THE PREPARATION OF STANDARD ACID HEMATIN SOLUTIONS FROM HEMIN*

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The need of a method which is simple, rapid, and accurate for the determination of blood hemoglobin is evident to all who have had occasion to make a large number of such determinations. The general use of the Newcomer method today in clinics and laboratories demonstrates that it has met to a large extent the requirements of such a method. The utilization of a piece of brown-colored glass of definite thickness as a permanent standard eliminates the necessity of preparing standard hematin solutions, a procedure entailing a considerable amount of work, especially when the solutions are made from blood.

Although the merits of the Newcomer method are recognized by most workers, there are certain disadvantages which must be considered. Robscheit (1), in a survey of the methods used for hemoglobin determinations, states, "Newcomer's method obviates many difficulties heretofore observed with other procedures and gives good results with the glass 0.96 mm. in thickness, although the color is quite pale." She found that the use of a heavier glass gave figures which were not so satisfactory. In view of these facts, the workers in her laboratory prefer to use liquid acid hematin standards. Osgood and Haskins (2) reached similar conclusions.

We have used the Newcomer method almost exclusively in our laboratory, although we have been aware of the disadvantages mentioned. We have also found that the filters placed on the market the past few years vary to a large extent and must be standardized before they can be used for accurate determinations.

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The Bausch and Lomb Optical Company (personal communication) recognizes the importance of calibrating all of the filters which they have furnished since they have sold four different lots of filters, each lot having been calibrated in a different way and at a different time. We have in our laboratory discs which vary as much as 12 per cent in the ultimate measurement of hemoglobin per 100 cc. of whole blood. In order to obviate any uncertainty as to the accuracy of the discs used in the different laboratories, a method for the rapid determination of the hemoglobin equivalent of these filters is needed. It is the purpose of this paper to outline a simplified method for the preparation of standard acid hematin solutions which may be used for the estimation of hemoglobin directly or for the standardization of any hemoglobinometer based on the acid hematin principle.

The acid hematin method, which entails the conversion of the hemoglobin in the blood sample to acid hematin with 0.1 N HCl and the comparison of the color produced with standard tubes of acid hematin, came into general use due to the work of Sahli (3). Cohen and Smith (4), who modified the original method by introducing the use of the colorimeter for comparing the intensity of the colors, also demonstrated that the acid hematin prepared from the blood of one species may be used for the determination of hemoglobin in the blood of a number of other species. Most of the standard acid hematin solutions used in the past have been prepared from blood obtained from any available animal, and the hemoglobin equivalent of each solution has been calculated from the oxygen capacity of the blood which is determined by the Van Slyke method. The calibration of the disc used in the Newcomer method (5) is also based on the oxygen capacity of blood. The standard solution, devised by Osgood and Haskins (2), which contains only inorganic material, is standardized in a similar manner.

Workers who have improved the acid hematin method have stressed the technique of making the estimations rather than the chemical changes involved in the formation of acid hematin. The term hematin has been used for over a century and it has generally been employed to designate the pigment part of the hemoglobin molecule. Anson and Mirsky (6) have suggested that the so called acid hematin formed when blood is treated with an acid cannot be the free iron compound; e.g., the hematin group.
unattached to other compounds. The evidence for their conclusion is based upon the fact that a soluble compound is formed when blood is treated with 0.1 N HCl and an insoluble compound is formed when a solution of hemin in NaOH is treated with acid of the same concentration. They call the free iron compound heme and believe the soluble compound (acid hematin) to be oxidized heme united with a nitrogen compound.

Keilin (7), however, has shown that if 0.2 per cent gum arabic is added to a solution of hemin in NaOH before acidification with HCl, the iron compound does not precipitate but remains in colloidal suspension protected from precipitation by the gum arabic. In his preparation the fluid remained clear and transparent, and gave the same absorption spectrum as acid hematin prepared from hemoglobin. It is, therefore, evident that the acid hematin produced from blood is a colloidal suspension of insoluble hematin protected from precipitation by the globin and that the introduction of a new term to distinguish a different iron compound is unnecessary.

It should therefore be possible to prepare a standard acid hematin solution from pure hemin, provided a protective colloid is used, and thereby eliminate the necessity of always preparing hematin solutions from blood. This procedure allows the hemoglobin equivalent to be calculated directly from the iron content of the hemin. When the iron content of the hemin preparation is once determined a large number of solutions may be prepared from the same sample, while when blood is used the oxygen capacity must be determined each time.

**EXPERIMENTAL**

The hemin used in this work was prepared by the following method: 2 liters of glacial acetic acid were placed in a 5 liter flask and 100 cc. of water and 0.25 gm. of NaCl were added. The flask was heated in a boiling water bath until the temperature of the acetic acid reached 90°. The flask was then removed from the bath and 850 cc. of defibrinated horse blood were added slowly from a dropping funnel. The flask was shaken continuously during the addition of the blood. The mixture was heated to 90° and kept at that temperature for 15 minutes after which the hemin crystals were allowed to settle overnight. The supernatant liquid
Standard Acid Hematin Solutions

was syphoned off and the crystals resuspended in dilute acetic acid. After allowing the crystals to settle overnight the clear liquid was syphoned off. This washing process was repeated with water, dilute HCl, and finally water. The washed crystals were filtered off and dried at room temperature.

The iron content of this preparation was found to be 7.4 per cent. The theoretical iron content calculated from the accepted empirical formula for hemin is 8.6 per cent. The lower iron content is largely due to the presence of a certain amount of moisture since the crystals were dried at room temperature, but the preparation may also contain a slight amount of other impurities since it was not recrystallized.

30 mg. of hemin were dissolved in sufficient dilute NH₄OH to bring about complete solution and the solution was diluted to 50 cc. A 10 cc. aliquot equivalent to 6 mg. of hemin was measured out, 5 cc. of a 5 per cent solution of gelatin were added, and the solution was diluted to 250 cc. with 0.1 N HCl. The solution of acid hematin prepared in this manner appeared to be very similar to that produced from blood. Since 1 cc. of blood contains approximately 6 mg. of hemin, the color intensity of the hematin solution was approximately the same as that obtained in the ordinary hemoglobin determinations when a dilution of 1 to 250 is made. The acid hematin solution was allowed to stand 24 hours and then the hemoglobin equivalent was determined by the Newcomer method.

Another 10 cc. aliquot was evaporated to dryness, ashed, and analyzed for iron. Since hemoglobin contains 0.335 per cent iron, the hemoglobin equivalent was calculated directly from the amount of iron found present. A large number of such determinations have been made but only a few typical results are given in Table I. Since the iron determinations were made on aliquots of each hemin solution no attempt was made to weigh exactly the 30 mg. of hemin used. The iron content of different solutions therefore varies slightly, but the duplicate determination on the same solution agrees very well.

The hemoglobin equivalent was determined with two different discs, and both results are recorded in Table I. The two filters were purchased from the Bausch and Lomb Optical Company. Filter 1 was purchased the early part of this year and Filter 2 was
It is readily seen from these results that the two discs vary in their hemoglobin equivalent to a considerable extent. Other discs in our laboratory show similar variations but not to such a great extent. Results also show that the hemoglobin equivalent for the various hematin solutions determined by the use of Filter 1 check very well with those calculated from the iron content. Filter 1 is the disc marketed by the Bausch and Lomb Optical Company at the present time. They have used a very elaborate procedure in determining the factor for this filter and, therefore, hope it is the most accurate of all the discs that have been placed on the market (personal communication).

**TABLE I**

_Hemoglobin Equivalents of Acid Hematin Solutions_

In each case a 10 cc. aliquot taken from solutions containing 30 mg. of hemin per 50 cc. was used for the iron determination and for the preparation of the acid hematin solution.

<table>
<thead>
<tr>
<th>Solution No.</th>
<th>Amount of hemin</th>
<th>Iron content</th>
<th>Hb calculated from iron</th>
<th>Newcomer method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>gm.</td>
<td>Filter 1</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>0.430</td>
<td>12.8</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.433</td>
<td>12.9</td>
<td>12.8</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.447</td>
<td>13.3</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.445</td>
<td>13.2</td>
<td>13.1</td>
</tr>
</tbody>
</table>

If the hemoglobin equivalent of a disc does not check with that of a standard acid hematin solution, the disc need not be discarded, but a factor may be introduced to convert the reading obtained to the correct hemoglobin value. For example, a factor of 1.17 will convert the results obtained with Filter 2 to values which agree with Filter 1 and the standard hematin solutions.

These results demonstrate that pure hemin may be used for the preparation of standard acid hematin solutions and that the hemoglobin equivalent of these solutions may be calculated from the iron content of the hemin used. In order further to substantiate this conclusion the hemoglobin content of several different samples of blood was determined by the following methods: (1) Newcomer, Bausch and Lomb Filter 1 with conversion table (H 236, 1 × 30)
being used, (2) standard acid hematin solution, and (3) oxygen capacity. About 10 cc. of blood were drawn from the animal and oxalated so that the same sample could be used for all methods. In the first and second methods the blood was diluted 1:250 with 0.1 N HCl and allowed to stand 40 minutes before making the colorimetric readings. The acid hematin solution used in the second method was the one designated as Solution 1 in Table I, and the value of 12.85 gm. of hemoglobin was taken as the hemoglobin equivalent.

The oxygen capacity of the various samples was determined by the use of Barcroft differential manometers. The manometers were properly calibrated and the flasks were thoroughly cleaned before being used. 2 cc. of ammonia solution (4 parts ammonium hydroxide, sp. gr. 0.88, in 1000 cc. of distilled water) were placed in each flask and 1 cc. of the blood sample was added. The contents of each flask were well shaken so as to lake the blood and saturate it with oxygen. In the right-hand flask a small vial (as used by Keilin (8) ) containing 0.2 cc. of saturated ferricyanide was suspended from the absorption tube. Both flasks were attached to the manometer and the apparatus placed in the water bath for 5 minutes. The taps were then closed, the manometer was read, and the ferricyanide upset into the flask. The apparatus was shaken in a horizontal direction, and the reading was taken when the constant maximal pressure was reached. Duplicate determinations at 20° and 37° were made for each sample of blood. The oxygen capacity of the blood was calculated by using the constant for each apparatus and by correcting for temperature and pressure. The hemoglobin content of the blood was obtained by dividing the oxygen capacity by 1.34.

The duplicate results obtained by the use of the three different methods on two samples of blood are given in Table II. These results are typical of a number of analyses. It is readily seen from this table that the values obtained by the three methods check very well. The figures obtained by the oxygen capacity method are slightly lower than those obtained by the other methods. However, the small discrepancy is probably due to the fact that Barcroft's differential method may give slightly lower results for the oxygen capacity of blood than the Van Slyke method (9). Acid hematin solutions prepared from hemin can therefore
be used as standards for hemoglobin determinations as well as for the standardization of the glass discs. Liquid standards have the advantage that the intensity of the color may be varied by changing the depth of solution in the colorimeter. The permanency of the liquid standards is naturally an important factor if they are to be used in hemoglobin determinations. Several solutions have

\[\text{TABLE II}\]

Hemoglobin Content of Various Samples of Blood Determined by Different Methods

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Newcomer, Filter 1</th>
<th>Standard acid hematin</th>
<th>Oxygen capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 a</td>
<td>10.7</td>
<td>10.7</td>
<td>10.5</td>
</tr>
<tr>
<td>1 b</td>
<td>10.5</td>
<td>10.8</td>
<td>10.5</td>
</tr>
<tr>
<td>2 a</td>
<td>12.6</td>
<td>12.6</td>
<td>12.3</td>
</tr>
<tr>
<td>2 b</td>
<td>12.6</td>
<td>12.5</td>
<td>12.4</td>
</tr>
</tbody>
</table>

\[\text{TABLE III}\]

Effect of Age on Hemoglobin Equivalents of Acid Hematin Solutions

<table>
<thead>
<tr>
<th>Date</th>
<th>Gm. of Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solution 1</td>
</tr>
<tr>
<td>Mar. 28</td>
<td>Prepared</td>
</tr>
<tr>
<td>&quot; 29</td>
<td>12.62</td>
</tr>
<tr>
<td>&quot; 30</td>
<td>12.91</td>
</tr>
<tr>
<td>Apr. 2</td>
<td>12.41</td>
</tr>
<tr>
<td>&quot; 6</td>
<td>12.62</td>
</tr>
<tr>
<td>&quot; 16</td>
<td>12.41</td>
</tr>
<tr>
<td>May 2</td>
<td>12.41</td>
</tr>
<tr>
<td>&quot; 22</td>
<td>12.41</td>
</tr>
</tbody>
</table>

been kept in the laboratory for 2 months with no definite change in the intensity of their color. Table III gives the hemoglobin equivalents for two of the solutions determined at various intervals.

There is no reason for displacing the Newcomer method with procedures entailing the use of liquid standards providing the glass discs used in the Newcomer method are properly calibrated. The method outlined in this paper is so simple that the calibra-
tation of the Newcomer discs in use becomes an easy procedure. The comparison of hemoglobin values obtained in different laboratories is only possible when all workers use a similar method and the standards used are properly calibrated.

SUMMARY

A method for the preparation of standard acid hematin solutions from hemin of known iron content is outlined. The hematin solutions may be used as a standard for the determination of hemoglobin or may be used to calibrate the glass discs used in the Newcomer method.

BIBLIOGRAPHY

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