell volume analyzer system is not artifactual. It is suggested, therefore, that these subpopulations be renamed as "mature" and "immature" RBC, rather than "A" and "B."

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Electronic Measurement of Cellular Volumes. VIII. Volumetric Change of Circulating Erythrocytes in WW^V Genetically Anemic Mice Implanted with w+w+ Fetal Liver

(C. C. Lushbaugh and E. S. Russell*)

INTRODUCTION

The genetically determined macrocytic anemia of WW^V mice can be "cured" by a single intraperitoneal injection of normal hematopoietic tissue contained in hepatic brei from 15-day w+w+ fetuses (1,2). As the number of red blood cells increases, the mean cell volume decreases to normal values, and the electrophoretically diffuse hemoglobin pattern changes to the "single" type of normal mice. Thus, the cure of the anemia appears to result from the actual replacement of the anemic cells by the implanted normal cells rather than from a change in the macrocytic cells of the host.

METHODS

In order to obtain additional support for this conclusion and to determine whether macrocytic WW^V RBC continue to be produced in chimeric implanted $W++/WW^V$ mice, the distribution of RBC volumes was studied in the anemic, donor, and chimeric animals using the cell volume analyzer system devised for the Coulter counter in Los Alamos (3). First, the blood

*From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

of implanted WW^{v} mice known by their electrophoretic hemoglobin patterns to be "failures" or "cures" was studied. When the feasibility of the method was thereby established, the blood of the implanted mice was analyzed at 3-week intervals following implantation in order to determine the time required for the implant to succeed or to fail. The mice were bled from the orbital plexus by capillary pipette. The blood was diluted to 1:1,000,000 and suspended in 0.9 per cent saline buffered at pH 6.0. The Coulter counter (Model A) settings were ApC VIII, g 2, for which the scaling factor for conversion of analyzer channel to cubic microns was 1.60 (3).

RESULTS

The results of these studies (Fig. 1) showed that the blood from both the anemic and normal mice had a bimodal curve of RBC volumes as previously described (4). The macrocytosis, as might be expected, was seen by this method as a displacement of the two populations to the right or large volume. In those mice in which the hemoglobin pattern had indicated implant failure, the RBC volumes did not decrease (Fig. 1c), while the volumes were almost identical with the normal volume distribution when the implant was successful (Fig. 1d).

The results of the second experiment (Fig. 2) showed

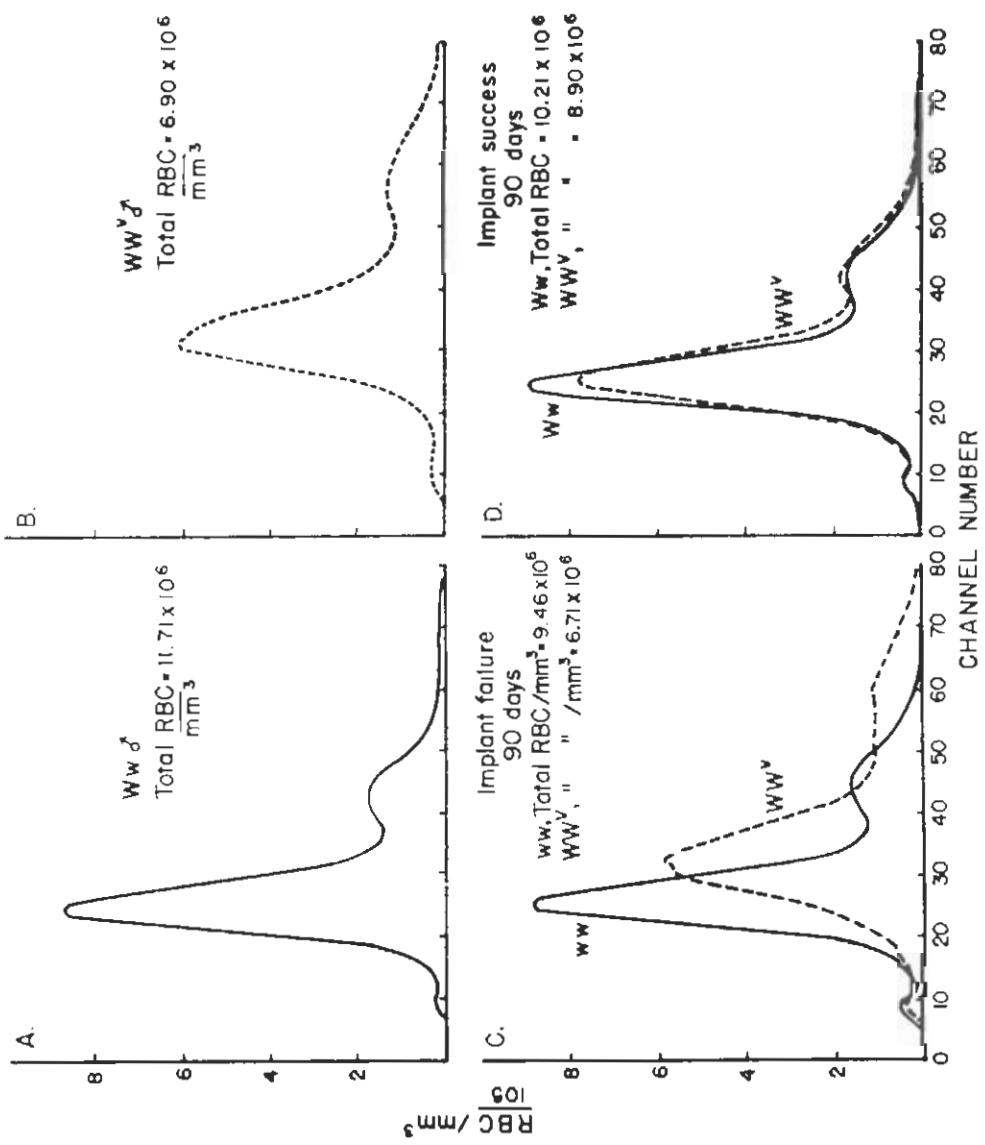

Fig. 1. Frequency RBC volume distribution profiles of (a) normal Ww mouse; (b) macrocytic anemic WW^v mouse; (c) normal WW mouse compared to a WW^v mouse (dotted line) in which the w+w+ implant failed; and (d) normal Ww mouse compared to a WW^v mouse (dotted line) in which the implant success is indicated by the shift of volumes to normal.

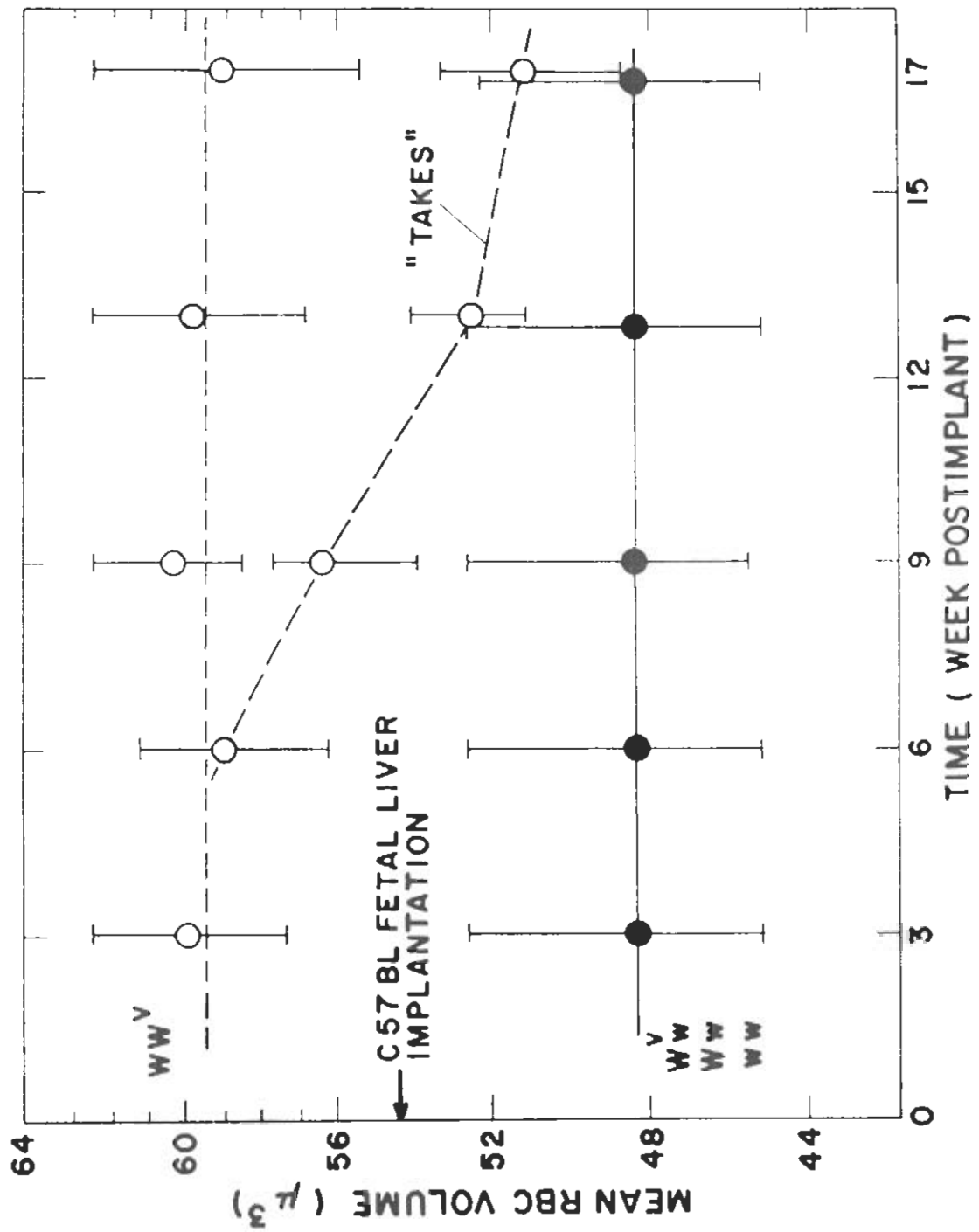

Fig. 2. Change in mean RBC volumes determined electronically in $W^V W^V$ anemic mice as a function of time after implantation with w+w+ hematopoietic tissue.

that RBC volumes commence decreasing measurably about 6 weeks after implantation and by 17 weeks are almost identical with those of normal mice (Table 1). The distribution profiles during this period of change first showed a decrease in the mode of the subpopulation of young RBC ("B"). This shift was followed by a shift in the mode of the old population ("A") until the profiles coincided as in Fig. 1d. Whether the resolution of the apparatus is not sufficiently precise, the variance of sizes too large, or the change in RBC size with age too rapid, the presence of two competing bone marrows (WW^V and w+w+) was not demonstrable by sizing their production of RBC using peripheral blood. The orderly change in frequency of the RBC and the volumes of the two subpopulations, however, confirmed morphologically the conclusion based previously on hemoglobin types that the w+w+ marrow supplanted the WW^V anemic marrow. An explanation of the failure to see two separate young populations at the stage when theoretically the normal (normocytic) and the abnormal (macrocytic) marrows are competing side by side may be that the presence of w+w+ hemopoietic cells suppresses the less vigorous WW^V marrow before the new smaller volumed RBC are delivered into the peripheral blood in sufficient numbers to be seen by this electronic method of measurement.

TABLE 1. COMPARISON OF SOME PARAMETERS OF THE DISTRIBUTION PROFILES OF THE RBC VOLUMES OF NORMAL AND "CURED" ANEMIC MICE

	Modal Frequency (per cent)		Mode (μ^3)		Variation (W = 1/2 H) (μ^3)	
	1*	2**	1*	2**	1*	2**
Population "A"	6.0	5.7	44.4	46.7	18.1	18.9
MCV			48.3	51.0		
Population "B"	1.8	1.7	79.0	81.0	23.5	25.3

*ww, Ww, W^vw mice.

**WW^v/w+w+ mice 17 weeks after implantation.

CONCLUSIONS

These findings imply that after successful implantation, the chimeric blood is derived entirely from the implant even though nothing was done experimentally to destroy the genetically determined macrocytic marrow of the host.

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