SPECTROPHOTOMETRIC DETERMINATION OF THE
OXYGEN SATURATION OF WHOLE BLOOD*

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A new approach to the spectrophotometric determination of blood oxygen saturation has been made which is based on the absorption maximum of oxyhemoglobin and the isosbestic point of oxyhemoglobin and hemoglobin. The precision of this new method is comparable to that of the manometric procedure in which the Van Slyke-Neill apparatus is employed; there is also good correlation between results obtained with these procedures.

The literature on the spectrophotometric method for the determination of blood oxygen has been reviewed by Hickam and Frayser (1). Since that publication the noteworthy advance has been made by Nahas who utilized the isosbestic point of hemoglobin for the determination of oxygen saturation of blood and designed a Lucite cuvette of short light path (2). In an investigation in which the simultaneous determination of oxygen tension and saturation was to be made we found the time limitation required for the procedure for oxygen saturation as described by Nahas unfeasible. Furthermore, the precision of that method was not great enough for our purpose.

Procedure

After the blood is drawn and mixed with heparin, 0.5 ml. is transferred into a 2 ml. syringe in the following manner: First, a short length of rubber tubing is tightly fitted over the tip of the 2 ml. syringe, protruding about 1.5 mm. All the dead space in this syringe and in the rubber tubing is filled with vaseline. A drop of blood is expelled from the sampling syringe and the tip is wiped off. Then the tip is fitted into the rubber connection of the 2 ml. syringe and 0.5 ml. of blood is gently pushed into the small syringe.

The blood is hemolyzed immediately by the addition of 0.01 ml. of a 50

per cent solution of saponin. The saponin solution is made up fresh about once a week or whenever it shows signs of deterioration and can be added conveniently by means of a syringe micro burette (Micro-Metric Instrument Company, Cleveland, Ohio) of 0.2 ml. per inch capacity with a needle (\frac{1}{8} inch, 21 gage) fitted onto the end. The addition may be made either by putting a rubber cap on the end of the syringe containing the sample and penetrating the cap with the needle or by simply putting the needle directly into the uncapped syringe, pushing the needle tip almost to the plunger, adding the saponin, and then capping the syringe. This latter method is easier and, if performed rapidly and carefully, will give the same results.

As soon as the saponin is added and the syringe capped without trapping air, the blood and saponin are mixed by rapidly rotating the syringe. It has been reported that if mixing is delayed a protein precipitate may form at the interface between the blood and saponin (1). We have not encountered this difficulty, since the mixing has always been done immediately. It takes less than 1 minute to complete the hemolysis, and with the conditions used no trouble with incomplete hemolysis has been encountered.

The hemolyzed blood is now forced into three cells of the short light path cuvette designed by Nahas (The Waters Corporation, Rochester, Minnesota), one cell being left filled with water to be used as a zero reference. The filling is done by placing on the end of the syringe a blunt needle with a rubber cap covering the lower part to make an air-tight connection with the cuvette opening. Some blood is pushed through the needle and discarded; then the needle is injected into each cell opening and an excess (3 times the cell capacity) is pushed through each cell as described by Nahas. It is essential that there be no air bubbles in the cells.

Readings are taken on the Beckman spectrophotometer, model DU, with the photomultiplier "sensitivity" switch set at 1, the attachment "load" at 2 (resistor 22 megohms), and a cooling water jacket around the tungsten lamp. Readings are taken at 576 and 505 m\mu with a slit width of 0.01 mm., the sensitivity being varied to set the reference cell at zero.

From the time the blood is placed in the cuvette the color begins to change rapidly; hence the readings must be made as soon as possible. The reading at 576 m\mu should be started within 1 minute of the time the cuvette is filled. The readings are then taken at 505 m\mu.

A blank reading must be taken at each wave-length on each cell. When the cells are cleaned and filled with water, readings are taken against the same zero reference used for blood, and the blood readings must be corrected accordingly. The blank may make an appreciable difference in the result and should be run very often, at least once a day.
Calculations

The extinction for each cell at each wave-length is corrected for the blank reading. Then the ratio of the reading at 576 m\(\mu\) to that at 505 m\(\mu\) is calculated separately for each cell. The average of the three ratios is taken, unless there is an obvious reason for discarding one (\textit{e.g.}, air in one cell).

From this average ratio the oxygen saturation of the blood is read directly from a graph. The graph has been constructed by plotting the ratio as abscissa and percentage saturation as ordinate; linearity between 0 and 100 per cent saturation has been established experimentally.

EXPERIMENTAL

First, an ideal line expressing the relationship between the oxygen saturation and the ratio of extinction readings at 576 and 505 m\(\mu\) was constructed (Fig. 1). For blood of 100 per cent saturation the reading at 576 m\(\mu\) represents the oxyhemoglobin concentration and that at 505 m\(\mu\) the total hemoglobin content. For blood of 0 per cent saturation containing no oxyhemoglobin, the reading at 576 m\(\mu\) represents only the extinction of hemoglobin at that wave-length, while the reading at 505 m\(\mu\) still represents the total hemoglobin content. If our assumption of a simple two-component system consisting of oxyhemoglobin and hemoglobin is valid, then the straight line linking the two points should represent all the blood saturations between 0 and 100 per cent saturation.

The 100 per cent saturation point is the average of the results from seven blood samples. Each sample was oxygenated immediately after being drawn by mixing for 10 minutes with an equal volume of pure oxygen at room temperature. The 0 per cent saturation point is the average of the results from eight blood samples. Each sample was degassed by extraction under vacuum in the Van Slyke-Neill apparatus (3). About thirty repeated extractions were made on each sample, a constant manometric reading for five consecutive extractions being the criterion of complete oxygen removal. The red cells were always intact after these extractions. To simplify this oxygen removal a chemical method instead of the physical one was attempted. The commonly used hydrosulfite was tried and was found to yield unsatisfactory results. Possible chemical change of hemoglobin by this reagent as suggested by Roughton \textit{et al.} (4) might have contributed to the failure.

A series of non-fasting blood samples was run both by the spectrophotometric and Van Slyke manometric procedures. The samples were all venous blood from healthy adults except for two arterial samples from patients with congenital heart disease. The oxygen saturations ranged from 12.3 to 100 per cent and the hemoglobins from 11.7 to 16.3 gm. per
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100 ml. The higher saturations (in vivo) were obtained by keeping the subjects at rest for one-half hour and having them breathe oxygen, the lower saturations by leaving the tourniquet on during sampling, and the points in between by variations of these two procedures.

Each blood sample was drawn in a 10 ml. syringe lightly greased with mineral oil and with the dead space filled with a 10 gm. per cent solution of heparin sodium. A porcelain disk made from a broken crucible was placed in the barrel of the syringe to facilitate mixing of the blood with the heparin. As soon as the sample was well mixed with the heparin, 0.5 ml. was transferred to a small syringe and the oxygen saturation was determined as described under “Procedure.” After the transfer the remainder of the sample was immediately placed in an ice bath where it was kept rotating. At one-half hour and 1 hour, it was run again.

Each sample was also analyzed for oxygen content by the Van Slyke procedure (5), the analysis being made in duplicate with 1 ml. samples. The following method was used for oxygenating the blood for the oxygen capacity determination: The blood is saturated in the syringe in which it was drawn. A volume of oxygen equal to the amount of blood in the syringe is added and the syringe is allowed to rotate at room temperature for 7 minutes. Then the oxygen is displaced and replaced with moist air. The syringe is rotated at room temperature for 7 minutes with the air; then that air is expelled and the syringe is refilled with moist air and allowed to rotate another 7 minutes. This procedure was adopted to prevent the loss of plasma which has been reported when the blood is saturated in a tonometer (6). The oxygen saturation is calculated from the content and the capacity values after each has been corrected for plasma oxygen.

The first spectrophotometer readings were made on each specimen between 5 and 8 minutes after the time the needle was withdrawn from the subject. On the one-half and 1 hour specimens the transfer of blood to the small syringe was started at 30 and 60 minutes and the first readings were made at about 35 and 65 minutes.

Several of the samples had fluoride added to them. The final concentration in the blood was approximately 0.012 per cent potassium fluoride and 0.4 per cent sodium fluoride. This was done because the method is to be used for another investigation in which the blood will have fluoride added to facilitate the measurement of blood oxygen tension (7).

Results

For the seven 100 per cent saturated blood samples the mean ratio of extinction values $E_{676 \text{ mC}}/E_{595 \text{ mC}}$ was 3.117 with a standard deviation of 0.0101 and the standard error of the mean of 0.0034.

For the eight 0 per cent saturated blood samples the mean ratio of the
extinction values $E_{576 \text{m} \mu}/E_{505 \text{m} \mu}$ was 2.025 with a standard deviation of 0.0107 and the standard error of the mean of 0.0041.

These two points were used to construct a calibration line as shown in Fig. 1, where the ratio of extinction values from 2.025 to 3.117 constitutes the abscissa and the per cent oxygen saturation from 0 to 100 per cent saturation the ordinate. The twenty-one points along the line are the results of comparison of the manometric method with the spectrophotometric procedure immediately after sampling. Table I presents the same

comparison for results obtained immediately, one-half hour, and 1 hour after sampling. The standard error of the mean of the triplicate runs of the spectrophotometric method was found to be 0.59 per cent $O_2$ saturation. The mean value of duplicate manometric analyses over the entire range of $O_2$ saturation encountered was found to have a standard error of 0.75 per cent $O_2$ saturation. The variance between the manometric and spectrophotometric methods for the twenty-one comparisons was calculated; it was $(1.14)^2$.

The standard error of the mean of the triplicate runs of the spectrophotometric method does not give a true picture of the precision of the method because it does not include any error during the hemolysis and
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handling of the sample or from variations in timing during the run. On the other hand, it is not possible to run true duplicates on the sample. It may be assumed that the standard error of the mean of any duplicate runs must be less than the standard deviation between the means of the zero and one-half hour specimens (assuming these specimens to be dupli-

\[\text{TABLE I} \]

Effect of Keeping Blood Sample in Ice Bath on Oxygen Saturation Value by Spectrophotometric Method

<table>
<thead>
<tr>
<th>Per cent oxygen saturation values</th>
<th>Manometric method</th>
<th>Spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr.</td>
<td>½ hr.</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------</td>
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</tr>
<tr>
<td>69.5</td>
<td>69.4</td>
<td>69.4</td>
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<td>24.0</td>
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<td>24.7</td>
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<td>16.3</td>
<td>15.8</td>
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<td>32.2</td>
<td>33.4</td>
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<td>67.3</td>
</tr>
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<td>26.6</td>
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</tr>
<tr>
<td>97.6</td>
<td>97.3</td>
<td>98.4</td>
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</table>

cates). This standard deviation has been estimated to be 0.53 per cent \(O_2\) saturation.

In a few cases in which the sample contained foam the spectrophotometric results were higher than the manometric ones, even when the amount of foam was very small. These results are presented separately in Table II.

The spectrophotometric procedure was applied to two blood samples from new-born infants. The Roughton-Scholander method (8) for blood oxygen was used to determine the \(O_2\) saturation because of the limited
amount of sample available. There seems to be no significant difference between the results; hence the method may also be used for blood containing fetal hemoglobin. In contrast, a large difference was observed when the same comparison was made on a blood sample from a patient with sickle-cell anemia, due probably to a different absorption spectrum of hemoglobin in this disease.

Addition of fluoride to the sample gave results indistinguishable from those without the addition. The samples from smokers, however, group on the same side of the calibration line, as shown in Fig. 1, even though the scatter is within experimental error.

**Table II**

*Effect of Foam in Sample on Oxygen Saturation Value by Spectrophotometric Method*

<table>
<thead>
<tr>
<th>Per cent oxygen saturation values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manometric method</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>0 hr.</td>
</tr>
<tr>
<td>19.3</td>
</tr>
<tr>
<td>46.0</td>
</tr>
<tr>
<td>73.3</td>
</tr>
<tr>
<td>50.5</td>
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</table>

**DISCUSSION**

The Roughton-Scholander method for blood oxygen determination has been adapted to measure blood oxygen saturation in this laboratory. The oxygen content of the sample before and after aeration allows one to calculate the per cent saturation by simple division. In the course of several years, occasional comparison of the values obtained by this method with those by the application of the Van Slyke manometric procedure for blood oxygen determination has shown good agreement. Since less time and sample are required with the Roughton-Scholander method, it has been used routinely and found satisfactory. However, at times the blood sample begins to coagulate slowly during the period when the sample is being aerated, thus leading to erratic values for oxygen capacity of the sample. To avoid this difficulty and to reduce the time and sample requirement the spectrophotometric procedure was investigated.

The spectrophotometric method is based on the theory that, in hemolyzed blood, oxyhemoglobin and hemoglobin may be regarded as true solutions obeying the law of Bouguer and Beer. The possible interfering substances, namely methemoglobin and ferrihemoglobin, are present in
normal blood in amounts less than 0.5 per cent (9); hence, their effect on the precision of the determination may be overlooked.

The distinctive absorption spectra of completely oxygenated hemoglobin and reduced hemoglobin are known. Assuming that the hemoglobin of a blood sample exists in either of the two forms, a number of different methods are possible for determining the per cent of oxyhemoglobin. By measuring two of the three quantities, oxyhemoglobin, hemoglobin, and total hemoglobin, the per cent of oxyhemoglobin may be calculated. Or, as a short cut, the ratio of a quantity related to oxyhemoglobin and a quantity related to total hemoglobin may be used for the calculation of per cent of oxyhemoglobin. The extinction value of hemolyzed blood at one of the maxima of oxyhemoglobin and the extinction value at the isosbestic point are such quantities. By constructing a calibration curve correlating the ratio to the per cent oxyhemoglobin no involved calculation would be necessary for the determination of blood oxygen saturation.

The total hemoglobin may be determined by first oxygenating or reducing the sample completely and measuring the total hemoglobin as 100 per cent oxyhemoglobin or hemoglobin. Or, as has been applied by Drabkin and Austin (10), the hemoglobin may be converted chemically into cyanmethemoglobin and the amount of the product measured. All the above mentioned approaches involve procedures which contribute to additional error of the method. The utilization of the isosbestic point of the hemoglobins by Nahas not only eliminated this source of error but also simplified the procedure for the oxygen saturation determination. In his method the total hemoglobin was read at 505 m\(\mu\) and hemoglobin at 605 m\(\mu\) in the same short light path cuvette.

However, two disadvantages were encountered when we attempted to apply the Nahas procedure. Namely, the limitation of time lapse of 8 minutes between sampling and the reading of extinction values and the insufficient precision of a standard deviation of 1.9 per cent oxygen saturation. The advantage of two absorption maxima of the oxyhemoglobin was then examined by us, since these maxima would permit the use of a narrow band width of the spectrophotometer while allowing some tolerance in the setting of the wave-length dial. The isosbestic point at 505 m\(\mu\) is preferred because of the proximity of the slopes of the absorption curves of oxyhemoglobin and hemoglobin.

The absorption maxima of oxyhemoglobin as determined by us were at 576 and 542 m\(\mu\). Both these maxima were investigated and tested against the Roughton-Scholander method. The peak at 576 m\(\mu\) gave much better correlation with the oxygen saturation as determined with the gasometric method; thus further work was continued with this wave-length only. At first a slit width of 0.17 mm. was used, but later it became possible to re-
duce it to 0.01 mm. with the attachment of a photomultiplier to the spectrophotometer.

The narrow slit width allows higher precision, but it should be carefully set and left undisturbed during a run. The wave-length settings must be made with even greater care, since a very small variation can cause a large difference in the reading. The timing after the introduction of the hemolyzed sample into the cuvette is perhaps the most crucial detail, since the readings are changing at unpredictable rates. On the other hand, keeping the sample in the ice bath for a period of 30 minutes does not seem to cause any significant change in the per cent saturation value.

The close correlation of the saturation values obtained by the spectrophotometric and the manometric procedures seems to bear out the assumption of a two-component system in blood hemoglobin, namely oxyhemoglobin and hemoglobin. In view of the additional evidence of a simple two-component system provided by Nahas’ work it may be said that, whatever limitation the theory of intermediates in oxygenation of hemoglobin as postulated by Roughton (11) may impose on the spectrophotometric approach, it is not detectable at the present.

After the conclusion of this work a paper by Klungsøyr and Støa (12) came to our attention. This modified spectrophotometric procedure for hemoglobin oxygen saturation seemed to have a high degree of reproducibility even though a significant difference was found between results obtained by this procedure and by the manometric method.

SUMMARY

A rapid spectrophotometric method is presented for the determination of blood oxygen saturation with 0.5 ml. of sample.

Twenty-one comparisons were made with the Van Slyke manometric method on blood samples with oxygen saturations varying from 12.3 to 100 per cent and hemoglobins ranging from 11.7 to 16.3 gm. per cent. The variance of the comparison was (1.14)².

BIBLIOGRAPHY

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