Technical methods

Red cell volume distribution histograms for establishing the normal range using the Coulter plotter model J

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In general haematology, a knowledge of the size distribution of the red cells can be of great value. In the past this information has been obtained by means of a Price-Jones curve (Price-Jones, 1911; 1920): the disadvantage of this technique is that the red cells are distorted during the preparation of a dry blood film.

The introduction of electronic counters has enabled an accurate estimation of the mean corpuscular volume (M.C.V.) to be more easily obtained (Brecher, Schneiderman, and Williams, 1956). Mixed cell populations in an abnormal specimen, however, can give rise to a normal M.C.V.

The development of the model J. Coulter automatic particle size distribution analyzer, used in conjunction with the Coulter counter model B, enables a histogram of the size and distribution of red cells in a blood sample to be made in 100 seconds (Brecher, Jakobek, Schneiderman, Williams, and Schmidt, 1962).

The following is a description of tests carried out in order to establish the type of pattern produced by normal blood samples, as a preliminary to ascertaining the suitability of the instrument for detecting early changes in red cell volume.

MATERIALS AND METHODS

Blood from normal individuals was collected into E.D.T.A. (Sequestrene) anticoagulant. Ideally the red cells should have been suspended in their own plasma, but this was impracticable (Brecher *et al.*, 1962). Although Eagle's basal medium diluted (9:1) with distilled water is a suitable diluent, the following diluent (Williamson, 1967) has been found to be satisfactory in this laboratory:—NaCl (A.R.), 90 g., was dissolved in 10 litres of distilled water, to which was added 20 ml. of 40% formaldehyde solution and 50 ml. of buffer solution. This was well mixed, the pH being 7.3. The solution was filtered by gravity through a Kieselguhr filter (grade Pasteur), and 1 ml. of 30% bovine albumin added. Buffer solution was made up of 53.4 g. Na₂HPO₄, 9.35 g. NaH₂PO₄, 1 litre of distilled water, and 15 ml. 40% formaldehyde (as a preservative). This fluid is easily

prepared and has excellent keeping properties. The Coulter A.P.S.D.A. model J provides a histogram, drawn in ink on special graph paper, which is divided on its horizontal axis into 25 equal increments (or windows), each of which represents a size range of red cells.

The Coulter model B (a modification of the model A) counts the number of cells in each size range which pass through the aperture in four seconds. This information is stored by the plotter and then registered on the histogram.

The counts in each window are registered so quickly in the glow counter that it would be difficult to record each reading. To make this easier, only alternate windows are registered, the glow counter being disabled during intervening counts. As each window is being accumulated, the pen, of which the vertical motion has been stopped by the height of the corresponding count of the previous window, traces a horizontal line at that level.

INSTRUMENT SETTING

The aim was to produce a histogram which would cover a size range from 0 to approximately 200 cubic microns. This could be obtained by using a suspension of particles of known mean volume. A small amount of a 'spore' powder (M.C.V. = 29.9 μ) was emulsified in approximately 1 ml. of wetting agent (Nonidet P42). The emulsion was added to the diluent until a suitable concentration of particles, as observed on the oscilloscope, was obtained. The amplification control and aperture switch were adjusted so that the majority of pulses occurred just above the centre of the screen. With the instrument used this was obtained with an amplification setting of 4 and aperture current of 1/4. Care was taken to avoid a high aperture current setting as this could cause boiling in the aperture. The lower and upper thresholds were adjusted to any setting, the lower always being below the upper, and the plotter was then brought into operation.

The window in which the highest peak of the histogram occurred was noted. This was found to be window 17. As the mean volume of the particles was 29.9 μ , each window was scanning a range of 1.8 μ . This indicated that the range covered by the selected settings was only from 0 to 45 μ . If the amplification of the impulses were reduced by a quarter, a range from 0 to 180 μ . would be obtained. Further histograms, with the amplification set at 1 and the aperture current at 1/4, produced a peak in window 4, indicating that the range now covered was 0 to 180 μ . These settings were confirmed by using another 'spore' powder¹ with a different M.C.V. (52.5 μ).

The correct settings having been established, a histogram of a 1 in 50,000 dilution of normal blood was traced. This produced the highest peak in window 11. As each window was equivalent to 7.2 μ , the volumes of the cells

¹Obtainable from Coulter Electronics, Ltd., Dunstable, Beds.

FIG. 1. Composite histograms of the maximum and minimum peaks in each window.

recorded and plotted in window 11 were from 72 to 79.2 μ .

ESTABLISHMENT OF NORMAL RANGE

The plotter has two controls, a recessed switch in front and a Vernier dial on top. These, when used in conjunction, can control the height of the peaks so that they correspond to a known number of cells. The fact that the Coulter B records the total number of cells in alternating windows facilitates this calibration. The settings chosen were those which gave a count of 60 cells for each division on the vertical scale of the special chart paper provided. As the height of the histogram was dependent on the number of cells in the suspension, it was decided that the concentration of the cell suspension should be standardized. A suitable concentration was found to be 50,000 cells per 0.5 ml. In each case a preliminary dilution of 1 in 50,000 was made and counted, the concentration being adjusted if necessary. The preliminary normal range was established by examining 50 samples of venous blood from normal adult males and females.

A composite histogram of the maximum and minimum peaks in each window was made from the results obtained (Fig. 1). The peaks which appeared in windows 1 to 4 were disregarded as being due to background noise, platelets, etc.

FIG. 2. Histograms showing a marked excess of microcytes (microcytic index greater than 2.5) with no excess of macrocytes (a); an excess of microcytes (microcytic index greater than 0.5) with no excess of macrocytes (b); normal histogram (c); an excess of macrocytes (macrocytic index greater than 0.75 and less than 1.00) with no excess of microcytes (d); a marked excess of macrocytes (macrocytic index greater than 1.0) with no excess of microcytes (e).

INTERPRETATION OF HISTOGRAM

In order to facilitate the detection of minor variations in cell-volume distribution, which may not be apparent on direct visual examination of the histogram, two indices were elaborated, macrocytic index and microcytic index. The macrocytic index was a ratio of the total numbers of the macrocytes to normocytes and the microcytic index the ratio of microcytes to normocytes. Microcytes were taken as those cells which peaked in windows 5 to 10 inclusive, *i.e.*, less than 72 μ . Normocytes were those cells which peaked in windows 11 to 14 inclusive, *i.e.*, 72 to 100.8 μ . Macrocytes were those cells which peaked in windows 15 and over, *i.e.*, greater than 100.8 μ . The indices were calculated by comparing the sums of the heights of the various peaks in each section. In the 50 normal histograms produced, the microcytic index was never greater than 0.50 and the macrocytic index never greater than 0.75.

To assess reproducibility a bulk dilution of red cells was made and divided into seven aliquots: histograms of each of these were traced and the macrocytic and microcytic indices were calculated (Table).

TABLE

Index	1	2	3	4	5	6	7	Average
Microcytic	0.68	0.68	0.68	0.67	0.64	0.64	0.63	0.66
Macrocytic	0.61	0.62	0.60	0.59	0.63	0.58	0.67	0.61

Figure 2 illustrates the different patterns of histogram obtained by screening a random sample of routine specimens, categorized according to a predominantly microcytic, normal, or macrocytic cell population.

We are indebted to the York 'A' Hospital Management Committee for arranging the purchase of this machine; and to Dr. G. A. C. Summers at whose suggestion the particular model was chosen. Our thanks are also due to members of the laboratory staff for donations of normal blood samples.

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SYMPOSIUM ON IMMUNOASSAY ON CLINICAL CHEMISTRY

The eighth West European Symposium of Clinical Chemistry, on immunology in clinical chemistry, will be held in Newcastle upon Tyne, England, on 28-29 March 1968. Sessions will be arranged on structure and functions of immunoglobulins, serological analysis, radio-immunoassay, and transplantation. For further details please write to the Organizing Secretary: Dr. T. R. C. Boyle, Department of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle upon Tyne, England.

Measurement of red cell diameter by image shearing

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METHOD

A description of the use of the Watson image shearing eyepiece (Wise)¹ in the haematological laboratory may be of interest. This instrument can be used to determine the mean red cell diameter and coefficient of variation in a stained blood film and can also provide a rapid method for screening films for macrocytosis. The Wise employs a dichroic beam splitter which enables the image to be sized by comparison with itself. The object forms red and green images within the eyepiece, which are superimposed in the zero position, but which separate in a vertical direction on rotation of the control knob. To make a measurement, rotation is continued until the images no longer overlap, but just touch edge to edge. A reading taken of the degree of shear required to achieve this, multiplied by a calibration factor previously obtained by observing the stage micrometer, enables the vertical dimension of the object to be measured.

To measure red cell diameters, a 2 mm. oil immersion objective and a $\times 10$ eyepiece are used. A polaroid screen is placed in the condenser light path and rotated to give optimum viewing conditions so as to restore the colour balance of the image pair, which would otherwise be upset by the red colour of the cells in the stained film.

The Wise is set at 7 μ as determined by a preliminary calibration. With this setting the image pairs from red cells, 7 μ in diameter, will be just touching, the pairs from cells greater than 7 μ will be overlapping, while the pairs from cells less than 7 μ will be separated. Two hundred cells are classified in this way. (Cells which are not circular present a difficulty, but can be included in the assessment because the Wise provides an estimate of the vertical diameter which can then be compared mentally with the horizontal and an average diameter assigned.) This procedure is repeated with the Wise set at 8 μ and again at 9 μ (Fig. 1). When 200 cells have been classified at each of three settings the figures for a Price-Jones curve can be worked out (Table I) and analysed in the usual way to give the mean cell diameter and coefficient of variation (Dacie, 1956).

Films showing anisocytosis over a considerable range (as in pernicious anaemia) and those showing microcytosis require modified procedures. When examining films showing considerable anisocytosis at the 9 μ setting, image pairs which overlap are not simply classified as 9.5 μ as in Table I but the degree of overlap is noted and a diameter of 9.5, 10.0, 10.5 etc., is assigned as seems appropriate to the degree of overlap. Similarly at the 7 μ setting, the degree of separation of the image pairs which

¹W. Watson and Son Ltd., Barnet, Herts.
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