A METHOD FOR THE DETERMINATION OF SUGAR IN NORMAL URINE.

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(Received for publication, February 1, 1918.)

Certain observations in this laboratory convinced the present writers some time ago that a quantitative study of the substances simulating reducing carbohydrate in the urine of normal animals would be of value, and the endeavor was made to construct a satisfactory quantitative method for this purpose. The procedure described below is the outcome of nearly 3 years of work on the subject and has been tested under widely varying conditions.

We have not attempted to differentiate glucose qualitatively, but have provided for determination of "total sugar," and the sugar fermentable by yeast, by difference. The non-fermentable portion, as determined in our method, is probably a true sugar, or is at least chiefly derived from carbohydrate in metabolism. These questions will be referred to again in subsequent papers.

The procedure we have adopted is an adaptation of the colorimetric procedure of Lewis and Benedict for determination of sugar in the blood. Myers has already reported an adaptation of the Lewis-Benedict method to urine, which provided for preliminary precipitation of most of the creatinine as picrate according to the suggestion of Folin. Our work with the Myers' modification has convinced us that results by this method are too high (due partly to imperfect removal of creatinine).

Indeed not infrequently the Myers method gives results at least 100 per cent too great. This is not surprising when we remember that nitrogenous compounds other than creatinine may react with picric acid in alkaline solution (allantoin, for instance, 0})

so reacts) and that creatinine yields about five times as much color with picric acid as does an equal weight of glucose. Polyphenols are another possible source of interference.2

The method which we wish to present may be considered under two divisions: (1) the preliminary precipitation of interfering substances from the urine, and (2) the determination of the sugar in the filtrate. We shall discuss these processes separately before describing the technique in detail.

In the preliminary precipitation we have deemed it desirable to remove creatinine completely, total nitrogen as completely as possible, glycuronic acid to such an extent at least that it should not interfere appreciably in normal urines, and finally to remove the polyphenols practically completely, since these compounds also react with alkaline picric acid, although not to such an extent as does creatinine or sugar.

We have found that urines can be almost entirely freed from the substances enumerated above by a single precipitation with excess of mercuric nitrate in presence of a slight excess of sodium bicarbonate. After subsequent removal of the mercury with zinc dust the filtrates are water-clear, and contain almost no measurable nitrogen, or at least not more than 0.1 mg. per cc.3 No creatinine can be detected in the filtrates. Folin's uric acid reagent gives only a trace of color (showing practical absence of polyphenols), and treatment of the final filtrate with basic lead

2 It is quite surprising to note the widely diverse results obtainable for sugar in urine after precipitations aimed only to eliminate creatinine. Thus Cole (Practical Physiological Chemistry, St. Louis, 4th edition, 1914, 249) used such a procedure for qualitatively detecting abnormal quantities of glucose in urine. Later Folin (J. Biol. Chem., 1915, xxii, 327) used a quite similar procedure and practically the same copper solution, for qualitatively detecting glucose in normal urine. Myers and Bailey then used the procedure for quantitative determination of small quantities of glucose in urine, and still later Hiller (ibid., 1917, xxx, 125) has used Folin's precipitation method (repeated word for word) to determine only pathological quantities of sugar in sugar in urine—normal urine showing no sugar by this method.

3 In most cases 20 cc. of final filtrate yield no more nitrogen by Kjeldahl than does a blank determination with the same reagents. Schöndorff (Arch. ges. Physiol., 1908, cxxi, 572) employed mercury in faintly acid solution for precipitation of urines prior to titration with Fehling's solution, and reported nitrogen-free filtrates.
acetate causes no change in the sugar content, thus showing absence of any appreciable amount of glycuronic acid. Interference from creatine in the original urine is negligible unless the creatine exceeds 0.3 per cent. Such a concentration of creatine is not often encountered in urines.

For the final determination of the sugar we have materially modified the original Lewis-Benedict procedure.

A study of the reaction between picric acid and sugar in alkaline solution was undertaken with a view to increase the flexibility of the method and to eliminate the objectionable feature of boiling to dryness in a test-tube.

The results of this study may be briefly summarized as follows. Sodium hydroxide causes secondary reactions which do not go to completion and hence cannot be employed in any concentration during the reaction between picric acid and sugar.

With a constant and only moderate excess of picric acid, the depth of color given by a constant amount of sugar will increase with increase of carbonate concentration almost indefinitely. This is apparently due to products of the primary reaction, reacting with the picric acid in increasing amount with increase of carbonate. In concentrated carbonate solution the color is not directly proportional to the quantity of sugar, hence the concentration of carbonate must be kept moderate and within fixed limits.

The reaction in presence of moderate concentration of carbonate goes to completion in a moderate excess of picric acid, providing the sugar concentration is not too low. If it is desired to have the reaction proceed in a quantitative fashion where the glucose concentration may be as low as 0.5 mg. in 9 cc., this can be accomplished by suitable increase in the concentration of picric acid.

The concentration of picric acid must however, for such conditions, exceed that of a saturated aqueous solution of picric acid. This may be attained either by addition of dry picric acid, or the addition of a solution of picric acid containing considerably more than does the ordinary saturated solution. The first of these procedures is objectionable because the picric acid would have to be weighed out each time, in order to be assured of enough to cause the reaction with sugar to go to completion, and to be sure
that enough was not added to cause precipitation of sodium picrate before the final reading could be made conveniently.

We therefore endeavored to prepare a solution containing about three times the picric acid present in an ordinary saturated solution. This has been accomplished by the help of a little sodium hydroxide. Only enough hydroxide is added to convert a portion of the picric acid into sodium picrate. With the help of this solution we may start with a dilute solution of glucose in water, and, by adding some of the strong picrate solution and carbonate, obtain a final solution containing a large excess of picric acid, and one in which the reaction proceeds to quantitative completion when heated in a boiling water bath for 10 minutes.

The special reagents used in the final method for the determination of sugar in urine are:

1. Mercuric nitrate solution. It is prepared as follows: To 160 cc. of concentrated nitric acid in a beaker add in small portion, 220 gm. of mercuric oxide. When all is dissolved heat the mixture to boiling, cool, and add 60 cc. of 5 per cent sodium hydroxide solution. Make up to 1 liter and filter. Keep in a brown bottle.4

2. Picrate-picric acid solution. To 500 cc. of 1 per cent sodium hydroxide solution in a liter flask or stoppered cylinder add 36 gm. of picric acid, and about 400 cc. of hot water. Shake occasionally until the picric acid is dissolved, cool, and dilute to 1 liter.5

The final solution is fully saturated with picric acid. A slight crystallization of picric acid on standing is of no account, but if abundant crystallization occurs (due to the solution becoming too cold for some hours) it is desirable to redissolve the picric acid by warming before using.

The procedure for the sugar determination is as follows: Measure 15 or preferably 20 cc. of urine into a 500 cc. beaker and

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4 The formula for this solution has been in the laboratory for a number of years. We have been unable to trace its origin.
5 The picric acid employed should be dry and free from lumps. On account of its explosive properties, the drying of the picric acid should be carried out at a moderate temperature (60°) and the dry and cool product freed from lumps by very gentle rubbing in a mortar.
6 If less than 15 cc. of urine are available 10 cc. may be used, but in this case the subsequent filtrations should be made on small filters and with the
add an equal volume of the mercuric nitrate solution and mix. Then add solid sodium bicarbonate in moderate quantities, with gentle shaking, until frothing ceases and the mixture reacts alkaline to litmus paper. Filter at once through a dry filter into a small dry flask or beaker. To the perfectly clear filtrate add a pinch of zinc dust and 1 or 2 drops of concentrated hydrochloric acid. Shake and let stand for about 5 minutes. Filter through a small dry filter into a dry test-tube or small beaker.

From 1 to 4 cc. of this final filtrate (the volume to be used depends on the sugar content of the original urine, from 0.5 to 2 mg. of sugar is the quantity desired) are measured into a large test-tube, which should be graduated to indicate 12.5 and 25 cc. Where less than 4 cc. of filtrate have been used enough water is added to make exactly 4 cc. and 1 cc. of 20 per cent (anhydrous) sodium carbonate solution is added. 4 cc. of the picrate-picric acid solution is now added, the tube plugged with cotton, and placed in boiling water for 10 minutes. The tube is then removed, cooled to room temperature with the help of running water, and the contents are diluted with water to the 25 cc. mark, or to 12.5 cc. if the quantity of sugar present was much below 1 mg. The aid of suction, in order to obtain a maximum of filtrate. If fairly concentrated, the original urine may, of course, be diluted before beginning the analysis.

Vigorous frothing occurs when the bicarbonate is first added—hence the use of the large beaker for this mixing. The bicarbonate may be added freely until the frothing ceases, when the mixture should be stirred or shaken only until alkaline to litmus. It should then be poured upon a filter at once.

This filtrate should be wholly free from mercury. Until thoroughly familiar with the method it is best to test a few drops with ammonium sulfide solution. If a dark or black coloration is obtained it is necessary to mix the filtrate again with the powdered zinc, and let stand for a few minutes.

Very satisfactory tubes for this purpose have been prepared for us by E. Machlett and Son of New York.

Where a number of determinations are to be made it is convenient to keep on hand four solutions of sodium carbonate of different strengths, as follows: No. 1, 5 per cent; No. 2, 6.7 per cent; No. 3, 10 per cent; and No. 4, 20 per cent. Where 1 cc. of final filtrate is used add 4 cc. of Solution 1, where 2 cc. of filtrate are used add 3 cc. of No. 2, for 3 cc. of filtrate add 2 cc. of No. 3, etc., in each case giving the final solution a volume of 5 cc., and a sodium carbonate (dry) content of 0.2 gm.
colored solution is then matched (within half an hour\textsuperscript{11}) in a colorimeter against a standard prepared by treating 1 mg. of glucose in 4 cc. of water, just as the final filtrate was treated; or against a permanent standard of picramic acid solution or potassium dichromate.

We have found that the best permanent standard is to be prepared from pure picramic acid, together with some of the picrate-picric acid solution used in the determination. This standard solution has the following composition: To 105 cc. of exactly 0.01 per cent picramic acid solution in 0.02 per cent sodium carbonate solution, add 0.5 cc. of 20 per cent sodium carbonate solution, and 15 cc. of the picrate-picric acid solution. Then dilute to 300 cc. with distilled water. Starting with pure picramic acid, the depth and quality of the color of this solution duplicate very exactly that obtained from 1 mg. of glucose dissolved in 4 cc. of water, treated as described for the final urine filtrates, and the colored solution diluted to 25 cc. The standard in the colorimeter should be set at a height of 15 mm.

The picramic acid originally prepared by the J. T. Baker Chemical Company was very satisfactory, but within the past 2 years we have seen samples of Baker's picramic acid which have yielded only about 30 per cent of the color given by an equal weight of pure picramic acid.

Where satisfactory picramic acid is not available, potassium bichromate may be employed as a standard. In our Duboscq colorimeters the dichromate solution matches the picramic acid solution standard almost exactly. In another instrument (Krüss) there is an appreciable difference in shade of color of the two solutions, but even in this instrument the dichromate standard can be employed with satisfactory results. To prepare the dichromate standard dissolve 0.536 gm. of pure potassium dichromate in water and dilute to 1 liter.

To determine the glucose or fermentable sugar in the sample of urine it is necessary to make a second determination on the sample of urine after fermentation. For fermentation we have adopted the following as a routine procedure. To 25 cc. of urine (free

\textsuperscript{11} Where 4 cc. of final filtrate are used, the final dilution is made to only 12.5 cc.; it is necessary to make the reading promptly to avoid interference from crystallization of sodium picrate.
from preservative) in a cylinder or test-tube add 20 to 25 mg. of glucose and about one-quarter cake of yeast. Mix well and allow to stand in the incubator at 35–38° for 18 to 20 hours. Decant 15 to 20 cc. of the urine and determine sugar as before fermentation. The difference between the two determinations gives the fermentable sugar.

The results obtained by this method for sugar determination in normal urine will be discussed in subsequent papers. For the present we may state that glucose added to urine is quantitatively recovered within a few thousandths of 1 per cent, and that we have considerable evidence to indicate that the method gives figures which represent very nearly the true sugar (reducing carbohydrate) content of normal urine. The procedure can be applied to diabetic urines, but most of such samples have to be diluted from 10 to 100 times before analyzing.

It is expected to apply the method to the determination of sugar in tissues in the near future. We are also studying the occurrence of polysaccharides in the urine by the help of the new method.

The glucose is added as a "check" on the activity of the yeast.
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