THE COLORIMETRIC DETERMINATION OF HEMOGLOBIN.*

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The method to be described for the determination of hemoglobin depends upon the comparison, in a colorimeter, of carbon monoxide hemoglobin solutions, one of which has a known hemoglobin content. Hoppe-Seyler (1) was the first to describe carbon monoxide hemoglobin and to make use of this stable combination for estimating the hemoglobin content of blood. He devised a "double pipette" for comparing the unknown carbon monoxide hemoglobin solution with the standard carbon monoxide hemoglobin solution, prepared from hemoglobin crystals; but the method never came into general use, because of the many technical difficulties involved. Haldane (2) suggested a much simpler method for comparing carbon monoxide solutions, using the apparatus employed by Gowers (3) for comparing oxyhemoglobin solutions with a picro-carmine standard. This apparatus was later employed by Sahli (4) who prepared an acid hematin standard by adding dilute hydrochloric acid to blood.

A critical discussion of the various methods in use for the estimating of hemoglobin is beyond the scope of this paper. As Haldane (2) has pointed out, artificially colored solutions and tinted glass present great difficulties in standardization with a definite strength of hemoglobin solution. With a certain strength of color solution or tinted glass, it is possible to imitate quite perfectly the tint of a given hemoglobin solution provided the quality of light remains the same. Any variation from these standard conditions, either in quality of light or strength of hemoglobin in solution leads to serious errors. Haldane at-

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tended to overcome the objection by preparing a carbon monoxide hemoglobin standard (1 per cent solution of a blood having an oxygen capacity of 18.5 per cent) which he considered permanent when kept sealed in a small test-tube in an atmosphere of carbon monoxide. 0.020 cc. of blood, placed in a similar sized and shaped graduated (on scale of 100) test-tube with a small amount of water saturated with carbon monoxide by means of ordinary illuminating gas, are diluted with water, drop by drop, until the unknown and standard tubes match in color, whence the percentage of hemoglobin in the unknown may be read off on the graduated scale. In principle, Haldane's method is sound; but certain practical difficulties arise. The standard is not so permanent as was at first thought, and, when water is used, it has been shown by Krogh (5) that the full color of the solution is slow in reaching its maximum. The further criticism is that the method is time-consuming and cumbersome—adding water, drop by drop, and shaking after each addition. Also the color comparison in the two tubes is not sharp. This method has one distinct advantage in that it may be used for the determination of hemoglobin in any species of animal.

Sahli employed Haldane's apparatus and technique; except that he used, as a standard, blood to which dilute hydrochloric acid had been added. There are three serious objections to Sahli's method: first, the standard is not permanent; second, there is considerable delay in the development of the maximum or permanent color, amounting, according to Meyer and Butterfield (6), in some instances to 20 per cent; and third, it cannot be used for the blood of different species.

The spectrophotometer has undoubted accuracy in the hands of skilled operators; but the expense and unavailability together with the difficult technique involved, make it impracticable for general use.

The great variety of methods and apparatus which have been proposed offer eloquent testimony to the unsatisfactory means for the determination of hemoglobin. There is great need for a rapid, accurate, and universally standard method for the estimation of hemoglobin in experimental work and the study of blood diseases in the clinics.

The method which we have found to fulfil the above conditions is as follows:
Method.

Blood is obtained in the usual manner by pricking the finger or lobe of the ear. A 1 per cent solution of blood is made by drawing 0.05 cc. into a special pipette and transferring into 5 cc. of 0.4 per cent ammonia solution—accurately measured with a calibrated pipette or burette into a 12 x 120 mm. test-tube. The blood pipette is rinsed out by drawing into it two or three times the ammonia solution. Ordinary illuminating gas is bubbled rapidly through the ammonia blood solution for 30 seconds, after which, it is compared in a Duboscq colorimeter with a standard carbon monoxide hemoglobin solution set at 10. The average of at least four readings is taken. The calculation is simple, 

\[
\frac{10}{R} \times 100 = \text{per cent hemoglobin.}
\]

Manner of Obtaining Blood.—With sufficient care the usual clinical method for obtaining small amounts of blood by pricking the ear or finger is satisfactory. A free flow is essential. Any undue manipulation or squeezing of the part should be avoided because an error of 5 or 10 per cent may be introduced by diluting the blood with tissue juice. Where there is marked anemia requiring larger amounts of blood than 0.05 cc., or where there is difficulty in obtaining blood from the ear or finger, venous puncture should be used, coagulation being prevented with oxalate or citrate salts. It is often practical and convenient to combine the determinations of hemoglobin with other blood analyses, where venous puncture is required. If blood has been drawn by venous puncture care must be taken that the corpuscles and serum are well mixed before filling the pipette. The blood should never be shaken violently before measuring, because it becomes filled with air bubbles. The mixture of corpuscles and serum may best be accomplished by first giving the receptacle a circular motion and finally stirring briskly with a glass rod or measuring pipette which is filled while stirring the blood.

Pipette for Measuring Blood.—The pipettes are made of millimeter glass tubing calibrated to contain 0.05 cc. and 0.10 cc. The pipettes are easily made in any laboratory from straight tubing, and require no blowing, the point being rounded off on an emery wheel. In this way time and expense are saved, since pipettes
Hemoglobin Determination

obtained from glass blowers require recalibration before use. It has been found that water may be used for this calibration, as pipettes which have been calibrated with both mercury and water check sufficiently well. The advantage of having the pipette calibrated to contain 0.10 cc. as well as 0.05 cc. is obvious. In bloods with a low hemoglobin content, 0.05 cc. may not be sufficient to give the color necessary for accurate color comparison in the colorimeter. A pipette of this type, and used in the manner described, is capable of measuring 0.05 cc. of blood with an accuracy of 0.2 per cent.

Ammonia Solution.—Ammonia solutions, containing 4 cc. of strong ammonia in 1 liter of water, suggested by Krogh (5) are used, because the full color of the carbon monoxide hemoglobin develops at once.

Saturation with Carbon Monoxide.—Ordinary illuminating gas as a source of carbon monoxide has proven entirely satisfactory. It was thought that there might be substances other than carbon monoxide in the gas which might form hemoglobin compounds and interfere with the determination. Accordingly, pure carbon monoxide was prepared by heating oxalic acid with concentrated sulfuric acid and passing the gas produced through sodium hydroxide to free it from carbon dioxide. As far as could be determined on comparison of the two solutions in the colorimeter the colors were identical. Oxyhemoglobin solutions are very unstable. Hence it is necessary, after transferring the blood to the ammonia solution, to saturate with carbon monoxide within an hour. After saturation with carbon monoxide, the solution may, on carefully stoppering and protecting from light, be placed in the ice box and the determination made at leisure. Saturation of the blood should be carried out under a hood. If the laboratory does not possess a hood, the saturation may be accomplished under a funnel, attached to a small water vacuum pump, to remove the gas.

Standard Hemoglobin Solution.—Haldane's standard of a 1 per cent solution of a blood having an oxygen capacity of 18.5 per cent is used. It has been shown by Haldane and Smith (7), Butterfield (8), Barcroft (9), and others, that the oxygen capacity of the blood depends upon its hemoglobin content. A blood of 18.5 per cent oxygen capacity contains approximately 14 gm.
hemoglobin per 100 cc. Although the blood of normal men in this country, as shown by Meyer and Butterfield (6), Williamson (10), and also as we have found by use of the method here described, contains on the average 16.6 gm. of hemoglobin, which would correspond to an oxygen capacity of about 22 per cent, it was thought best to keep Haldane's standard for the present. It is a simple matter to compute the gm. of hemoglobin in any given blood from the results obtained.

The standard hemoglobin solution is prepared as follows: A quantity of defibrinated human or ox blood is obtained. The oxygen capacity is determined by the method of Van Slyke (11). The blood may also be standardized by a spectrophotometer or solutions made from hemoglobin crystals prepared in the manner described by Butterfield (8). We have checked several times our standard and found that the oxygen capacity method for standardization is most convenient and satisfactory. The blood is diluted with 0.4 per cent ammonia solution so as to make a 20 per cent solution of a blood with an oxygen capacity of 18.5 per cent. This 20 per cent blood solution is then saturated with carbon monoxide by bubbling through it illuminating gas for 10 minutes. A drop of caprylic alcohol prevents troublesome foaming. The glass tube through which the gas is passed into the blood solution is withdrawn slowly and the bottle stoppered immediately. Rubber corks must not be used in connection with hemoglobin solutions. The cork should be sealed in with paraffin and the solution, protected from light, kept in the ice chest. Such a solution thus protected will keep for months. Several solutions now nearly a year old prepared in this manner have shown no deterioration. 5 cc. of this 20 per cent blood solution made up to 100 cc. with 0.4 cc. of ammonia solution and saturated with carbon monoxide, make the 1 per cent standard for use in the colorimeter and may be prepared from time to time as desired. The 1 per cent standard for routine use may be kept in a dark glass or black painted aspirator bottle, the lower opening of which is provided with a cork, through which passes a glass tube with a ground glass cock for withdrawing small amounts of solution. A glass tube is put through the cork in the top of the bottle and extends to the bottom. Both corks should be sealed with paraffin. This glass tube is connected with an open
gas fixture in order that when solution is withdrawn from the bottom, gas rather than air will enter to replace it. Solutions thus prepared may keep for several weeks; but, as a precaution, it is advisable to make fresh 1 per cent solutions frequently; i.e., every 2 or 3 weeks. It should be remembered that dilute hemoglobin solutions are less stable than concentrated solutions; and that hemoglobin solutions keep best in the cold and protected from light. The first indication of solution deterioration is a change in color from the characteristic cherry-red of carbon monoxide hemoglobin to a red with a brownish tinge, due to the formation of methemoglobin.

Comparison in Colorimeters.—The Duboscq or Kober colorimeters have proven to be by far the most accurate and satisfactory instruments for this colorimetric work. Other colorimeters, however, may be used. The difficulties encountered are those inherent in all colorimetric work and in this connection reference to Kober's (12) article may be made. The color of the carbon monoxide hemoglobin, because of the relatively low stimulus threshold for the eye, is admirably suited to colorimeter comparison, slight differences being easily detected. We prefer to use the daylight from a north window. Satisfactory results are, however, obtainable with artificial light when "daylight glass" is used between the source of light and the solution. Considerable experimentation with light filters has failed to improve on the accuracy with which the comparison may be made. No difficulty should be experienced in making the readings check within 0.2 of a single division on the colorimeter scale.

Color comparisons are most accurate when the unknown hemoglobin solution reading falls between 9 and 11 on the colorimeter scale. If the reading of the unknown falls below 8 or above 12, another sample should be taken and the dilution made such that the reading will fall within these limits. This is easily accomplished by varying the amounts of blood and ammonia solution, making the necessary correction in the colorimeter. 2 cc. of solution is adequate for the Kober instrument and 5 cc. for the Duboscq. If a Duboscq or a Kober colorimeter is not available, the Hellige instrument may be used. The 1 per cent hemoglobin standard may be sealed with paraffin into the wedge and the wedge, when not in use, kept in the ice box.
and protected from light. Attention should be called to the fact that the scales of the Hellige colorimeter are often inaccurately placed. The standard solution must be checked against itself and the scale adjusted so as to read 100 when the color in the cup and wedge match. We have found more difficulty in obtaining accurate checks with this instrument than with the Duboscq or Kober colorimeters; but with care the error should not exceed 2 per cent. 2 cc. of solution is sufficient for the Hellige cup, hence the Sahli pipette, which contains 0.020 cc. of blood, may be used with this colorimeter. As large errors have been found in the calibration of the Sahli pipettes, it is necessary to recalibrate them before using.

Accuracy of the Method.—The accuracy of the method depends to a large extent on the care of the operator in carrying out the various details of the technique. The several steps involved in the method are common chemical procedures with known limits of accuracy. With care duplicate determinations are close and the error should not be more than 1 per cent. A series of ten determinations was made on the same blood. In eight of the ten, the first reading of the colorimeter was in every case exactly the same. In the two remaining a difference of 0.1 of a division on the colorimeter scale occurred.

In the following table are presented a few determinations by the method described using the Duboscq and Hellige colorimeters and comparing with the values estimated from the oxygen

<table>
<thead>
<tr>
<th>Blood sample</th>
<th>Hemoglobin determinations.</th>
<th>Difference between Duboscq and Hellige oxygen capacity.</th>
<th>Percentage difference</th>
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<td>Hellige</td>
<td>Oxygen capacity.</td>
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<td>108.1</td>
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capacity of the blood. The first ten blood samples of a large series were chosen and illustrate the error in estimation which may be expected. Except in blood Sample 6, no variation between the colorimetric determination (Duboscq) and the oxygen capacity method is greater than 1.0 per cent.

Advantages.—(1) Single determinations may be carried through in 2 minutes. (2) An accuracy within 1 per cent is easily obtained. (3) The standard solution is easily and adequately controlled. (4) Similar solutions are used for comparison, making the color fields, within the limits of the colorimeter, identical. (5) The apparatus required is found in any well equipped laboratory.

SUMMARY.

A method for the determination of hemoglobin colorimetrically with an accuracy of 1 per cent is described.

BIBLIOGRAPHY.

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