DINITROSALICYLIC ACID: A REAGENT FOR THE ESTIMATION OF SUGAR IN NORMAL AND DIABETIC URINE.

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The methods used for the quantitative determination of sugar in urine, including the polariscope, give good results when the amount of sugar present is considerable, but there is always a point where the urine contains so little sugar that the results are dubious. Special methods have been published for the determination of sugar in normal urine. Many of these give results that are too high, and others, like the method of Benedict and Osterberg,[1] are too long for clinical work.

It appeared that some reagent might be found which could be used both qualitatively and quantitatively and which could be applied to both normal and diabetic urine. The author believed that the most suitable substance would be a compound similar to picric acid in that it would be reduced by glucose to form a highly colored nitroamino compound, but dissimilar to picric acid in that it would neither form colored compounds with acetone or creatinine nor be reduced by urinary constituents other than the sugars.

Two nitro compounds have been found which meet most of these requirements; these are 4, 6-dinitroguaiacol and 3, 5-dinitrosalicylic acid.[2] Both of these compounds can be used successfully for the estimation of glucose in diabetic urine, but while they are not reduced in the presence of sodium carbonate by any urinary constituent yet tried, with the exception of the

[1] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

[2] Hübner, H., *Ann. Chem.*, 1879, cxcv, 45.

reducing sugars, they are reduced by such substances as uric acid and polyphenols when glucose is also present. Moreover, they are reduced by the urine as a whole even after the sugar present has been destroyed. The results obtained by the use of these reagents for the sugar in normal urine may be as much as 100 per cent too high, or even more in some cases.

The author with Miss V. A. Graham recently read a paper on the use of dinitroguaiacol at the meeting of the American Society of Biological Chemists and will not describe the use of dinitroguaiacol in this paper because he believes dinitrosalicylic acid is a far better reagent. While both compounds, under the same conditions of heating and alkalinity, give approximately the same results with normal urine, dinitrosalicylic acid possesses the advantages of being more soluble in the form of its sodium salt, of being lighter in color, and cheaper to prepare. In addition dinitrosalicylic acid is an excellent protein precipitant and on this account can probably be used for the determination of blood sugar.

Using dinitrosalicylic acid as a reagent, the author has found a way to obtain the true values for the sugar in normal urine as follows: 1 cc. of urine is heated in boiling water with 1 cc. of 3 per cent sodium hydroxide for 15 minutes. This treatment destroys the reducing sugars completely, provided the amount present is less than 1 mg. Either 0.5 or 1 mg. of glucose, depending upon the quantity of glucose estimated to have been present originally, is now added to the cooled solution, after which 1 cc. of dinitrosalicylate solution is added and the test-tube heated for 5 minutes. Any reduction exceeding that given by the added glucose is caused by substances which are not sugars. The reducing value of these substances is subtracted from the total reducing value of the urine, giving the value for sugar by difference. It has been found that the quantity of glucose added must be approximately equal to the amount destroyed by heating with alkali, otherwise an error is introduced.

That glucose when present in small amounts can be completely destroyed by heating with alkali has been carefully proved, using the sensitive copper and phosphomolybdic reagents used by Folin and Wu[3] for the determination of blood sugar. With

[3] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

these reagents it was found that heating 1 cc. of a solution of 1 mg. of glucose with 1 cc. of 3 per cent sodium hydroxide solution for 15 minutes destroyed all but 2 to 3 per cent of the sugar. Heating for 20 minutes destroyed all of the sugar. In practice the author heats the urine with alkali for 15 minutes and uses diluted urine when the reduction due to both sugar and other reducing substances is equivalent to more than 1.5 mg. The reduction due to sugar alone is usually about 60 per cent of the total reduction.

That the material in normal urine destroyed by heating with alkali is mostly sugar has been proved by fermenting urine with yeast. The yeast treatment is as follows: 50 cc. of urine together with a little paraffin and a few glass beads are weighed in a 100 cc. Erlenmeyer flask, acidified with a drop or two of glacial acetic acid if not already acid, and boiled for 5 minutes to remove dissolved toluene. The flask is cooled and water is added to restore the original weight after which the contents are well mixed with half a cake of Fleischmann's compressed yeast. The flask is stoppered and kept in an incubator at 32-33°C. for about 20 hours. Blanks containing pure glucose were fermented occasionally. It was found that with a glucose concentration of 1 mg. per cc. all the glucose was removed.

Table I shows the reduction given by unfermented urine before and after heating with alkali and by fermented urine before and after heating with alkali. A correction has been made for the water content of one-half a cake of yeast. This was found to amount to about 4.5 gm.

These results show plainly that normal urine contains something that reduces dinitrosalicylic acid, that is readily destroyed by heating with sodium hydroxide, and that is largely fermented by yeast. The results show also that the reducing material in normal urine which is not destroyed by heating with alkali is not fermented by yeast.

Shaffer and Hartmann[4] have recently published a paper in which they claim that normal urine contains little or no fermentable sugar. We are obliged to disagree with this statement.

Preparation of 3, 5-Dinitrosalicylic Acid (the Author's Method).—Mix 75 gm. of concentrated sulfuric acid with 15 gm. of concen-

[4] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 365.

trated nitric acid and cool in ice water. When well cooled add with shaking 15 gm. of salicylic acid in small portions at a time, keeping the preparation well cooled. When all the salicylic acid has been added the material is poured into about 800 cc. of water, cooled for some time, and filtered off by suction. The crystals of dinitrosalicylic acid are dissolved by heating in dilute sodium carbonate solution, filtered, and salted out by adding a large excess of saturated sodium carbonate solution, followed by cool-

TABLE I.

Percentage Reduction of Normal Urine.

Urine No.	Before fermentation.		After fermentation.	
	Total reduction.	After heating with alkali.	Total reduction.	After heating with alkali.
1	0.041	0.022	0.022	0.025
2	0.106	0.061	0.065	0.061
3	0.074	0.032	0.034	0.032
4	0.088	0.031	0.044	0.029
5	0.126	0.050	0.061	0.050
6	0.165	0.065	0.087	0.057
7	0.120	0.046	0.053	0.055
8	0.090	0.040	0.053	0.042
9	0.232	0.083	0.123	0.080
10	0.087	0.033	0.034	0.033
Average....	0.113	0.046	0.058	0.046

Total sugar..... 0.067 (0.113–0.046)
Fermentable sugar..... 0.055 (0.113–0.058)
Unfermentable sugar..... 0.012 (0.058–0.046)

ing. The sodium salt is filtered off by suction and washed with saturated sodium carbonate solution. The material is now dissolved in hot water, filtered, and precipitated as the free acid by adding a large excess of strong hydrochloric acid. After cooling, the dinitrosalicylic acid is filtered off, pressed, recrystallized once from a small amount of boiling water, and dried to constant weight at 100°C.

Preparation of Dinitrosalicylate Solution.—Dissolve 2 gm. of dinitrosalicylic acid in about 70 cc. of hot water with the aid of 10 cc. of 20 per cent sodium carbonate solution. The solution is cooled and made up to 100 cc. volume.

Sodium Hydroxide Solutions.—Two solutions of sodium hydroxide are required, containing 1.5 and 3.0 per cent of sodium hydroxide, respectively.

Glucose Solutions.—Solutions containing 1 and 0.5 mg. of glucose per cc. are prepared and preserved with toluene.

Method.

Pipette 1 cc. of urine into a 1.5 by 15 cm. test-tube; add 1 cc. of the 2 per cent dinitrosalicylate solution and 2 cc. of the 1.5 per cent sodium hydroxide solution. Mix, plug with cotton, and heat in boiling water for 5 minutes. Cool and dilute to 25, 50, or 100 cc. volume according to the depth of color. Mix and compare in colorimeter against standard. If the sugar content of the urine is greater than 0.4 per cent the test must be repeated with diluted urine. If the unknown solution contains an appreciable quantity of precipitate it should be centrifuged.

The above procedure gives with normal urine results that are too high because of the reducing action of uric acid and polyphenols. The reduction due to these substances is determined as follows: Pipette into a test-tube 1 cc. of urine and 1 cc. of 3 per cent sodium hydroxide solution. Mix, plug with cotton, and heat in boiling water for 15 minutes. If the total reduction has amounted to more than 1.5 mg. per cc. calculated as glucose, diluted urine must be used. After heating for 15 minutes cool and add 1 cc. of a solution containing either 1 or 0.5 mg. of glucose, according to whether the total reduction of the urine was more or less than 1 mg. per cc. calculated as glucose. Add 1 cc. of 2 per cent dinitrosalicylate solution, mix, plug with cotton, and heat for 5 minutes in boiling water, proceeding as above. After subtracting from the result obtained the amount of glucose added the remainder will be the value for the uric acid and polyphenols. This last is subtracted from the original reducing value of the urine and gives as the difference the value of the sugars. With diabetic urine this procedure is not necessary unless the sugar content is low.

Standard.—Heat 1 cc. of a solution containing 1 mg. of glucose with 1 cc. of the dinitrosalicylate solution and 2 cc. of the 1.5 per cent sodium hydroxide solution for 5 minutes; cool, dilute to 25 cc. volume, and mix. This standard will keep for several hours.