CALIBRATION OF AN ELECTRONIC COUNTER AND PULSE HEIGHT ANALYZER FOR PLOTTING ERYTHROCYTE VOLUME SPECTRA

63-8

FEDERAL AVIATION AGENCY
AVIATION MEDICAL SERVICE
AEROMEDICAL RESEARCH DIVISION
CIVIL AEROMEDICAL RESEARCH INSTITUTE
OKLAHOMA CITY, OKLAHOMA

MARCH 1963

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2. Erythrocyte
3. Cell Volume

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Hematology Section
BIODYNAMICS BRANCH

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FOREWORD

Certain studies of blood samples drawn from large numbers of airmen, are hampered by the lack of rapid and efficient means of obtaining quantitative data on red cells. The widely used optical techniques are slow and fraught with opportunities for error. This report presents information concerning a new technique for calibrating electronic sizing instruments. These instruments measure cell volume spectra with a greater degree of accuracy and speed than conventional techniques.

ACKNOWLEDGMENTS

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The development of electronic instruments for counting blood cells with unprecedented accuracy (1) has revived interest in this hematological variable, not only for clinical studies, but also in other research areas requiring the most exact measurements. A recent study (2) has indicated that electronic instruments of certain design can be calibrated to yield cell volume data accurate enough for the detection of subtle differences in erythrocyte populations. However, the employment of expensive pulse-height analyzing systems and computing equipment (3) for calibrating the instrument seems to place such research outside the scope of the ordinary laboratory. The recent development of a research model counter with a companion pulse height analyzer makes available a system which can be easily calibrated. The principles employed are simple (3, 4), but no detailed descriptions or evaluations of calibration techniques have appeared in the literature. This report offers a technique for calibration and considers the limiting factors for obtaining precise calibration.

METHODS

The system used in these studies was a Coulter Counter Model B with its companion Particle Size Distribution Plotter.* This system has been described by Brecher, *et al* (4). The same aperture tube was used throughout the experiment; its opening measured approximately 100 μ in diameter by 75 μ in length.

Forty to 50 ml. of venous or heart blood were withdrawn through 20 gauge needles into siliconized syringes and immediately mixed in a plastic centrifuge tube with 0.2 ml. of sodium heparin solution. This mixture constituted the "sample," and portions of it were used for the measurment of Evans blue hematocrits, centrifuge hematocrits, erythrocyte counts, and for the recording of erythrocyte volume spectra.

Evans Blue Hematocrit

The method used was essentially that of Shohl and Hunter (5). Three 5.0 ml. portions of the sample were drawn into volumetric pipets (calibrated "to contain") and transferred to plastic centrifuge tubes containing 1.0 ml. of a solution of Evans blue dye in 0.9% NaCl. After thorough mixing, each 5.0 ml. transfer pipet was rinsed in the dye-blood mixture, and after a second mixing the tubes were centrifuged at approximately 2500 × G for 15 minutes at 20°C. Another portion of the sample was also centrifuged and the plasma was transferred with calibrated 2.0, 3.0, and 5.0 ml. pipets into 1.0 ml. portions of the dye solution. The plasma-dye mixtures obtained from whole blood and those obtained from known quantities of

* Coulter Electronics Co., Hialeah, Florida.

plasma were placed in matched quartz cuvets and their optical densities measured in a Beckman model DU spectrophotometer at 600 mμ (slit width = 0.073 mm.). In the dye concentrations employed, adherence to Beer's law was demonstrated.

The optical density determined in the plasma standards was plotted so that the 5:1 ml. of plasma:dye mixture represented 0 ml. of cells and the 3:1 and 2:1 ml. mixtures 2 and 3 ml. of cells, respectively. The values for the mean optical density of the three plasma-dye mixtures obtained from the whole blood were then superimposed on the established calibration line. Thus, the total erythrocyte volume contained in 5 ml. of the sample was determined. The Evans blue hematocrit amounted to 100/5 of the value obtained.

RISA Hematocrit

In one experiment the Evans blue hematocrit technique was evaluated by mixing portions of identical whole blood or plasma samples with the dye and with a dilution of radio-iodinated human serum albumin (RISA).† Sixty milliliters of venous blood were withdrawn from a fasted human subject and mixed immediately with 0.4 ml. of heparin solution. The Evans blue technique was performed on two 5.0 ml. portions of this blood, and three portions of 3.0 ml. each were taken at the same time to be thoroughly mixed with 100 microliters (λ) each of RISA solution (approximately 2.4 μc/ml.). Plasma samples of 1.0 and 3.0 ml. were also mixed with 100 λ of the RISA solution. After centrifuging the blood-RISA and blood-dye mixtures, the dye concentrations were measured as usual and 500 λ portions of the RISA-plasma samples were counted for two minutes each in a scintillation counter with two facing thallium-activated NaI crystals (each 4 inches in diameter by 3 inches thick).

The calculations for RISA hematocrit were based on the relative concentrations of I^{131}-albumin in the plasma and whole blood samples.

Centrifuge Hematocrit

Small portions of each sample were taken into heparin-coated capillary tubes* and centrifuged for four minutes on a hematocrit "Electrofuge."** The hematocrits were read directly from these tubes with an International Micro-Capillary Reader, Model CR.

Recording of Erythrocyte Volume Spectra

Precision grade micro pipets* and a Machlett-type auto pipet (25.0 ml.)** were used to make all dilutions: Fifty λ of the sample were mixed with 25.0 ml. of a filtered 0.9% NaCl solution; 250 λ of this dilution were then mixed with another 25.0 ml. of the NaCl solution. This final dilution of 1:50,000 was used for the recording of volume spectra and for the cell counting procedure. Three determinations were made of each sample.

The 1:50,000 dilution, in a glass vial of appropriate size, was quickly placed under the aperture tube of the counting assembly and a volume distribution spectrum was obtained: the plotter mechanim was engaged, the manometer stopcock was opened, and the "start" and "record" switches were actuated. Previously, blood samples from various species of interest had been studied to determine the optimal settings for amplification, aperture current, and plotter scale factor.

The aperture current gain control was set on 30. *(Care must be taken that the gain control setting is recorded and that this setting is not changed during and after calibration!)* Because of shifts in volume spectra which occurred at about 10 minutes following the second dilution, recordings were always made within three minutes after dilution.

Erythrocyte Counts

After the volume spectrum had been recorded, the same dilution was used to obtain the erythrocyte count. Examination of the volume spectrum revealed a suitable counting threshold, i. e. a "lower threshold" setting midway between the lowest channel which is free from noise and the first channel which shows

† Abbot Laboratories, Oak Ridge, Tennessee.

* Progressive Laboratory Specialties Corp., Jamaica 35, New York.

** Chicago Surgical and Electrical Company, Chicago, Illinois.

* Corning Glass Works, Corning, New York

** Fisher Scientific Company, New York 14, New York.

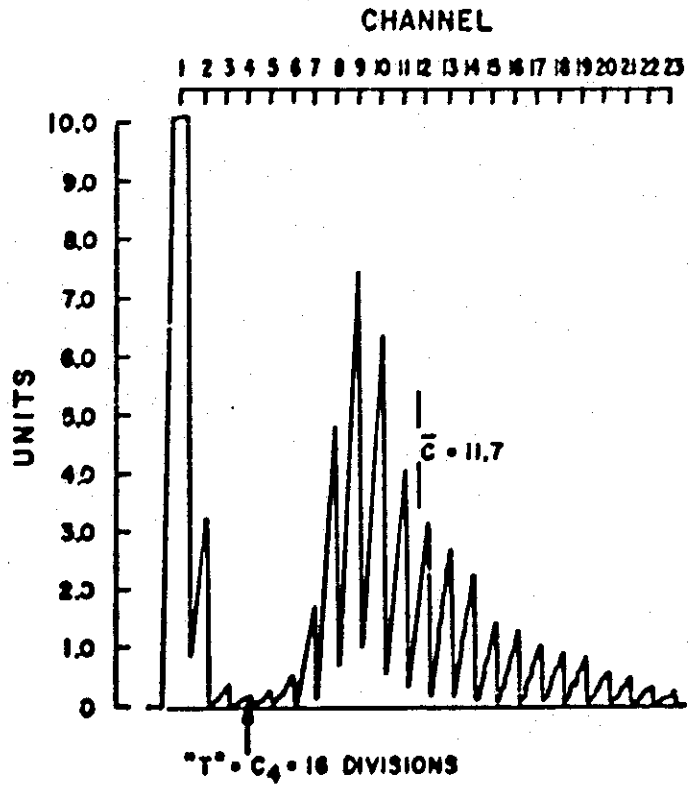

FIGURE 1. Plotter Read-Out from a sample of rat blood: Note selection of lower counting threshold ("T"). The mean channel ($\bar{C}$) is calculated from the equation (C) page 4.

a pen excursion greater than the background (See Fig. 1).

The "upper threshold" setting was completely locked out and the plotter was disengaged. A count was then recorded in the usual manner: the manometer stopcock was opened, the reset switch was snapped in either direction, and the manometer cock closed. The first three digits of the count were recorded and corrected for coincidence by referring to a statistical chart.* The counting procedure was performed on each dilution and the mean of the three counts was obtained.

Calculations

Per Cent of Total Count

In order to project volume spectra on a comparable scale they were plotted as per cent of total cell count (above threshold) vs. cell volume at channel mid-point (4). The former was calculated for each channel:

$$(A) \qquad \% \text{ Total Ct.} = \frac{U_i}{\sum_{i=T}^{i=25} U_i}$$

When U_i is the number of units of pen excursion for any given (i^{th}) channel; and

$$\sum_{i=T}^{i=25} C_i U_i$$

is the sum of all units in all channels from the threshold setting (T) through Channel No. 25, including the i^{th} channel.

* Coulter Electronics Co., Hialeah, Florida.

Mean Corpuscular Volume (MCV):

$$(B) \qquad MCV = \frac{Hct}{Ct} \times 10^{7};\ \text{e.g.}$$

$$\frac{50}{5 \times 10^{6}} \times 10^{7} = 100\ \mu^{3}/\text{cell}$$

When Hct is the hematocrit in Vol. % and Ct is the erythrocyte count per mm^3.

Mean Channel ($\bar{C}$):

$$(C) \qquad \bar{C} = \sum_{i=T}^{i=25} C_i U_i \Big/ \sum_{i=T}^{i=25} U_i$$

$\sum_{i=T}^{i=25} U_i$

When $i = T$ is the product of the channel number and the units of pen deflection for any given (i^{th}) channel and the denominator is the same denominator used in equation (A).

Volume Calibration Factor (a):

$$(D) \qquad a = \frac{MCV}{\bar{C}} = \mu^{3}/\text{channel}$$

Using the raw data from the plotter (Fig. 1) (and) the hematocrit and the erythrocyte count a volume calibration factor (a) is calculated. This factor may be used to express any channel number in volumetric dimensions.

For example, once a is obtained for given settings of aperture current and amplifications it may be used to obtain the MCV of an unknown sample by employing the formula

$$(E) \qquad MCV = a\bar{C}$$

When volume spectra are graphically projected it is advantageous to consider the cells recorded in a given channel as having a volume corresponding to the channel mid-point (4).

The channel mid-point is derived by subtracting 0.5 from the channel number; e.g. channel number 1 is considered to be channel number 0.5, number 2 is then 1.5, etc.

Hence, the mean corpuscular volume at mid-channel will be

$$(F) \qquad (a)\ (\bar{C} - 0.5) = \text{MCV at mid-channel}$$

RESULTS

Hematocrits – Replication of the Evans blue method for estimation of hematocrits gave satisfactory results; the coefficient of variation ranged from 2.2% to 12.8% (Table I-D). The samples exhibited little hemolysis or lipid turbidity. There was a slight tendency for centrifuge hematocrits to be lower than those obtained with the dye technique, but there was no evidence of a statistically significant difference (Table III-A). In a single experiment (Man-3) the RISA hematocrit of 47.3% compared favorably with the dye hematocrit of 48.0%.

Erythrocyte Counts are summarized in Table I-A. These results were typical for those obtained with the electronic counter (1); the pooled standard error of the measurement was only ± 44,000 counts and the coefficient of variation ranged between 0.5 and 4.5%.

MCV–Table III-B presents the mean corpuscular volumes as calculated from the electronic counts and the centrifuge and dye hematocrits. No statistically significant difference was found.

Volume Spectra and Volume Calibration Factors – In Fig. 2 volume spectra are plotted as per cent of total count vs. channel. Volume spectra were obtained from triplicate samples at two aperture currents (1/.707 and 1/.5); thus, the mean channels as expressed in Table I-B and I-C represent the average of

TABLE I

SUMMARY OF RESULTS OF THREE REPLICATIONS OF MEASUREMENTS ON VARIOUS SPECIES

I-A *Erythrocyte Counts × 10^6 per mm^3*

*Species**	M-1	M-2	M-3	R-1	R-2	C-1	C-2	C-3	C-4	Range for All Species
X	5.26	4.56	5.21	4.77	4.40	8.38	4.04	8.05	8.89	—
SD.	0.03	0.09	0.24	0.04	0.04	0.17	0.06	0.05	0.05	—
C.V.(%)	0.48	1.91	4.51	0.74	0.80	2.04	1.93	0.62	0.80	0.5 - 4.5

I-B *Mean Channel ($\overline{C}$) Current = $\frac{1}{0.707}$*

*Species**	M-1	M-2	M-3	R-1	R-2	—
X	12.4	13.6	12.8	9.6	9.1	—
SD.	0.12	0.61	0.12	0.46	0.08	—
C.V. (%)	0.9	4.5	0.9	4.8	0.8	0.8 - 4.8

I-C *Mean Channel ($\overline{C}$) Current = $\frac{1}{0.50}$*

*Species**	C-1	C-2	C-4	C-3	R-2	M-3	—
X	10.23	10.03	10.96	10.86	14.03	17.00	—
SD.	0.208	0.231	1.211	0.153	0.751	0.721	—
C.V. (%)	2.0	2.3	11.0	1.4	5.3	4.2	2.0 - 11.0

I-D *Hematocrit (Per Cent) — Dye Method*

*Species**	M-1	M-2	R-1	C-1	C-2	C-4	—
X	43.9	45.0	33.6	39.3	25.6	43.0	—
SD.	1.3	1.0	1.2	5.0	2.1	2.0	—
C.V. (%)	2.9	2.2	3.4	12.8	8.1	4.7	2.2 - 12.8

*M = Man; R = Rabbit; C = cat; X = mean; SD_x = standard deviation; C.V. = coefficient variation.

TABLE II

SUMMARY OF THE THREE ESTIMATES OF *a* FROM THE APPROPRIATE THREE REPLICATIONS OF ERYTHROCYTE COUNT, MEAN CHANNEL, AND DYE-HEMATOCRIT

$$CURRENT = \frac{1}{0.707}$$

*Species**	M-1	R-1	R-2	M-2	M-3	—
X	6.70	7.31	7.17	7.27	7.06	—
SD,	0.27	0.49	0.14	0.34	0.37	—
C.V. (%)	4.00	6.74	0.20	4.72	5.23	0.2 - 6.7

$$CURRENT = \frac{1}{0.50}$$

*Species**	C-1	R-2	C-2	C-4	M-3	C-3	—
X	4.57	5.11	5.99	4.40	5.27	4.13	—
SD,	0.44	0.06	0.87	0.61	0.36	0.06	—
C.V. (%)	9.63	1.11	14.46	13.92	6.84	1.55	1.1 - 14.5

*M = Man; R = Rabbit; C = cat; X = mean; SD_x = standard deviation; C.V. = coefficient variation.

Range for All Species

TABLE III – A

COMPARISON OF TECHNIQUES FOR ESTIMATION OF HEMATOCRIT

Subject	*RISA Hct.*	*A. Centrif. Hct. (Mean of two)*	*B. Dye Hct. (Mean of three)*	*Diff = B-A*
Dog		52.3	59.0	+6.7
M-1		47.5	44.0	−3.5
M-2		45.3	45.0	−0.3
M-3	47.3	45.8	48.0*	+2.2
R-1		32.5	33.4	+0.9
R-2		30.5	30.6	+0.1
C-1		35.0	38.0	+3.0
C-2		17.8	25.0	+7.2
C-3		38.0	35.2	−2.8
C-4		42.3	44.0	+1.7
				d = +1.52

$t_{\text{paired-matched experiments, } d=0} = 1.367$; $p > 0.10$

M = man; R = rabbit; C = cat

*Mean of two

TABLE III — B

COMPARISON OF ESTIMATES OF MEAN CORPUSCULAR VOLUME

Subject	*A. MCV from Centrif. Hct.*	*B. MCV from Dye Hct.*	*Diff = B-A*
Dog	75.8	85.5	+9.7
M-1	90.3	83.7	−6.6
M-2	99.3	98.7	−0.6
M-3	87.1	91.5	+4.4
R-1	68.1	70.0	+1.9
R-2	69.3	69.5	+0.2
C-1	41.8	45.3	+3.5
C-2	44.0	61.7	+17.7
C-3	47.3	43.8	−3.5
C-4	48.7	50.6	+1.9
			$\bar{d}$ = +2.9

'(paired control experiment, df = 9) = 1.323; p > 0.10

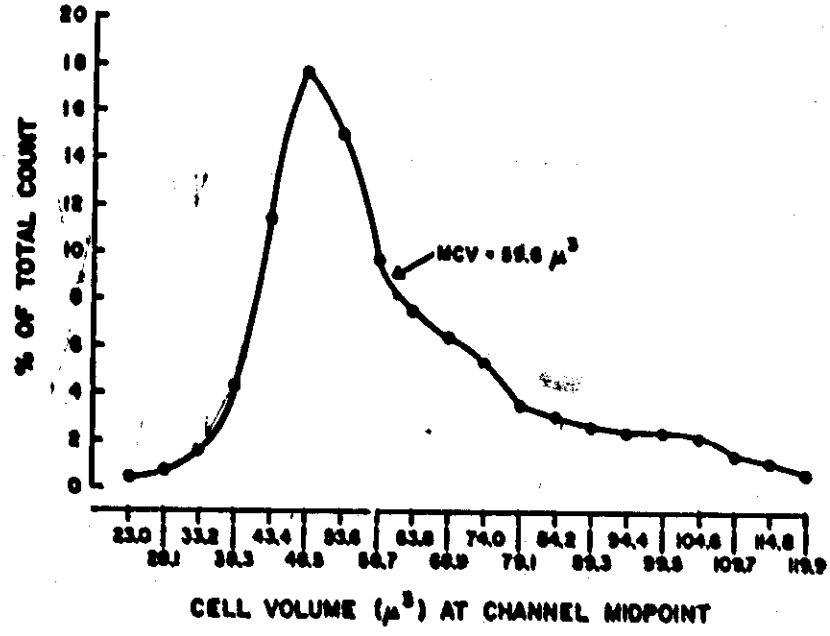

FIGURE 2. Volume spectrum taken from a sample of rat blood (see Fig. 1 and equations on page 4) cell volumes at channel mid-point and % of total count were calculated from the raw data given in Fig. 1.

triplicate measurements. The range of coefficients of variation was greater when the higher current was used, and in general there was less reproducibility with the higher current. Consistent with these results, the estimation of the volume calibration factor (a) was more reproducible at the lower current (Table II).

DISCUSSION

The results of this study indicate that erythrocytes may be counted by electronic means with an impressive reproducibility. The erythrocyte counts presented in Table I-A indicate that populations as small as 4.04×10^6 cells/mm^3 and as large as 8.69×10^6 cells/mm^3 may be estimated within a reasonable error of measurement.

One might suspect that the limiting factor in estimating the volume calibration factor (a) is the measurement of hematocrit. Examination of the coefficients of variation suggests that the mean channel determinations at the higher current may introduce almost as much error as the hematocrit (Tables I-C and I-D). This is consistent with the report of Brecher, *et al*, (4) that high aperture currents may alter the size of mammalian erythrocytes; the coefficients of variation obtained when a was calculated from the cell count, dye hematocrit, and $\bar{C}$ support this statement.

The characteristics of the size distribution plotter used in these experiments require that the pulse heights fall within a certain voltage range for proper analysis. It is not necessary, however, to use higher aperture currents in order to satisfy this criterion. As a rule, changes in pulse height amplification serve to bring the pulse pattern within the range of the plotter; too high an amplification (e.g. 1/.5 or above), however, may result in an excess of electronic noise (4). A good balance between amplification and aperture current intensity is best obtained by trials with the erythrocytes to be used in a particular experiment.

Ponder (6) stated that the Evans blue dye method for hematocrit estimations is almost ideal in the sense that the dye is neither adsorbed to the erythrocyte surface nor taken into the cell. In one experiment the Evans blue hematocrit of 48.0% agreed more closely with a RISA hematocrit of 47.3% than the estimation obtained by centrifugation (45.8%). The latter technique, however, yields estimates of the mean erythrocyte volume which compare satisfactorily to those obtained by the more elegant dye technique (Table II).

The calibration factors obtained in these experiments have been used for two months in our laboratories for the plotting of erythrocyte volumes in rats and we have observed no drift in the spectra obtained from control animals.

SUMMARY

A simple technique is presented for calibrating an electronic system used in the plotting of erythrocyte volume spectra. The calibration factors, once obtained, apparently remain applicable for some time. Precise estimates of calibration factors appear to be limited by errors in measuring hematocrit and/or mean channel. For consistent determination of the latter, the selection of a sufficiently low aperture current appears to be of importance.

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