CXXIX. A COLORIMETRIC METHOD FOR THE ESTIMATION OF SUGAR IN BLOOD.

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INTRODUCTORY.

WITH the recent introduction of insulin in the treatment of diabetes, the necessity for methods of determining accurately the sugar in small quantities of blood has increased. For clinical purposes, colorimetric methods have found favour, owing to their reasonable accuracy and comparative ease of manipulation. Most of the methods which have been used for the colorimetric estimation of sugar in blood have involved the production of colour by the reduction of some aromatic organic compound.

Wacker [1910] proposed *p*-phenylhydrazinesulphonic acid and caustic soda as a colorimetric reagent, whilst Lewis and Benedict [1915] employed picric acid and sodium carbonate as a reagent, the glucose in the blood, if of sufficient concentration, reducing it to the highly coloured salt of picramic acid at a temperature of 100°. Pearce [1915] showed that the reduction could be carried out in more dilute solution, if the higher temperature of an autoclave were employed.

Although the Lewis-Benedict method found great favour at first, and displaced most of the older methods, it was soon recognised that it gave results higher by about 30–50 %, than those obtained by other methods. Hunter and Campbell [1917], de Wesselow [1919] and others have shown conclusively that the method is unreliable on the following grounds:

1. Picric acid is readily reduced, under the conditions used, by substances in the blood other than sugar. Thus it is reduced by such substances as creatine, creatinine, and uric acid, so that the results are higher than those obtained by a titrimetric method devised by MacLean [1919]. This would not be of so much importance as would at first appear, if the results were consistently high, but the quantity of these other reducing substances in blood varies probably as much as the sugar content.

2. The colorimetric comparison is reliable only when the intensities of the tints employed are approximately the same.

Sumner [1921] introduced 3–5-dinitrosalicylic acid as a new reagent for the colorimetric determination of sugar in urine. He pointed out that, unlike

picric acid, this substance is not reduced by creatine, creatinine, or acetone, but that it is reduced by uric acid and other urinary constituents.

It has been found that the sodium salt of dinitrosalicylic acid is a sensitive reagent for the colorimetric estimation of sugar in blood, and it will be shown that the errors due to other reducing substances present are for practical purposes negligible. Moreover, it has been found possible to work accurately with 0.5 cc. of blood, even when carrying out the colour comparison in 25 cc. Nessler cylinders.

The reducing action of creatinine was tested carefully. In some experiments the colour produced under standard conditions by 1 mg. of glucose, was compared with that obtained with the same weight of glucose and ten times the maximum quantity of creatinine likely to be present in 1 cc. of blood. The increase in the results due to the presence of creatinine was not more than 4 %.

In a similar series of experiments the maximum amount of uric acid to be found in 1 cc. of human blood was used. The colour obtained with 1 mg. of glucose and this quantity of uric acid, was never more than 2 % deeper than that obtained from 1 mg. of glucose alone.

It was found essential to compare colours of approximately the same depth of tint, that is, within 25 % of each other, but it is advisable to have them as nearly equal as possible. When the coloured solution was examined by means of a spectrometer it was found that there were no well-defined absorption bands in the visible spectrum but that the solution cut out the whole of the violet and blue and part of the blue-green. As the solution was diluted it cut out less and less of the blue-green and blue.

Lastly, it may be added that unlike many of the other colorimetric methods, no error due to back oxidation through atmospheric oxygen, is involved.

EXPERIMENTAL.

Preparation of 3-5-dinitrosalicylic acid. In the preparation of dinitrosalicylic acid, the nitration of salicylic acid was carried out as recommended by Sumner, although the purification was effected in a slightly different manner. 75 g. of concentrated sulphuric acid were mixed with 15 g. of concentrated nitric acid and the mixture cooled to 0°. 15 g. of dry powdered salicylic acid were added gradually, the mixture being kept well cooled. The whole was poured into water, and after solidification, filtered and washed once with a little cold water. The purification was not carried out by the method of Sumner involving the preparation of the sodium salt, as it was found possible to free the dinitrosalicylic acid from accompanying mononitro acids by extracting the mass with cold water. On evaporating down the filtered extract, crystals of pure dinitrosalicylic acid separated out. The crystals were then dried at a moderate temperature, and their purity determined by their m.p. (173°).

Preparation of reagents. Three standard solutions are required in addition to "dialysed iron" and saturated sodium sulphate solution. It is doubtful whether dialysed iron can be obtained free from chlorine. Specimens of

dialysed iron (B.D.H., B.P. strength) were found to contain appreciable amounts of chlorine, although they gave no precipitate with silver nitrate. In testing for chlorine the following procedure was adopted. To the dialysed iron a few drops of a solution of silver nitrate in dilute nitric acid were added. The solution was then made alkaline with ammonia, and the precipitated ferric hydroxide filtered off. The filtrate invariably gave a precipitate of silver chloride when acidified with nitric acid. All attempts to obtain dialysed iron free from chlorine led to the precipitation of the colloid, before a negative test for chlorine could be obtained. Since the dinitrosalicylic acid gives a red colour with ferric chloride, this is of importance, as the possibility of this affecting the colorimetric estimation of the sugar in the blood filtrate must be guarded against by the use of an excess of alkali in the colour reaction. The three standard solutions are: (1) 3 % caustic soda solution; (2) glucose solution. It is convenient to make up a solution from pure glucose, containing about 2 mg. per cc., which can be standardised accurately against Fehling's solution. Such a solution keeps unchanged for a long time, in the presence of toluene, and can be diluted 1:2 or 1:4 according to the intensity of colour required in the standard. (3) Dinitrosalicylic acid solution. This is prepared according to the directions of Sumner. 2 g. of dinitrosalicylic acid are dissolved in about 70 cc. of hot water containing 10 cc. of 20 % sodium carbonate solution. The solution is cooled and made up to 100 cc. If the laboratory temperature falls too low, the salt tends to crystallise, but the crystals redissolve on warming.

Procedure. 1 cc. of blood is measured carefully by means of an accurate pipette into a 50 cc. flask. The pipette is rinsed free from blood with distilled water into the flask. 2 cc. of saturated sodium sulphate solution are added, and sufficient water to make up the volume to about 25 cc. The flask is heated for a few minutes in a boiling water-bath, until the protein is coagulated, when 10 cc. of dialysed iron (sp. gr. 1010) are added. The flask is cooled and the volume made up to 50 cc. with the addition of one drop of alcohol to disperse the froth. The liquid is filtered through a Whatman filter paper, and the filtrate used for the estimation.

25 cc. are measured out and run into a 50 cc. beaker, which is suspended by means of a ring, in a boiling water-bath, until all the water is evaporated. The white residue is washed down from the sides of the beaker with 1 cc. of distilled water, and 1 cc. of 3 % caustic soda solution, after which 1 cc. of the dinitrosalicylic acid solution is pipetted into the beaker. The beaker is immersed for exactly five minutes in a boiling water-bath, and the resulting solution quickly diluted with cold water, and filtered through glass wool into a Nessler cylinder. The glass wool is washed free from colour, and the filtrate made up to a suitable volume (50 cc.). Blank experiments showed conclusively that there was no absorption of colour during the filtration through glass wool.

The standard, which may be prepared simultaneously, is obtained in the following way: 1 cc. of glucose solution containing 0.5 mg. of glucose is measured into a similar 50 cc. beaker; 1 cc. of caustic soda solution and 1 cc. of dinitro-

salicylic acid solution are added, and the beaker immersed for five minutes in a similar boiling water-bath. The solution is diluted and made up to 50 cc. in a Nessler cylinder as before, omitting the filtration.

If the sugar is present in the blood to a normal extent, the two solutions are comparable in tint, and the colours can be compared in the usual way, by removing a measured quantity of the solution with the deeper tint, until the tints are matched. When carrying out a large number of estimations, it is convenient to arrange the standard in such a manner that its tint is always slightly lighter than that of the unknown solution; so that the same standard can be used many times. If the percentage of sugar in the blood be higher than normal, it is convenient to prepare the standard from 1 cc. of glucose solution containing 0.1 % of glucose. If the blood contain more than 0.3 % glucose, it is best to dilute to 100 cc. as the tint is then too deep at a dilution of 50 cc. for accurate comparison. The standard is then prepared from 2 mg. of glucose. A series of standards may be prepared, and can be kept for at least two days without any visible change of colour. When an estimation is carried out with 0.5 cc. of blood, the volume should be made up to 25 cc., and 12.5 cc. evaporated, after which the procedure is the same. The colours can be compared accurately in suitable 25 cc. Nessler cylinders.

Effect of reducing substances other than sugar present in blood.

1. *Creatinine*. 1 cc. of glucose, containing 1 mg. of the sugar, was heated for five minutes with 1 cc. of caustic soda and 1 cc. of dinitrosalicylic acid solution, and the colour compared with that obtained in a similar experiment wherein 2 mg. of creatinine were added. When the tints of the two solutions were compared in Nessler cylinders, it was found that that of the solution containing the creatinine was deeper only to the extent of 4-5 %. This value was never exceeded in a number of experiments, and therefore the error due to creatinine is negligible.

2. *Uric acid*. A solution containing 0.35 g. of uric acid per litre was made with the addition of a little caustic soda. 1 cc. was measured out into a beaker and evaporated to dryness. 1 mg. of glucose was then added, and the colour obtained by the ordinary procedure compared with that obtained by the use of 1 mg. of glucose alone. The following figures represent the number of cc. of the two solutions when matched after making up to 50 cc.

0.35 mg. uric acid + 1 mg. glucose	1 mg. glucose
cc.	cc.
48	50
49	50
49	50
49.5	50

Average difference, 2 %.

EXAMPLES OF SUGAR ESTIMATIONS IN BLOOD.

The following are some of the results obtained by the above method with different specimens of blood, all of which were laked with sodium fluoride.

Source of blood		Result with		
		2 cc.	1 cc.	0.5 cc.
Ox	1	0.070 %	0.076 %	—
	2	0.069 %	0.073	—
Normal human	3	—	0.10	—
		—	0.094	—
" "	4	—	0.088	—
		—	0.090	—
" "	5	—	0.078	—
		—	0.082	—
Diabetic human	6	—	0.138	0.142 %
		—	0.134	0.138
" "	7	—	0.121	—
		—	0.119	—
" "	8	—	0.132	—
		—	0.138	—
" "	9*	—	0.571	0.570
		—	0.565	0.580

* This specimen was obtained from a patient suffering from acute glycosuria.

Distribution of glucose between serum and corpuscles in ox blood.

A series of estimations of reducing sugar in ox blood, and in the serum derived from the blood, were carried out in order to determine the distribution of the sugar between the serum and the corpuscles. With fresh ox blood and the serum derived from it, the following results were obtained:

	Blood	Serum from blood
1	0.071 %	0.062 %
2	0.067	0.060

After keeping the blood and the serum under aseptic conditions for a fortnight, it was found that the sugar content of the serum had diminished to a very low figure, whereas there had been little change in the sugar content of the blood.

Percentage of glucose in the two specimens of ox blood after two weeks.

	Blood	Serum
1	0.065	0.01*
2	0.063	0.02*

* Too small for accurate estimation.

This result was confirmed by a further experiment. It would seem then, that the conversion of reducing sugar to non-reducing polysaccharide proceeds rapidly in the absence of the corpuscles, which probably retard this change.

With the above method something like half a dozen determinations can be easily carried out in two hours, provided that there is accommodation for evaporating the filtrates simultaneously in separate beakers. It may be added that the evaporation process, which may be regarded by some as an objectionable feature of the method, takes less than three-quarters of an hour, and, given

a suitable water-bath, does not require attention. If desired, the time may be further curtailed by making up in the first place 1 cc. of blood to 25 cc. instead of 50 cc. -

It is doubtful whether the measurement of small quantities of blood in a pipette is desirable on the score of accuracy, as the blood adheres slightly to the glass surface and cannot be removed readily by washing with cold water. It is probable that the accuracy of blood sugar estimations would be materially increased if the sugar concentration were expressed in grams per cent. instead of per 100 cc.

Finally I must associate myself with many others in the opinion that the determination of the concentration of reducing sugar may not be an absolute guide in the treatment of diabetes, as it leaves out of account the significance of the non-reducing carbohydrate known to be present in the blood. There is no doubt that an intimate relation exists between the reducing and the non-reducing carbohydrates. The latter have so far received little attention, and it is proposed to study the problem in the near future.

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