THE ESTIMATION OF SUGAR IN BLOOD AND NORMAL URINE.

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About a year ago the writer (1) described technique for the determination of sugar in blood which yields figures appreciably lower than those obtained by the use of the older methods. Since the publication of that paper two investigators, employing widely differing procedures, have reported results which indicate the correctness of the lower figures obtained with the new method. Harned (2), after completely precipitating the nitrogenous constituents from the blood by the use of mercuric nitrate and sodium bicarbonate, obtained figures for the sugar by use of the Folin-Wu method which agree quite closely with those obtained by the Benedict procedure on the tungstic acid filtrate.

Folin (3) has proposed new reagents for the determination of sugar in blood and urine. The figures reported by the new procedure show essential agreement with those obtained by the new Benedict method. In this latest contribution to the subject of blood sugar determination Folin has subjected the new Benedict procedure to a very searching critique. Indeed Folin's paper would seem to lead to the rather anomalous conclusion that while the new Benedict method yields figures which are probably correct, the method itself is quite unsatisfactory. Obviously the main point of interest is the question as to what figures most nearly represent the true glucose content of the blood. Of much less importance is the particular technique employed to obtain such figures. It is therefore only because we feel reasonably confident that our new technique, especially in the modified form described below, is somewhat more convenient and decidedly safer for general use in biochemical determinations of sugar than is the new method described by Folin, that the following discussion is presented.
Early in his paper (3) Folin remarks, "Benedict seems to believe that the only essential feature of his qualitative sugar reagent is the fact that its alkali is a carbonate instead of a hydroxide. This concept I think is not quite correct." A little later in the same paper Folin suggests a simple experiment to prove his contention that the citrate reagent is less sensitive than is a tartrate reagent. That the present writer held no such mistaken opinion as Folin would imply is shown by the following quotation from our recent paper (1). "This [tartrate] reagent is more delicate than the citrate reagent subsequently proposed, but was not so stable nor so satisfactory, because of its great sensitiveness as a general qualitative reagent for sugar in urine." In view of the fact that we thus clearly recognized and called attention to the greater delicacy of the tartrate solutions (although, as we pointed out, the sensitiveness is gained at the cost of a loss in specificity) it is not clear why Folin should have so elaborated on this point.

It is quite likely that any marked gain in specificity for glucose by an alkaline copper solution will result in production of less cuprous oxide from a given amount of glucose under fixed conditions. This is as true of Folin's new copper solution as it is of our citrate reagent. In the case of Folin's new reagent the expedient resorted to to gain a normal yield of cuprous oxide is to increase the period of heating almost 100 per cent. We have employed sulfite to accomplish the same purpose. From the practical standpoint the sulfite offers the better solution to the problem, and we fail to find any theoretical reason against its use as we employ it.

Folin has objected on several grounds to the use of the complex tungstic acid reagent which we employ for color production. Thus he says that the acidity of the final mixture is so low that "the mixture will develop a blue color with uric acid." We suggest that anyone interested in the practical bearing of this criticism try the effect of uric acid added in quantity equal to ten or more times the amount ever present in blood filtrates, before color development. Such a simple experiment should convince anyone that there is no practical basis for the criticism. Discussing the color reagent still further Folin raises objection because the cuprous oxide dissolves very slowly and says, "Benedict . . . . intended to provide for this flaw in his method by inserting a waiting period of 10 minutes between the addition of the uric acid reagent . . . . and the dilu-
tion with water.” In these days when speed is one of the prime considerations in the use of an analytical method the waiting 10 minutes for color development may be something of a drawback in the use of the method, but we are quite unwilling to admit that there is any flaw at this point. By imposing conditions which the new technique was never intended to meet, Folin showed that some cuprous oxide might remain undissolved during the 10 minute period. Such results are never obtained when the method is used as directed. It is perhaps interesting to note that both of Folin’s criticisms of the color reagent should, if valid, result in high figures for the blood sugar, a defect which the method does not appear to exhibit in practical use. The actual figures yielded by the method seem to give ample vindication of our use of the complex tungstic acid reagent.

In the modified form of the method given below the use of the tungstic acid color reagent has been retained, in spite of the fact that long ago we developed, and for a time used, a molybdc acid reagent which gave a satisfactory color yield with the citrate copper reagent. Although Folin so strongly champions the complex molybdc acid reagent for color development, and has described a new reagent of this type which is prepared in quantity only with some difficulty, it is our opinion that the molybdc reagents are perhaps not so desirable as those containing complex tungstic acid. Folin states that even his new molybdc reagent is not very satisfactory for color development with our new copper reagent, because of the “huge” citrate content of the latter. Yet as a matter of fact the citrate in this copper reagent can be reduced some twenty or thirty times and still show a marked effect upon color development with the molybdc reagent. If so seemingly innocuous a substance as sodium citrate, which has neither oxidizing nor reducing properties, can seriously impair color development with the molybdate reagents, it would seem that for biochemical work in general, where unknown constituents are practically always present in one solution and not in the standard, it would be safer to adopt the tungstic acid reagent, which is not so susceptible to the effects of foreign compounds. Especially is this so since there appears to be no reason against adoption of this reagent. The latter reagent yields an intense pure blue coloration with cuprous oxide, while the molybdc reagents yield a greenish
blue shade at first, the greenish tint rapidly disappearing on standing, so that a freshly diluted solution cannot be compared in a colorimeter with one which has stood for even a relatively short time.

Folin has called attention to one serious defect in the method which we presented; viz., that the sulfite content of the reagent becomes rapidly oxidized so that the mixed solution is useful for only a very limited time. This criticism is fully justified, and we much regret the error which at this point appeared in the published form of the method. There would be little gained by going into a detailed account of how this trouble arose. It will be sufficient to say that for more than 2 years prior to publication the reagent had been prepared at intervals and as so prepared contained nearly 2 per cent of "sodium bisulfite" as contained in a bottle of Kahlbaum's product which had been in the laboratory for some years. After the paper was in the form of page proof we discovered that fresh sodium bisulfite could not be used in nearly the quantity indicated in the older formula. The only opportunity for change in the paper then was to alter the quantity of bisulfite to that which we knew could be added without detrimental results. Subsequent work has shown that sodium bisulfite is not a wholly definite product, some samples containing a relatively large proportion of meta-bisulfite, and others very little. In the modified form of the method detailed below we have therefore abandoned the use of bisulfite. Sodium sulfite is now used in an amount allowing for a considerable excess of this salt, and proper precautions are taken so that under even the most adverse conditions of keeping the reagent as used will always contain an adequate amount of sulfite.

Folin's new bicarbonate-containing copper reagent owes its specificity to decreasing the alkali content to a very minimum. Consequently this new reagent has not sufficient alkali to take care of even minute amounts of acid, nor can more than traces of alkali be added without wholly upsetting the hydrogen ion content of the solution. Folin frankly admits these facts, and yet he rather minimizes the necessity for neutralization of the blood filtrates prior to the sugar determination. According to our experience with the new reagent exact neutralization of all filtrates is quite essential if the figures obtained are to have any significance. It is
doubtful whether such a delicately balanced solution is best for
general use. The lack of any reserve alkali whatever in the solu-
tion is apt to lead even very careful workers into error when the
ground of operations is changed in any way. As will develop
later in this paper, Folin has himself apparently fallen into serious
error in assuming that his new copper reagent can be applied to the
determination of sugar in normal urine along the lines of accepted
procedure for this analysis.

The following technique for the determination of blood sugar is
essentially the same as that previously proposed (1), with modifi-
cations which make for greater speed and convenience in the
analysis, and with provision for always having present an adequate
amount of sulfite. Through addition of an ammonium salt to the
copper reagent the reduced copper is held in solution, so that color
development is practically immediate after addition of the color
reagent. We believe it to be at least a theoretical advantage to
have the reduced copper in the same condition in both standard
and unknown. In all other copper methods with which we are
familiar the cuprous oxide is always in a more finely divided
form in the blood filtrate than in the standard, and this introduces
the chance of greater reoxidation in one than in the other, or of a
difference in relative intensity of action upon the color reagent.
The complex tungstic acid color reagent has been somewhat
changed in composition. Owing to the increased quantity of
sulfite in the copper reagent the formaldehyde has been increased
in the color reagent. Sodium chloride has been added to increase
the specific gravity of the solution so that it mixes more promptly
with the other solution after reduction of the latter, and hydro-
chloric acid has been added to provide for higher acidity of the
total mixture. A portion of the hydrochloric acid is, however,
neutralized by the additional carbonate content of the copper
reagent. The carbonate in the latter reagent has been somewhat
increased—slightly more than enough being added to react with
the ammonium salt now contained in the reagent. While our
reagents yield a slight blank, the color under such conditions is
so slight that we have made no effort to improve the method in
this respect. Anyone who may feel that the color from a blank
may be significant should try the experiment of running a blank
side by side with a standard. When the two are diluted it will be
readily seen that the color of the blank is so faint as to be entirely unobjectionable, especially since it is always present in both standard and unknown.

The procedure for the method follows.

Reagents.

1. Alkaline Copper Solution.—

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Pure crystallized copper sulfate</td>
<td>6.5 gm.</td>
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<tr>
<td>Sodium citrate</td>
<td>200.0 &quot;</td>
</tr>
<tr>
<td>carbonate (anhydrous)</td>
<td>60.0 &quot;</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>9.0 &quot;</td>
</tr>
<tr>
<td>Distilled water to make</td>
<td>1000 cc.</td>
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</tbody>
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Dissolve the citrate and carbonate together in about 800 cc. of water. Dissolve the copper sulfate separately in about 100 cc. of water and add to the other solution with stirring or shaking. Add the ammonium chloride, dilute to 1 liter, and mix.

Not more than 1 month before use add to each 100 cc. of the reagent 2.5 to 3.0 gm. of pure anhydrous sodium sulfite. This can be conveniently done as follows: Select a bottle holding about 100 cc., and when desired fill it from the main reagent bottle. Select a porcelain spoon or other convenient measure which will hold 2.5 to 3 gm. of sodium sulfite and use this to measure the sulfite for addition to the small reagent bottle. Even a quite wide variation (nearly a whole gm.) in the quantity of sulfite added will not appreciably affect the results with the method. Where only occasional sugar determinations are to be made it may be more convenient to add the sulfite from a 20 per cent solution kept in a dropping bottle. Where this method of addition of sulfite is used, 5 drops of the 20 per cent sulfite solution should be added to each sugar tube prior to adding the other solutions. The strong sulfite solution will keep satisfactorily for at least 4 months in well filled tightly stoppered bottles, and probably two or three times this length of time. We have not yet had opportunity to find out just how long the sulfite-containing solutions will keep. The periods given are minimal.

2. Complex Tungstic Acid Color Reagent.—This is prepared as follows: 100 gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water. 50 gm. of pure
arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes. After cooling add 60 cc. of commercial formalin, 45 cc. of concentrated hydrochloric acid, and 40 gm. of sodium chloride. Dilute to 1 liter and mix.

The technique of the method is as follows: 2 cc. of the 1:10 tungstic acid filtrate are measured into a Folin-Wu sugar tube, followed by 2 cc. of the copper reagent. The contents are mixed by side to side shaking for a moment, and the tubes placed in boiling water for 5 minutes. The tubes are then cooled by immersion in cold water and 2 cc. of the complex tungstic acid color reagent are added. After 1 to 2 minutes the contents of the tubes are diluted to 25 cc. with water, thoroughly mixed, and compared with a standard in a colorimeter. As standard solution, pure glucose solution containing 0.1 mg. of glucose per cc. is employed. This can conveniently be prepared by 1:10 dilution of a 0.1 per cent solution of pure glucose in water, preserved with 1 or 2 cc. of toluene. This solution will keep indefinitely so long as any toluene remains upon the surface of the solution. The diluted standards will also keep for long periods in the presence of a few drops of toluene.

1 In our previous paper we suggested the substitution of an ordinary graduated test-tube and the use of benzene, as an alternative for the use of the Folin-Wu sugar tubes. Folin (3) thinks that such substitution cannot safely be made, and offers a fanciful, and quite incorrect explanation of our findings in this matter. Apparently we were not specific enough in our directions. There are two important questions involved. These are the width of the tube and the quantity of benzene employed. If one uses a wide tube and a minimum of benzene, reoxidation is little affected. With a moderately narrow tube (15 mm. or less, internal diameter) and enough benzene to leave a trace on the surface of the solution at the end of the heating, reoxidation is as low as when the Folin-Wu sugar tubes are used when used with the technique we previously proposed. The common impression that there is no reoxidation when the Folin-Wu sugar tubes are employed is not correct. By comparing results with sugar tubes which have been evacuated with a good suction pump (and the vacuum maintained during the period of heating and cooling) with those not so treated, it will be found that as usually employed the Folin-Wu sugar tubes permit 6 to 7 per cent reoxidation, against more than 20 per cent in the wide straight tubes first used in the Folin-Wu method. We consider the Folin-Wu tubes a very valuable contribution to sugar analysis, and advocate their use in the procedure described in the present paper.
The degree of accuracy of the method is satisfactory where the solution analyzed is not more than twice as strong or half as weak as the standard. Results by the modified method for blood sugar are in quite close agreement with those previously reported (1).

Folin has advocated application of his new bicarbonate copper solution to the determination of normal urine sugar in Lloyd's filtrates, and reports figures where the new reagent is employed which are as low as one-half to one-third those obtained on the same filtrates with the Folin-Wu reagent. It seems, even at first glance, doubtful whether these excessively low figures reported by Folin for urines can be correct. We therefore first tried the effect of Lloyd's reagent upon the standard glucose solution, using the Lloyd's exactly as Folin and Berglund (4) employed it, and Folin's new copper reagent in place of the Folin-Wu solution.

When Folin's new reagent is substituted for the Folin-Wu copper solution, treatment of the standard glucose solution with Lloyd's reagent results in an apparent loss of glucose up to 30 per cent. Apparently the loss is more or less proportional to the length of time of shaking the mixture with Lloyd's, or to the length of time it remains on the filter. Hence we infer that the amount of Lloyd's which dissolves determines the sugar loss. Doubtless the loss is due to withdrawal of hydroxyl and carbonate ions through precipitation which occurs when the filtrate is heated with the copper reagent. It is not due to the faint acidity which remains after treatment with acid and Lloyd's. The latter reagent itself neutralizes most of the added acid, and exact neutralization of the trace of acid left does not appreciably affect the results. A single experiment on recovery of sugar added to urine by the new Folin method showed a loss of about 25 per cent of the 40 mg. added. Obviously the new Folin procedure is not adapted to the determination of sugar in normal urine. This finding serves to emphasize the caution necessary in the use of the new Folin bicarbonate copper reagent.

For determination of sugar in normal urines the reagents described earlier in this paper probably yield the most nearly correct results. The method is carried out exactly as described by Folin and Berglund with the use of Lloyd's reagent, save that the new reagents are substituted for the Folin-Wu copper and molybdic acid reagents. Results are slightly lower than where the Folin-
Wu reagents are employed, but there is not a great deal of difference between figures by the two methods.

BIBLIOGRAPHY.

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Stanley R. Benedict