MICRODETERMINATION OF OXYHEMOGLOBIN,
METHEMOGLOBIN, AND SULFHEMOGLOBIN
IN A SINGLE SAMPLE OF BLOOD*

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The purpose of this paper is to present a simple, accurate photo-
electric method for the determination of oxyhemoglobin (HbO₂),
methemoglobin (MHb), and sulfhemoglobin (SHb) in a single
sample of finger blood.

Methods

Methemoglobin—When sodium cyanide is added to a solution
of MHb (Fig. 1), the characteristic absorption band at 635 mμ is
almost completely abolished by the conversion of MHb into cyan-
methemoglobin (MHbCN). The resulting change in optical
density is directly proportional to the concentration of MHb.
This change in optical density may be measured on the photo-
electric colorimeter (1) with a color filter which transmits a nar-
row spectral band in the vicinity of 635 mμ (Fig. 1).

Sulfhemoglobin—The depth of the 620 mμ absorption band of
SHb (Fig. 2) is unchanged by the addition of cyanide, hence the
concentration of SHb in a solution containing HbO₂, MHb, and
SHb is proportional to the residual optical density of the solution
after the MHb has been converted into MHbCN by addition of
cyanide. This measurement can be made on the photoelectric
colorimeter with a suitable filter (Curve 620 of Fig. 2) on the same
solution used for the MHb determination. A correction must of
course be made for the small, though not negligible, absorption
of HbO₂ and MHbCN at 620 mμ.

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Oxyhemoglobin—The concentration of HbO₂ is obtained by subtracting the values for MHb and SHb from the concentration of total hemoglobin determined by a modification of the method of Austin and Drabkin (3). In this method the various forms of hemoglobin are converted into MHbCN and the concentration of this pigment is determined by measuring the optical density of the solution in the photoelectric colorimeter, with a filter which transmits a spectral band in the vicinity of the MHbCN absorption maximum at 540 μ (Fig. 1).

Reagents—
2. M/60 phosphate buffer of pH 6.6 prepared from the above by dilution as required.
3. 20 per cent aqueous potassium ferricyanide.
4. 10 per cent aqueous sodium cyanide.
5. A neutralized solution of sodium cyanide prepared within 1 hour of the time of use by mixing equal parts of 10 per cent sodium cyanide and 12 per cent acetic acid.

6. Concentrated ammonium hydroxide.

Reagents 3 to 6 should be kept in dropper bottles which deliver approximately 25 drops to the cc.

**FIG. 2.** Spectrophotometric curves of oxyhemoglobin (0.1 gm. per 100 cc.), sulfhemoglobin (0.15 gm. per 100 cc.), and Filter 620. The curve for SHb was drawn from the data of Drabkin and Austin (2). Abscissa, wavelength in μ; ordinate, percentage light transmission.

**Procedure**

0.1 cc. of fresh whole blood (finger blood, or venous blood to which not more than 2 mg. of potassium oxalate per cc. have been added) is delivered into 10 cc. of M/60 phosphate buffer of pH 6.6 in a colorimeter tube. The solution is allowed to stand for 5 minutes, and a reading is made with Filter 635,1 after the galvanometer is first adjusted to 100 with a blank tube containing

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1 The photoelectric colorimeter and all accessories such as filters and absorption test-tubes can be obtained from the Rubicon Company, 29 North Sixth Street, Philadelphia.
water only. The optical density\(^2\) is recorded as \(L_1\). 1 drop of the neutralized sodium cyanide is then added to the solution to convert any MHb into MHbCN, and after 2 minutes a second reading (\(L_2\)) is made with the same filter and blank tube. The difference (\(L_1 - L_2\)) is proportional to the concentration of MHb. The solution, which up to this time will have been very slightly turbid, is now cleared for the SHb determination by addition of 1 drop of concentrated ammonium hydroxide, and a reading (\(L_3\)) is made with Filter 620 and a blank tube containing water only. Finally 2 cc. of the solution are pipetted into a second colorimeter tube containing 8 cc. of \(\frac{m}{15}\) phosphate buffer of pH 6.6 and 1 drop of 20 per cent potassium ferricyanide. The tube is allowed to stand for 2 minutes in order to allow all the \(\text{HbO}_2\) to be converted into MHb; then 1 drop of 10 per cent sodium cyanide is added to convert the MHb into MHbCN for the total hemoglobin determination. At the end of 2 minutes a reading (\(L_4\)) is made with Filter 540 and a blank tube containing 10 cc. of water and 1 drop each of 20 per cent potassium ferricyanide and 10 per cent sodium cyanide. The concentrations of the various hemoglobin derivatives are calculated from the following equations in which \(T\), \(M\), and \(S\) represent gm. per 100 cc. of blood of total hemoglobin, MHb, and SHb respectively.\(^3\)

\[
T = \frac{100 \times L_1}{2.38} \quad (1)
\]

\[
M = \frac{100 \times (L_1 - L_2)}{2.77} \quad (2)
\]

\[
S = \frac{1000 \times L_3 - (8.5 \times M + 4.4 \times T)}{100} \quad (3)
\]

\(^2\) Throughout this paper the symbol \(L\) will be used to refer to the \textit{approximate} optical density which is measured when one uses the photometric colorimeter, instead of a spectrophotometer which measures the true monochromatic optical density. With the filters used by us, however, the \(L\) value of a solution behaves just like the true optical density in that it is directly proportional to the concentration of the colored substance, and may therefore be used in the equation \(L = K \times C\) where \(K\) is a constant and \(C\) is the concentration of the colored substance.

\(^3\) At the conclusion of the hemoglobin determination there remain 8 cc. of diluted blood which may conveniently be used for the determination of sulfanilamide by a simple photometric modification of the method of Marshall (4).
The value for $T$ obtained from Equation 1 is not strictly accurate when the blood contains SHb, because it is not possible to convert SHb into MHbCN with the reagents used. Fortunately this error is not very serious, because the absorption (at 540 m$\mu$) of the compound formed from SHb by the action of ferricyanide is almost as great (78 per cent) as that of a corresponding amount of MHbCN. Since the error introduced in the total hemoglobin determination is directly proportional to the concentration of SHb, the true value of $T$ may be calculated from the formula

$$\text{Corrected } T = T \text{ (calculated from Equation 1)} + 0.22 \times S \quad (4)$$

The concentration of oxyhemoglobin is obtained from the equation

$$\text{HbO}_2 = \text{corrected } T - (M + S) \quad (5)$$

The numerical values of the calibration constants which appear in the above equations apply only to the particular type of photoelectric colorimeter used by us, but the form of the equations will be the same for all photoelectric colorimeters, and no difficulty should be encountered in adapting the method to some other instrument.

**Results**

Data on the accuracy of the determination of the various hemoglobin derivatives are summarized in Table I. The measurements were made on artificial mixtures of known amounts of pure solutions of the individual pigments. Pure SHb was not available but solutions containing about 70 per cent SHb were prepared, and the concentration of SHb as determined by the method of Drabkin and Austin (2) was used in calculating the concentration of SHb in the final mixtures. From this data the following conclusions may be drawn.

**Total Hemoglobin**—When no SHb is present, the error of this determination does not exceed 0.2 gm. per 100 cc. When Equation 4 is employed to correct for the systematic error due to the presence of SHb, the error from this source can be kept below 0.4 gm. per 100 cc. even when the concentration of SHb is as high as 6 gm. per 100 cc. The highest concentration of this pigment which we have observed in four cases of sulfhemoglobinemia caused
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by sulfanilamide is 1.1 gm., and in five cases of sulfhemoglobinemia due to other causes 2.5 gm. per 100 cc.

**Methemoglobin**—The error in this determination does not exceed 0.2 gm. per 100 cc. and is usually about 0.1 gm. The lower limit of sensitivity is about 0.2 gm. per 100 cc., and this limit is partially due to the slow spontaneous formation of MHb which occurs when a 100-fold dilution of blood is allowed to stand.

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<th>Methemoglobin Calculated</th>
<th>Sulfhemoglobin Calculated</th>
<th>Total hemoglobin Calculated</th>
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* Evans' blue was added to blood in a concentration of 1.5 mg. per 100 cc. This corresponds to 5 times the maximum concentration used in the determination of blood volume by the method of Gibson and Evans (5). A patient who had received this amount of dye would be intensely cyanosed.

† To a sample of this blood was added the purple pigment obtained by exposing a solution containing 10 mg. of sulfanilamide per 100 cc. to the sun for 60 minutes. This pigment is either identical with or at any rate closely related to the pigment which occurs in the blood of patients receiving sulfanilamide, and the amount added in this experiment was sufficient to produce a much greater degree of cyanosis than is seen clinically.

‡ Phenolsulfonephthalein was added, 0.5 mg. per 100 cc. of blood.
Since the measurement is based on the difference of two optical densities, the accuracy is not affected by the presence in the solution of other pigments, even if they absorb light at 635 m, as long as their absorption is not altered by addition of cyanide.

Sulphemoglobin—The sensitivity of the method is about 0.1 gm. per 100 cc. It is difficult to make an estimate of the absolute accuracy of the determinations, since the value of the calibration constant which appears in Equation 3 is obtained from the admittedly approximate data of Drabkin and Austin (2). It is probable, however, that the error in the concentration of SHb does not exceed 10 per cent of the value obtained by the above procedure. The reproducibility of the determination is almost as good as that of the total hemoglobin and MHb measurements, and the absolute accuracy can easily be improved at any future time if a method of preparing pure SHb should be discovered.

Effect of Extraneous Pigments—By the method described above any pigment which absorbs light at 620 m (other than the hemoglobin derivatives themselves which are allowed for in Equation 3) will obviously be measured as sulphemoglobin. A negative SHb test is always reliable, but a false positive test might conceivably be obtained on a blood which contained some other colored substance. This possibility is particularly important in the blood of patients receiving sulfanilamide, because this substance frequently gives rise in vivo to blue- and purple-colored derivatives which produce intense cyanosis. Fortunately, however, the interfering effect of such substances is almost entirely eliminated, since the blood is diluted 100-fold before the colorimetric measurements are made. Table I shows that the addition to blood of amounts of blue dyes several times greater than that required to produce alarming cyanosis has no appreciable effect on the accuracy of the determinations; therefore it seems fairly safe to conclude that the specificity of the method is adequate for use in human blood. If necessary, it might be possible to remove the interfering substance by using washed red blood cells instead of whole blood for the determination. It is recommended, however, that all bloods giving a positive SHb test be examined spectroscopically for the 620 m band which is not affected by addition of cyanide and shifts its position when the blood is equilibrated with carbon monoxide. As an illustration of the satisfactory use of the method...
in clinical investigation, it might be mentioned that in over 100 photoelectric examinations of blood from patients receiving sulfanilamide (in whom the indication for requisitioning the determination was obvious cyanosis) only four positive SHb tests were obtained (0.3 to 1.1 gm. per 100 cc.), and none of these was a false positive, since all four were confirmed spectroscopically. The incidence of methemoglobinemia was much higher, practically all the patients showing at least a trace of methemoglobin, but the amounts found were usually of the order of 0.5 to 1.5 gm. per 100 cc., and in only five cases over 2.5 gm. per 100 cc. The complete lack of correlation between the MHb concentration and the intensity of the cyanosis shows clearly that the usual cause of the cyanosis is the presence of colored derivatives of the sulfanilamide itself. Since this latter type of cyanosis is capable of masking that due to MHb completely, the determination of MHb is still of definite value, because methemoglobinemia may undoubtedly reach serious proportions in certain cases.

SUMMARY

1. A simple photoelectric method is described for the determination of oxyhemoglobin, methemoglobin, and sulfhemoglobin on a single 0.1 cc. sample of blood.

2. The determination of methemoglobin is subject to an error of not more than 0.2 gm. per 100 cc., and this also represents the smallest amount which can be detected with certainty.

3. As little as 0.10 gm. of sulfhemoglobin can be detected, but the absolute accuracy of the measurement is somewhat less than that of the methemoglobin determination.

BIBLIOGRAPHY

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