A RAPID SPECTROPHOTOMETRIC METHOD FOR THE
DETERMINATION OF METHEMOGLOBIN AND
CARBONYLHEMOGLOBIN IN BLOOD

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Little use has thus far been made of the near infra-red spectrum for the
identification and determination of hemoglobin and its derivatives. By
means of infra-red photography Eggert (1) established the transparency of
carbon monoxide-saturated blood and used this property for the qualitative
detection of carbon monoxide poisoning. Eggert's observations were
extended and placed on a more quantitative basis by Merkelbach (2). A
further advance was made by Matthes and Gross (3) who developed a
method for the determination of the carbon monoxide content of blood
in vivo in which the absorption of red and infra-red radiation by flowing
blood was measured in the ear lobe. A band for reduced hemoglobin at
7550 A has been described by Sidwell, Munch, Barron, and Hogness (4)
and employed by these workers for the spectrophotometric determination
of the degree of oxygenation of hemoglobin solutions.

In the near infra-red region of the spectrum, between 7000 and 10,000 A,
the hemoglobin derivatives have absorption bands which, because of their
lower specific absorption, become apparent only when solutions more
concentrated than those generally employed for spectrophotometric work
are examined. A detailed description of these bands is contained in a
previous publication (5). Despite the lower absorption, the large
differences between the spectra of methemoglobin and cyanmethemoglobin
and between oxyhemoglobin and carbonylhemoglobin make this region
well suited for analytical purposes. On the basis of these considerations
we have developed a spectrophotometric method for the determination of
methemoglobin and carbonylhemoglobin in blood. The method is rapid
and accurate and requires only a few manipulations. It has an advantage
over existing methods in that it permits the determination of the total
hemoglobin, carbonylhemoglobin, and methemoglobin content in a single
blood sample.

Principle of Method

For each sample of blood to be analyzed, three absorption measurements
are made. From the first of these measurements, in the infra-red, a density
$D_1$ is obtained which may be expressed as follows:
DETERMINATION OF MHB AND HBCO

\[ D_1 = (\epsilon^1_{\text{HbO}_2} C_{\text{HbO}_2} + \epsilon^1_{\text{MHB}} C_{\text{MHB}} + \epsilon^1_{\text{HbCO}} C_{\text{HbCO}}) \frac{L}{d_1} \]

where \( \epsilon^1_{\text{HbO}_2} \), \( \epsilon^1_{\text{MHB}} \), and \( \epsilon^1_{\text{HbCO}} \) are the extinction constants for the wavelength interval used and \( C_{\text{HbO}_2} \), \( C_{\text{MHB}} \), and \( C_{\text{HbCO}} \) are the concentrations in the original blood sample of oxyhemoglobin, methemoglobin, and carbonylhemoglobin, respectively, \( d_1 \) is the dilution factor for the sample measured, and \( L \) is the length of the light path through the absorption cell.

The sample in the absorption cell is then treated with KCN to convert the methemoglobin present to cyanmethemoglobin, and the density measured at the same wavelength interval.

\[ D_2 = (\epsilon^1_{\text{HbO}_2} C_{\text{HbO}_2} + \epsilon^1_{\text{MHB}} C_{\text{MHB}} + \epsilon^1_{\text{HbCO}} C_{\text{HbCO}}) \frac{L}{d_2} \]

where \( \epsilon^1_{\text{MHB}} \) is the extinction constant for cyanmethemoglobin at the wavelength used. From these two equations the concentration of methemoglobin in the blood sample may be computed.

Essentially the same principle has been employed by Evelyn and Malloy (6) for the photoelectric determination of methemoglobin, with a spectral band in the vicinity of 6350 Å. The infrared region is to be preferred because the absorption bands are relatively broad and carbonylhemoglobin and cyanmethemoglobin are more transparent.

The sample is then diluted and a third determination made in the visible region.

\[ D_3 = (\epsilon^2_{\text{HbO}_2} C_{\text{HbO}_2} + \epsilon^2_{\text{MHB}} C_{\text{MHB}} + \epsilon^2_{\text{HbCO}} C_{\text{HbCO}}) \frac{L}{d_3} \]

where \( \epsilon^2_{\text{HbO}_2} \), \( \epsilon^2_{\text{MHB}} \), and \( \epsilon^2_{\text{HbCO}} \) are the respective extinction constants for the second spectral interval and \( d_3 \) is the second dilution factor. Since \( C_{\text{MHB}} \) is already known, a solution of Equations 1 and 3 will yield the values of \( C_{\text{HbO}_2} \) and \( C_{\text{HbCO}} \).

Instruments—The method has been applied to the Coleman spectrophotometer, model 10S, with a 5 μ slits, and to several portable photometers especially designed for this purpose. These photometers are alike in general design but differ somewhat in optical features. In every case the light beam is split into two parts each of which ultimately activates one of

* The molecular extinction coefficient \( \epsilon \) is defined by the equation \( D = \log_{10} I_0/I = \epsilon C L \) where \( C \) is the concentration in equivalents per liter and \( L \) the cell length in cm. This differs somewhat from the notation used in a previous publication (5) in which the absorption coefficient \( \alpha \) was used to designate the coefficients obtained when \( C \) was expressed in equivalents per cc. It would appear to be more consistent with general practice to reserve the absorption coefficient \( \alpha \) for constants defined in terms of natural logarithms, \( \log_e I_0/I = \alpha C L \).
a pair of matched photocells which are mounted in a balanced electrical circuit according to Hanson (7). One light beam passes through the absorption cell containing the sample. The second beam traverses some arrangement for reducing the light intensity to equal that transmitted by the absorption cell. This consists of either a neutral wedge of density 1.0 attached to a vernier scale, or a spiral disk designed to reduce the aperture of a diaphragm linearly with rotation of the disk. The results described below were obtained with a photometer containing the neutral wedge; similar results have also been obtained with other models in which the spiral disk is employed.

Selection of Wave-Lengths—Absorption measurements on the Coleman spectrophotometer were made at dial settings of 4965 and 8000 A. 8000 A is sufficiently close to the maximum for alkaline methemoglobin to afford a high sensitivity and in addition is isobestic for oxyhemoglobin and reduced hemoglobin; the presence of reduced hemoglobin will therefore not interfere with the determination. For these reasons 8000 A is to be preferred to the absorption maximum for oxyhemoglobin at 9200 A, which would be somewhat more sensitive for the carbonylhemoglobin determination.

The spectral interval at 4965 A constitutes an isobestic point for oxyhemoglobin and carbonylhemoglobin, so that loss of CO during the final dilution will not affect the results. At each of the spectral intervals selected the absorption curves for all the components concerned are relatively flat, insuring a minimum of deviation from Beer's law.

In the selection of filters for the photometers various combinations were tested in a search for spectral intervals isobestic for oxyhemoglobin and carbonylhemoglobin in the visible and for oxyhemoglobin and reduced hemoglobin in the infra-red region. Because the effective spectral interval is in part a function of the phototube and lamp characteristics, different combinations must be tested in every case. The particular photometer used in this study, equipped with RCA No. 919 and No. 917 phototubes, contained the following Corning filters: Set 1, No. 3385, 1.1 mm.; No. 5030, 6.0 mm.; No. 255, 2.0 mm.; Set 2, No. 338, 4.0 mm.; No. 4303, 5.1 mm.; No. 5543, 2.6 mm. Set 1 isolates a suitable band in the infra-red region in the vicinity of 8000 A and Set 2 a suitable band in the visible region near 5000 A. Although oxyhemoglobin and carbonylhemoglobin are not perfectly identical in absorption in the region isolated by Set 2, they are sufficiently alike for the purposes of the method.

Analytical Procedure

Solutions—Since the absorption spectrum of methemoglobin is dependent upon the pH of the solution, it is necessary to work with buffered solutions. We have found it most convenient to use borate buffer at pH 9.4. 24.8 gm.
of $\text{H}_3\text{BO}_3$ were dissolved in $0.20 \text{ N} \text{NaOH}$ to a final volume of 1 liter; 80 cc. of this solution were then mixed with 20 cc. of $0.20 \text{ N} \text{NaOH}$ to form the stock $0.2 \text{ N} \text{buffer}$.

A hemolyzing solution was prepared by dissolving 0.3 gm. of saponin (Eimer and Amend, pure white grade) in 10 cc. of the $0.2 \text{ N}$ borate buffer and diluting to 100 cc. This hemolyzing solution was freshly prepared each morning, since it becomes turbid on standing.

Absorption Measurements—Blood samples were collected by venipuncture and treated with approximately 0.81 volume of 25 per cent potassium oxalate. For the absorption measurements, the blood sample was accurately diluted 1:10 with the hemolyzing solution and allowed to stand a minute or two to insure complete hemolysis. The solution was then transferred to the absorption cell and the transmission ($T_1$) measured at 8000 A on the Coleman spectrophotometer and the density ($D_1$) with filter Set 1 on the photometer. The solution in the cell was then treated with a few crystals of KCN, stirred, and the transmission ($T_2$) and density ($D_2$) again measured on each instrument at the same wave-length and filter settings. Finally, 1 cc. was withdrawn from the absorption cell, diluted accurately to 25 cc. with water, and the transmission ($T_3$) and density ($D_3$) with filter Set 2 on the photometer.

Calibration of Instruments—For the computation of results from Equations 1 to 3, it is necessary to know the molecular extinction constants for each hemoglobin derivative at each spectral interval. These were determined with samples of normal blood from non-smoking individuals, the total hemoglobin content of which was determined independently by spectrophotometric analyses. Each sample was divided into three portions: one portion was completely oxygenated, a second was saturated with CO, and the third converted to methemoglobin by the addition of $\text{K}_3\text{Fe(CN)}_6$ (30 mg. per cc. of blood). Each of these samples was then analyzed in the manner described above. For the absorption measurements on the methemoglobin solutions the comparison cells were filled with solutions containing equivalent concentrations of $\text{K}_3\text{Fe(CN)}_6$. The transmission values obtained from the Coleman spectrophotometer ($T_1$, $T_2$, and $T_3$) were converted to density units ($D_1$, $D_2$, and $D_3$) by the following equation.

$$D = \log \frac{1}{T}$$

From the calibration samples the extinction constants were computed by applying the relation

$$D = \varepsilon CL$$
where \( \epsilon \) is the molecular extinction constant for the particular spectral region and derivative employed, \( C \) is the concentration in equivalents per liter on an Fe basis, and \( L \) the length of the absorption cell in cm.

In Table I are listed the extinction constants obtained for each of these instruments.

The constants obtained with the Coleman spectrophotometer, because of the relatively narrow effective slit width used, are similar to those previously published for precise spectrophotometric work. The constants obtained with the photometer, however, differ widely from those obtained with monochromatic light, because of the difficulty of obtaining narrow intervals by means of filters.

**Table I**

<table>
<thead>
<tr>
<th>Filter region</th>
<th>Instrument</th>
<th>Extinction constants, ( \epsilon \times 10^{-a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbO(_2)</td>
<td>HbCO</td>
</tr>
<tr>
<td>Visible</td>
<td>Coleman</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>Photometer</td>
<td>6.59</td>
</tr>
<tr>
<td>Infra-red</td>
<td>Coleman</td>
<td>0.235</td>
</tr>
<tr>
<td></td>
<td>Photometer</td>
<td>0.252</td>
</tr>
</tbody>
</table>

From Equations 1 to 3 the following equations may be developed for the computation of the results.

\[
C_{\text{MHb}} = \frac{(D_1 - D_2) d_1}{(\epsilon_{\text{MHb}} - \epsilon_{\text{MHbCN}}) L}
\]

\[
R = \frac{D_1 - \frac{L}{d_1} C_{\text{MHb}} \epsilon_{\text{MHb}}}{D_2 - \frac{L}{d_2} C_{\text{MHb}} \epsilon_{\text{MHbCN}}}
\]

\[
f_{\text{HbCO}} = \frac{R_{\text{HbO}_2} - R}{R_{\text{HbO}_2} - R_{\text{HbCO}}}
\]

\[
C_{\text{HbO}_2} + C_{\text{HbCO}} = \frac{d_2}{L} \left( \frac{D_2 - \frac{L}{d_1} C_{\text{MHb}} \epsilon_{\text{MHbCN}}}{(\epsilon_{\text{HbO}_2} + (\epsilon_{\text{HbCO}} - \epsilon_{\text{HbO}_2}) f_{\text{HbCO}})} \right)
\]

\[
C_{\text{total}} = C_{\text{MHb}} + (C_{\text{HbO}_2} + C_{\text{HbCO}})
\]

\[
C_{\text{HbCO}} = f_{\text{HbCO}} (C_{\text{HbO}_2} + C_{\text{HbCO}})
\]

where \( C_{\text{MHb}}, C_{\text{HbO}_2}, \) and \( C_{\text{total}} \) are the respective concentrations expressed in equivalents per liter, \( d_1 \) and \( d_2 \) are the dilution factors, \( L \) is the length of
the cell, and \( R \) is the ratio of the infra-red to the visible absorption, after these have been corrected for the absorption due to methemoglobin. \( R_{\text{HbO}_2} \) and \( R_{\text{HbCO}} \) are the ratios for solutions containing these derivatives only and may be computed from the calibration constants. \( f_{\text{HbCO}} \) is the fraction of ferrous hemoglobin present as HbCO. The dilution factors \( d_1 \) and \( d_2 \) were equal to 10 and 250 respectively. The cell lengths \( L \) were 1.00 cm. for the wedge photometer and 1.30 cm. for the Coleman spectrophotometer.

Substitution of the appropriate values for \( \epsilon \) from Table I in Equations 4 to 7 yields the following equations for the particular instruments used in these studies.

**Coleman Spectrophotometer**

\[(4, \ a)\]  
\[C_{\text{HbO}_2} = (D_1 - D_2)16.9 \times 10^{-3}\]

\[(5, \ a)\]  
\[R_{\text{obs.}} = \frac{D_1 - 68.2C_{\text{HbO}_2}}{D_2 - 37.8C_{\text{HbO}_2}}\]

\[(6, \ a)\]  
\[f_{\text{HbCO}} = \frac{1.070 - R_{\text{obs.}}}{0.830}\]

\[(7, \ a)\]  
\[C_{\text{HbO}_2} + C_{\text{HbCO}} = \frac{(D_2 - 37.8C_{\text{HbO}_2})250 \times 10^{-3}}{7.15 + 0.25f_{\text{HbCO}}}\]

**Photometer**

\[(4, \ b)\]  
\[C_{\text{HbO}_2} = (D_1 - D_2)28.1 \times 10^{-3}\]

\[(5, \ b)\]  
\[R = \frac{D_1 - 42C_{\text{HbO}_2}}{D_2 - 35C_{\text{HbO}_2}}\]

\[(6, \ b)\]  
\[f_{\text{HbCO}} = \frac{0.954 - R}{0.776}\]

\[(7, \ b)\]  
\[C_{\text{HbO}_2} + C_{\text{HbCO}} = \frac{(D_2 - 35C_{\text{HbO}_2})250 \times 10^{-3}}{6.59 + 0.34f_{\text{HbCO}}}\]

When the corrections for the small differences in absorption between HbCO and HbO\(_2\) in the visible regions are neglected, Equations 7, a and 7, b reduce to

\[(7, \ a')\]  
\[C_{\text{HbO}_2} + C_{\text{HbCO}} = (D_2 - 37.8C_{\text{HbO}_2})35.0 \times 10^{-3}\]

\[(7, \ b')\]  
\[C_{\text{HbO}_2} + C_{\text{HbCO}} = (D_2 - 35C_{\text{HbO}_2})37.9 \times 10^{-3}\]

The error introduced by this simplification is negligible for sublethal concentrations of HbCO.

The computation of results may be greatly simplified by the use of suitable nomograms in place of the equations presented above. Such nomograms have been prepared for the Coleman spectrophotometer and the wedge photometer used in these studies.
Results

A number of samples of human blood were treated with varying amounts of NaNO₂ and pure CO to bring about partial conversion of these samples to methemoglobin and carbonylhemoglobin. For the same samples the CO content was determined by the method of Horvath and Roughton (8) and the total and active hemoglobin contents were determined according to the procedures described in Peters and Van Slyke (9). In Table II we compare the results of the spectrophotometric analyses with those obtained by the gasometric methods. The nomograms referred to above were used for the computation of the spectrophotometric data; essentially the same values are given when the equations are used.

The concentrations of the hemoglobin components in Table II are given in terms of milliequivalents per liter. These may be converted to gm. per 100 cc. by multiplying by 1.67, and to volumes per cent by multiplying by 2.24.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Total hemoglobin</th>
<th>Methemoglobin</th>
<th>Carbonylhemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coleman Photom.</td>
<td>Van Slyke Photom.</td>
<td>Van Slyke</td>
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<tr>
<td>1</td>
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<td>8.29</td>
<td>8.41</td>
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<td>2</td>
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<td>8.41</td>
</tr>
<tr>
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<td>4</td>
<td>9.66</td>
<td>9.77</td>
<td>9.73</td>
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<tr>
<td>5</td>
<td>10.18</td>
<td>10.27</td>
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</tr>
<tr>
<td>6</td>
<td>10.24</td>
<td>10.27</td>
<td>10.27</td>
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<td>7</td>
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<td>9.23</td>
<td>9.27</td>
<td>9.13</td>
</tr>
</tbody>
</table>

DISCUSSION

An examination of Table II discloses that the results of spectrophotometric analysis compare very favorably with results obtained with the
Van Slyke apparatus. Standard deviations from the Van Slyke data have been computed and turn out to be 0.07 for total hemoglobin, 0.10 for methemoglobin, and 0.14 for carbonylhemoglobin, for both of the instruments used. The accuracy is thus about 0.7 per cent for total hemoglobin, 0.9 per cent for methemoglobin, and 1.5 per cent for carbonylhemoglobin. Attention is drawn to the unusually large discrepancies observed in the MHB determinations in Samples 9 and 10. These results as well as the carbonylhemoglobin determination on the Coleman spectrophotometer for Sample 2 have been omitted from the computation of standard deviations. The discrepancies in the methemoglobin determinations are undoubtedly due to a failure of the standard Van Slyke method for total hemoglobin with samples containing high concentrations of MHB. The reproducibility of the Van Slyke method for such samples is very poor, and may be attributed to a failure to obtain complete reduction of the MHB with Na₂S₂O₃. It will be observed that the results obtained from the two photoelectric instruments are in good agreement.

In the visible region the absorption contributed by blood components other than hemoglobin is negligible (5). In the infra-red region, however, an appreciable contribution is made by absorption and scattering from material in the blood plasma and from red cell fragments. If normal blood is treated uniformly, this contribution is sufficiently constant to permit accurate determinations. It has been observed, however, that the infra-red absorption is influenced by the manner in which the blood is collected. Thus, solutions prepared by immediately hemolyzing freshly drawn blood without the addition of anticoagulant will be appreciably more transparent than solutions treated with oxalate in the manner described above. Any convenient method may be used, provided the calibration constants are determined in the same way and the same procedure is followed throughout. While the presence of unusually large or small amounts of scattering or absorbing material has little effect on the determination of methemoglobin and total hemoglobin, the CO determination is more sensitive and may be in error by substantial amounts. For this determination particular care must be exercised. It is recommended that no samples be taken for several hours after a moderately heavy meal. The results described in Table II were obtained with samples taken in the forenoon, the subjects having consumed an unrestricted breakfast.

If analyses of the methemoglobin content alone are desired, the method may be simplified by eliminating the determination in the visible region. In such case the total hemoglobin may be estimated from the absorption of the original solution, with an accuracy which will depend on the CO content of the blood.

Scholander and Roughton (10) have recently described a micro gaso-
metric method for the determination of CO in blood which is simple and accurate and suitable for field use. It has important advantages over the present method in that no photometer is required and smaller blood samples can be analyzed. It does, however, require a considerable number of reagents, whereas the spectrophotometric method requires but one readily prepared solution. The present method can be used for the simultaneous estimation of total hemoglobin and methemoglobin, as well as carbonylhemoglobin, and is exceedingly simple and rapid. The method can readily be adapted to any photometer which extends into the near infrared region. In view of the wide spectral intervals isolated by the filter systems employed in the wedge photometer it is felt that the method can be successfully applied with sufficient accuracy to any spectrophotometer which isolates a band no broader than about 300 or 400 Å.

The authors are indebted to Dr. John S. Kirby-Smith for the construction of the photometer, to Dr. H. L. Andrews for helpful suggestions, and to Mr. William Pricer for the Van Slyke analyses.

SUMMARY

1. A simple spectrophotometric method for the simultaneous determination of methemoglobin, carbonylhemoglobin, and total hemoglobin is described.
2. Details of the method as applied to the Coleman spectrophotometer and to a special photometer constructed for this purpose are given.
3. Results obtained by this method are compared to Van Slyke gasometric determinations. The method is accurate to about 1 per cent.

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