THE DETERMINATION OF HEMOGLOBIN IN MINUTE AMOUNTS OF BLOOD BY WU’S METHOD*  

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Despite the multiplicity of methods for the determination of the hemoglobin concentration of the blood, there appears to be no procedure available that requires less than 10 to 20 c.mm. While such volumes are usually considered quite small, it may at times be difficult to secure even these amounts by skin puncture, without at least being tempted to squeeze the surrounding tissues in an effort to bring more blood to the surface. Any manipulation that involves "milking" or otherwise forcefully expressing the blood may cause surprisingly large errors in the hemoglobin determination and, contrary to what might be supposed, the errors usually lead to high rather than low values. Thus, an anemic rat, bled by snipping the end of the tail, may through faulty technique show apparently normal values. We have found that the volume of the smallest drop of blood that can be conveniently obtained even from the tail of a new born mouse, weighing less than 1.5 gm., is about 0.5 c.mm. From slightly larger animals 1.0 c.mm. can be easily obtained. On these minute samples of blood the hemoglobin concentration can be accurately determined by means of the method described herein. Because of the small volume required, it enables one to exercise the best technique in the collection of the sample, such as wiping off the first drop that appears after skin puncture and filling the pipette with freely flowing blood.

* A preliminary account of these experiments was presented at the Meetings of the American Society of Biological Chemists in Montreal, Canada, April 11, 1931.

The data in this paper are taken from a dissertation to be presented by Reginald W. Baker in partial fulfillment of the requirements for the degree of Master of Arts, Western Reserve University, 1931.
Hemoglobin Determination

The method is essentially the pseudoperoxidase reaction described by Wu (1) in 1923. Wu developed the well known benzidine test for blood into a quantitative procedure and showed that the reaction was due entirely to hemoglobin. The method does not appear to have been used much and, indeed, we were unable to get check results by following the original directions. A thorough study was therefore made of the factors affecting the color development and as a result an improved technique was developed that we feel should render Wu's unique method as useful to others as it has been in some of our studies. It is necessary to adhere to the following directions exactly, as a perusal of the paper of Clark, Cohen, and Gibbs (2) on the oxidation of benzidine will show that many factors affect the color formation, and all of these must be standardized in order to obtain accurate results.

Solutions Required

Benzidine Reagent—Dissolve 2 gm. of a good quality benzidine in 20 cc. of glacial acetic acid with the aid of gentle heat. Allow the mixture to cool and dilute it to 100 cc. with distilled water. Decolorize by shaking with 0.5 to 1.0 gm. of decolorizing carbon and allow it to stand with occasional stirring for about 15 minutes. Filter and store in a brown bottle. If the solution becomes dark it may again be treated with carbon. For this reagent it is essential to use a good grade of benzidine. To ascertain whether a sample is suitable, prepare the reagent as described, place 2 cc. in a test-tube, and add 1 cc. of 3.0 per cent H₂O₂. No color should develop in 1 hour. While poor samples of benzidine may be improved by being dissolved in twice their weight of warm 95 per cent alcohol, by addition of charcoal and refluxing for 15 minutes, pouring into 5 volumes of recently boiled and cooled (below 60°) distilled water, filtering, and drying in the vacuum oven, it is better to purchase a good brand at the beginning.

20 Per Cent Acetic Acid—This is made simply by diluting glacial acetic acid with 4 volumes of water.

Hydrogen Peroxide Solution—The ordinary 3 per cent solution is titrated with potassium permanganate (U. S. P.)¹ to make certain that it is the proper strength. For use, this is freshly

diluted with 4 volumes of water to give a 0.6 per cent solution. The strength may vary from 0.5 to 0.75 per cent H₂O₂.

**Standard Blood Solution**—A sample of about 10 cc. of oxalated or defibrinated blood is analyzed for its oxygen capacity, according to the method of Van Slyke and Neill (3). The figure for oxygen capacity multiplied by the factor 0.746 is called hemoglobin. The blood is diluted with 1 per cent boric acid to give a solution having 20 mg. of hemoglobin per cc. A total volume of 25 cc. of solution is more than enough to last 1 month. For use, the standard blood is shaken and 0.5 cc. diluted to 200 cc. with distilled water. This dilute standard must be prepared fresh daily from the stock solution, and the latter should be kept at about 5°. The use of boric acid as a blood preservative was introduced by Brown and Hill (4).

**Procedure**

A blood sample of suitable volume is secured from a free flowing source and diluted by blowing into 2000 volumes of water. The pipette should be filled several times with the water and emptied, according to the usual technique. For human subjects, one may use a blood counting pipette if it has been previously calibrated to the 0.5 mark, this volume being about 0.005 cc. The pipette may be calibrated by weighing the amount of mercury delivered or, more conveniently, by comparison of the amount of blood delivered with an amount delivered by an accurately calibrated 1.0 cc. pipette.

If the volume of blood taken for analysis is 0.005 cc., the sample is blown into 10 cc. of water. If the volume is 0.001 cc., the blood may be blown into 2 cc. of water, or even 3 cc., in the latter case giving a dilution of 1:3000 but allowing duplicate determinations to be made upon the one sample. In every case 1 cc. of the thoroughly mixed and diluted blood is used for the determination. This is added to 2 cc. of the benzidine reagent, that has been previously carefully measured into a test-tube graduated at 25 cc. 1 cc. of 0.6 per cent H₂O₂ is then added. The solution turns blue in color, gradually increases in intensity, and becomes purple. The reaction should be permitted to proceed for at least 1 hour, at the end of which time the mixture is diluted to the mark with 20 per cent acetic acid, stoppered with a paraffined cork, inverted several times to mix, and, after 8 minutes, read in a colorimeter.
against a standard set at 10.0 mm. The standard is prepared in exactly the same way, 1 cc. of dilute blood solution containing 0.05 mg. of hemoglobin being used. The calculations are very simple.

\[
\text{Concentration in gm. per 100 cc. blood} = \frac{S}{100} \times \frac{\text{amount of Hb in standard}}{\text{volume of sample in cc.}} \times \frac{100}{\text{total volume of diluted blood sample}}
\]

where \( S \) = reading of standard and \( R \) = reading of unknown. With a standard containing 0.05 mg. of Hb, set at 10.0 mm., and a blood sample of 0.005 cc. diluted to 10.0 cc., the equation reduces to: concentration in gm. per 100 cc. = \( \frac{100}{R} \).

**DISCUSSION**

*Order of Addition of Reagents*—Wu laid considerable stress upon the necessity of adding the blood to the benzidine and the \( \text{H}_2\text{O}_2 \) to the mixture of the benzidine and blood, because low values were obtained when, instead, the benzidine was added to the blood. Probably a greater error, we find, occurs when \( \text{H}_2\text{O}_2 \) comes in contact with dilute blood unmixed with benzidine. For example, 5 cc. of 3 per cent \( \text{H}_2\text{O}_2 \) were mixed with 5 cc. of blood diluted 2000 times, and 1 cc. immediately pipetted into 2 cc. of the benzidine reagent in another tube. The time required for the mixing and the transference could not have been more than about 30 seconds, but no color developed even when more \( \text{H}_2\text{O}_2 \) was added. The substitution of 0.6 per cent \( \text{H}_2\text{O}_2 \) does not entirely obviate this danger, for after about 30 seconds contact the color was found to be two-thirds of the normal value, and after 3 minutes contact no color developed. The fact that dilute \( \text{H}_2\text{O}_2 \) destroys the pseudoperoxidase activity of dilute blood solutions indicates that under no circumstances should one allow drops of dilute blood to cling to the sides of the tube above the benzidine reagent where they run the danger of being “washed down” by the peroxide.

*Effect of Concentrations of Reacting Substances on Time and Intensity of Color Development*—The color produced is roughly proportional to the volume and concentration of the benzidine reagent.
Therefore, this solution must be measured accurately. Also, in order to have the hemoglobin concentration as the single variable, the volume of the reaction mixture should be kept constant by using the same amounts of benzidine, diluted blood, and H₂O₂ solutions for all determinations.

Wu used a 3 per cent H₂O₂ solution for the reaction, and allowed the unknowns and standard tubes to stand 15 minutes before dilution. If the digestions were allowed to proceed longer than 30 minutes the benzidine was destroyed by the H₂O₂ and higher values were obtained. The necessity of diluting the tubes at a particular time is very inconvenient when many determinations are to be made. Our procedure involves the substitution of a much more dilute H₂O₂ solution and necessitates a longer period of standing. We were led to this change quite by accident because a particular bottle labelled 3 per cent H₂O₂ was found to work very satisfactorily and gave perfect checks when eight to twelve tubes were made up with the same amount of blood in each. It was later found that the actual strength of the particular H₂O₂ solution was only 0.7 per cent by permanganate titration. Accordingly, the effect of concentration of the H₂O₂ was investigated. It was found that concentrations of 0.5, 0.6, and 0.75 per cent gave similar good results, but that 0.25 per cent required longer to develop the full color, while 1.0 and 2.0 per cent solutions had no apparent advantage. The smoothness of the reaction when 0.6 per cent H₂O₂ is used is indicated by the curve in Fig. 1. This was constructed by plotting the apparent hemoglobin concentration against time. It is to be seen that the color increases gradually until at 1 hour the maximum has been attained. No change was noted even in tubes allowed to stand 3 hours. Thus, there is no destruction of the benzidine with 0.6 per cent H₂O₂ at room temperature within this time.

The greater accuracy that we have obtained with the use of 0.6 per cent H₂O₂ overcomes the disadvantage of requiring a longer time for the reaction. The fact that no attention is required to avoid letting the reaction go too long is also an advantage. We feel these better results are due in part to a lessened tendency for the destruction of the hemoglobin activity by stronger peroxide solutions and in part to the avoidance of the direct oxidation of the benzidine. Incidentally, the use of dilute H₂O₂ eliminates the
occasional turbidity due to the precipitation of the traces of H$_2$SO$_4$ that are to be found as a preservative in some samples of 3 per cent peroxide.

Under the conditions of the reaction the hemoglobin concentration of the diluted blood is the only variable factor. To ascertain the relationship between hemoglobin concentration and the color development a series of proportionality tests was made by diluting one sample of blood to different volumes with water and using 1 cc. of each dilution for analysis. The results are shown in Fig. 2. It is to be noted that only one sample, that containing 0.05 mg. of hemoglobin, was used as the standard. Theoretical proportionality was obtained from 0.01 mg. to 0.10 mg. of hemoglobin. Below the lower limit the colors are too dilute to match conven-
iently unless a weaker standard is used, and above the 0.10 mg. limit the results tend to be low. This range represents concentrations of hemoglobin varying from 2.0 to 20.0 gm. per 100 cc., if the blood is diluted 2000 times. It covers the extremely low figures that may be found in severe experimental anemias, and the

![Diagram](http://www.jbc.org)

**Fig. 2.** The diagonal line represents true proportionality between hemoglobin present and color produced. The black circles represent actual observations, and show that with a single standard containing 0.05 mg. of hemoglobin the range of accuracy is from 0.01 mg. to 0.10 mg. of hemoglobin. Above this range the results tend to be low, and below 0.01 mg. the color is too dilute to match conveniently.

high values obtained in polycythemia, blood concentration, and early infancy. For hemoglobin determinations outside this range the dilution may be altered or standards selected that more nearly match the color of the unknown.
Other Blood Pigments—Wu proved that oxyhemoglobin, hemoglobin, methemoglobin, and carboxyhemoglobin all gave color values equivalent to hemoglobin. Our results have completely confirmed this, and also the peculiar observation that acid hematin gives only about 50 to 80 per cent of the color of an equivalent quantity of hemoglobin. If a large excess of potassium ferricyanide is present in a methemoglobin preparation it will itself affect the reaction.

Temperature—Increase of temperature does not speed up the time required for attainment of maximum color value. Tubes kept in a bath at 40° match exactly those made up at 20°, if both have been allowed to stand for 1 hour. A temperature of 60° gives an apparent increase in color without changing the slope of the time curve. This seems to be due to a destruction of the benzidine, because such solutions develop a brownish and somewhat turbid color.

Hydrogen Ion Concentration—The hydrogen ion concentration of the mixture affects the nature and the depth of color. The reaction mixture is ordinarily purple and has a pH of about 3.0. Addition of small amounts of strong alkali to the solution tends to give an increased color, addition of strong acid changes the color to a weak purple.
brown, which is also much weaker. When the usual blood-benzidine-H₂O₂ mixture is diluted to 25 cc. with 20 per cent acetic acid a crystal clear wine-red solution is produced, having a pH of about 2.3. If the dilution is made with water or a glycocoll-HCl buffer of pH 3.0, a purple solution is obtained, and on standing a purple precipitate slowly settles out. Dilution with glacial acetic acid yields a clear brown solution. Dilution with 0.1 N HCl also produces a brown color, and dilution with 0.005 N HCl gives a red solution with a faint cloudiness. The acetate ion, therefore, exerts an influence on the solubility of the colored products of the reaction.

The color change on dilution with 20 per cent acetic acid is not instantaneous. Observation has shown that 3 minutes are required for all trace of the purple hue to disappear. The color value continues to diminish, as shown in Fig. 3, but reaches an equilibrium in 8 minutes. Thereafter, the color remains constant for at least 24 hours. There is no such change in intensity if the dilution is made with water or a glycocoll-HCl buffer at pH 3.0, indicating that the diminution in color is associated with the change of hydrogen ion concentration. However, other factors are also involved for, with some samples of benzidine we have recently tried, a dark red instead of a purple color is obtained in the reaction mixture, and no alteration in color value occurs after dilution. This factor we are studying further. Because of the great extent of the color change usually obtained after dilution, it seems advisable

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Standard blood</th>
<th>Hb in gm. per 100 cc.</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>O₂ capacity × 0.746</td>
</tr>
<tr>
<td>Mouse</td>
<td>Human I</td>
<td>12.4</td>
</tr>
<tr>
<td>Rooster</td>
<td>&quot; I</td>
<td>13.7</td>
</tr>
<tr>
<td>Man</td>
<td>&quot; II</td>
<td>9.3</td>
</tr>
<tr>
<td>Rabbit</td>
<td>&quot; II</td>
<td>12.2</td>
</tr>
<tr>
<td>Man</td>
<td>&quot; II</td>
<td>5.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot; II</td>
<td>11.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>Rabbit I</td>
<td>12.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>Human III</td>
<td>12.9</td>
</tr>
</tbody>
</table>
either to test for this phenomenon with each sample of benzidine, or to allow at least 8 minutes before reading in the colorimeter. There appeared to be no material advantage from attempting to control the acidity further by the introduction of a buffer solution.

Comparison with Oxygen Capacity Method—The present colorimetric method was checked against the values obtained by multiplying the oxygen capacity figures, determined according to the technique of Van Slyke and Neill, by 0.746. The blood samples were oxalated specimens from normal persons, hospital patients, and different species of animals. The results are presented in Table I and show a very good correspondence of the two methods.

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Body weight (gm.)</th>
<th>Hb in gm. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tail sample 0.00091 cc.</td>
</tr>
<tr>
<td>1</td>
<td>7.1</td>
<td>7.6</td>
</tr>
<tr>
<td>2</td>
<td>9.3</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>15.5</td>
<td>8.7</td>
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<tr>
<td>4</td>
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<td>8.7</td>
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<tr>
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<td>9.7</td>
<td>9.7</td>
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<tr>
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<td>11.0</td>
<td>10.6</td>
</tr>
<tr>
<td>8</td>
<td>11.7</td>
<td>11.2</td>
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<tr>
<td>9</td>
<td>8.2</td>
<td>11.2</td>
</tr>
</tbody>
</table>

For these colorimetric estimations 1.0 cc. of blood was diluted to 2 liters and duplicate determinations made upon the diluted sample. Incidentally, these determinations also showed that each sample of blood when preserved with boric acid kept unaltered for 1 month. Probably, standard solutions could be kept for longer periods, but we have made no attempt to use any solution beyond 1 month.

Accuracy of Hemoglobin Estimations on Minute Samples of Blood—Preliminary experiments satisfied us that 1.0 c.mm. of blood could be accurately measured, blown into 2.0 cc. of water, and the hemoglobin content measured upon 1 cc. of the laked blood. In these trials, special micro pipettes were used. We wish to express
our appreciation of the help of Dr. A. T. Shohl of Western Reserve University in making these small pipettes. To demonstrate the accuracy of measuring minute volumes of blood, samples that happened to be 0.00091 cc. were taken from the tails of small mice and the hemoglobin concentration determined. The mice were 14 and 21 days old, the body weights ranging from 7 to 15 gm., depending upon age and the number of young permitted in the litter, and all the animals showed varying degrees of nutritional anemia. The mice were then etherized, an incision made through the neck muscles, and the carotid artery and jugular vein cut. A large drop of blood was collected on a watch-glass, and 0.0079 cc. immediately taken and blown into 20 cc. of water. Determinations of the hemoglobin concentration were made in duplicate upon this sample and compared with the values obtained with the tail sample. The results of these measurements are presented in Table II. They show that tail samples, when obtained by allowing the blood to flow freely, have the same concentration as systemic blood. This has been demonstrated by McCay (5) with rats. Because it has been shown that the supposed differences between systemic and peripheral blood do not ordinarily exist in rats and mice, one is inclined to ascribe also to faulty technique at least some of the discrepancies that have been reputed to occur in normal persons.

Applications—The method described herein has been found particularly valuable for determining the hemoglobin concentration of rats suffering from anemia brought about by an exclusive milk diet. Accurate determinations can be made upon small volumes of blood even when the concentration of hemoglobin is below 3 gm. per 100 cc. Moreover, if desired, samples can be taken daily with a minimal loss of blood to the animal. The method permits the estimation of the hemoglobin concentration in small animals such as baby rats and mice without sacrificing them. It has also been applied to the estimation of low hemoglobin concentrations in human subjects. In cases having as little as 5 gm. of hemoglobin per 100 cc. an easily obtained sample of 0.005 cc. of blood from the finger suffices for duplicate determinations.

CONCLUSIONS AND SUMMARY

Wu's method for estimating hemoglobin in blood by means of benzidine and $\text{H}_2\text{O}_2$ has been studied, and an improved technique
Hemoglobin Determination

described. Sources of error in the application of the method have been pointed out. The method has been checked by parallel determinations with the oxygen capacity procedure of Van Slyke and Neill, normal and pathological human blood being used, and blood from several species of animals. By means of the present method, estimations of the hemoglobin concentration can be made upon samples of blood that measure as little as 0.001 cc. The maximum error should not be more than ± 4 per cent.

The authors desire to express their indebtedness to the continued advice and criticisms of Professor Victor C. Myers during the progress of these experiments.

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