CLXXII. THE DETERMINATION OF GLUCOSE AND MALTOSE IN AQUEOUS SOLUTION AND IN BROTH AND PEPTONE SOLUTIONS.

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NUMEROUS methods have been worked out by different investigators for the estimation of small amounts of reducing sugars in watery solution and also in biological fluids such as blood and urine. Throughout the work about to be described, a method based on the "cuprous" method of Shaffer and Hartmann [1920] has been used. The method is modified according to the medium in which the sugar is dissolved, and has been extended for use with maltose and with mixtures of maltose and glucose.

Estimation of glucose or maltose in aqueous solution.

The sugar solution is diluted till 0.5–2.0 mg. sugar are contained in 5 cc. 5 cc. quantities are then measured into boiling-tubes and 5 cc. Shaffer-Hartmann reagent added (the composition of the reagent is given on p. 378 of their paper). The tubes are rotated to mix the contents, plugged with cotton-wool and immersed in a bath of briskly boiling water for 40 minutes. After this, they are cooled to room temperature and 10 cc. *N* sulphuric acid are added to each. The contents are stirred and after 2 minutes are titrated with *N*/200 thiosulphate exactly as described by Shaffer and Hartmann.

All estimations are carried out in duplicate and "blanks" are performed on the reagents.

Time of heating. The only significant modification of the Shaffer-Hartmann technique was in the period of heating. It was found that the amount of reduction increased with the time of boiling, rapidly at first and then more slowly. After about 35 minutes the increase is negligible. The chosen period of boiling thus combines a high reduction per mg. of sugar with relative insensitivity to small changes in heating time and hence a uniformity of results. Moreover, maltose reduces rather more slowly than glucose and when estimating mixtures of the two sugars it is desirable to have a standard period of heating which is the same for both.

The results obtained with known solutions of glucose and maltose (B.D.H. A.R. reagents, dried over P_2O_5) are shown in Fig. 1. Both the glucose and the maltose curves are the results of three sets of determinations done at different times and by two different observers. The curve obtained by Shaffer and

Hartmann for glucose is given for comparison. On examination of Fig. 1, it will be seen that our glucose curve has a rather steeper slope than that of Shaffer and Hartmann. This is no doubt owing to the longer period of heating.

Fig. 1. Titration of glucose and maltose in aqueous solution with thiosulphate.

For glucose the relationship between the amount of thiosulphate used up and the amount of sugar present is a linear one within the range 0.3–2.0 mg. glucose. For maltose the relationship is linear within the range of 0.5–2.5 mg. Amounts of maltose greater than 2.5 mg. were not investigated. With very small amounts of sugar, the relationship is no longer linear; presumably some sugar is destroyed by the heat before it can reduce the copper. With the longer period of boiling the amount destroyed is somewhat increased. It is probably not feasible to estimate amounts of sugar less than 0.3 mg. with great accuracy by this method.

Estimation of mixtures of maltose and glucose.

If conditions can be found under which maltose will hydrolyse quantitatively to glucose without producing other reducing by-products, then the analysis of mixtures of maltose and glucose can be carried out by the Shaffer-Hartmann method.

Maltose does not hydrolyse with such readiness as lactose or sucrose. It

was found that in order to obtain complete hydrolysis, the solution must be at least 1.6 *N* acid and the heating time must be at least 35 minutes.

The procedure adopted is to measure 5 cc. of the solution to be hydrolysed into a boiling-tube and to add 1 cc. of 10*N* sulphuric acid. The tubes are plugged and immersed in a bath of boiling water for 40 minutes. After this time they are cooled and one drop of cresol red is added to each. The solutions are now neutralised by the addition of 40 % sodium hydroxide drop by drop, and then just acidified by the addition of *N* sulphuric acid (2 or 3 drops usually suffice). Now 5 cc. Shaffer-Hartmann reagent are added and reduction and titration are carried out as usual. The end-point is slightly more difficult to read in the presence of the indicator but presents no serious difficulty once the observer is familiar with the colour change. Simultaneous estimations are carried out on the sugar solutions without hydrolysis.

The additions of sulphuric acid and sodium hydroxide do not affect the blank value so that one determination of the reagent blank suffices for the hydrolysed and unhydrolysed portions.

This method was tested by estimating a solution of maltose before and after hydrolysis.

Before hydrolysis, maltose recovered = 1.62 mg.

After hydrolysis, glucose recovered = 1.65 mg.

Calculation of the amounts of glucose and maltose in mixtures.

As has been stated above, with both maltose and glucose a certain amount of sugar is destroyed without causing reduction of the copper. In mixtures, the amount of each sugar destroyed is proportional to its concentration in the mixture and the total amount destroyed is 0.23 mg. under the specified conditions.

A computation of the actual amounts of glucose and maltose present is somewhat complex, but to a first approximation the values can be calculated as follows:

Let the number of mg. of maltose present be *X*.

„ „ glucose „ *Y*.

Let *t*₁ cc. *N*/200 thiosulphate be used in titrating before hydrolysis and let *t*₂ cc. *N*/200 thiosulphate be used in titrating after hydrolysis.

Now over the straight portions the slope of the glucose curve corresponds to 8.00 cc. *N*/200 thiosulphate per mg. glucose, and the corresponding figure for the maltose curve is 3.15 cc. per mg. If the straight lines are produced they cut the base line at a point corresponding to 0.23 mg. sugar approximately. If the values of sugar recovered are taken not from the origin but from the point where the lines cut the base line, two simultaneous equations can be obtained. Let these new values be *X'* and *Y'* respectively.

After hydrolysis all the sugar (*X' + Y'*) is present as glucose, therefore

$$t_2 = 8 (X' + Y') \quad (1).$$

Before hydrolysis *X'* is present as maltose, *Y'* as glucose, therefore

$$t_1 = 3.15 X' + 8 Y' \quad (2).$$

t_1 and t_2 are determined experimentally and hence X' and Y' can be found. Now the true values for X and Y can be calculated to a first approximation thus:

$$X = X' + \frac{0.23 \times X'}{X' + Y'}$$

and

$$Y = Y' + \frac{0.23 \times Y'}{X' + Y'}$$

In practice the calculation is not nearly so tedious as it appears to be on paper.

That the formula represents the facts with reasonable accuracy may be seen by the following estimations of a known mixture of maltose and glucose.

Sugar added		Sugar recovered	
Maltose	Glucose	Maltose	Glucose
%	%	%	%
0.300	0.15	0.311	0.151

Estimation of glucose and maltose in peptone water or tryptic digest medium.

Separation of the sugar from other reducing substances. Bacteriological culture media made from muscle digest or peptones present peculiar difficulties when it is desired to estimate the reducing sugar contained in them. For such media contain a large proportion of non-sugar reducing substances which are not completely removed by the ordinary methods of protein precipitation.

Precipitation with phosphotungstic acid removes a larger proportion of these substances than tungstic acid or any of the recognised mercury reagents, but even so the filtrate after precipitation contains 15–20 % of its original nitrogen and has a moderate reducing power. Animal charcoal and Lloyd's reagent do not remove these reducing substances.

Kerly [1931] gives a method for the estimation of glucose in muscle, in which the proteins are first removed and the glucose in the filtrate is then precipitated with copper hydroxide and lime by the method of Van Slyke [1917]. Using the technique described by Kerly, we have repeated this process for two broths A and B containing glucose, with simultaneous estimation of the total nitrogen content at the various stages. The results, corrected for the dilution involved, are as follows:

	Percentage original nitrogen remaining	
	A	B
Original broth	(100)	(100)
Phosphotungstic acid filtrate	15.2	20
Filtrate after 1st copper-lime precipitation	4.6	3.5
Filtrate after 2nd „ „	2.3	2.1

The final filtrate is shown to be relatively free from nitrogenous material, the actual nitrogen content being 0.01–0.014 mg. N per cc. The medium used was a tryptic digest of horse muscle, which is very rich in nitrogenous material. With peptones the percentage nitrogen remaining after the above process would probably be less.

Estimation of the separated sugar. Kerly re-dissolved her "copper-lime" precipitate in acid, boiled it with alkaline tartrate and used the reduced copper to reduce a complex phosphomolybdate mixture with the production of an intense blue colour. This in its turn was converted into a colourless compound by titration with standardised permanganate.

This method was tried by us but found to be inaccurate for the following reasons.

- (1) The instability of the blue compound which necessitates very rapid work.
- (2) The difficulty of reading the end-point accurately when working rapidly.
- (3) The insensitivity of the method.

Attempts to measure the blue colour by means of a photoelectric colorimeter also failed owing to the rapid and eventually complete fading of the colour even when kept in stoppered bottles in the dark.

Shaffer-Hartmann method. Since the Van Slyke method for precipitating sugars involves the addition of copper sulphate, it seemed desirable to use one of the alkaline copper tartrate methods for estimating the sugar. The Shaffer-Hartmann small-scale method was therefore tried. These workers point out the necessity for keeping the concentration of Cu^{++} as low as possible in order to avoid a spontaneous back reaction with conversion of some of the excess cupric to cuprous salts. In Kerly's technique, the amount of cupric ions present in the final filtrate is four times as great as that recommended by Shaffer and Hartmann. We found that the concentration of cupric salt recommended by Kerly can be halved without deleterious results but that further reduction in the amount causes incomplete precipitation of the sugar.

When half Kerly's concentration of copper is used, the concentrations of the tartrate, oxalate and carbonate in the Shaffer-Hartmann reagent must be doubled to allow for the extra concentration of copper. This doubling of the concentration of the reagent proved to have no effect on the amount of reduction caused by the glucose.

The procedure for estimating glucose or maltose in peptone or broth is thus as follows.

Special reagents.

Phosphotungstic acid. 8 % in 1.7 N HCl. The HCl solution is better for this purpose than that in H_2SO_4 as with the latter solution calcium sulphate tends to precipitate.

Alkaline tartrate reagent.

	%
Anhydrous sodium carbonate	8.0
Tartaric acid	1.5
Potassium oxalate ($\text{1H}_2\text{O}$)	3.68
Potassium iodide	1.0
Potassium iodate	0.07

Milk of lime 5 %. 110 g. calcium chloride are dissolved in 500 cc. distilled water and 100 cc. 40 % NaOH added slowly with stirring. The precipitate is separated on the centrifuge, washed twice with 1 litre of water and finally suspended in water to make a volume of 1 litre.

Method.

10 cc. of the broth containing sugar are measured into a boiling-tube and 19.5 cc. phosphotungstic acid and 0.5 cc. 10N HCl added. The contents are stirred, left 2 minutes and filtered through dry paper. 15 cc. of the filtrate are measured into a 50 cc. volumetric flask, and 5 cc. 10 % CuSO_4 are added. 40 % NaOH is added, drop by drop, until the copper just begins to precipitate, then 10 cc. milk of lime are added and the contents made up to the mark. The mixture is left for half an hour, then 40 cc. are measured into a centrifuge-pot and spun for 3 minutes. The precipitate is re-dissolved by the addition of 20 cc. N HCl and the volume made up to 40 cc. 20 cc. are transferred to a centrifuge-pot and 20 cc. of 0.5 N NaOH added. The contents are stirred, left for half an hour and spun. The precipitate is re-dissolved in 5 cc. N HCl and the volume made up to 20 cc. During the above process, the solutions can safely be left in the acid state but should not be allowed to stand longer than the specified time in the alkaline state, and the precipitate should be dissolved immediately after spinning.

For the reduction, 5 cc. of the final solution are measured into each of two boiling-tubes and into two more tubes, which are used as blanks, 0.5 cc. 10 % CuSO_4 and 5 cc. water are measured. To all tubes 5 cc. alkaline tartrate are added and the tubes are plugged and plunged into a bath of boiling water for 40 minutes. They are then cooled, acidified and titrated exactly as for aqueous solutions. To be on the safe side, a small quantity of solid potassium oxalate is added immediately before titration as insufficient oxalate prevents a clear end-point being obtained, and in these cases the calcium present combines with some of the oxalate.

Fig. 2. Titration of glucose and maltose from broth and peptone solution with thiosulphate.

The method was tested for 2 % Difco proteose peptone and also for tryptic digest broth by determining the reducing value of the medium with and without the addition of known amounts of glucose and maltose, and constructing graphs as for glucose in water. The reducing value when there is no added

sugar must be subtracted from the values when sugar is added to obtain comparable graphs.

These graphs are shown in Fig. 2.

Each graph has been constructed from three separate sets of readings, the result of observations by two different experimenters, and in the case of broths on different batches of broth. Though the duplicates do not agree quite so closely as in the glucose-water estimations, there is never any substantial difference between the three sets of results.

It will be seen that glucose and maltose in digest broth and maltose in Difco peptone give practically the same reducing value per mg. sugar as the same sugars in aqueous solution, *i.e.* there appears to be no loss of sugar during precipitation of bases, *etc.*

When glucose in Difco peptone is used, however, the recovery is only about 85 %. Apparently in this case some sugar is lost either in the phosphotungstic acid precipitate or elsewhere. Since the graph has been reproduced from 3 sets of observations, there seems no reason for doubting that this is a true result. It follows that in estimating glucose in Difco peptone solution care must be taken to read from the glucose-peptone curve in Fig. 2. It has not been ascertained whether the low results given by 2 % Difco proteose peptone containing glucose would also be obtained if other peptones were substituted.

Fermentation of the medium.

For peptone solution, the reducing value in the absence of added sugar is very small (0.47 cc. *N*/200 thiosulphate).

For peptone-meat extract or tryptic digest broth, this blank value is much higher. For the maker of culture media, it is of interest to ascertain what portion of this blank value is due to sugar present in the finished medium and what portion is due to non-sugar reducing substances.

This can be ascertained by estimating the reducing value before and after fermentation with yeast.

A sample of baker's yeast is washed three times with a large volume of distilled water to remove any adhering sugar and then pressed. The cake can be preserved moist at 4° for upwards of 14 days without losing its activity. 0.2 % yeast is added to one sample of the medium and incubated at 35–37° for 5 hours (2½ hours proved insufficient). After this period, the reducing value of the unfermented and fermented samples is determined and the difference gives the sugar content of the original broth.

The following results may be of interest:

Type of broth		Glucose equivalent		Glucose in broth %
		Before fermenting %	After fermenting %	
Tryptic digest of horse muscle	1	0.031	0.028	0.003
	2	0.044	0.016	0.028
	3	0.065	0.028	0.037
2 % Difco veal extract	1	0.034	0.018	0.016
	2	0.108	0.034	0.074

It will be noted that the sugar content of the broth is quoted as glucose. As a matter of fact, no experiment was made to determine the nature of the reducing sugar present.

Mixtures of maltose and glucose in peptone and broth.

If the culture medium contains a mixture of maltose and glucose the hydrolysis is carried out immediately after precipitation with phosphotungstic acid.

15 cc. of the acid filtrate are measured into a boiling-tube and 1 cc. concentrated HCl added. The tubes are plugged and immersed in a bath of boiling water for 1 hour. (The longer period of hydrolysis is made necessary by the larger volume of liquid being used.) After hydrolysis, the contents of the tube are transferred to a 50 cc. volumetric flask, the tubes are rinsed with water, the rinsings added to the main liquid and 5 cc. of 10 % copper sulphate are added. 15 cc. of the unhydrolysed phosphotungstic acid filtrate are measured into another 50 cc. flask and the contents of both are put through two "copper-lime" precipitations. Reduction and titration are carried out as usual.

Maltose in broth or 2 % Difco peptone can be recovered quantitatively as glucose after hydrolysis in this manner.

SUMMARY.

1. The method of Shaffer and Hartmann for the determination of small amounts of glucose has been extended to the determination of maltose and of mixtures of maltose and glucose.

2. The method has been modified for the determination of maltose and glucose in peptone and digest culture media.

3. In such media, 0.1 %–0.4 % glucose or maltose can be recovered with an error not exceeding 5 % of the amount estimated.

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