THE STANDARDIZATION OF BLOOD HÆMOGLOBIN DETERMINATIONS*

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THE inadequacy of the commonly employed methods for determination of blood hæmoglobin has led a number of workers recently to propose improved methods, and to seek a convenient absolute standard for these determinations. Rimington[1] has recommended the pyridine hæmochromogen method of Roets,[2] based upon pure hæmin as a standard. Clegg and King[3] have proposed an alkaline hæmatin method, also based upon pure hæmin. A number of workers, including Myers and Eddy,[4] and Karr and Clark,[5] have relied upon total iron determinations, which have been found to agree with estimations based upon oxygen capacity. A recent report to the Medical Research Council[6] recommends, as a standard, iron determinations upon washed erythrocytes.

The determination of blood hæmoglobin involves: (1) a convenient and precise method for routine measurements; (2) an absolute standard for calibrating the measurements. Our experience with various procedures, arising out of a study of phenothiazine anæmia, is outlined in the following experiments.

EXPERIMENTAL

The determinations were carried out upon human and dog blood. In each case venous blood was oxalated and was then diluted 1:10 with water, in order to facilitate precise sampling; all analyses were carried out on this resultant solution of hæmoglobin, using the same pipette throughout. Colorimetric measurements were made in a Lumetron model 401 photoelectric colorimeter, using ½ inch test-tubes and the green filter B530; transmission values were converted to extinctions by the formula $E = -\log T$. The results reported are averages of repeated measurements with a mean deviation of less than 1%. The results obtained by the various methods are illustrated in Table I, giving values for a sample of human blood and one of dog's blood.

Iron determinations.—Total iron was estimated upon 0.5 ml. samples of the diluted blood, using the wet ashing method of Myers and Eddy.[4] The pure iron standards (30 μg. Fe) for colorimetric comparison were always made up in the same amount of sulphuric acid as was used in the digestion. Since Bernhart and Skeggs[7] have found that pure hæmoglobin contains 0.340% iron, this figure was used in calculating hæmoglobin concentrations.

TABLE I.

COMPARISON OF HÆMOGLOBIN DETERMINATIONS

Method	*Hb. gm. per 100 ml.*	
	Human	*Dog*
Fe by mercaptoacetate	16.4	16.9
Pyridine hæmochromogen	16.6	16.7
Alkaline hæmatin (not corrected)	19.9	20.5

Blood iron has customarily been estimated by the thiocyanate method, but this is very unsatisfactory for precise work (Hoffman[8]). Good results have been obtained with the mercaptoacetate method, following the procedure of Koenig and Johnson.[9] This reagent has the advantage of cheapness, relative freedom from interference, and acts as its own reducing agent. The method was applied to a sample of pure hæmin kindly furnished by Dr. E. J. King, and gave the same value, within 0.5%, as reported by him for Delory's[10] titanous sulphate titration. Mercaptoacetate is somewhat less sensitive than *o*-phenanthroline or dipyridyl, giving about two-thirds the colour intensity under our conditions. The use of phenanthroline in determining blood iron was found to give results about 2% too low, while dipyridyl gave, in one case, a value 10% too high.

Preparation of hæmin.—Pure hæmin was prepared from washed dog erythrocytes by the method of Delory.[10] The iron content of the crystals was found, by the colorimetric method described above, to be 8.27%. Recrystallization by Delory's method resulted in a product containing the theoretical amount of iron, 8.57%. This pure hæmin was used as an absolute standard in the pyridine hæmochromogen and alkaline hæmatin methods described below. The hæmin crystals supplied by Dr. King (containing 8.45% iron) were also used in these two methods and gave identical values, within 1%.

Pyridine hæmochromogen method. — The method of Roets[2] was modified as follows. To about 7 ml. of N/10 NaOH in a 10 ml. cylinder

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was added 0.20 ml. of diluted blood and to this was added 2 ml. of pyridine. Standing 30 minutes after mixing was found to be necessary for full colour development; then 5 to 10 mg. of sodium hydrosulphite was added, the solution was diluted to 10 ml. with NaOH and the intensity of the red colour was measured.

As an absolute standard, the pure hæmin was dissolved in N/10 NaOH at a concentration of 65 mg. per 100 ml., equivalent to 0.001 M. Of this stock solution, 0.20 ml. was treated as above. As pointed out by Drabkin and Austin,[11] the absorption spectra of the reduced pyridine hæmochromogens from hæmoglobin and from hæmin are not identical. The difference is, however, so small as to cause a virtually negligible error.

Alkaline hæmatin method.—The procedure of Clegg and King[3] was followed. Of the dilute blood, 0.5 ml. was made up to 10.0 ml. in N/10 NaOH and this was heated 5 minutes in a boiling water bath; the solution was then cooled and read in the colorimeter. As a standard, 0.5 ml. of the stock hæmin solution was diluted to 10 ml. with the NaOH. As pointed out by Clegg and King, the colour intensity of alkaline hæmatin from hæmoglobin is appreciably greater than that derived from pure hæmin. They accordingly added a correction of 30% to the observed colour intensity of the alkaline hæmatin from hæmin.

In order to compare the absorption spectra, solutions of alkaline hæmatin were prepared from human blood and from hæmin, in equivalent concentration (based on iron) of 0.050 mM per litre. The extinctions of the two solutions were measured with four filters in the photoelectric colorimeter, and also with the eight Ilford spectral filters in a visual colorimeter against a gray disc of optical density 0.74.

The results are recorded in Table II and confirm the observation of Clegg and King that the two spectra diverge considerably. Virtually the same ratio as they obtained was observed with the green filter in the visual colorimeter. With the photoelectric colorimeter, however, the ratio in the green was only 1.22; this value was obtained repeatedly with both human and dog's blood. (Dr. King's hæmin gave ratios of 1.21 and 1.34 in the photoelectric and visual colorimeters respectively.) It must further be noted that the absorption spectrum of alkaline hæmatin changes rapidly, even when the solution is kept in the dark. The extinction in the green remained constant, but the absorption in the blue and red increased markedly.

TABLE II.

LIGHT ABSORPTION OF ALKALINE HÆMATIN SOLUTIONS

Filter	*Extinction*		*Ratio, Hb./ hæmin*
	Hb.	*Hæmin*	
Photoelectric, (0.05 mM)			
Blue, B420	0.758	0.513	1.48
Green, B530	0.362	0.296	1.22
Yellow, B590	0.294	0.250	1.17
Red, B660	0.128	0.090	1.42
Visual, (0.10 mM)			
Violet, 450 mμ	1.16	0.62	1.87
Blue, 470 mμ	0.84	0.52	1.62
Blue-green, 490 mμ	0.70	0.51	1.37
Green, 520 mμ	0.60	0.44	1.36
Yellow-green, 550 mμ	0.51	0.34	1.50
Yellow, 575 mμ	0.48	0.32	1.50

It is clear that this method is not suitable for absolute standardization of hæmoglobin determinations, since the ratio, and therefore the correction factor, will depend upon the spectral characteristics of the light source, the filter, and the photocell or other detecting device.

Routine determinations.—Both the iron and the pyridine method are too complicated for routine hæmoglobin determinations. Clegg and King's alkaline hæmatin method was found to give consistent results, if standardized by an absolute method, but the brown colour is not ideal for colorimetric comparison. We have preferred to adopt the cyanmethæmoglobin method, as recommended by Drabkin and Austin,[12] who state: "MHbCN is an ideal pigment for the determination of total concentration".

The procedure is essentially the same as that recently described by Sunderman.[13] Into about 9 ml. of water in a 10 ml. cylinder or volumetric flask is pipetted 0.02 ml. of blood; to this is added 1 drop of 5% potassium ferricyanide and about 1 mg. of solid KCN (estimated on the tip of a spatula). The solution is made up to 10 ml., mixed, and read immediately in the colorimeter with a green filter. The method is simple, rapid, and relatively safe. With a sample of blood of known Hb concentration a calibration curve is constructed. We have found that extinction plotted against concentration deviated slightly from a linear relationship.

Before the photoelectric colorimeter became available, a visual colorimeter was used as a photometer, as described by Sunderman.[13] An Ilford neutral gray disc of optical density 0.74

was placed in the left-hand cup, with an Ilford yellow-green (550 mμ) spectral filter on the eyepiece. Under these conditions blood containing 15 gm. Hb per 100 ml., diluted 1:200, gave a reading of about 1.8 cm., *i.e.*, the extinction (1 cm. depth) was 0.74/1.8 = 0.41.

This cyanmethæmoglobin method, while eminently suitable for routine hæmoglobin determinations, does not, of course, give absolute values unless a spectrophotometer be available for measuring absolute extinction coefficients. (On a previous occasion an opportunity to use a Coleman Universal Spectrophotometer to measure the extinction coefficient of MHbCN at 540 mμ gave results for Hb concentration which were about 10% too low, compared with iron values. This was presumably due to the fact that the 35 mμ band of the instrument is broader than the peak of the absorption band of MHbCN.)

For calibration of the MHbCN method, the hæmoglobin content of several samples of human and dog blood was determined by total iron and by the pyridine hæmochromogen method, as described above and illustrated in Table I. The average values from these two methods were used to obtain the calibration factor for MHbCN. For example, the sample of human blood in Table I was taken to have a hæmoglobin concentration of 16.5 gm. per 100 ml. The MHbCN factor (Hb conc. ÷ extinction) was found to be 62.7. For the dog blood, 16.8 gm. Hb per 100 ml., the factor was 62.0.

DISCUSSION

The experiments described have involved four methods of determining blood hæmoglobin concentration: total iron, pyridine hæmochromogen, alkaline hæmatin, and cyanmethæmoglobin. The iron method gives absolute values for Hb (assuming a negligible amount of non-Hb iron). The pyridine hæmochromogen method also gives absolute values, when calibrated against pure hæmin as a standard. These two methods gave results agreeing within 1%, values which can therefore be assumed to be essentially correct. The procedures are, unfortunately, too involved for routine Hb determinations; but they appear to be very suitable for accurate standardization.

Both the alkaline hæmatin and the cyanmethæmoglobin method are useful for routine determination of total pigment. The latter method is preferred for simplicity and rapidity, greater intensity and stability of the colour. Absolute standardization of these procedures may be accomplished through either the iron or the pyridine method, as described above.

The method of Clegg and King for standardizing alkaline hæmatin against pure hæmin was found to be unreliable since the absorption spectra of the alkaline hæmatins from Hb and from hæmin diverge so markedly. No constant correction factor, applicable in all laboratories, can be used for the hæmin values, because the correction ratio depends upon the spectral distribution of the light used in the colorimetric measurement.

SUMMARY

1. The cyanmethæmoglobin method was employed for routine determination of blood hæmoglobin concentration (total pigment).

2. The method was calibrated, using both human and dog blood, by determination of total iron, and by the pyridine hæmochromogen method against pure hæmin. The two methods gave results agreeing within 1%.

3. The alkaline hæmatin method was found unsuitable for the determination of absolute hæmoglobin concentration, since pure hæmin gave an absorption spectrum markedly different from that obtained with blood.

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It is stated officially that a remedy for seasickness has been worked out in Canada as the result of work carried out by the late Sir Frederick Banting, Surgeon Captain C. H. Best, Royal Canadian Navy, and Dr. Wilder Penfield, Montreal Neurological Institute. The remedy, which has experimentally prevented seasickness in about 75% of persons normally susceptible to it, is given in capsule form.—*J. Roy. Inst. Pub. Health & Hyg.*, 1943, 6: 303.