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THE SAMPLING ERROR IN HAEMOGLOBIN DETERMINATION

BY

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Of the numerous methods for the estimation of haemoglobin the one which gives the most generally reliable results is that using oxyhaemoglobin in the Medical Research Council neutral grey wedge photometer (Macfarlane, King, Wootton, and Gilchrist, 1948 ; King, Wootton, Donaldson, Sisson, and Macfarlane, 1948). An experiment using this method at various levels of haemoglobin showed that in a routine laboratory it gave results on single samples of blood with an average standard deviation of 1.6% of haemoglobin (see experiment I). This remarkably small technical error suggests that a difference of 5% between two readings on one patient would usually indicate that a real change in haemoglobin level had occurred. In other words, if two readings on one patient were 100 and 95% on 19 out of 20 occasions, these readings would indicate a real change in haemoglobin level.

In the routine use of the M.R.C. photometer it is soon apparent that this precision is not reached. Much larger differences in haemoglobin than 5% may result from random variation. This increased variability is not due to technical inaccuracy but to the differences between two samples of blood. The haemoglobin level of the blood in one normal person is not a static and unchanging figure. The distribution of cells and plasma in different 0.02-ml. samples taken from the same person at the same time is not constant. The haemoglobin level changes during the course of the day and from one day to another. This inherent variability in the haemoglobin readings of normal people cannot be distinguished by the clinician from purely technical errors. The clinician wishes to know whether a difference between two readings on a single patient may be attributed with reasonable certainty to the progress of disease or the effects of treatment. The fact that the haemoglobin level of normal people is not constant must be taken into account in assessing the results of the test.

The variability of haemoglobin readings in normal people is well recognized. In 1941 Sörensen showed that samples of capillary blood taken at the same time had a different haemoglobin content and that the variation was greater with blood from the ear than that from the finger. McCarthy and Van Slyke (1939), Mole (1945), Renbourn (1947), and others all showed that the haemoglobin level in normal people varies through the day. In most people there is a progressive fall in haemoglobin between 6 a.m. and 10 p.m. and a rise during sleep. Between 9 a.m. and 5 p.m. when routine blood samples will usually be taken, the change is not large, amounting to a change of 2 to 3% of haemoglobin. Renbourn (1947) showed that

the haemoglobin level of normal people varies from one day to another independently of any diurnal variation and that this daily variation is larger than the diurnal changes.

With the introduction of a reliable method for haemoglobin determination into routine use it is important to recognize the extent of the normal fluctuation in readings to be expected, and to determine whether or not this source of variation can be reduced, for example, by the use of venous blood samples for haemoglobin estimations. Experiments were undertaken to determine the probable extent of natural variation in haemoglobin.

Methods

Collection of Blood.—Heparinized venous blood samples and capillary blood samples from the finger were used.

Haemoglobin Determination.—Whole blood, 0.02 ml., is pipetted into 4 ml. of ammoniated distilled water and the level of haemoglobin is read with the M.R.C. neutral grey photometer. This instrument is graduated in percentage of haemoglobin taking 100% to represent 14.8 g. in 100 ml. of blood.

Haematocrit.—The microtechnique for the determination of packed cell volume was worked out in collaboration with Dr. Marmion.

Anticoagulant.—About 0.05 g. of dried heparin is ground in a mortar with 5 ml. of refined liquid paraffin. The suspension is thoroughly shaken before use because the heparin sediments to the bottom.

Haematocrit Tubes.—Uniform bore, thick-walled barometer tubing is cut into sections about 4 in. in length. These are ground to a tapered point at one end. The internal diameter of the tubing is about 0.8 mm.

Technique.—Immediately before use the anticoagulant is drawn into the tube and blown out to leave a coating on the internal surface. Blood is drawn up to an arbitrary level and below this a short column of mercury is drawn up to seal the lower end. The tube is encircled with a stout rubber band and enclosed in a test-tube with a rubber buffer in the bottom, and is centrifuged at 3,000 r.p.m. for 30 minutes. The paraffin separates as a layer on top, and the length of the column of packed cells and plasma can be measured.

With one sample of venous blood this method gives a coefficient of variation of about 1.5%.

Experimental Results

Experiment I: The Technical Error of the M.R.C. Photometer.—Four separate experiments were made using a range of different haemoglobin pipettes and employing five observers to make the readings. In each experiment 50 subsamples of 0.02 ml. of blood were taken from one venous blood sample and the level of haemoglobin was measured. The average haemoglobin levels of these samples were 98.1%, 69.8%, 32.0%, and 17.2%. In these experiments the total variability of the readings was represented by standard deviations of 1.66, 1.37, 2.04, and 1.28% of haemoglobin.

The extent of technical variability, including differences between observers, was remarkably small, having an average value of 1.59% of haemoglobin. An interesting feature of this technical error was that it appeared to be independent of the level of haemoglobin. At low levels of haemoglobin therefore the technical error is relatively

more important; at 17% of haemoglobin the technical error gave a proportional error (coefficient of variation) of 7.44%.

Experiment II: The Variability of Haemoglobin Readings on Capillary Blood Samples Taken at One Time—Since the technical error of the method does not account for the variability of haemoglobin readings in practice, an attempt was made to see whether or not the variability could be attributed to differences between capillary blood samples. Four experiments were carried out. In each of these 20 samples of capillary blood were taken in rapid succession from one normal individual using 20 different pipettes. The haemoglobin levels in the diluted samples were read by two observers. The mean haemoglobin levels and the total standard deviations for each experiment are shown in Table I. It is obvious that an important

TABLE I

MEAN HAEMOGLOBIN LEVEL AND TOTAL STANDARD DEVIATION AND COEFFICIENT OF VARIATION IN EXPERIMENT II

Experiment No.	Mean Haemoglobin (%)	Standard Deviation (%) of Haemoglobin	Coefficient of Variation
IIa	96.4	4.09	4.24
IIb	105.3	2.60	2.47
IIc	100.5	5.35	5.32
IId	88.4	5.98	6.76

increase in variability is introduced by the use of capillary samples. Whereas the mean technical error was 1.6% of haemoglobin, that for the experiments using capillary blood was 4.5%.

Experiment III: The Variability of Haemoglobin Readings on Capillary Blood Samples Taken on Different Days.—To extend the results of experiment II haemoglobin readings were made on 20 normal subjects and 20 patients on six or seven successive days. In each normal individual or patient tested five capillary samples were taken at the same time on each day. In this way a total of 30 or 35 estimations were made for each subject. The levels of haemoglobin in the patients covered a wide range from 23 to 100%. In the normal people the range was from 83 to 120%. In this way it was hoped that it might be possible to make an approximate estimate of the extent of random variability to be expected at different haemoglobin levels. In the normal people there was no consistent trend of haemoglobin change from one day to another, but in many of the patients an increase or decrease in haemoglobin occurred over the test period. In the anaemic patients an improvement followed appropriate treatment. In seven patients in the puerperium there was a rise in haemoglobin presumably due to readjustment of the blood volume following delivery. In a patient with aplastic anaemia there was a fall in haemoglobin associated with the failure of normal erythropoiesis. Since the experiment was designed to assess the random variation to be expected from capillary haemoglobin samples the influence of these consistent trends in haemoglobin levels had to be eliminated from the results.

The results were calculated for each individual by analysis of variance. In this way the error due to variations in haemoglobin from day to day could be separated

from random variability. The variation from one day to another was then separated into two components, one that could be attributed to linear regression, i.e., the consistent change in haemoglobin level, and one due to deviations from the regression or random variations from one day to another. The components of random variation were combined to give the total error. An example of the calculations involved is given in Table II.

In nine subjects five microhaematocrit readings were made on each day of the experiment. The haematocrit method has a small technical error, and the results

TABLE II

THE HAEMOGLOBIN LEVELS EXPRESSED AS PERCENTAGES OBSERVED IN ONE PATIENT IN EXPERIMENT III AND THE CALCULATIONS TO DETERMINE THE TOTAL OF RANDOM ERROR

Sample	Day of Experiment 1	2	3	4	5	6	7
1	38	48	49	51	47	48	60
2	40	45	47	52	50	51	60
3	42	47	50	54	51	51	61
4	45	43	52	48	51	52	60
5	46	44	49	52	50	53	58
Total	211	227	247	257	249	255	299

Sum = 1745 mean = 49.86
n= 35

Total sum of squares

$$= (38^2 + 40^2 - 60^2 + 58^2) - \frac{(1745)^2}{35}$$

$$= 1030.29$$

Sum of squares referable to differences between days

$$= 1/5(211^2 + 227^2 - 299^2) - \frac{(1745)^2}{35}$$

$$= 906.29$$

Sum of squares due to linear regression calculated from the formula

$$\frac{(Sxy - \frac{SxSy)^2}{n}}{Sx^2 - \frac{(Sx)^2}{n}}$$

$$= 740.60$$

Deviation from regression = 906.29 − 740.60
= 165.69

ANALYSIS OF VARIANCE

Source of Error	D.F.	Sum of Squares	Mean Square	Standard Deviation
Total	34	1030.29		3.30
Regression	1	740.60		
Deviations from regression	5	165.69	33.14	2.40
Random error	28	124.00	4.43	2.10

of the test should give readings which vary in the same way as the haemoglobin readings. These additional tests were carried out to ensure that the variation in haemoglobin readings could not be attributed to some unidentified technical error in the haemoglobin method. The haematocrit values showed day-to-day variations parallel to the haemoglobin readings (Fig. 1), and the random variability, to be attributed to sampling errors, was similar to that of the haemoglobin estimations. The observed variations in haemoglobin percentage must therefore be attributed to differences between capillary samples and random changes in haemoglobin from one day to another.

The extent of the observed variation was wide, ranging from a standard deviation of 1.65 to 6.96% of haemoglobin. Expressed as a percentage of the mean haemoglobin readings (coefficient of variation) the error tended to increase with lower levels of haemoglobin (Fig. 2). Fig. 2 shows that there is no close correlation between the extent of the

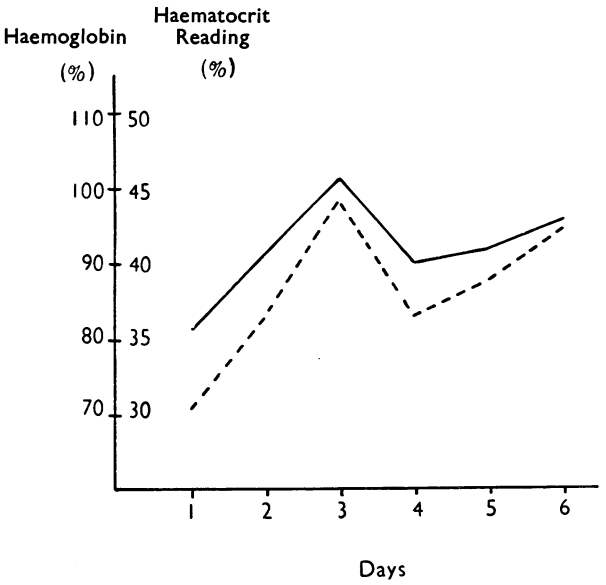

FIG. 1.—The haemoglobin readings of a patient in the puerperium as a mean value for five daily estimations made on six consecutive days (solid line). The haematocrit values as a mean of five estimations made on the same six days and at the same time as the haemoglobin readings (broken line).

TABLE III

ERROR IN ESTIMATING PERCENTAGE OF HAEMOGLOBIN USING THE M.R.C. NEUTRAL GREY WEDGE PHOTOMETER

Haemoglobin Level (%)	Calculated Range of 19/20 Observations on One Individual	Significant Difference (% Hb)
120	113–127	10
110	102–118	11
100	91–109	12
90	81–99	13
80	71–89	13
70	61–79	13
60	51–69	12
50	42–58	11
40	33–47	10
30	24–36	8
20	16–24	6

In the second column is indicated the range within which separate observations on one individual should fall. In the third column is shown the difference between two isolated haemoglobin readings on one person which would suggest that a real change had occurred (calculated from the formula $2\sqrt{2S^2}$)

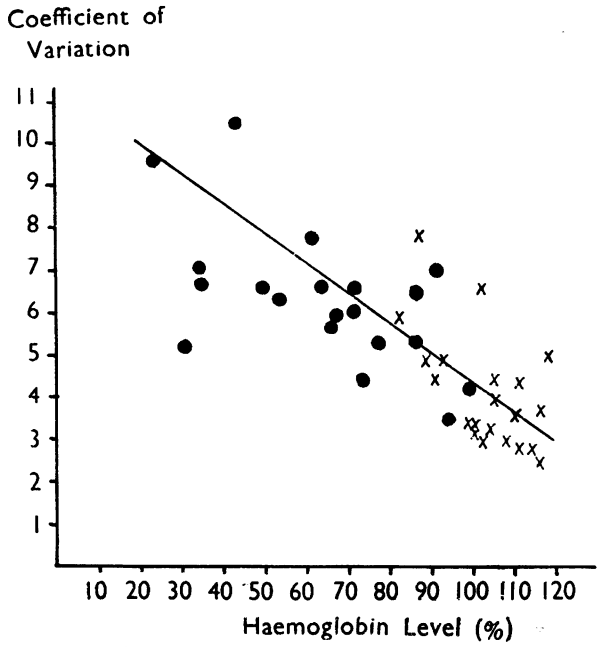

FIG. 2.—The coefficients of variation for the haemoglobin readings of the patients and normal people tested in experiment III plotted against the level of haemoglobin. (Experiments with normal individuals, X. Experiments with patients, ●).

error and the haemoglobin level but that the error does tend to increase with the lower values. This increase in error is due in part to the technical error which appears to be constant at different haemoglobin levels and, though small, will become proportionately large at low values of haemoglobin (see experiment I).

It is clear from Fig. 2 that no exact limits of variability to be expected in routine haemoglobin readings can be laid down, but as a rough guide to the general order of these changes an approximate mean value for the coefficient of variation at different haemoglobin readings was obtained from Fig. 2 and from these the figures in Table III were obtained. The limits of variability given in Table III correspond reasonably well with the random fluctuations in haemoglobin encountered in the routine laboratory.

Experiment IV: The Variability of Venous and Capillary Blood Samples on Different Days.—It seemed possible that some variability might be a feature of capillary blood and that venous blood might be more consistent in its constituents. An experiment was designed to compare the variability of venous and capillary samples on different days.

In this experiment two 0.02-ml. samples of capillary blood and one venous sample were taken from five normal subjects on ten consecutive days. The errors

TABLE IV

VARIABILITY OF HAEMOGLOBIN READINGS OF VENOUS AND CAPILLARY BLOOD

	Day-to-day Variation (%)	Random Variation (%)	Total Variation (%)
Error determined from capillary blood samples ..	4.40	4.05	6.01
Error determined from venous blood samples ..	4.62	3.65	5.83
Error of microhaematocrit	4.30	4.50	6.29
Error of M.C.H.C.	1.91	3.85	4.30

expressed as coefficients of variation calculated by analysis of variance are given in Table IV. The variability of the capillary and venous samples was similar, and each showed a significant difference from day to day.

In addition the packed cell volume was measured by the micro-method on both venous and capillary blood for six days of this experiment. The packed cell volume showed a variability similar to that of the haemoglobin. As would be expected the mean corpuscular haemoglobin concentration showed a random variation similar to that of haematocrit and haemoglobin estimations, but there was little variation from one day to another. This experiment shows that the differences between venous samples are as great as those between samples derived from capillary blood. There can be no advantage in using venous blood samples for haemoglobin determination. For the estimation of the mean corpuscular haemoglobin concentration it is obviously advisable to use one sample of venous blood. In this way the random variability of different samples will be avoided.

The Diurnal Variation in Haemoglobin.—The extent of the diurnal variation in haemoglobin for the hours during which samples of blood are taken for examination in the routine laboratory is not large (Renbourn, 1947). The labour involved in taking serial blood samples at the same time of day would be great and would not increase the precision of the method noticeably.

Summary

These experiments have shown that the variation in haemoglobin readings to be expected in the routine use of the M.R.C. neutral grey photometer is much greater than that which can be attributed to the technical error of the method. This increased variability is due to differences between one capillary blood sample and another and to changes in the level of haemoglobin from one day to another. No reduction in error can follow the use of venous blood samples because venous samples show differences as great as those between capillary samples. The increased precision which might follow the collection of serial samples at one time of day is small in relation to the other unavoidable errors. If it is desired to determine the mean corpuscular haemoglobin concentration the haemoglobin and haematocrit readings should be made on the same sample of venous blood, when sampling errors will be avoided. In the routine use of the M.R.C. photometer the errors to be expected at different haemoglobin levels are shown in Table III. These figures are compiled from the results of experiment II and give an approximate guide to the general variability of the method.

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