XIV. AN APPLICATION OF THE METHOD OF HAGEDORN AND JENSEN TO THE DETERMINATION OF LARGER QUANTITIES OF REDUCING SUGARS.

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THE method of Hagedorn and Jensen [1923] has become widely used for the estimation of reducing sugar in blood. The method depends upon the fact that potassium ferricyanide is reduced to ferrocyanide when heated in alkaline solution with certain reducing substances. In the Hagedorn and Jensen procedure the ferrocyanide formed in this way is precipitated as the double potassium zinc salt, and the residual ferricyanide is estimated by adding an excess of potassium iodide and acidifying. The ferricyanide is reduced quantitatively by the iodide, and equivalent iodine is liberated according to the equation:

The iodine is titrated with thiosulphate.

This method based on ferricyanide as the substance reduced has an inherent advantage over copper methods which will be referred to later. In the original form it was devised for estimating the reducing sugar in the filtrates from 0.1 cc. samples of blood. The capacity is accordingly very small, the upper limit being 0.385 mg. of glucose. Small volumes of solutions are used, and the essential reactants are present in low concentration so that the introduction of impurities may cause relatively large errors. For the purposes of my own work the range of the original method was inconveniently narrow, and the present modification makes possible the estimation of about ten times as much sugar. The new procedure has been standardised for maltose as well as for glucose. No essential change from the original method has been made, except as regards the concentrations and volumes of the various reagents employed.

The following solutions are used in the modified method. The chemicals used were of the "Analytical Reagent" quality, as supplied by the British Drug Houses, Ltd., unless otherwise stated.

Solution A.

Potassium ferricyanide	...	...	8.25 g.
Anhydrous sodium carbonate	...	...	10.6 g.

Make up to 1 litre with distilled water. This solution should be kept for 2 or 3 days before use and it must be stored in a bottle provided with an opaque jacket. In making the solutions for the standardisation data the potassium ferricyanide was twice recrystallised but as no significant difference is found when the untreated "A.R." product is used, this is not considered necessary.

Solution B.

Potassium iodide	...	...	12.5 g.
Zinc sulphate	...	...	25.0 g.
Sodium chloride	...	...	125.0 g.

Make up to 500 cc. with distilled water. Traces of iodine appear in this solution on storing. This iodine should be removed before using by filtering through two thicknesses of filter paper.

Solution C.

5 cc. glacial acetic acid, diluted to 100 cc. with distilled water.

Solution D.

Soluble starch solution, saturated with sodium chloride. 1 g. Merck's soluble starch in about 20 cc. cold water is washed into 60 cc. of boiling water. This is boiled for 2 minutes, 20 g. sodium chloride are added and the solution is allowed to cool. It is then made up to 100 cc. This solution keeps for several months.

Solution E.

An approximately *N*/75 sodium thiosulphate solution which is used in a 10 cc. burette, graduated into 0.02 cc. divisions. In practice it is found convenient to make up about 10 litres of this solution (containing 3.33 g. sodium thiosulphate per litre) using boiled-out water. It is then stored in a bottle, protected from atmospheric carbon dioxide by a soda-lime tube, and the solution is run through a siphon to the burette. When stored in this way the solution keeps well. At first it should be standardised each day but after the first week it need only be standardised at intervals of 3 or 4 days.

The thiosulphate solution is most conveniently standardised against a potassium iodate solution. This solution is made up by weighing out carefully a quantity of about 0.80 g. of desiccated potassium iodate and making it up to a volume of 1 litre in a volumetric flask standardised at the temperature obtaining. A 5 cc. pipette of slow delivery is standardised by weighing four or five deliveries of water at a known temperature. (The tip should, of course, be drained against the side of the collecting vessel for a standard time.) With this pipette 5 cc. of the standard iodate solution are run into a boiling-tube. To this are added 5 cc. of a 2 % solution of potassium iodide and then it is acidified by the addition of 3 cc. of 5 % acetic acid (solution C). The iodine liberated is titrated with the thiosulphate solution, adding a drop of the

starch solution D when the colour becomes a pale yellow, and titrating to the disappearance of the blue colour. The volume of thiosulphate required when the normality is 0.01333 (*N*/75) is about 9 cc. The value for the normality is found from the expression

$$\frac{\text{g. K iodate per litre} \times \text{vol. of pipette (cc.)}}{35.67 \times \text{vol. of thiosulphate required (cc.)}}$$

The results from this method were found to give exact agreement with the method described by Cole [1928] in which the thiosulphate is standardised against a standard acid.

PROCEDURE FOR DETERMINATION OF SUGAR.

The estimations are carried out in boiling-tubes, 1 × 7 in. Glass bulbs with about an inch of tubing left attached are used as covers for the boiling-tubes.

5 cc. of solution A (alkaline ferricyanide) are pipetted into a boiling-tube, allowing the pipette to drain a standard time. To this are added 5 cc. of the solution whose reducing power is to be determined. (Note: if less than 5 cc. is added, sufficient water is added to make up the difference in volume.) A water blank is now made up by pipetting 5 cc. of solution A into a second tube and adding 5 cc. of distilled water. It is important that any drops of liquid adhering to the walls of the tubes should be mixed in, and this is best done by inclining the tube and slowly rotating it.

The tubes are now covered with glass bulbs and placed for 15 minutes in a boiling water-bath 2 or 3 in. deep. After this they are cooled for 3 minutes in cold running water.

The remaining operations are concerned with the estimation of the residual ferricyanide. 5 cc. of solution B are added. This is conveniently run in from a rapid pipette as the volume need not be precise. A white flocculent precipitate appears and at the same time iodine is set free. 3 cc. of solution C are now added (also from a rapid pipette). The reduction of the ferricyanide is now completed and the iodine liberated is titrated against the standardised thiosulphate.

The titration is carried out in the same boiling-tube. Mixing during the titration is effected by shaking the tube with a circular motion. The titration should be carried out in a regular manner. Thiosulphate is run in slowly until the liquid has a pale yellow colour; 3 drops of the starch indicator are then added and thiosulphate is added drop by drop until the sudden disappearance of the blue colour¹. The end-point is very sharp and it is advantageous to fit the burette with a fine tip which has been coated with paraffin wax as this makes the drops smaller and prevents the thiosulphate from wetting the tip.

¹ An "overshot" end-point may be regained by adding counted drops of iodine (about *N*/75 in KI) from a fine dropping pipette until the blue colour returns, titrating to the end-point with thiosulphate, and deducting from the reading the thiosulphate equivalent of the added iodine which is found by repeating the operation.

The difference between the water blank value (WB) and the reading obtained with the experimental solution (R) gives the thiosulphate equivalent of the ferricyanide reduced by the experimental solution. These reduction values have been determined for different amounts of added reducing sugars, maltose and glucose, and the results are shown in the standardisation graph, Fig. 1. For convenience in calculation the results are given in terms of $N/100$ thiosulphate, although approximately $N/75$ is used in the titration. Thus if the normality of the thiosulphate used for a particular estimation is 0.01351, then the $N/100$ thiosulphate equivalent of the ferricyanide reduced is $(WB - R) \times 0.01351$ cc. The amount of sugar corresponding to this reduction is either read from the graph, or in the case of maltose it may be calculated from a factor.

In practice the water blank value need be determined only once or twice a day. Estimations can be done conveniently in batches of five or six and in this way it is possible to make 18 estimations an hour when facility in the technique has been acquired.

Fig. 1.

The heating period for reduction.

In order to follow the reduction after different periods of heating, five boiling-tubes were set up each containing 5 cc. of solution A and 5 cc. of a maltose solution containing 2.5 mg. These were placed in the boiling water-bath and allowed to remain for 5, 10, 15, 20, and 30 minutes respectively. On removal each tube was cooled for 3 minutes and the residual ferricyanide was estimated in the usual way. The results are given in Fig. 2 in which the amounts of ferricyanide reduced ($WB - R$) are plotted against time of heating.

It is evident that during the first 15 minutes most of the reduction has taken place. Actually the ferricyanide reduced at 20 minutes is only 1.5 % more than at 15 minutes (with this amount of sugar present). The 15-minute period was adopted as the standard heating time.

THE STANDARDISATION GRAPHS.

Maltose.

The maltose used was a sample of Kahlbaum's pure maltose which had been recrystallised three times from 60 % alcohol and then dried in warm air. For this specimen I am indebted to Mr S. W. Cole of the Biochemical Institute, Cambridge. When dried *in vacuo* over concentrated sulphuric acid for 2 hours the sugar lost 0.1 % in weight. The optical activity was $[\alpha]_D^{20} + 130.7^\circ$ using a solution of about 9 %. This falls within the range of published values for maltose monohydrate, although it is somewhat greater than the average given by Tollens [1914] as $+ 129^\circ$ – 130° .

Fig. 2.

Owing to the difficulty of preparing anhydrous maltose, this was not attempted, but the data have been obtained from the hydrate and calculated in terms of the anhydrous sugar by multiplying weights of the hydrate by the factor 342/360.

Maltose solutions of known strength were prepared by weighing out quantities of the sugar and making up to a known volume in a standardised volumetric flask. A set of standardised Ostwald pipettes, from 0.5 to 5.0 cc. capacity was used to measure out portions of these solutions into boiling-tubes containing the standard 5 cc. of solution A, and the amount of ferricyanide reduced after 15 minutes' heating was determined in the way described. These data have been used to construct the standardisation graphs (Fig. 1). The reliability of the procedure may be seen from the fact that the points which were obtained from three different maltose solutions all lie very nearly on the same line. The experimental data on which these points are based are given in an appendix.

In the case of maltose the relationship between ferricyanide reduced and the amount of sugar is a linear one. 1 cc. of *N*/100 thiosulphate is equivalent to 0.414 mg. anhydrous maltose over the entire range and it is therefore convenient to employ this factor for the conversion of reduction values ($WB - R$) into maltose.

Glucose.

The glucose used was a sample of "Dextrose A.R." (British Drug Houses). It was desiccated *in vacuo* over concentrated sulphuric acid for 48 hours by which time it was found to have attained a constant weight. The specific rotation of this desiccated sugar was $[\alpha]_D^{20} + 52.2^\circ$ in a 10 % solution. Theoretical value for anhydrous glucose $+ 52.5^\circ$.

The glucose standardisation data were obtained as for maltose. The points from three solutions lie very nearly in the same line, but the relationship is not linear here as it is for maltose. This difference between the two sugars was investigated. The reduction progress curves for small and large amounts of glucose or maltose have different forms. When plotted as percentages of the reduction after 30 minutes' heating against time of heating, the curves intersect: those for maltose at 15 minutes, for glucose at 10. Consequently after the standard heating (15 minutes) the reduction by maltose is proportional to the maltose added, but this is not so for glucose since at 15 minutes the percentage progress curve for a small amount of sugar lies below the curve for a large amount. For glucose then, reduction values are most conveniently converted into sugar values by reading from a graph. Table I gives figures for constructing this.

Table I. *The glucose standardisation graph.*

Mg. glucose	cc. <i>N</i> /100 thiosulphate	Mg. glucose	cc. <i>N</i> /100 thiosulphate
0.20	0.58	2.00	3.40
0.50	1.45	2.50	7.38
0.80	2.32	3.00	8.93
1.00	2.91	3.50	10.55
1.50	4.40	3.80	11.53

Note. These figures have been read at convenient intervals from a large scale graph of the experimental data (Fig. 1).

THE STABILITY OF THE ALKALINE FERRICYANIDE (SOLUTION A).

It was thought important to investigate the keeping qualities of solution A in view of the fact that certain authors have suggested that the alkaline ferricyanide solution of the original Hagedorn and Jensen method should not be kept long. One modification of the original method has been proposed in which the ferricyanide and the alkali are made up separately, to be mixed immediately before the estimation (Pattersen, quoted by Cole [1928]). Accordingly lots of solution A were made up at different times and stored in black-jacketed bottles at room temperature, to be compared at a later date. The results are given in Table II for the reducing values found for 5 cc. of a maltose

solution containing 3.86 mg. maltose, using these solutions. The change after the second day is very slight and it must be concluded that storing the solution for 3 months causes practically no deterioration. During the first 2 days of storage, however, there seems to be an appreciable change in the reduction, solution A should therefore be prepared 2 or 3 days before using. The solution A used in the standardisation estimations had been made up 6 to 10 days.

Table II.

Values in cc. 0.01290 *N* thiosulphate

Time of storing solution A	(a) Water blank	(b) With 3.86 mg. maltose	(c) Thiosulphate equi- valent of reduced ferricyanide
10 min.	9.59	2.39	7.20
2 hrs	9.61	2.40	7.21
2 days	9.61	2.32	7.29
8 "	9.64	2.34	7.30
28 "	9.60	2.35	7.25
34 "	9.54	2.21	7.33
90 "	9.63	2.30	7.33

THE EFFECT OF DISSOLVED OXYGEN.

I have found previously¹ that when copper methods are used a considerable error may be involved in estimating the reducing power of sugar solutions with different amounts of dissolved oxygen. Thus, with the Shaffer and Hartman micro-method, two samples of the same sugar solution, one free from oxygen and the other saturated with oxygen, give reducing values differing by as much as 25 %. The lower the concentration of dissolved oxygen, the higher becomes the reducing value obtained. This was shown to be due to the re-oxidation of cuprous to cupric copper by dissolved oxygen in the early stages of the reduction heating.

The Hagedorn and Jensen method (and the present modification) are found to be entirely free from this defect, as is shown in the following data, which were obtained by taking two portions of a solution of maltose, saturating one with oxygen by bubbling the gas through it and washing the other with a stream of pure nitrogen. The reducing values were then determined.

Mg. maltose found in 5 cc. sample.

	(a) Free from oxygen	(b) Saturated with oxygen
Solution 1	4.05	4.02
" 2	1.54	1.54

Since the values found are very nearly the same it is evident that there is no appreciable re-oxidation of the ferrocyanide by dissolved oxygen under the conditions of the procedure. It is much safer, therefore, to use a ferricyanide method when dealing with solutions whose oxygen equilibrium may have been disturbed by any previous treatment such as boiling, filtering under reduced pressure, or washing with a gas stream other than air.

¹ Hitherto unpublished.

THE EFFECT OF THE PRESENCE OF STARCH.

The present method was worked out primarily for the purpose of following the progress of sugar production during the hydrolysis of starch; it was necessary therefore to find out whether the presence of an excess of starch interfered with the final iodine titration. It is found that although the titration is slightly more difficult in that there is a tendency to overshoot the end-point, a little practice overcomes this difficulty. The stirring must be more vigorous and towards the end of the titration the thiosulphate must be added more slowly. Experiments were done in which known amounts of maltose were added to solutions of soluble starch and amylose of various concentrations. The latter were undialysed and had small reducing powers themselves. Values found for added maltose, subtracting the reduction due to the starch or amylose, were in all cases in close agreement with the amounts added.

SUMMARY.

The range of the Hagedorn and Jensen sugar method has been extended to enable the estimation of about ten times the amount of reducing sugar. The solutions and procedure for the modified method are described and standardisation data are given for glucose and maltose. An important advantage over copper methods is pointed out in the fact that variations in the amounts of dissolved oxygen in sugar solutions do not affect the reducing values arrived at by the ferricyanide method.

In conclusion I wish to express my thanks to Mr S. W. Cole of the Biochemical Institute, Cambridge, for his very generous help and criticism.

REFERENCES.

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Hagedorn and Jensen (1923). *Biochem. Z.* 135, 46.
Tollens (1914). *Handb. der Kohlenhydrate*.

APPENDIX.

The standardisation data.

Water blank values: 9.62, 9.63, 9.625, 9.605, 9.615, 9.61, 9.62, 9.61, 9.62 cc.
Thiosulphate used: 0.01290 N.

Mg. glucose	3.71	3.39	3.08	2.49	2.05	1.50	1.25	0.829	0.749	0.622	0.414	0.213
Ferricyanide reduced*	9.72	7.90	7.11	5.69	4.64	3.42	2.83	1.83	1.69	1.39	0.88	0.46
(WB - R)	9.68	7.92	7.12	5.70	4.64	3.40	2.83	1.84	1.69	1.37	0.89	0.48
Mg. maltose	4.86	4.44	3.93	3.86	3.13	2.47	1.96	1.89	1.56	0.980	0.780	0.382
Ferricyanide reduced*	9.12	8.29	7.40	7.32	5.86	4.65	3.69	3.59	2.92	1.81	1.46	0.70
(WB - R)	9.09	8.28	7.39	7.31	5.92	4.66	3.69	3.57	2.92	1.82	1.47	0.71

* Expressed in terms of cc. of 0.01290 N thiosulphate.