V. ESTIMATION OF SUGAR IN THE BLOOD.

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THE principal object of this note is to point out a fallacy occurring in this estimation as commonly practised.

As in the course of our experiments we found that certain details must be very carefully attended to if correct results are to be expected, it will be convenient to mention these first.

Four years ago Folin and Wu [1919] published a method for the estimation of blood sugar in which the cuprous oxide formed by reduction of alkaline copper tartrate by the sugar was converted into a deep blue solution by means of a reagent containing phosphomolybdates and phosphotungstates in strongly alkaline solution. This method was criticised on the grounds (1) that the cuprous oxide had opportunity to be partially oxidised by the air before the blue colour was formed; (2) that the blue colour due to any unreduced copper tartrate introduced an error which could not be allowed for; (3) that the phosphotungstic-phosphomolybdic reagent gives a blue colour with the so-called phenols of blood.

To meet these objections Folin and Wu [1920] modified their method (1) by conducting the reduction in constricted test-tubes, thus lessening the possibility of atmospheric oxidation, and (2) by using a new reagent which does not react with phenols, but does give a blue coloration with cuprous oxide. As this reagent is used in acid solution, any error due to unreduced copper tartrate is eliminated. It is this newer method which has been used by numerous recent workers, including Calvert [1923].

In working with it we soon noticed that duplicate standards made at the same time often differed from one another very considerably. It was at first thought that this might be due to the standards not having been prepared absolutely at the same instant, but although we find that the blue colour fades appreciably with time, the difference is not perceptible when the interval of time is small. A standard made one hour after another one is identical with it, but one made three hours afterwards is distinctly darker. This change of tint with time has been noticed by others [Fleury and Boutot, 1923]. In using this method it is therefore necessary to perform the colorimetric measurement within an hour of the time at which the blue colour is produced.

A factor of much greater importance, which turned out to be the explanation of the observed differences, is the temperature of the liquid at the moment when the blue colour is formed. The colour is darker the lower the temperature. Folin and Wu seem to be aware of this, for they recommend that the tubes be immersed in cold water for two or three minutes to bring them to the same temperature before adding the reagent, but they do not emphasise the point as being of the great importance we have found it to be, and many of those who have used the method in one modification or another are in the habit of adding the reagent to the hot solution. It is impossible to obtain concordant results if this be done, for the temperature of the last of the batch of tubes is bound to be very much lower than that of the earlier ones. The length of time for which it is necessary to keep the tube in the cold bath will depend, of course, on the size of the tube, and must be determined for any given quantities by seeing, with a thermometer, how long it takes. We use very small tubes, which at the material time contain only 2 cc. of solution, and with these we find an immersion of $3\frac{1}{2}$ minutes in water at about 20° to be necessary. With larger tubes the time will be considerably longer.

The following experiment indicates the magnitude of the difference caused by different temperatures. Two dextrose solutions containing the same amount (20 cmgm.) of dextrose were treated exactly as described by Calvert [1923], except that in one case the reagent was added immediately after the tube was removed from the boiling water, whilst in the other the addition was made after the tube had been cooled in water at about 20° for five minutes. On comparing in the dilution colorimeter it was found that, taking the "cold" tube as standard (20), the "hot" tube had a value of only 15. The shades of colour were so different as to make more than an approximate comparison impossible. The colour produced at the higher temperature is an indigo blue, quite different from the pure deep blue obtained in the cold.

We can confirm the necessity of using tubes with a constriction, and the results quoted below show that no re-oxidation of cuprous oxide then occurs.

In our hands, therefore, the process takes the following form. The solutions are those described by Folin and Wu in their second paper. 1 cc. of the solution containing dextrose and 1 cc. of alkaline copper tartrate are placed in a small test-tube such that 2 cc. come to the middle of the constriction. A standard is similarly prepared from 1 cc. of pure dextrose solution containing 0.1 mg., or, as we prefer to term it, 10 centimilligrams (abbreviated cmgm.) of pure dextrose. The tubes are heated in a large, vigorously boiling water-bath for six minutes. They are supported in a cage of wire gauze made so that they do not actually touch the bottom of the bath. At the end of six minutes the tubes are immersed in a beaker of water at room temperature for $3\frac{1}{2}$ minutes. 1 cc. of Folin's reagent is next added to each tube and, after a pause of two minutes, 3 cc. of water. After mixing, 5 cc. is pipetted off from each tube and compared in the dilution colorimeter. Should the un-

known contain much dextrose, a smaller quantity, such as 2 cc. or 1 cc., is taken for the colorimeter. The following results show the degree of accuracy obtained.

Table I.

Taken	Found	Taken	Found
5.0	5.32	17.5	17.6
7.5	7.52	20.0	21.0
10.0 (standard)	—	25.0	25.0
12.5	12.56	30.0	30.2
15.0	14.8	40.0	40.85

There is for any coloured substance at a given thickness of layer a particular concentration at which the colour comparison is most sensitive. Using a 10 mm. layer we find the most sensitive point is in the neighbourhood of the blue solution resulting from 10 cmgm. of dextrose, and for that reason we use it as standard. Below that concentration the solution pales very rapidly, and this is why the estimation of the 5 cmgm. sample in Table I shows a bigger error than most of the others. This is, however, a case that does not often occur in practice, as by suitably arranging the quantity of unknown material taken it can nearly always be ensured that the unknown solution shall be stronger than the standard. The actual colorimetric comparison then takes place at the concentration of the standard. At this point a difference of 1 % can be detected with certainty, that is, the addition of one drop (0.05 cc.) of water to 5 cc. of solution makes a perceptible difference. This being so, some explanation is required of why some of these results show an error of as much as 5 %. In all cases where the error has exceeded 1 or 2 %, it is such that the amount found is *more* than it should be, and we think the only explanation of these discrepancies is in the occasional contamination of tubes, pipettes or the like with dust of organic origin. In the worst case in Table I the absolute value of the error is only 1 cmgm., and it is easy to imagine enough contamination to account for that. The tubes, pipettes etc. are kept clean with distilled water and dried in a hot oven. Periodically they are treated with chromic acid. Drying with alcohol and ether should be avoided at all costs.

One other observation deserves mention. As Calvert remarks [1923], a mixture of equal volumes of the alkaline copper tartrate and phosphomolybdate solutions should give a colourless liquid. Now, we find that, although the mixture is at first colourless, a blue coloration gradually develops on standing. It is first noticeable after a few minutes, and after an hour or two it is quite marked. We are unable to suggest any explanation for this phenomenon, but the exactness of the results given in Table I shows that, if anything of the kind happens to the unreduced copper tartrate in the course of an ordinary estimation, the error occasioned is too small to matter.

A fallacy in Calvert's results. In his paper already cited, Calvert is chiefly concerned with replacing the standards actually prepared from glucose by suitably selected Lovibond Tintometer glasses. In this way the labour involved

in continually making up fresh standards is avoided. In test experiments with this arrangement, using a series of dextrose solutions of known dextrose content, Calvert observed that the amounts of dextrose found did not agree with the amounts taken. Plotting his results on a curve he found that at concentrations below that of the standard the values found were less than they should be, whilst at concentrations above that of the standard the values found were greater than they should be. The error so involved is very considerable, especially when the unknown solution is much stronger or weaker than the standard. For instance, using Calvert's units of measurement, when 0.05 is found, the real value should be about 0.075, that is, the result is 33 % too low. Similarly when 0.1 is found, the real value is 0.125 (error 20 %). Calvert's standard is 0.2 in his units, and there, for obvious reasons, the value found agrees with the true value. Above that point the divergence steadily increases with concentration. A "found" value of 0.4 corresponds with a true value of 0.34 (error 18 %). A found value of 0.5 corresponds with a true value of 0.4 (error 25 %). A found value of 1.0 corresponds with a true value of 0.7 (error 40 %).

Calvert supposes these differences to be due to the fact that the copper is not proportionately reduced, and his procedure therefore is to convert his "found" values into true values by reference to the curve. The results quoted in Table I above, however, show that the copper *is* proportionately reduced. Indeed, in view of the small quantities dealt with, the agreement is remarkable.

It remains to give an explanation of Calvert's results. As it is more than ten years since one of us [Stanford, 1913] pointed out that *all* colorimeters of the Duboscq type were liable to give very inaccurate results excepting in the special case where the concentrations of known and unknown were nearly equal to begin with, we thought at first that Calvert's curve was merely an elegant demonstration of the errors of his colorimeter, but the figures given in Table II below show that this is not the case. It appears that, in this particular instance, the colorimetric errors [Stanford, 1913] happen to be equal in amount as well as opposite in direction, at any rate in the neighbourhood of the standard. As we happen to have a Duboscq instrument, we made

Table II.

Taken	Found		
	Duboscq colorimeter		Dilution colorimeter
	at 10 mm.	at 15 mm.	
5.0	5.15	5.15	4.95
7.5	7.41	7.50	7.40
10.0 (standard)	—	—	—
12.5	12.8	12.7	12.4
15.0	16.4	14.8	14.8
20.0	21.4	19.8	19.6
25.0	26.3	25.9	25.0
30.0	31.2	30.6	30.9
40.0	41.7	41.7	39.95
50.0	50.0	49.7	49.5

experiments with that. A series of dextrose solutions of known concentration was prepared, ranging from 5 to 100 cmgm. per cc. The colorimetric comparisons of the blue solutions were made

- (a) with the Duboscq colorimeter, using a 10 mm. layer for the standard;
- (b) with the same, using a 15 mm. layer as standard;
- (c) with the dilution colorimeter.

The results are shown in Table II.

Reference has already been made to the important influence of the temperature at which the blue colour is produced on its intensity and shade. To examine the possibilities of this source of error, the experiments shown in Table III were made. The figures in the columns marked 1, 2 and 3 were obtained by following Calvert's procedure exactly, that is, by adding the reagent to the hot solution. The figures in the columns marked 4, 5 and 6 were obtained by the same procedure, excepting that the reduction mixture was cooled to about 20° before the reagent was added. In each case the colorimetric comparison was performed in three ways:

- (a) with Duboscq colorimeter and daylight;
- (b) with Duboscq colorimeter illuminated by a 100 w. half-watt lamp having a piece of "Dalite" glass in front of it;
- (c) with the dilution colorimeter.

Table III.

Taken	Hot			Cold		
	Duboscq daylight	Duboscq Dalite glass	Dilution colorimeter	Duboscq daylight	Duboscq Dalite glass	Dilution colorimeter
100	—	143	148	115	107	113
80	120	111	108	100	94	97
60	86	81	74	79	73	74
40	49	47	47	46	46	43
20	Standard					
Curves	1	2	3	4	5	6

These figures are plotted in Fig. 1. In this diagram the curve marked *TT* is the true curve showing that the values found are the same as those taken. It is therefore a straight line inclined at 45°. The data plotted are those of Table I, and the curve shows that the values found agree with the quantities taken within the limits of experimental error.

The curve marked *CC* is Calvert's curve, transposed from his paper. In between this curve and the straight line will be seen the six curves resulting from Table III. As to these it may be remarked that No. 1, obtained with the Duboscq and daylight, hot, approximates very closely to Calvert's curve.

All the Duboscq curves show that the colorimetric comparison is noticeably affected by the nature of the source of light used, as was to be expected. Actually, the comparisons were more exact with the electric lamp and "Dalite" glass than with genuine daylight.

At this point it still remained to explain the reason for Calvert's curve.

It seemed possible that the time of heating had to do with it. Accordingly,

a series of experiments was undertaken in which the time of heating was increased to 12 minutes, instead of the usual six. The curve numbered 12 (Fig. 1) resulting from these experiments is intermediate between Calvert's

Fig. 1. Dextrose estimations.

curve and the true one, but it shows that the real source of error is not to be found there.

The most striking feature of Calvert's careful work is that, at concentrations above the standard, he always found *more* than he took, whilst at

concentrations *less* than the standard, he found *less* than he took, and yet our experiments shown in Table I prove that the reaction is quantitative.

The only other difference between Calvert's conditions of reaction and those of Folin and of us is in the matter of concentration. In the experiments from which he drew his curve, Calvert (apart from doing it in the hot, more or less) was working with solutions which in respect of both the alkaline copper tartrate and the Folin reagent were very much weaker than those used by Folin and by us.

Experiments increasing the amount of reagent showed no difference from the results of the method as used by Calvert. When, however, the amount of alkaline copper tartrate was increased (in proportion) to that used by Folin and by us, quantitative results were obtained, as shown in Table IV.

Table IV.

Dilution colorimeter Taken	Calvert's standard 20 Found
20	Standard
40	39
60	59
80	80
100	100

This proves that the curve given by Calvert is based on a fallacy and is due to his not having had a sufficient concentration of the alkaline copper tartrate. It would appear that at concentrations in the neighbourhood of his standard the reaction is not quantitative unless a large excess of the alkaline copper tartrate is present. When the relative quantities given by Folin, and used by us, are employed, accurate results are obtained.

Tests of the method in the conditions which we have indicated above show that added dextrose is then quantitatively recovered from a blood filtrate, and also that the content in reducing sugar of a blood filtrate remains sensibly unchanged after two days.

SUMMARY.

1. The blue coloration obtained by the action of phosphomolybdic acid on cuprous oxide in acid solution fades with time.
2. The temperature at which the blue coloration is produced must be the same for the standard and for the unknown to be compared with it.
3. The comparisons must be carried out in a dilution colorimeter.
4. The reacting substances (especially the tartrate) must not fall below the concentrations given by Folin.

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