CIX. ESTIMATION OF SUGAR IN THE BLOOD.

BY EDWIN GEORGE BLEAKLEY CALVERT.

Assistant, Medical Clinical Unit, St Mary's Hospital, W.

(Received June 23rd, 1924.)

THE object of this paper is briefly (1) to describe a method of estimating the sugar-content of the blood—a method that is accurate and yet so simple as easily to be carried out by medical practitioners; (2) to reply to Stanford and Wheatley's criticism [1924] of my method of estimating the blood-sugar [1923].

First of all let me say that I am indebted for this criticism in that it has led to a profitable re-examination of the subject; their findings, however, have in no way invalidated the accuracy of the method. The points to which Stanford and Wheatley chiefly direct attention are:

(a) That in working with duplicate standards (glucose solutions) made at the same time, and using the Folin and Wu method [1919, 1920] or my technique, results were obtained that often varied very considerably one from another.

(b) That my contention that, in dealing with the small amounts of glucose used in the estimations, copper is not quantitatively reduced is fallacious and due to the relative concentration of alkaline copper tartrate being considerably lower than that employed by Folin and Wu and by them.

These workers attribute the diversity of results obtained with duplicate standards to the different temperatures at which the blue colour is produced by the addition of phosphomolybdic acid. They state that it is impossible to obtain concordant figures when the reaction is carried out in the "hot," as the temperature of the last of a batch of tubes is bound to be very much lower than that of the earlier ones. This difficulty does not arise if the tubes are put into the boiling water-bath at 20–30 seconds intervals and each one removed therefrom at the end of 6 minutes and the phosphomolybdic acid added immediately. I always do this and do not fail to get accurate results.

Experiment to show the effect of cooling in the air before adding the phosphomolybdic reagent. 5 cc. of the same weak glucose solution were used in each instance; also 2 cc. of the copper solution and 2 cc. of the phosphomolybdic acid.

Tube No.	Interval (minutes) before adding the reagent	Colorimeter readings against a fixed standard	Readings half- an-hour later
1	0	12.6	12.7
2	1	12.7	12.6
3	2	12.7	12.8
4	3	12.8	12.8
5	4	13.0	12.9
6	5	13.9	13.8
7	7	15.1	15.0

The readings, verified by a number of workers in the laboratory, were taken in each case about 5–10 minutes after the formation of the blue colour. In the case of the first four tubes, full development of colour had taken place within this period, but with the remaining tubes a longer time was required, e.g. about half an hour for the 7 minute tube. Therefore, in using the quantities and the method I employ, no error is introduced by carrying out the reaction in the "hot."

As regards their second point, I would remind them that the relative concentrations of alkaline copper tartrate and sugar in my standard [1923] are the same as those of Folin and Wu, but the absolute concentrations are of course much weaker. I have, however, increased the strength of the copper solution ($\times 1.75$) so that its concentration, when mixed with the 5 cc. sugar solutions, is equal to that in Folin's method and have repeated the experiments described in the construction of my "curve of correction" [1923]. A copper solution stronger than this has also been employed—one twice the strength instead of 1.75 times. All the estimations have been carried out both in the "hot" and at the 20° recommended by Stanford and Wheatley.

The double-concentration copper solution may be dismissed with the remark that it gave the same results as the "1.75" copper solution (hereafter referred to as "the copper solution").

It was found that the reduced copper solution (7 cc. in vol.) was lowered in temperature to 20° by immersion in cold water for $3\frac{1}{2}$ minutes or less, depending on the temperature of the water; running water will considerably shorten this period. However, I have cooled the solutions in the way Stanford and Wheatley suggest. Some interesting results have emerged from these experiments which will now be described.

In the way indicated in my paper [1923] a duplicate series of 5 cc. solutions, containing amounts of the purest dry glucose ranging from 0.025–0.800 mg., were prepared. 2 cc. of the copper solution were then added and two tubes of equal sugar concentration were simultaneously placed in a vigorously boiling water-bath. At the end of 6 minutes 2 cc. of phosphomolybdic acid were added to one immediately, while the other was cooled to 20° by immersion in cold water for $3\frac{1}{2}$ minutes before the addition of the acid. Both solutions were then made up to the 12.5 cc. mark. On holding them up to the light, the contrast in colour is very striking. The one formed in the "hot" is a pure blue (or a blue with a slight indigo tint), while the "cooled" solution is a blue with a marked greenish tinge in it. It is impossible to match the colours in the colorimeter. Now the first set of colours (the "immediate" colours) can be at once matched easily and accurately in intensity, and also in tint, with my fixed standards in glass. The readings remain the same for at least a couple of hours and then slowly have to be increased to maintain the match. On the other hand, the colours produced by the cooling method change slowly in the deep solutions and comparatively rapidly in those of light intensity, until the colour produced in the hot solution is reached. Thus in the case of two 5 cc.

solutions each containing 0.10 mg. of glucose, the "cooled" one will change from its greenish-blue colour to that of the practically stable "immediate" blue of the "hot" solution in about 20 minutes, while the colour of a "cooled" solution containing 0.80 mg. will require about 2-2½ hours to develop the "immediate" colour. The interesting feature is that the solutions are then equal in colour-intensity. This shows that the degree of oxidation that occurs during a short period of cooling in suitably constructed boiling-tubes may be considered as negligible. The cooling process introduces a time-consuming factor (the slow development of colour) and is apt to lead to error. It is better to treat the solutions in the "hot," avoiding any possible inaccuracy due to oxidation by spacing the times of heating as already indicated. It is worth noting that the colour of the water-cooled solutions may be changed rapidly to that of the "hot" solutions by re-heating in the water-bath. Further, the greenish tint does not seem to occur with similar short periods of air-cooling, presumably because the lowering of temperature is much slower and therefore much less pronounced.

Curve of correction for "found" values. The results of the experiments referred to above show that, using a colorimeter of the Kober or Duboscq type and the new copper solution, quantitative findings are not obtained except for solutions only a little removed in concentration from that of the standard. [See *Table of Correction*, p. 844.] The estimations have been repeated many times and the utmost care exercised on every occasion.

The effect of multiplying the strength of the original copper solution by 1.75 has been to increase the intensity of colour developed for any given amount of glucose. This has enabled me to use a standard containing a smaller quantity of glucose, namely, 0.15 mg. in 5 cc. of distilled water, and has also permitted of the employment of as small an amount of blood as 0.16 cc. The curve is also less divergent from the line of quantitative reduction.

A SIMPLE AND ACCURATE METHOD OF ESTIMATING THE SUGAR-CONTENT OF BLOOD: SUITABLE FOR MEDICAL PRACTITIONERS.

To avoid the expense of the torsion balance and platinum capsule of my previous method, a pipette has been introduced for the collection of the blood. The pipette is similar to that of MacLean; it is smaller and calibrated to measure 0.16 cc. The nose of the pipette is longer and narrower in lumen, while the bore of the stem is also much finer. These modifications enable one readily to collect the blood by means of suction and for this purpose a piece of rubber tubing is attached to the pipette. It is important that the bulb of the pipette be spindle-shaped in order to prevent the trapping of air. The pipettes must be kept thoroughly clean and dry; this can be accomplished by washing successively with water, absolute alcohol and ether, and then passing dry air through them. Clotted blood may be removed by the use of hot nitric acid.

The 0.16 cc. of blood is easily obtained from a single prick of the finger or thumb, preferably the latter, as the blood is less liable to overrun its broad dorsal surface. Swinging the arm and the application of a piece of rubber tubing around the base of the digit ensure an ample supply of blood. *Without delay* the specimen is washed out of the pipette into 7.3 cc. of distilled water which is contained in a short wide test-tube (say $1'' \times 3\frac{1}{2}''$).

Before proceeding further, it is necessary to describe the other apparatus required¹:

(1) Several special resistance-glass boiling-tubes with 7 cc. bulbs and graduated for 12.5 cc.

(2) Pipettes of the following capacities: two 1 cc. pipettes (in $\frac{1}{10}$ ths) for the tungstate and H_2SO_4 solutions; two 2 cc. ordinary bulb-pipettes for the copper and phosphomolybdic acid solutions; one 5 cc. pipette for the blood filtrate.

(3) 25 cc. burette.

(4) Water-bath (fairly large).

(5) Whatman starch-free filter papers, No. 30 (7 cm. diameter).

(6) The following solutions:

(a) 10 % sodium tungstate.

(b) $2N/3 \text{ H}_2\text{SO}_4$.

(c) *Copper Solution.* Dissolve 70 g. pure anhydrous sodium carbonate in about 500 cc. distilled water in a litre flask. Then add 13.125 g. tartaric acid, and when the latter has dissolved and effervescence has ceased, add 7.875 g. pure crystallised copper sulphate; dissolve without the aid of heat and make up to one litre with distilled water. Keep in a dark-coloured bottle. If any sediment occurs during the first couple of weeks, then transfer the clear solution to another bottle.

(d) *Phosphomolybdic acid solution.* 35 g. pure molybdic acid are dissolved in 200 cc. of 10 % NaOH and 200 cc. of water added. The whole is now boiled for 20–40 minutes, or longer, until all traces of ammonia have been driven off. Cool and dilute to about 350 cc. with water and then add 125 cc. of phosphoric acid (85 % strength) and water to 500 cc.; 2 cc. of this solution when added to 2 cc. of the copper solution should produce complete decolorisation.

¹ The entire outfit may be obtained from Baird and Tatlock (London), Cross St, Hatton Garden, E.C.

(e) *Stock sugar solution.* Dissolve 4 g. of absolutely pure powdered glucose in 1 litre of distilled water, a few drops of toluene being added as a preservative.

The *Standard sugar solution* is prepared by placing 7.5 cc. of the stock solution in a litre volumetric flask and making up to the mark with distilled water. 5 cc. of this solution contain 0.15 mg. glucose.

Solutions (a), (b), (c), (d) seem to keep indefinitely while the stock sugar solution will, with ordinary care, remain unaltered for several months.

(7) A reliable colorimeter. For use in general medical practice this need not be an expensive one. Indeed, with a little practice one can tell with the *naked eye*, from the depth of blue colour developed (to be described presently), whether the blood-sugar content is normal or not; for comparison the colour derived from a normal fasting blood or from a sugar solution of known strength (e.g. 0.1 mg. in 5 cc. water) may be employed.

Technique of estimating the blood-sugar. This will be dealt with briefly as details are to be found in my paper referred to.

(a) To the solution of 0.16 cc. blood in the 7.3 cc. distilled water are added 0.3 cc. 10 % sodium tungstate and then 0.3 cc. $2N/3$ H_2SO_4 . This makes the volumes up to 8 cc. (0.1 cc. being allowed for blood, less evaporation during experiment). A rotatory movement of the test-tube mixes the solutions which turn to a chocolate colour in a couple of minutes. The proteins have now been completely coagulated. The solution is next filtered through the 7 cm. Whatman filter-paper; this must be done carefully in order to avoid the escape of protein, which event would spoil the estimation.

(b) 5 cc. filtrate are pipetted into a boiling tube, 2 cc. of copper solution added, and the tube placed for exactly 6 minutes in a vigorously boiling water-bath. The addition of 2 cc. of phosphomolybdic acid solution immediately on withdrawing the tube from the bath produces the blue colour. The standard blue colour for comparison is obtained by treating similarly 5 cc. of the *standard sugar solution*. After standing for about 4 minutes the solutions are cooled under the tap and made up to the 12.5 cc. mark with distilled water. Inversion of the tubes mixes the solutions, which are then compared in a colorimeter. Reference to my paper [1923] will show how the trouble of making standard solutions may be avoided by employing glass discs which match the standard blue. The "7.5 B" discs equal in colour intensity the standard blue solution set at 13.7 mm. reading, and as the level is optional, within certain limits, at which one may set the standard the use of the discs means a considerable saving in time and labour. The 13.7 figure has been obtained with the purest glucose and this invests the discs with a further advantage when glucose solutions are of doubtful quality.

An example of the calculation of results: 0.16 cc. sample of blood taken from a normal person (fasting). The colorimeter reading of the unknown blue solution when matched against the standard blue solution, set at 13.7 (or, what is equivalent, against the discs) was found to be 20.3.

The standard colour or the discs (i.e. 13.7) represents 0.15 mg. glucose, therefore the unknown (or 5 cc. of blood filtrate)

$$= \frac{0.15 \times 13.7}{20.3} = \frac{2.055 \text{ (a constant factor)}}{20.3} = 0.101 \text{ mg. glucose.}$$

Now 5 cc. of blood filtrate

$$= \frac{5}{100} \text{ amount of blood taken (0.16 cc.)} = 100 \text{ cmm.,}$$

∴ 100 cmm. blood contain

$$= 0.101 \text{ mg. glucose (uncorrected),}$$

∴ normal "fasting" blood-sugar

$$= 0.102 \% \text{ (corrected from Table).}$$

Table of Correction.

Values mg. glucose		Values mg. glucose		Values mg. glucose	
Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
0.020	0.029	0.130	0.130	0.340	0.324
0.030	0.040	0.150	0.150	0.360	0.342
0.035	0.045	0.170	0.170	0.380	0.358
0.040	0.048	0.190	0.190	0.400	0.377
0.045	0.052	0.195	0.195	0.420	0.395
0.050	0.056	0.200	0.199	0.440	0.409
0.055	0.061	0.205	0.204	0.460	0.427
0.060	0.065	0.210	0.209	0.480	0.443
0.065	0.070	0.215	0.213	0.500	0.459
0.070	0.075	0.220	0.218	0.520	0.476
0.075	0.080	0.225	0.223	0.540	0.492
0.080	0.085	0.230	0.228	0.560	0.510
0.085	0.089	0.240	0.237	0.580	0.525
0.090	0.093	0.250	0.246	0.600	0.542
0.095	0.098	0.260	0.255	0.650	0.583
0.100	0.102	0.270	0.265	0.700	0.622
0.105	0.107	0.280	0.274	0.750	0.661
0.110	0.111	0.290	0.283	0.800	0.699
0.115	0.115	0.300	0.291	0.850	0.747
0.120	0.120	0.320	0.307	0.900	0.775

These figures may be plotted as a curve which will facilitate the process of correction.

SUMMARY.

The paper consists in

- (a) the description of a method of estimating the sugar-content of blood, so simple and accurate as to appear ideal for general medical practice;
- (b) a refutation of the criticism by Stanford and Wheatley of my article [1923].

REFERENCES.

- Stanford and Wheatley (1924). *Biochem. J.* **18**, 22.
 Calvert (1923). *Biochem. J.* **17**, 117.
 Folin and Wu (1919) *J. Biol. Chem.* **38**, 106.
 ——— (1920) *J. Biol. Chem.* **41**, 367