A COLORIMETRIC METHOD FOR THE DETERMINATION
OF FRUCTOSE IN BLOOD AND URINE*

BY JOSEPH H. ROE

(From the Department of Biochemistry, School of Medicine, George Washing-
ton University, Washington)

(Received for publication, July 6, 1934)

In 1926 Campbell and Hanna (1) published a method for the
determination of fructose based upon the reduction of molybdenum
in phosphoric acid solution and the reoxidation of the reduced
molybdenum with potassium permanganate. This method is not
entirely specific for fructose; other substances in blood and urine
have some reducing action upon the molybdic acid reagent. The
method will not permit microapplications of the same magnitude
as are possible with colorimetric methods, the regular procedure
being developed to estimate 1 mg. quantities. The time required
for reduction is 90 minutes, which limits the usefulness of the
method.

In 1927 a method based upon the Seliwanoff reaction was pub-
lished by Kronenberger and Radt (2). These authors depro-
teinized blood with trichloroacetic acid and treated the filtrate
with resorcinol and HCl. They stated that normal blood filtrates
gave a brownish color with their reagents; and they experienced
difficulty in determining fructose in urine because of interfering
substances. They abandoned their procedure and adopted the
method of Van Creveld (3), which was published the same year.

Van Creveld’s method makes use of the blue color produced
when fructose is dehydrated with HCl in the presence of diphenyl-
amine, a reaction first observed by Ihl and Pechmann (4). In
this method blood is deproteinized with HgCl₂, the filtrate is
mixed with HCl and diphenylamine in a tube and placed in a boil-
ing water bath for 15 minutes, the color obtained is extracted
with amyl alcohol, and comparison is made with standards pre-

* Some of the data in this paper are taken from a dissertation presented
by Joseph H. Roe in partial fulfilment of the requirements for the degree of
Doctor of Philosophy, Yale University, 1934.
pared by dissolving fructose in mercury blood filtrates. Corley (5) has modified the Van Creveld method and applied it to the determination of fructose in tungstic acid blood filtrates, and in urine. He found a greenish color produced by the Van Creveld reagents with tungstic acid blood filtrates, but was able to use an aqueous fructose standard for colorimetric comparison by introducing a correction for the interfering color. Both Van Creveld and Corley reported difficulty in applying this procedure to urine.

A study of the color reactions for fructose mentioned above was made, and it was found that a greater depth of color could be obtained with resorcinol than with diphenylamine under the same dehydration conditions. The Seliwanoff reaction was therefore selected as the basis of the method which has been developed and will be described below.

In his original procedure Seliwanoff (6) used 12 per cent HCl. It was found that the sensitivity of the reaction could be improved by increasing the concentration of HCl to 18 per cent, although it is not safe to go much above this concentration of acid. The dehydrating conditions of the reaction were improved further by introducing ethyl alcohol. Alcohol also acts as a solvent for the red product formed, and thus serves to produce a much better and a more stable color than is obtained with aqueous solution. Under these conditions a slow reaction with glucose occurs at the temperature of the boiling water bath. This objection was overcome by warming the reaction mixture in a water bath at 80°. At this temperature there is no color produced by glucose with the proposed reagents and technique when glucose is present in amounts less than 300 mg. per 100 cc. This makes it possible to determine fructose in normal blood and urine without interference from glucose. By these adjustments of the conditions of the Seliwanoff reaction a color is produced which is 13 times as intense as that obtained when the original Seliwanoff reagents are applied to a fructose solution at 80°.

When the reagents of this method are applied to tungstic acid blood filtrates, a brownish color is obtained which is apparently the result of overdehydration of the fructose. The Somogyi zinc hydroxide deproteinizing method (7), however, produces blood filtrates which yield colors that are entirely satisfactory for comparison with the colors obtained with aqueous fructose standards.
Considerable difficulty was experienced in applying this method to urine. The problem with urine is the removal of pigments and cellular debris. Adsorbent silicates, and lead, mercury, silver, uranium, and zinc salts were tried as clarifying agents without success. The difficulty was overcome by using an acid-washed, reactivated, decolorizing charcoal. Washing the charcoal with acid is necessary to remove basic constituents. The urine mixture must have a reaction acid to pH 3.1 to prevent the adsorption of fructose by the charcoal. The proper pH for removing the interfering substances, and not removing the fructose, cannot be produced with certainty unless the charcoal is washed reasonably free of basic constituents. The charcoal must also be reactivated by heating it to redness for some time.

Reagents

0.1 per cent alcoholic resorcinol. Dissolve 0.5 gm. of resorcinol in 500 cc. of 95 per cent ethyl alcohol. This reagent should be made up fresh once every 2 months.

30 per cent HCl. To 1 part of distilled water add 5 parts of concentrated HCl, sp. gr. 1.19. Concentrated HCl may be used, but the addition of some water makes a reagent that fumes less and is more pleasant to work with.

Standard fructose solutions. Prepare a stock standard by dissolving 1 gm. of C.P. fructose in 100 cc. of saturated benzoic acid solution. Three working standard solutions, containing 0.1, 0.05, and 0.025 mg. per cc., are made by diluting the stock standard with saturated benzoic acid. Fructose keeps well in benzoic acid solution.

Somogyi deproteinizing reagents. Solution I, 10 per cent solution of ZnSO₄·7H₂O. Solution II, 0.5 N NaOH. The two solutions must be so related that when 10 cc. of Solution I, diluted to 50 or 75 cc. with water, are titrated with Solution II, 10.8 to 11.2 cc. of the latter are required to produce a permanent pink color with phenolphthalein.

Acid-washed, activated charcoal. Place 100 gm. of a good grade of adsorbent charcoal on a filter and pour over it 500 cc. of 10 per cent acetic acid. Wash with 1000 cc. of water. Remove the charcoal to a vessel suitable for heating to high temperatures. Apply heat slowly at first until the water has evaporated and then heat the charcoal to redness for 15 minutes. When treated by
Fructose in Blood and Urine

this procedure, nor it was found to be entirely satisfactory for clarifying urine preliminary to the determination of fructose.

Procedure

For Blood—To 1 part of blood add 7 parts of distilled water. Allow a few minutes for hemolysis, then add 1 part of 10 per cent ZnSO₄·7H₂O and 1 part of 0.5 N NaOH. Filter. Place 2 cc. of the filtrate in a test-tube. Place in each of three similar test-tubes 2 cc. of standard fructose solution, the standards used containing 0.1, 0.05, and 0.025 mg. of fructose per cc. To each of the tubes add 2 cc. of 0.1 per cent alcoholic resorcinol solution and 6 cc. of 30 per cent HCl. Mix by shaking the tubes vigorously and place in a water bath adjusted to maintain a temperature of 80°. Keep in the water bath for 8 minutes. Remove the tubes to a beaker and cool with running water. Compare in a colorimeter in the usual manner, the standard which most closely matches the unknown being used.

The three standards selected permit the accurate determination of fructose in quantities ranging from 25 to 100 mg. per 100 cc. of blood. They will permit the determination of 10 to 200 mg. per 100 cc., with slight errors due to deviations from Beer's law. If quantities around 10 mg. per 100 cc. are encountered, somewhat greater accuracy may be obtained by making a blood filtrate diluted 1:5. If the blood contains 200 mg., or more, of fructose per 100 cc., it is best to dilute the 1:10 filtrate appropriately and repeat the determination with a smaller equivalent quantity of blood.

Calculation—\((S/U) \times S_1 \times (100/0.2) = \text{mg. of fructose per 100 cc. of blood.} \) \((S = \text{reading of standard}; U = \text{reading of unknown}; S_1 = \text{mg. of fructose in standard tube selected for comparison.})\)

For Urine—Place 2 cc. of urine in a small Erlenmeyer flask and add 18 cc. of 1 per cent acetic acid. Add approximately 0.2 gm. of acid-washed, activated charcoal. Shake vigorously, then allow to stand for 5 minutes with occasional shaking. Filter through a good grade of retentive filter paper. Place 2 cc. of the filtrate in a test-tube and prepare three standard tubes, the standards used containing 0.1, 0.05, and 0.025 mg. of fructose per cc. The standard solutions should be made by diluting the stock fructose solution with 1 per cent acetic acid in order to have the
same concentration of acetic acid in the standards as in the unknown. (The fructose standards used for blood may be used for urine, but failure to adjust the acetic acid concentration of the standards results in an error of 3 per cent.) Add to each of the tubes 2 cc. of 0.1 per cent alcoholic resorcinol and 6 cc. of 30 per cent HCl. Place the tubes in a water bath at 80° for 8 minutes. Remove the tubes and cool promptly in running water. Compare in a colorimeter, the standard which most closely matches the unknown being used. If the urine has a high fructose content, dilute the filtrate with 1 per cent acetic acid to a value that will permit comparison with the 0.1 mg. per cc. standard and repeat the above procedure. The formula for calculation is the same as given above for blood.

If protein is present in the urine, it may be removed by treating with the Somogyi reagents in the same manner as outlined above for blood. To each 10 cc. of deproteinized urine filtrate add 0.1 cc. of glacial acetic acid and 0.1 gm. of the adsorbent charcoal and proceed the same as with normal urine.

DISCUSSION

It is necessary to measure all reagents exactly. The depth of color obtained is dependent upon the amount of alcohol and HCl present. It is therefore very important that the same amounts of alcohol and HCl be added to the standards and the unknown. Excesses of resorcinol above the amount used do not make any difference. The tubes should be placed in the water bath exactly simultaneously and the same precaution should be followed in removing them from the bath to cold water. The color produced is dependent upon the velocity of a reaction at an elevated temperature; hence identical heating and cooling conditions must be imposed upon each tube. After cooling to room temperature the color obtained does not change in character for 24 hours or more.

The cherry-red color obtained in this procedure is of excellent quality for colorimetric work. It has a high degree of proportionality to the amount of fructose present. Good agreement with Beer's law was observed in studies upon this relationship. It is best, however, to use a group of standards in practical work, and to compare the unknown with the standard that most closely matches it. When the standard is set at 20 mm. in the colori-
Fructose in Blood and Urine

meter, readings of unknown solutions between 15 and 25 mm. are acceptable.

In determining fructose in urine, it is important to realize that fructose is adsorbed by the clarifying charcoal if the reaction is alkaline to pH 3.1. The relation of pH to the adsorption of fructose by acid-washed, activated norit was studied as follows: norit was added to aqueous fructose solutions of varying hydrogen ion concentrations, the mixtures were shaken intermittently for 15 minutes and filtered, the pH of the filtrate was determined by the quinhydrone electrode, and the fructose was estimated by the colorimetric method. Adsorption of fructose occurred only in the mixtures with a pH on the alkaline side of 3.1. As a result of experiments of this kind it was decided to use a 1 per cent acetic acid solution (approximate pH, 2.9) for diluting the urine preliminary to treating with norit.

To determine whether concentration of fructose might influence the adsorption of the latter by norit, an experiment was performed in which fructose was added to urine to produce concentrations varying from 0.025 per cent to 10 per cent. The urines were then diluted with 9 parts of 1 per cent acetic acid, treated with norit, and filtered. The filtrates were diluted as necessary and their fructose content was determined. The results are shown in Table I. The data of this experiment do not show any adsorption of fructose, and they are also of interest in that they are representative of the recoveries that may be obtained by this method when fructose is added to normal urine secreted during fasting.

<table>
<thead>
<tr>
<th>Fructose added (gm. per 100 cc.)</th>
<th>Fructose recovered (gm. per 100 cc.)</th>
<th>Per cent error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>0.026</td>
<td>+4.0</td>
</tr>
<tr>
<td>0.100</td>
<td>0.105</td>
<td>+5.0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.972</td>
<td>-2.8</td>
</tr>
<tr>
<td>2.0</td>
<td>2.007</td>
<td>+0.35</td>
</tr>
<tr>
<td>5.0</td>
<td>4.967</td>
<td>-0.66</td>
</tr>
<tr>
<td>8.0</td>
<td>7.936</td>
<td>-0.80</td>
</tr>
<tr>
<td>10.0</td>
<td>10.085</td>
<td>+0.85</td>
</tr>
</tbody>
</table>
If normal human urine secreted under fasting conditions is examined by this method, an entirely negative blank test is obtained. The urine secreted after a meal which includes certain fruits and sucrose contains enough fructose to give a positive qualitative test; and in some instances there is enough fructose in a postprandial sample of urine from a normal subject to permit quantitative determination by this method. The evidence for assuming that fructose is present in the urine of normal subjects is that positive tests are obtained upon postprandial samples of urine with our modified Seliwanoff technique and that these tests become negative after fermentation of the urine with yeast. Evidence has been obtained by Tashiro and Tietz (8) and Harding and Selby (9) which indicated that a part of the sugar of normal human urine may be fructose. With this method it has been possible to confirm the supposition of these authors.

Data are given in Table II which show the amount of additive error when fructose is determined by this method in the presence of glucose or galactose. From these data a correction curve may be constructed for use with this procedure when glucose in amounts greater than 300 mg. per 100 cc., or galactose in quantities greater than 500 mg. per 100 cc., is present.

Furfural gives a green color when submitted to the conditions of this procedure. The interference due to pentoses is thus more serious than that due to hexoses, and correction data cannot be used when pentoses are present in concentrations sufficient to produce a perceptible green color. Xylose was found to give a

<table>
<thead>
<tr>
<th>Fructose present</th>
<th>Glucose added</th>
<th>Fructose obtained</th>
<th>Per cent error</th>
<th>Galactose added</th>
<th>Fructose obtained</th>
<th>Per cent error</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>300</td>
<td>101</td>
<td>+1.0</td>
<td>500</td>
<td>101.4</td>
<td>+1.4</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>102.2</td>
<td>+2.2</td>
<td>1000</td>
<td>104.0</td>
<td>+4.0</td>
</tr>
<tr>
<td>100</td>
<td>1000</td>
<td>106.4</td>
<td>+6.4</td>
<td>2000</td>
<td>111.2</td>
<td>+11.2</td>
</tr>
<tr>
<td>100</td>
<td>2000</td>
<td>113.3</td>
<td>+13.3</td>
<td>3000</td>
<td>110.0</td>
<td>+10.0</td>
</tr>
<tr>
<td>100</td>
<td>5000</td>
<td>141.3</td>
<td>+41.3</td>
<td>5000</td>
<td>127.6</td>
<td>+27.6</td>
</tr>
</tbody>
</table>
negative blank test when present in amounts up to 1000 mg. per 100 cc. and a green color in concentrations above the latter amount. Accurate results may not be expected, therefore, when fructose is determined in the presence of more than 1000 mg. of xylose per 100 cc. of solution; and the method should not be applied to the determination of fructose in the presence of other pentoses when the latter are present in concentrations that will give a green color in a blank test.

BIBLIOGRAPHY

A COLORIMETRIC METHOD FOR THE DETERMINATION OF FRUCTOSE IN BLOOD AND URINE
Joseph H. Roe