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Electronic Measurement of Cellular Volumes. I. Calibration of the Apparatus

(C. C. Lushbaugh, J. A. Maddy, and N. J. Basmann)

INTRODUCTION

Diagnosis and treatment of anemia are based in part on the number and mean cellular volume (MCV) of circulating erythrocytes. Routinely these data are not obtained accurately because of inherent difficulties in the indirect hematocrit method used in determining the MCV. Fifty years ago, Price-Jones (1) showed that the frequency distribution of erythrocyte diameters varied in a diagnostically useful manner in some diseases, but this technique proved too laborious for routine use. Recently the development of electronic particle counting has enabled routine red and white blood cell counting to be done rapidly with less than 1 per cent error by unskilled persons (2). Because the amount of change in electrical resistance occurring during the passage of a cell through the counting aperture is proportional to the volume of solution displaced, this method can also be used for determining the volume of a cell directly. This report describes the progress made in combining the Coulter electronic particle counter with a LASL pulse height analyzing system affording an immediate visual display of the frequency distribution of erythrocyte volumes.

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METHODS

A commercially available (Coulter) electronic particle counter and glassware with a 100-micron aperture were used in this study. The signal was obtained from the cathode follower circuit of the device and fed into a single-channel analyzer with upper and lower gate discriminators that allowed selection of pulse heights of from 4 to 104 volts. The chosen pulses were sorted in a 100-channel analyzer and their numbers collected in its memory storage unit whose capacity was limited by means of a scaler and count control unit which stopped the analysis when 100,000 cell passages were analyzed and stored. A cathode display unit allowed constant visualization of the distribution of the collected cell volumes, enabling rapid calibration of a Moseley autograph plotter to fit the frequency distribution curve being obtained. The data were graphed automatically as well as printed out on a computing tape recorder (Fig. 1).

The erythrocyte number per centimeter (RBC) of heparinized blood was determined with the Coulter counter in the accepted manner (2). Human, rabbit, and guinea pig blood were diluted 1:800,000; mouse, horse, and goat blood 1:1,600,000; reptilian and fowl blood 1:400,000 in order that a concentration of about 6000 RBC per ml of saline resulted. These high dilutions obviated correction of the

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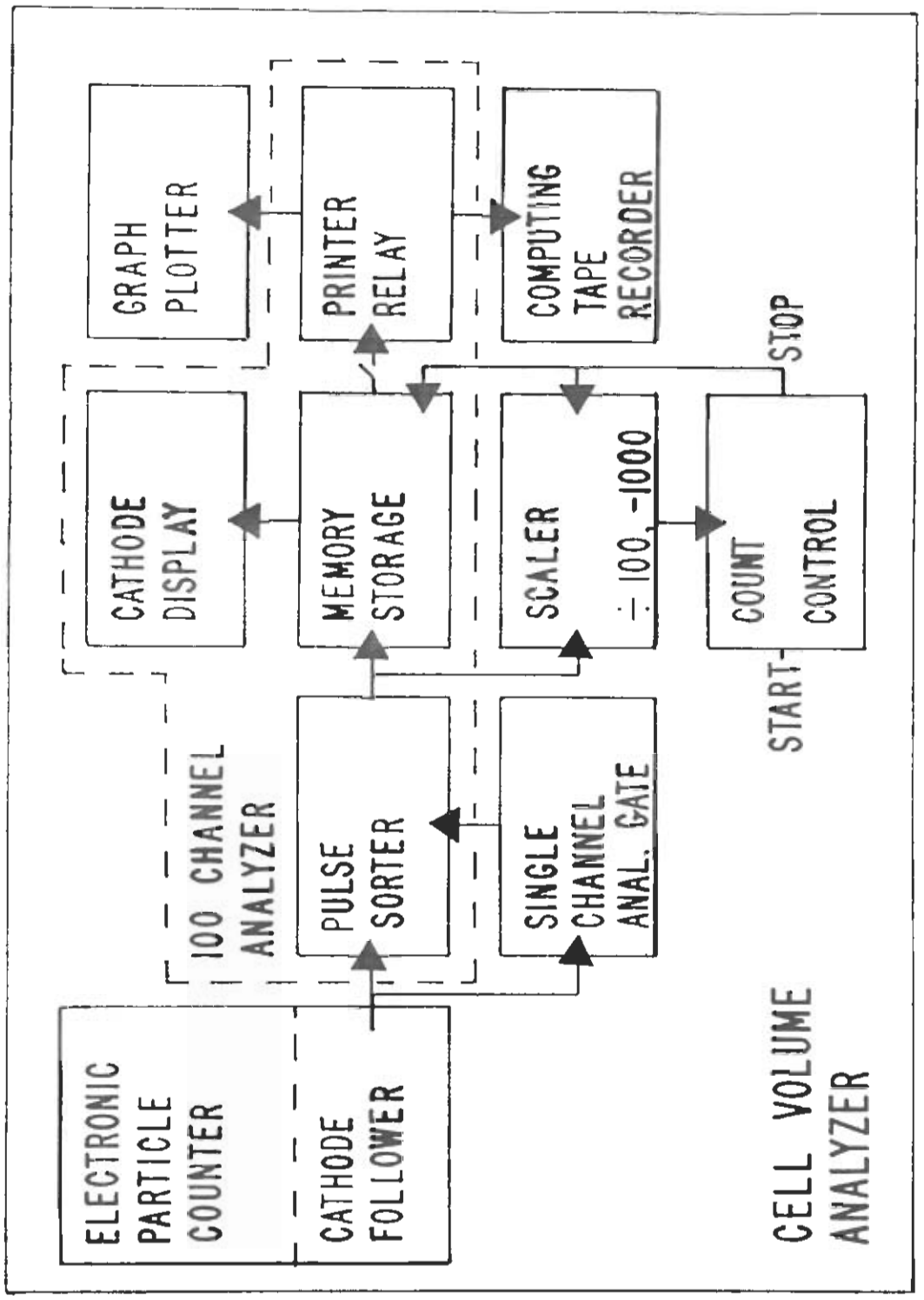

Fig. 1. Simplified scheme of arrangement of components of cell volume analyzer.

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curves for coincidence error and analyzer "dead time" loss. Microhematocrits (Hmct) were done simultaneously with the RBC and the mean corpuscular volume (MCV) determined for each sample by the formula

$$\frac{\text{Hmct} \times 10}{\text{RBC}} = \text{MCV}.$$

The analyzer channel in which the mean cell volume appeared to fall electronically was found by determining mathematically the weighted mean of the frequency distribution curve (Fig. 2). This mean channel number plus 4* equalled the mean pulse height in volts (mean pulse voltage = MPv). The MCV of each sample was then plotted against the MPv to obtain best fit lines that could be used to convert pulse height voltage readings into cubic microns. In this part of the study, the blood of 8 mice, 6 rabbits, 4 guinea pigs, 25 men, 10 chickens, and 5 ducks was used because the means of the MCV of the cells of these species were widely spaced when plotted against their respective MPv (Fig. 3).

By altering the aperture current settings (ACS) of the Coulter counter, the amount of current passing through the counting aperture can be varied, so that pulses caused by

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*No pulses less than 4 volts in height were measured. The first analyzer channel counted pulses of 4 volt strength, the second channel 5 volt pulses, and so on.

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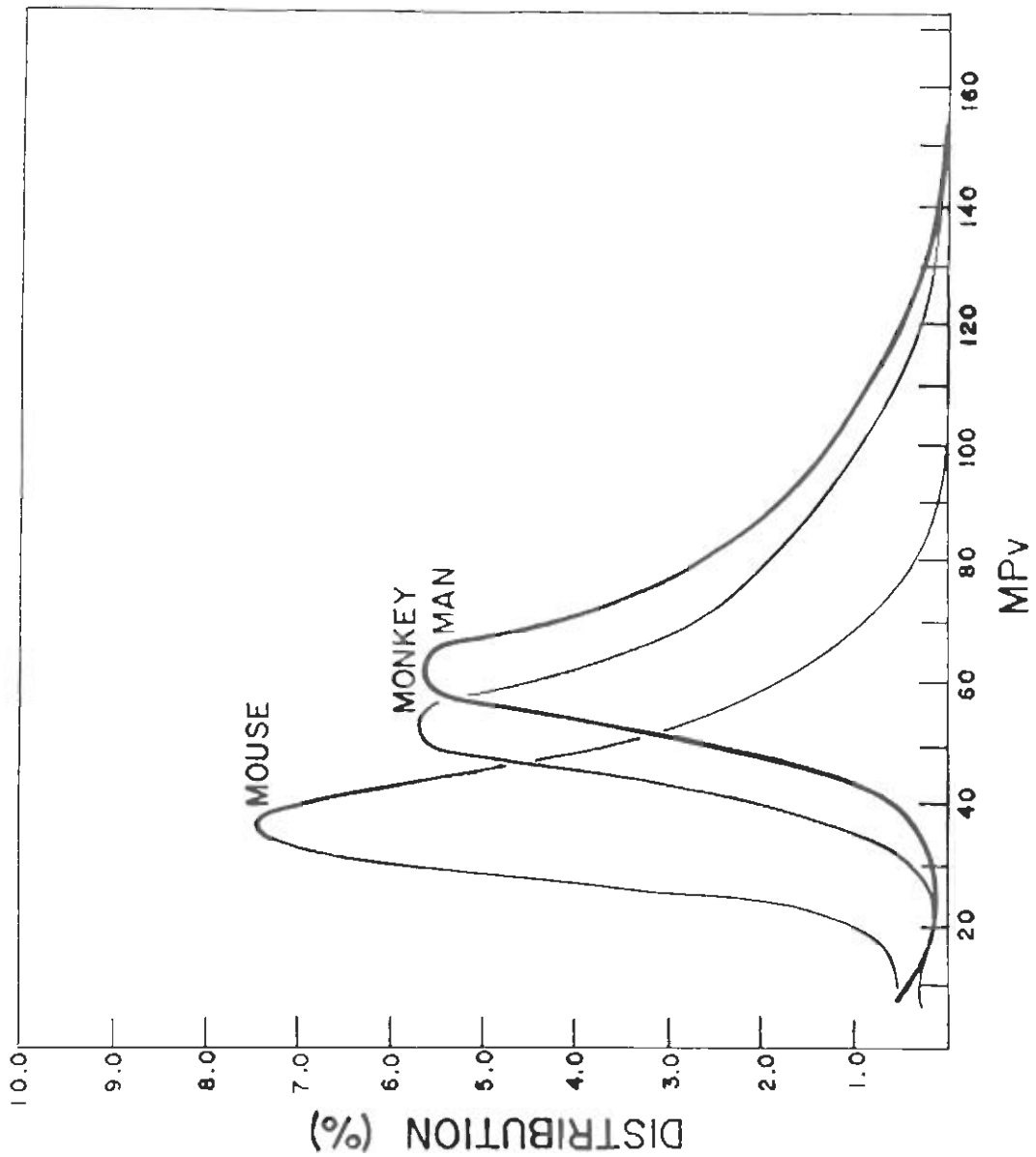

Fig. 2. Frequency distribution profiles of erythrocyte populations of mouse, monkey, and man determined electronically.

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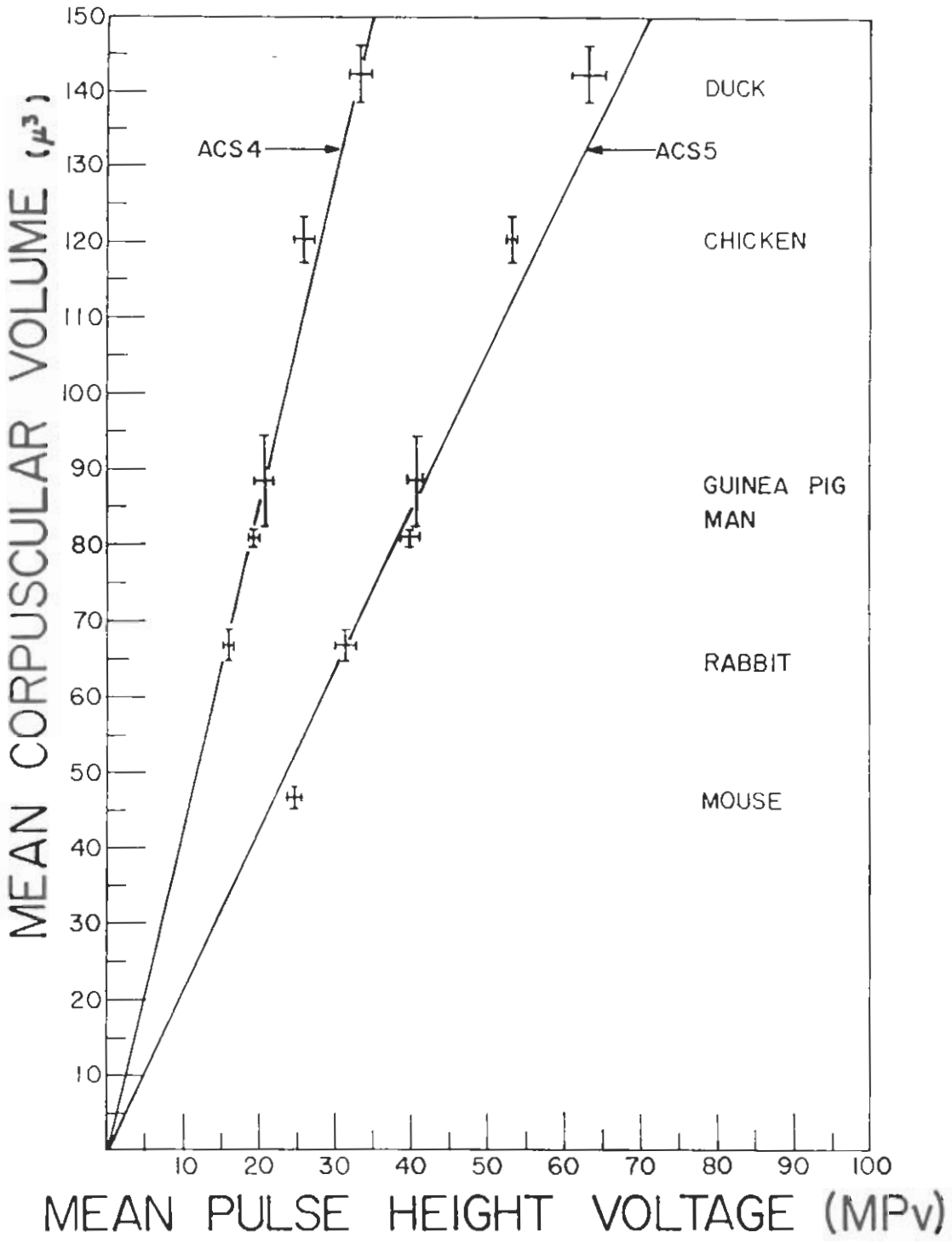

Fig. 3. Best fit lines showing relationship of MCV to MPv for ACS4 and ACS5.

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populations of large cells are decreased and by populations of small cells are increased. In this manner, most cell populations can be made to produce pulses varying from 4 to 104 volts so that the entire frequency distribution profile can be contained within the limits of the 100 analyzer channels. In this calibration study, aperture current settings of 4 and 5 were used routinely to analyze each sample. When available, populations with very small cells (e.g., horses, goats, and cats) were re-analyzed at ACS6, ACS7, and ACS8, and those with very large cells (e.g., birds and reptiles) were re-analyzed with ACS1, ACS2, and ACS3. This procedure was followed to determine the alteration of apparent size caused by changes in aperture voltage and thereby relate the 8 ACS's of the Coulter counter to one another. By means of the effect of ACS on pulse heights of cells of constant volume, mathematical factors were determined for converting pulse height voltage to cubic microns for ACS1 through ACS8.

RESULTS

Typical frequency distribution profiles for the RBC of mouse, monkey, and normal man are shown in Fig. 2. The populations were found to be asymmetrical so that the mean cell volume and the most frequent cell volume (mode) were not identical, making it necessary to determine the MCV or MPv mathematically.

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In Table 1, the experimental data have been tabulated in order to show the differences in the MCV of the RBC of the 6 species studied and the mean MPv obtained for these volumes with ACS4 and ACS5. The relationships of these data are shown in Fig. 3. The mouse point was omitted at ACS4 because many of the smaller mouse RBC's did not produce pulses greater than 4 volts so that an erroneously high MPv was obtained. While the mouse point was still skewed slightly to the right of the line at ACS5, the duck and chicken points were skewed to the left of the line at ACS5 because at this aperture current the largest RBC of these species created pulses greater than 104 volts and were, therefore, not analyzed. The analyzer loss of these large cells caused an apparent decrease in the mean of these populations. The modification in the relationship of cell volume to pulse height voltage affected by aperture current is shown in Fig. 4. The data illustrated in these 2 figures and in Table 1 enabled the determination of the conversion factors tabulated in Table 2. It was found that the apparent volume indicated by the channel width of the analyzer varied with amount of aperture current so that, for example, at ACS1 a single channel indicated a volume of 17.33 μ^3 while at ACS6 the indicated volume was 1 μ^3. These volumes have been designated at Factor 1 in Table 2, and can be used as factors to convert the analyzer channel

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TABLE 1. RELATIONSHIP OF AVERAGE MCV OF RBC OF SIX SPECIES WITH THE AVERAGE MPv OF THEIR FREQUENCY DISTRIBUTION CURVES AT ACS4 AND ACS5

Species	No.	MCV* (μ^3)	MPv (ACS4) (volts)	MPv (ACS5) (volts)
Mouse	8	46.9 ± 1.5**	--	24.9 ± 0.6
Rabbit	6	66.9 ± 2.0	16.0 ± 0.8	31.5 ± 1.4
Man	25	81.1 ± 1.2	19.3 ± 0.6	40.0 ± 1.3
Guinea pig	4	88.6 ± 6.4	20.7 ± 1.2	40.6 ± 0.9
Chicken	10	120.3 ± 3.2	25.8 ± 1.3	53.3 ± 0.5
Duck	5	142.5 ± 3.7	33.2 ± 1.4	63.1 ± 2.7

*$\frac{\text{Hmct x 10}}{\text{RBC}}$ = MCV.

**Standard error.

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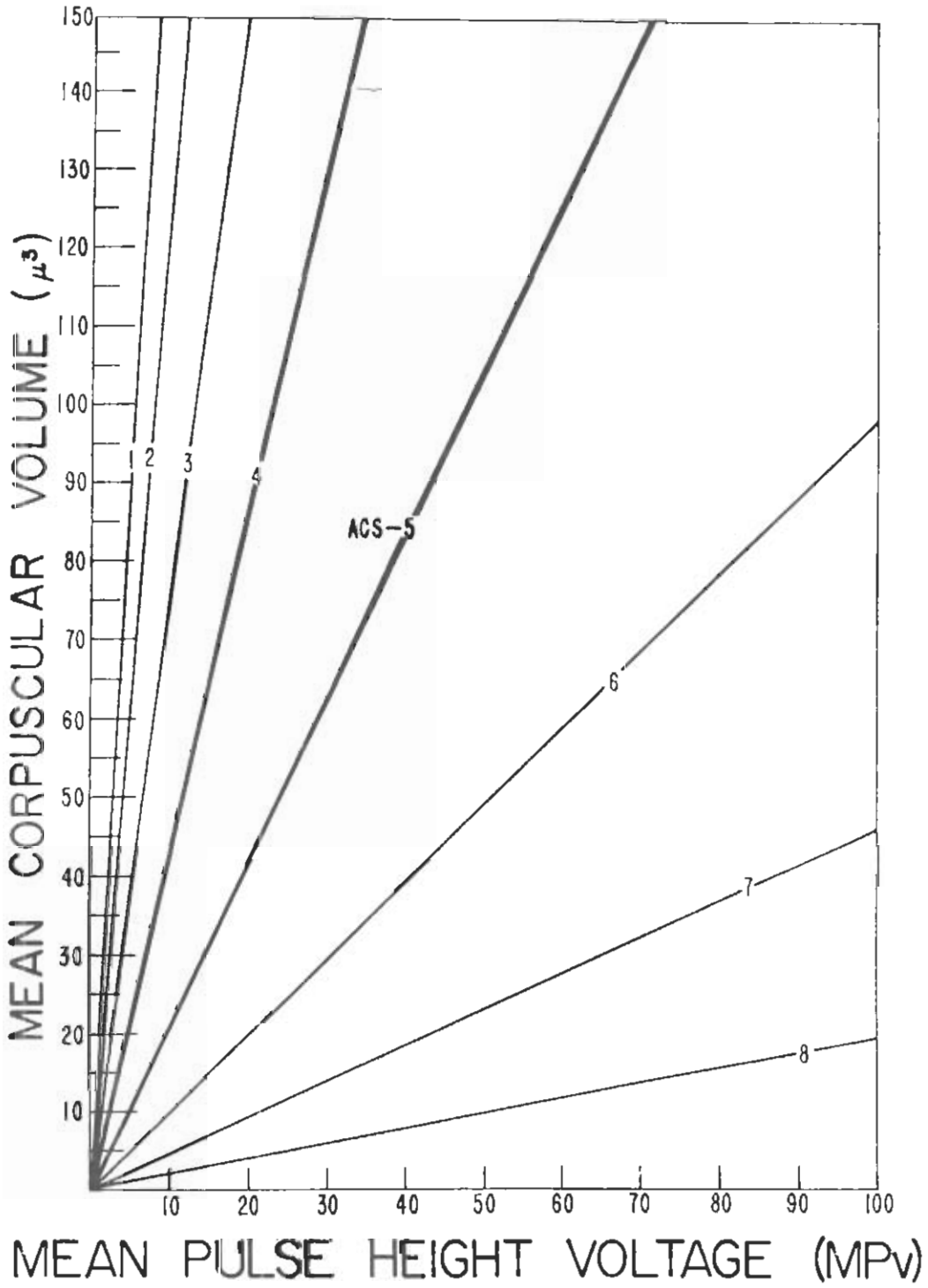

Fig. 4. The effect of aperture current setting (ACS) upon the relationship of particle volume to pulse height voltage.

TABLE 2. CONVERSION FACTORS FOR CHANNEL NUMBER TO CUBIC MICRONS (ACS5)

ACS No.	F_1 Anal. Channel Volume (μ^3)	F_2 Pulse Height $\hat{=}$ 1 μ^3 (volts)	F_3 Channel Volume Relative to ACS5	F_4 Approximate Magnification Relative to ACS5
1	17.33	0.06	8.10	0.1
2	11.30	0.09	5.28	0.2
3	7.55	0.13	3.48	0.3
4	4.33	0.23	2.02	0.5
5	2.14	0.47	1.00	1.0
6	1.00	1.00	0.47	2.0
7	0.46	2.17	0.29	3.5
8	0.19	5.26	0.09	11.0

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number or voltage into volume and to construct scales for the visual conversion of channel number (or voltage) to volume, as shown in Table 3 for ACS5. Factor 2 in Table 2 designates the pulse height voltage equivalent to 1 μ^3 at the 8 aperture current settings and can be used to compute the effect of volumetric change upon pulse height and subsequent analyses. Factor 3 in Table 3 can be used to convert the volumetric scale for ACS5 (Table 3) to a scale for any other ACS setting and thus to approximate cell volumes relative to the human erythrocyte. Similarly, Factor 4 enables the use of the aperture current settings as microscope objectives and shows the magnification produced by the apparatus in relation to human erythrocytes at ACS5. For example, an object measured at ACS8 appearing on the viewer in channel 32 is magnified 11 times relative to a human erythrocyte in that channel and, therefore, has a volume of about 7 μ^3 instead of 77 μ^3 (channel 32 + 4 = 36 volts; 36 x 2.14 = 77 μ^3, or 36 x 0.19 = 6.8, or 77/11 = 7).

DISCUSSION

Variation in resistances and internal gain adjustments produce sufficient differences in pulse heights to make it necessary to calibrate each instrument in the manner described here. Calibration of a second instrument could be greatly

TABLE 3. VISUAL CONVERSION OF PULSE HEIGHT VOLTAGE (CHANNEL NUMBER + 4) TO CUBIC MICRONS AT ACS5

Pulse (volts)	Volume (μ^3)	Pulse (volts)	Volume (μ^3)	Pulse (volts)	Volume (μ^3)	Pulse (volts)	Volume (μ^3)
1	2.1	26	55.6	51	109.1	76	162.6
2	4.3	27	57.8	52	111.3	77	164.8
3	6.4	28	59.9	53	113.4	78	166.9
4	8.6	29	62.1	54	115.6	79	169.1
5	10.7	30	64.2	55	117.7	80	171.2
6	12.8	31	66.3	56	119.8	81	173.3
7	15.0	32	68.5	57	122.0	82	175.5
8	17.1	33	70.6	58	124.1	83	177.6
9	19.3	34	72.8	59	126.3	84	179.8
10	21.4	35	74.9	60	128.4	85	181.9
11	23.5	36	77.0	61	130.5	86	184.0
12	25.7	37	79.2	62	132.7	87	186.2
13	27.8	38	81.3	63	134.8	88	188.3
14	30.0	39	83.5	64	137.0	89	190.5
15	32.1	40	85.6	65	139.1	90	192.6
16	34.2	41	87.7	66	141.2	91	194.7
17	36.4	42	89.9	67	143.4	92	196.9
18	38.5	43	92.0	68	145.5	93	199.0
19	40.7	44	94.2	69	147.7	94	201.2
20	42.8	45	96.3	70	149.8	95	203.3
21	44.9	46	98.4	71	151.9	96	205.4
22	47.1	47	100.6	72	154.1	97	207.6
23	49.2	48	102.7	73	156.2	98	209.7
24	51.4	49	104.9	74	158.4	99	211.1
25	53.5	50	107.0	75	160.5	100	214.0

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simplified, however, by referring it to the first by simultaneously determined measurements of the same sample in both instruments. It would also not be necessary to compare MCV with MPv, since the comparison of the MPv or of the modal Pv in both machines would enable the conversion of the various calibration factors.

The results of this calibration study indicate that a rapid, simple calibration could be done using only human erythrocytes and treating each case separately. For standardization purposes, human red blood cells from normal young adults are more uniform and have a more constant volume distribution than any commercially available particles of this size range. Most commercially available particles are either too small, as in the case of Latex, or too small and variable, as in the case of puff balls, to be used as a standard for either finite calibration or the daily control of analyzer drift. The human red cell proved in this study to be quite adequate for this purpose.

A preliminary study of the frequency distribution curves of red blood cell populations using these calibrations and techniques is to be reported later (5).

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Electronic Measurement of Cellular Volumes. II. Frequency Distribution of Erythrocyte Volumes

(C. C. Lushbaugh, N. J. Basmann, and B. Glascock)

INTRODUCTION

The apparatus, technique, and calibrations described previously (1) were used to study the frequency distribution of erythrocyte volumes in man and in other animals. These analyses were done in order to determine whether such population curves might vary in a manner that might prove useful either theoretically or practically in clinical or descriptive hematology.

METHODS

Blood samples were obtained in dry, heparinized capillary tubes. Five lambda of the sample was diluted with 10 ml of saline. One-half ml of this suspension was then diluted with 200 ml of saline, agitated thoroughly, and then counted in the usual manner in the Coulter counter. The second dilution was changed if the resulting count did not fall between 3000 and 4000 cells per 0.5 ml in 13 seconds. When the standard counting rate was obtained, the frequency distribution of the volumes of the cells was determined using the 100-channel pulse height analyzer unit previously described (1). The resulting curves were compared visually

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and then analyzed as a spectral curve and as a composite of 2 populations of normally distributed (Gaussian) particles.

ANALYTICAL PROCEDURES

In Fig. 1, a stylized population profile (frequency distribution of volumes) for circulating mammalian erythrocytes has been drawn. It defines the terms used in the first type of analysis done in this study. In this analysis, the curve was considered a spectral peak whose resonance or resolution could be expressed mathematically by its modal frequency, mode, mean, width at half-height, and fractional width. The term "fractional width" was used because this seemed to be less confusing than the spectrographic one of "resolution" and was determined by dividing width by mode. These numbers were used as channel volts without conversion to cubic microns.

In the second mathematical analysis, a 704 IBM computer was used;* after proper programming, the computer attempted to fit 2 (or sometimes 3) normal Gaussian curves to the population profile curve as it was obtained by the 100-channel analyzer. The data obtained were then expressed graphically as in Figs. 2 and 3, and mathematically as proportional areas under the total curve with standard deviations of the Gaussian curves enclosing these areas.

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*This computer program was arranged and operated for us by H. Israel of Group H-6.

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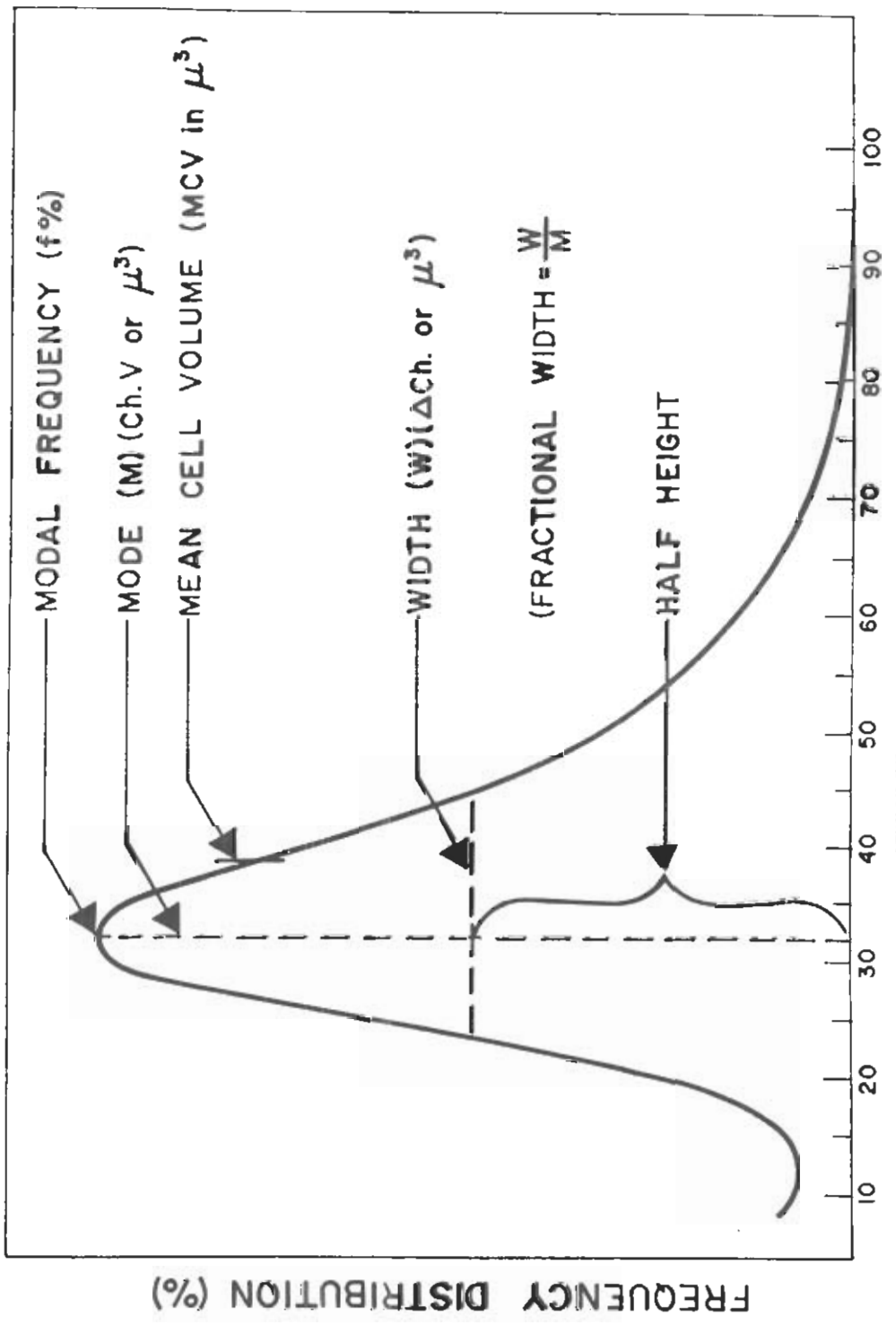

Fig. 1. Stylized frequency distribution of erythrocyte volumes defining the terms used in the "spectral" type of analysis.

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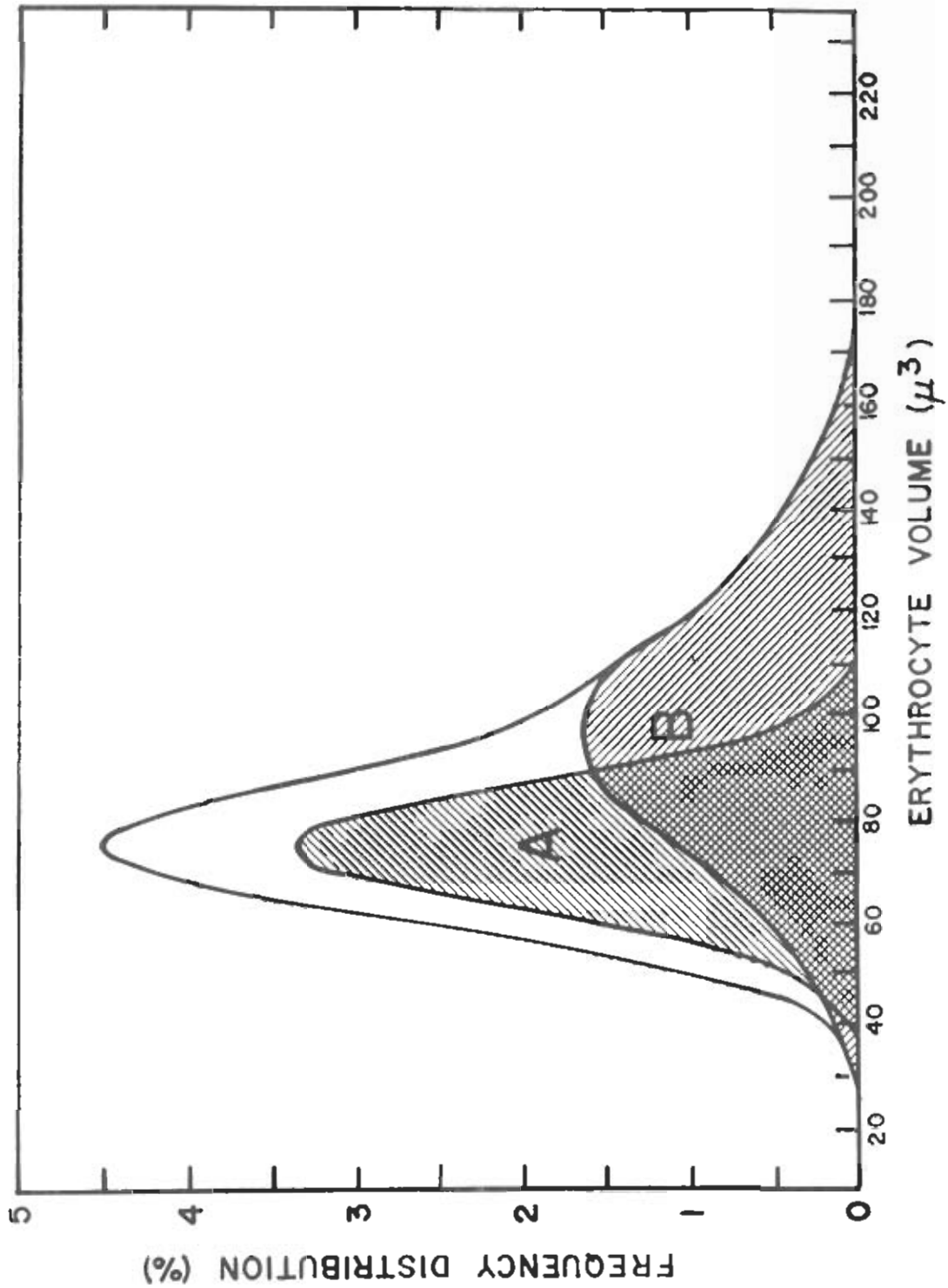

Fig. 2. Frequency distribution curve of erythrocyte volumes of human blood and its representation by 2 cell populations (A and B), each with a normal Gaussian distribution.

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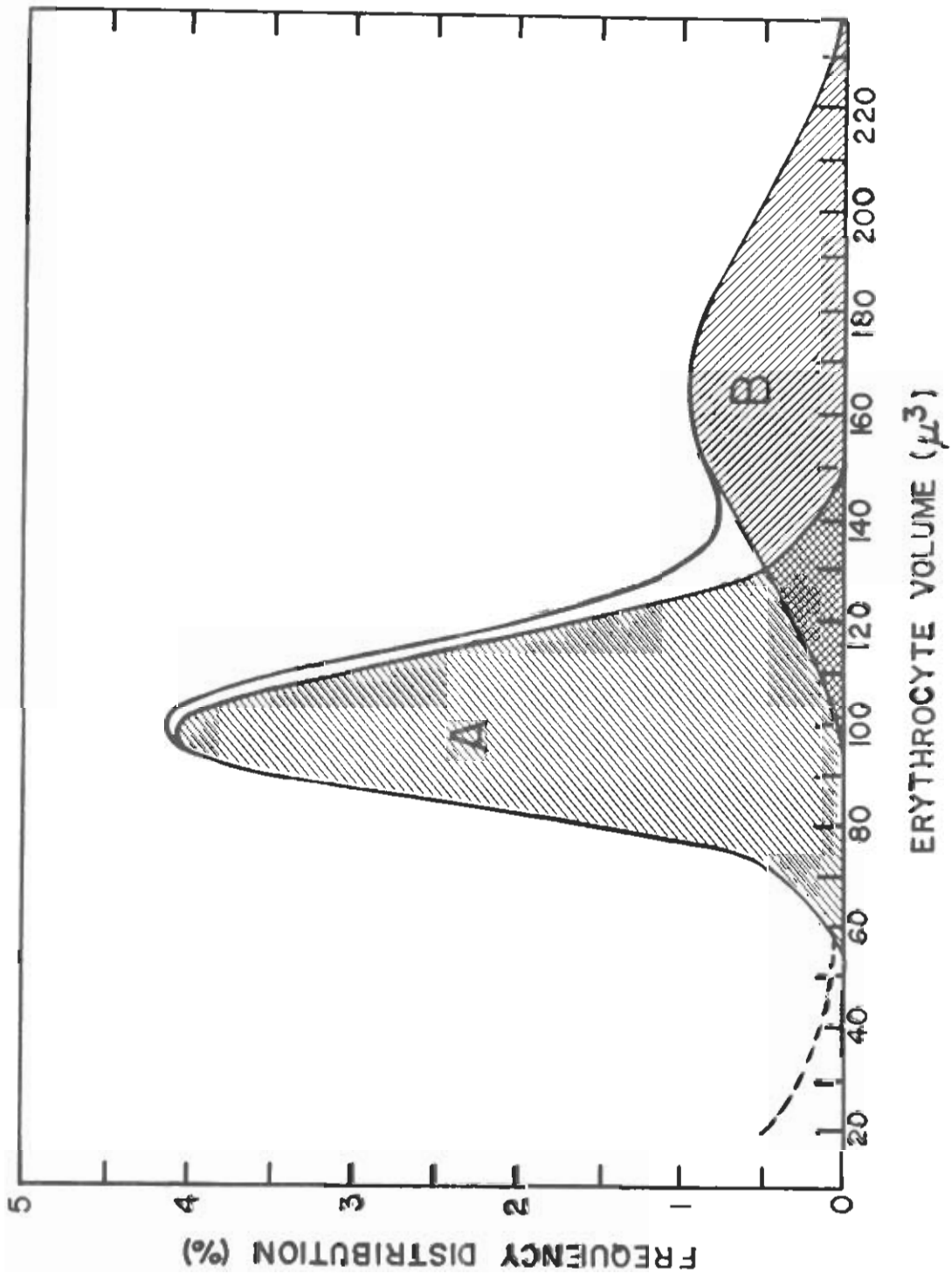

Fig. 3. Frequency distribution curve of erythrocyte volumes of chicken blood and its representation by 2 cell populations (A and B), each with a normal Gaussian distribution.

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RESULTS

The results of the spectral analysis of these population profiles are tabulated in Table 1. Their fractional width was found to vary between 0.40 and 0.60 in healthy man, mouse, monkey, chicken, frog, and toad. In several questionably sick horses, as well as in 12 patients suffering from various anemias, the width of the RBC population profile was increased by a skewing of the curve to the large side of the mode so that a large increase in fractional width occurred. In man, if anemia was present, this measurement was always found to be greater than 0.6; the mean W/M was 0.80.

It was also found that as the mode became smaller its frequency increased, the width of the peak became narrower, and the mean moved closer to the mode. The mean never coincided with the mode because of the skew to the larger volume. In birds and reptiles especially but in occasional ill human beings, this skew could be seen visually to be the result of an overlapping large-volume population of cells. Examination of Wright-stained blood films of such specimens proved that if such a larger population did exist, it was not possible to determine it microscopically, although it was possible to determine that the bulge to the right was not caused by leukocytes.

It was quite gratifying, therefore, to have the IBM 704

TABLE 1. FRACTIONAL WIDTHS OF RBC POPULATION PROFILES (ACS5)

Species	No.	Frequency (per cent)	Width (ΔCh.)	Mode (Ch.)	Mode (μ^3)	Fractional Width (W/M)
Normal Man	14	4.5	17.3	33.0	70.6	0.52
Pathologic Man	12	3.6	26.2	33.3	71.6	0.80
Normal Mouse	8	7.8	11.0	18.0	38.5	0.61
Normal Chicken	13	4.0	16.3	38.6	82.6	0.42
Normal Monkey	1	5.6	16.0	27.0	57.8	0.59
Normal Frog*	1	3.1	22.0	40.0	85.6	0.55
Normal Toad*	1	3.8	18.0	39.0	83.5	0.46
Normal (?) Horse	5 3**	6.8 4.0	12.9 21.0	12.8 28.0	27.4 28.0	1.00 0.75

*ACS = 3.

**ACS = 6.

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computer analysis produce de novo perfect 2 Gaussian population fits to the curves, as shown in Figs. 2 and 3. Figure 2 shows the 2 overlapping populations of RBC in a normal young girl, a picture which has become the rule as these 704 computer analyses have continued, with population A comprising 45.6 per cent and population B 54.4 per cent of the total area or number of RBC analyzed. In Table 2, the other parameters of these 2 normal human RBC populations are listed. They show that population A is more uniform and volumetrically smaller than population B, although comprising about half of the total circulating red blood cells.

In Fig. 3, a similar analysis is depicted for the blood of a chicken. Population B is so far to the right of population A that it is rather well defined by the total curve. One can see that the 2 populations of chicken RBC have fewer cells with identical volumes than is the case with human blood.

DISCUSSION

The fact that there are 2 distinct populations of RBC circulating at 1 time in the same individual has been surmised before on the grounds of different sensitivities to various hemolysins (2) but has never obtained good morphologic support before.

If the 2 populations of red blood cells, demonstrated here

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TABLE 2. AVERAGE PARAMETERS OF ERYTHROCYTE POPULATIONS IN NORMAL HUMANS

Population	(per cent)	σ (Width) (μ^3)	Mode (μ^3)
A	45.6	11.7 ± 2.4	72.8 ± 11.6
B	54.4	28.5 ± 5.4	95.0 ± 14.8

B/A = 1.36 ± 0.07.

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mathematically, correspond physiologically with the 2 kinds of cells that can be differentiated by hemolysins, one would expect population A to consist of older, more fragile cells than population B, since it is well known that reticulocytes and relatively new red cells are larger than senile cells which are also hemolysin-sensitive. If so, this method should show in a hemolytic anemia a shift in favor of population B. In Figs. 4 and 5 this conjecture appears to be substantiated. It depicts 2 cases of the hemolytic anemia, erythroblastosis fetalis, that were transfused with normal adult blood. The preponderance of population B can be seen before transfusion in both cases, and its diminution afterwards. In the more severely affected case (Fig. 4), only population B appears to have been present before the exchange transfusion was done.

This hypothesis that young, newly formed cells comprise population B would seem to be susceptible to experimental verification. Such experiments are contemplated. If they support this hypothesis, the size of population B could be interpreted as the animal's response to his disease and would explain the increase in fractional width seen by us so far in all anemic disease states.

These preliminary studies of the variation and characteristics of RBC population profiles, as measured and analyzed electronically, have been sufficiently fruitful and the

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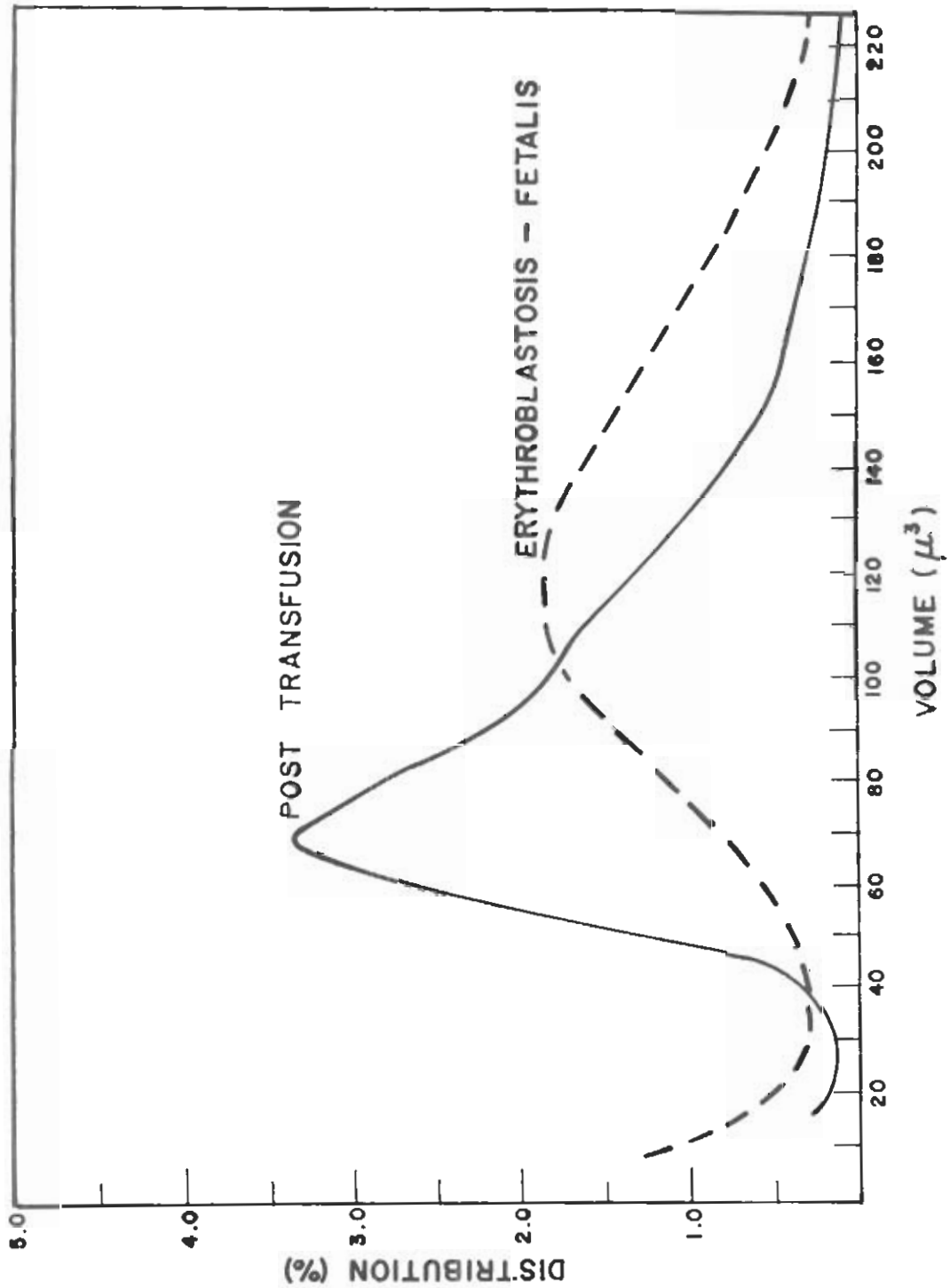

Fig. 4. Pre- and post-transfusion red blood cell profiles in a case of erythroblastosis fetalis showing the large cell preponderance in the presence of active hemolytic disease.

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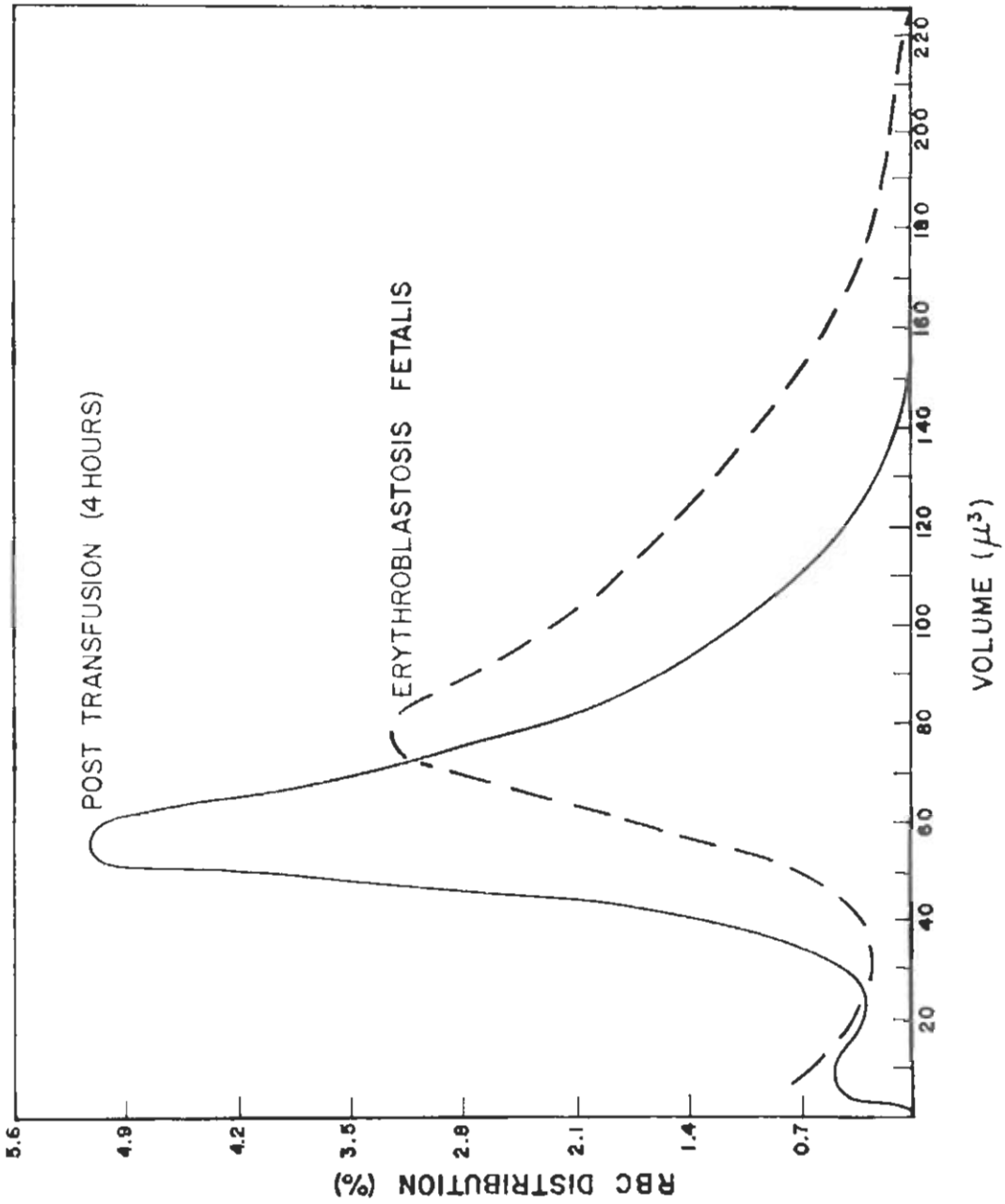

Fig. 5. Pre- and post-transfusion red blood cell profiles in a case of erythroblastosis fetalis showing the large cell preponderance in the presence of active hemolytic disease.

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results provocative enough to warrant their continuation. Studies are in progress now that promise to explain as well as define the changes in RBC populations that commonly accompany various hematologic diseases.

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