A MICRO TIME METHOD FOR DETERMINATION OF REDUCING SUGARS, AND ITS APPLICATION TO ANALYSIS OF BLOOD AND URINE.

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(Received for publication, July 10, 1929.)

The author and Van Slyke (5) have recently published a method for sugar determination in which the time required to decolorize a standard ferricyanide solution was used as a measure of the sugar content. The convenience which this method demonstrated in clinical work stimulated attempts to widen its range of usefulness by developing a micro modification. By carrying out the reaction in small test-tubes heated in a porcelain casserole, which provides a brilliant white background, it has proved possible without decrease in accuracy to make duplicate determinations on a 0.2 cc. sample of blood.

We regret that at the first publication of the time method (5) we overlooked the fact that Cole (1) had previously employed for urine analysis the principle of measuring sugar content by the time required for reducing a colored reagent. The latter was a copper solution, and has apparently not been adapted to the small amounts of sugar encountered in blood.

DETERMINATION OF REDUCING SUGAR IN BLOOD.

Reagents.

Potassium Ferricyanide Reagent.—This reagent is the same used by Hawkins and Van Slyke (5), except that the present solution

1 Although apparently not published until 1926, the method was reported by Professor Cole to the Biochemical Society in 1922.
contains only 0.5 gm. of potassium ferricyanide, instead of 1.0 gm. per liter.2

_Tungstic Acid Solution (Mixed Reagents of Folin and Wu (3))._
—The solution is the same as that previously used by Van Slyke and Hawkins (6) except that 1 volume of 10 per cent sodium tungstate and 1 volume of $\frac{2}{3}$ N sulfuric acid are mixed with 8 volumes of distilled water.

_Procedure for Blood Sugar Determination._

_Precipitation of Blood Proteins._—When samples of capillary blood are to be analyzed, a sufficient number of rubber-stoppered tubes (15 $\times$ 100 mm.) are prepared each containing exactly 2 cc. of the tungstic acid precipitating reagent described above. The blood drops forming on the incised finger or ear lobe are drawn by capillary attraction into a 0.200 cc. capillary pipette, which is at once emptied into one of the test-tubes. The pipette is rinsed twice by drawing the tungstic acid up into it. The test-tube is then stoppered and shaken. The blood is diluted 1:11 by this procedure. After 2 minutes the mixture is filtered through a dry filter paper (4.5 cm.) into a test-tube (15 $\times$ 100 mm.).

If the blood is known to be hyperglycemic, a portion of the filtrate is diluted with an equal volume of water.

When blood analyses are to be made which require a Folin-Wu filtrate where the blood is diluted 1:10, this filtrate can be used instead of the 1:11 blood filtrate.

The 0.200 cc. pipette is made from a capillary tube of about 1 mm. bore, and is calibrated by weighing 2.71 gm. of mercury in the dry pipette.

_Decolorization of Ferricyanide by Blood Filtrate._—0.5 cc. of filtrate is pipetted into a Pyrex test-tube, of 9 $\times$ 90 mm. outside measure3 followed by 0.5 cc. of ferricyanide solution. Both

2 The potassium ferricyanide should not contain potassium ferrocyanide. The potassium ferricyanide may be tested as follows. To 10 cc. of 0.5 per cent potassium ferricyanide add 0.5 cc. of a 0.5 per cent solution of ferric chloride and 1 drop of N HCl. If ferrocyanide is present in quantity sufficient to be 0.1 per cent of the ferricyanide, under these conditions, a green color appears on the addition of the ferric chloride. C.P. ferricyanide reagents we have tested did not develop any green color.

3 The tubes were made to order for us from standard thin wall Pyrex glass tubing, internal diameter 8 mm. The thickness of the glass wall is 0.6 mm.
solutions must be measured accurately from Ostwald bulb pipettes with capillary stems. The ferricyanide should be added last, in order to facilitate its mixture with the lighter filtrate. The tube is shaken to mix the two solutions and is then immersed in water in a white glazed casserole in which the water is boiling gently. The casserole has a diameter of 95 mm. so that the tubes rest with their mouths on the edge, but cannot slip into the water. Two or three tubes may be heated at once. A tube containing distilled water is immersed with the other tubes to facilitate by comparison the detection of the moment when the ferricyanide solutions are decolorized. The time in seconds for each tube is taken, preferably with a stop-watch, from the moment the tube is immersed in the boiling water until the last trace of yellow disappears.

With the prescribed amount of ferricyanide and size of test-tubes, 50 mg. of sugar per 100 cc. of blood decolorize the reagent in 520 seconds, and 300 mg. of glucose per 100 cc. of blood will decolorize it in 76 seconds, when the blood filtrate represents a 1:11 dilution of the blood. If blood filtrate represents a 1:10 dilution for blood, the decolorization times are correspondingly shorter, as shown by Fig. 1. It is possible to work to the above time limits. However, if the reagent is decolorized in less than 110 seconds (equivalent to more than 150 mg. per cent of blood sugar for blood diluted 1:11, and to more than 140 mg. per cent of blood sugar for blood diluted 1:10) it is best to dilute another portion of blood filtrate with an equal volume of water and to repeat the analysis with the diluted filtrate in order to obtain a longer decolorizing period and more exact results.

\[ \text{Graphic Calculation of Results of Blood Analysis.} \]

The number of mg. of sugar per 100 cc. of blood is read directly from the appropriate curves shown in Fig. 1 when the 0.5 cc. of filtrate used represents a 1:11 or a 1:10 dilution of the blood. When the filtrate is twice as dilute (hyperglycemic blood) the blood sugar content indicated by the appropriate curve is doubled.

DETERMINATION OF REDUCING SUGAR IN URINE.

The procedure outlined below is designed for use with the same apparatus and reagents with urine that are employed in the above
micro blood analysis. Such a micro method is not required for urine, but we describe it for the reason that it makes available for urine the same technique and apparatus used for blood. The urine analysis is planned for use with urines, such as those encountered in diabetes, in which the glucosuria is so gross that its significant variations can be satisfactorily shown by measurement of the total reducing substances. The method is accurate to within 0.1 gm. of glucose per 100 cc. of urine. Unusually concentrated normal urines may have reducing substances equivalent in reducing power to as much as 0.4 per cent glucose. The normal reducing substances, however, have been found by previous authors (2, 4, 7) to be almost entirely non-fermentable, and to have no apparent relationship to carbohydrate metabolism. Accordingly the technique is regulated to determine reducing substances in concentrated urine in concentrations of 0.5 per cent and above, and in dilute urine in concentrations of 0.25 per cent and above.

Reagents for Urine Sugar.

Ferricyanide Solution.—Same as for blood sugar.

Procedure for Urine Sugar.

Dilution of Urine.—Ordinarily 1 cc. of urine is diluted with water to 100 cc. In urine so diluted the ferricyanide reagent will determine up to 3 per cent of glucose. In case the sugar content is known to be above 2 per cent, 1 cc. of urine is diluted to 200 or 400 cc., so that glucose up to 6 and 12 per cent respectively can be determined. On the other hand, if the urine as voided is presumably of low sugar content, it is best to dilute only 25-fold. Dilution is the only preliminary treatment of the urine required, as even albumin does not affect the determination.

Decolorization of Ferricyanide by Diluted Urine.—0.5 cc. of diluted urine is pipetted into a Pyrex test-tube (9 × 90 mm. outside measurement) followed by 0.5 cc. of ferricyanide solution. The procedure from this point is exactly the same as in the blood sugar method described above.

The amount of sugar in the urine is found by use of the curve in Fig. 1. If the urine has been diluted 200-fold instead of 100-fold, the sugar concentrations indicated by the curve are doubled.
On the other hand, they are halved if the dilution has been only 50-fold.

EXPERIMENTAL.

In order to determine the rate at which glucose reduces ferricyanide, the reagent was heated with standard glucose solutions of various concentrations covering the ranges encountered in blood and urine analyses. 1 volume of reagent was mixed with 1 volume of glucose solution. The results are shown in Fig. 1, and Tables I and II. Table I gives the results obtained under the conditions

**TABLE I.**

Determination of Time Required by Various Concentrations of Glucose to Decolorize Ferricyanide Reagent under Conditions of Micro Blood Determination, When Blood is Diluted 1:11.

<table>
<thead>
<tr>
<th>Glucose concentration in standard solutions, mg. per cc.</th>
<th>0.2727</th>
<th>0.1818</th>
<th>0.1364</th>
<th>0.0682</th>
<th>0.0455</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose represented in portion of solution used for determination, mg.</td>
<td>0.1364</td>
<td>0.0909</td>
<td>0.0682</td>
<td>0.0455</td>
<td>0.0341</td>
</tr>
<tr>
<td>Time required to remove color from reagent, sec.</td>
<td>75</td>
<td>98</td>
<td>126</td>
<td>184</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>98</td>
<td>120</td>
<td>183</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>98</td>
<td>123</td>
<td>185</td>
<td>270</td>
</tr>
<tr>
<td>Average</td>
<td>76</td>
<td>98</td>
<td>123</td>
<td>184</td>
<td>269</td>
</tr>
</tbody>
</table>

**TABLE II.**

Determination of Time Required by Various Concentrations of Glucose to Decolorize Ferricyanide Reagent under Conditions of Blood Determination, When Blood is Diluted 1:10.

<table>
<thead>
<tr>
<th>Glucose concentration in standard solutions, mg. per cc.</th>
<th>0.3</th>
<th>0.2</th>
<th>0.15</th>
<th>0.10</th>
<th>0.075</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose represented in portion of solution used for determination, mg.</td>
<td>0.15</td>
<td>0.1</td>
<td>0.075</td>
<td>0.05</td>
<td>0.0375</td>
<td>0.025</td>
</tr>
<tr>
<td>Time required to remove color from reagent, sec.</td>
<td>70</td>
<td>91</td>
<td>114</td>
<td>164</td>
<td>240</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>91</td>
<td>115</td>
<td>168</td>
<td>236</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>91</td>
<td>113</td>
<td>166</td>
<td>238</td>
<td>455</td>
</tr>
<tr>
<td>Average</td>
<td>71</td>
<td>91</td>
<td>114</td>
<td>166</td>
<td>238</td>
<td>451</td>
</tr>
</tbody>
</table>
Fig. 1. Time curves of reduction of ferricyanide by standard glucose solutions under conditions of blood and urine determinations. Abscissa represent time in seconds required by glucose standards to decolorize ferricyanide reagent. Each cross represents the average of several determinations with glucose standards. Ordinates represent per cent of sugar in urine when urine is diluted 1:100, or mg. of sugar per 100 cc. of blood when blood is diluted 1:10 or 1:11. If other dilutions are used, sugar contents represented by the ordinates are multiplied or divided accordingly. E.g., if urine dilution is 1:50 instead of 1:100, multiply by 0.5 the sugar content indicated by the curve.
described for the micro blood method, where 0.2 cc. of blood is added to 2.0 cc. of tungstic acid reagent. Table II gives the results obtained under the conditions described for the urine method, and for the blood method with filtrate representing a 10-fold dilution of blood. The results indicate that a reproducible curve is obtained by means of which it is possible to determine the amount of glucose in a solution from the time taken to decolorize the ferricyanide reagent.

Fig. 2. Comparison of sugar found in blood by the Van Slyke-Hawkins gasometric and the time methods. Ordinates represent mg. of sugar per 100 cc. of blood. Each symbol represents the average of duplicate determinations.
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Comparison with Gasometric Blood Sugar Method.

Comparison of results obtained by the Van Slyke-Hawkins gasometric method with those yielded by the present blood sugar method in analyses of twenty bloods, normal and pathological, is shown in Fig. 2. The two methods agreed, usually within a few mg. per 100 cc. In these analyses the dilutions of blood filtrate, either 1 volume of blood and 10 volumes of precipitant, or 1 volume of blood and 9 volumes of precipitant were used.

### TABLE III.

<table>
<thead>
<tr>
<th></th>
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<tr>
<td>1</td>
<td>2.89</td>
<td>2.80</td>
<td></td>
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<tr>
<td>2</td>
<td>2.42</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.60</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.57</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.25</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.38</td>
<td>0.33</td>
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<tr>
<td>8</td>
<td>0.35</td>
<td>0.33</td>
<td></td>
</tr>
</tbody>
</table>

Comparison with Gasometric Urine Sugar Method.

In Table III are given the results of analyses of diabetic urines and the comparison with the Van Slyke-Hawkins method. The two methods agree within 0.1 gm. of sugar per 100 cc. of urine.

**SUMMARY.**

The method of Hawkins and Van Slyke (5), in which sugars are estimated from the time they require to reduce a known amount of yellow ferricyanide solution completely to colorless ferrocyanide, has been refined so that 0.2 cc. of blood suffices for duplicate analyses.

The accuracy, ± 5 per cent of the amount determined, and the time required (75 to 300 seconds) are the same as with larger samples and the previous technique.

Use for urine analyses of the same reagents and apparatus employed for blood sugar determination is described.
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J. Biol. Chem. 1929, 84:69-77.

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