CXVIII. THE FERRICYANIDE METHOD FOR THE DETERMINATION OF REDUCING SUGARS.

A MODIFICATION OF THE HAGEDORN-JENSEN-HANES TECHNIQUE.

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THE modification of the Hagedorn and Jensen [1923] method for the estimation of reducing sugars introduced by Hanes [1929] has the advantage over copper reduction methods that much smaller amounts of sugar can be accurately estimated. Also, as Hanes showed, this method, unlike the copper reduction methods, gives results that are not appreciably affected by the oxygen tension in the solution being estimated. He employed the method for the estimation of glucose and maltose, the latter sugar giving a linear relation between the amount of sugar and amount of ferricyanide reduced while glucose did not give such a linear relation under Hanes's conditions.

Callow [1930] applied the Hanes method to the estimation of invert sugar, but the method has not been standardised for fructose.

In order to use this method for a study of carbohydrate metabolism in green leaves, employing small quantities of material, it was first necessary to investigate the sugar/ferricyanide relation for fructose and for varying mixtures of the reducing sugars (glucose, fructose and maltose). It was hoped also that it might be possible to find a set of conditions under which the sugar/ferricyanide relationship would be linear for all the reducing sugars. Under such conditions one might expect that the total reducing power would be the simple sum of the reducing values of the component sugars. This would enormously simplify the analysis of the extracts. From the results of Hanes and Callow it seemed likely that this might be attained by altering the time of boiling, or the volume or both. As the results of this paper show, this possibility has been realised by changing the volume alone.

EXPERIMENTAL.

Hanes's technique for the actual oxidation of the sugar by ferricyanide and the titration of the excess of ferricyanide with thiosulphate was carefully followed with the exception that the volume of sugar solution taken was 10 cc. in place of 5 cc.

The thiosulphate solution (approximately $N/75$) was standardised against standard potassium iodate by the method given by Hanes. It was found advisable to check the strength of this thiosulphate solution every 2 or 3 days, especially in warm weather. The chemicals used were of the A.R. quality as supplied by the British Drug Houses, Ltd.; the proportions used were exactly as given by Hanes.

Sugar samples.

Glucose. The glucose used was Dextrose A.R. (British Drug Houses), which had been dried *in vacuo* over phosphorus pentoxide to constant weight. The sugar so treated had an optical rotation of $[\alpha]_D^{20°} + 52{\cdot}78°$ in an 8 % solution; the calculated rotation was $[\alpha]_D^{20} + 52{\cdot}68°$. In order to check the dryness of the sample the true weight of glucose in a solution made by dissolving 5 g. of the above sample in distilled water and then making to 100 cc. was ascertained by making use of the density tables of Brown, Morris and Millar [1897]. By this method it was found that exactly 5 g. of glucose were present in this solution.

Invert sugar. This sugar was prepared from Saccharose A.R. (B.D.H.) which was ground to a fine powder and dried under diminished pressure over phosphorus pentoxide to constant weight, by the method of Lane and Eynon [1923]. A 10 % unneutralised solution had $[\alpha]_D^{20°} - 20{\cdot}62°$; the calculated value, allowing for the acid present, was $[\alpha]_D^{20°} - 20{\cdot}72°$. The rotation of the sucrose from which the invert sugar was prepared was $[\alpha]_D^{20°} + 66{\cdot}39°$ in a 2 % solution; calculated value $[\alpha]_D^{20°} + 66{\cdot}42°$.

Fructose. The fructose used was a sample of Kahlbaum's "Fructose kryst" which was ground to a fine powder and dried under diminished pressure at 60° over phosphorus pentoxide to constant weight. It had $[\alpha]_D^{27°} - 86{\cdot}40°$. This rotation is within the range of published values for fructose but is rather low compared with the theoretical value calculated from the formula suggested by Vosburgh [1920]. This fact tended to show that, although dried to constant weight under the conditions described, the fructose was not completely dry. This was proved by the fact that a solution containing 5 g. of the sample in 100 cc. of solution was found, from density determinations (as for glucose above), to contain actually only 4·930 g. of dry fructose. Hence apparent weights of fructose must be multiplied by 4·93/5·00 to give the true weight of dry fructose. This correction is embodied in all the results given for fructose; its application leads to the following optical rotation for the fructose sample used: $[\alpha]_D^{27°} - 87{\cdot}68°$, the theoretical value being $- 88{\cdot}08°$.

Maltose. The sample used was a Kahlbaum preparation of pure maltose monohydrate which had been recrystallised three times from 60 % alcohol and dried *in vacuo* as above to constant weight. The $[\alpha]_D^{20°}$ of an 8 % solution was $+ 130{\cdot}02°$; calculated value $+ 130{\cdot}07°$ [Parcus and Tollens—see Landolt, 1898].

The calculated values for the optical rotations of the sugars were obtained from the formulae given by Landolt [1898] unless otherwise stated.

Sugar solutions.

Two different methods of delivering the required amounts of sugar in solution were employed.

(*a*) Sugar solutions of 0·01 and 0·05 % were prepared and transferred to the boiling-tubes containing the ferricyanide from a 10 cc. burette (graduated to 1/50 cc. and fitted with a fine waxed jet) in the required quantities.

(*b*) Sugar solutions of 0·01, 0·02, 0·25 and 0·05 % were prepared and transferred to the boiling-tubes by means of accurate 2, 5 and 10 cc. pipettes.

In both cases the final volumes of sugar solution were made to 10 cc. with distilled water using a graduated pipette, when necessary. Water volume differences of 0·01 cc. had no measurable effect on the results.

Both methods (*a*) and (*b*) were used indiscriminately although in most cases duplicate estimations using the two methods were carried out, good agreement being obtained. In all cases the total number of duplicates was four and the maximum variation in cc. thiosulphate ($N/75$ approx.) among replicates was 0·03 cc.

Standardisation data.

Table I gives the mean values obtained for glucose, invert sugar, fructose and maltose. The actual readings of approximately $N/75$ thiosulphate have been converted to their $N/100$ equivalents for the sake of uniformity.

Table I.

Sugar mg.	cc. $N/100$ thiosulphate $-$ $(WB - R) \times$ normality factor (mean of four estimations)			
	Glucose	Invert sugar	Fructose	Maltose
0·20	—	—	0·54	0·38
0·25	0·69	0·69	0·67	—
0·50	1·44	1·43	1·43	1·05
0·75	2·17	2·16	2·12	—
1·00	2·90	2·89	2·88	2·18
1·25	—	—	—	2·70
1·50	4·32	4·36	4·36	3·24
1·75	—	—	—	3·79
2·00	5·83	5·87	5·82	4·34
2·50	7·31	7·36	7·30	5·42
3·00	8·76	8·82	8·72	6·545
3·50	—	—	—	7·66

Above 3·0 mg. in the case of glucose, fructose and invert sugar and above 3·5 mg. in the case of maltose the results are not sufficiently accurate. This is due, no doubt, to the large percentage of the total ferricyanide used in the oxidation of the sugar in these cases.

The sugar-thiosulphate reaction.

From inspection of the glucose results it is clear that equal increments in sugar give approximately equal increments in thiosulphate, 0·25 mg. glucose corresponding on the average to 0·735 cc. thiosulphate. The observed value for an initial 0·25 mg. glucose is, however, 0·69 cc. so that although the relation is approximately linear the line will not pass through the origin. The relationship between sugar and thiosulphate may therefore be put in the form

$$S = b\,(T + a),$$

a representing an amount in cc. thiosulphate to be added to the observed thiosulphate value, T, and b the conversion factor for thiosulphate to sugar, S. A similar relation holds for all the sugars under investigation. In future thiosulphate value T will be distinguished from the value $(T + a)$ by designating the former "observed thiosulphate value" and the latter "reducing value," T and a being expressed in terms of $N/100$ thiosulphate unless otherwise stated.

Fig. 1 shows the experimentally determined points for glucose and maltose (two separate series of determinations) and the lines corresponding to the calibration equation finally adopted, *i.e.* for glucose $S = 0{\cdot}340\,(T + 0{\cdot}05)$—see later.

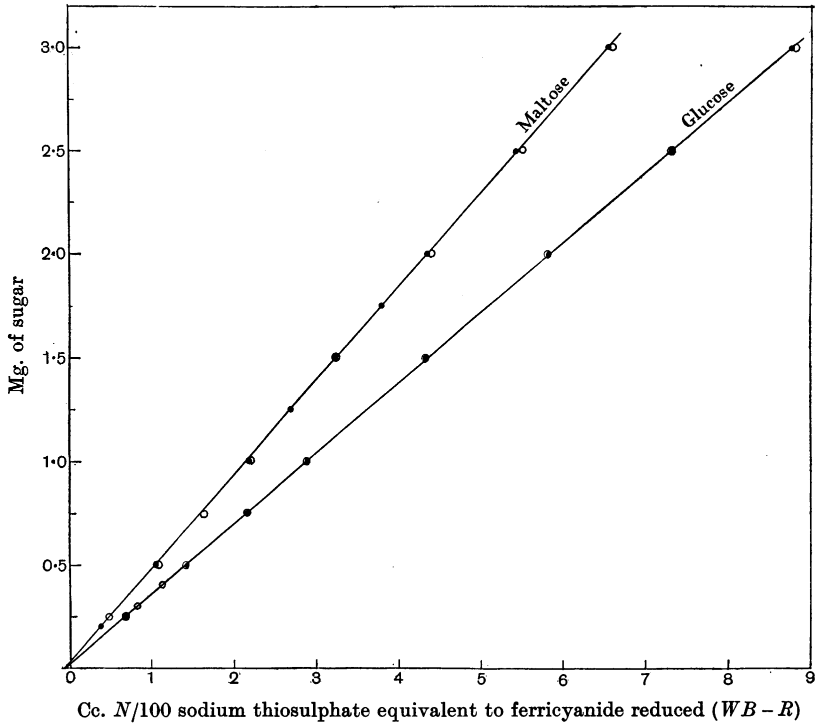

Fig. 1.

Values of a and b were obtained by the method of least squares taking the calculations to the third and fourth decimal place respectively. The values obtained are given in Table II.

As a measure of the accuracy with which these values fit the observations

Table II.

Sugar	*a*	*b*	S.D.$_c$
Glucose	0·041	0·3409	0·0065
Fructose	0·052	0·3410	0·0063
Invert sugar	0·059	0·3377	0·0046
Maltose	0·053	0·4545	0·0063

we give also, as S.D.$_c$, the standard deviation of calculated from observed values in mg. sugar.

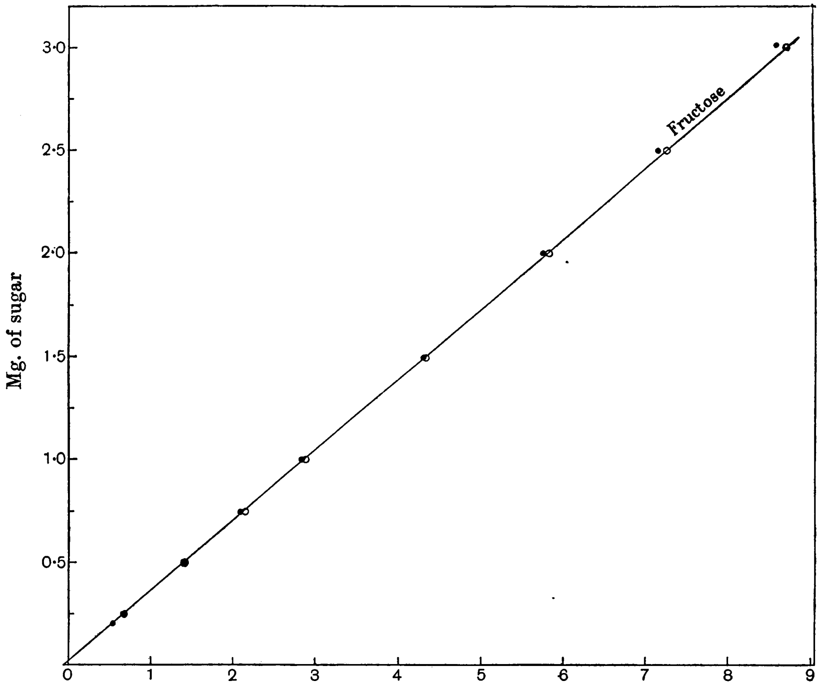

Fig. 2.

Consideration of factors a and b.

As it was impossible to read the thiosulphate burette, which was graduated in 0·02 cc. divisions, to a greater accuracy than 0·01 cc. the factors *a* and *b* can only have significance to the second and third decimal place respectively. Further, it is clear that the values of *a* are not significantly different for the different sugars and the mean value 0·05 can be used without loss of accuracy. Using this value of *a* and taking *b* to the third decimal place, the following rationalised values were obtained (Table III):

Table III.

Sugar	*b*	S.D.$_c$	S.D.$_e$
Glucose	0·340	0·0071	0·0033
Fructose	0·341	0·0067	0·0034
Invert sugar	0·338	0·0053	0·0028
Maltose	0·455	0·0077	0·0033

The figures show that by this rationalisation the standard deviation of calculated from observed is not appreciably increased. Hence the a factor of 0·05 with the b factors given in the above table are accepted as the standard values for the experimental conditions used. It will be seen that the fructose and glucose factors differ by less than 0·3 %, a fact of obvious advantage. The slightly lower value obtained for invert sugar will be discussed later.

The value S.D.$_e$ in Table III is the standard deviation in mg. sugar of the mean of four repeated determinations. It was calculated for each sugar from the expression:

$$\text{S.D.}_e = b\sqrt{\Sigma d/(f \times n)},$$

where d is the deviation in cc. $N/100$ thiosulphate from the mean of four repeated determinations, f is the total number of degrees of freedom (3 for each set of 4 repeated determinations), n the number of repeated determinations in each set (4), and b is the thiosulphate conversion factor for the sugar in question. Although the deviation from calculation (S.D.$_c$) is rather greater than the error due to successive estimations (S.D.$_e$), the scatter of the deviations about the sugar-thiosulphate lines calculated from the standard values of a and b does not suggest any significant departure from linearity. Moreover, it is clear that the linear relation fits the observations to within 0·5 % of the mean amount of sugar estimated (1·5 mg.).

Significance of the "a" factor.

A factor corresponding to the a factor (*i.e.* reduction curves not apparently passing through the origin) is common in copper reduction methods but had not been observed previously with the ferricyanide method; Hanes's curves, obtained for a sugar volume of 5 cc., passed through the origin. This fact, for 5 cc. sugar volumes, has been confirmed by the present authors so that the factors giving rise to a cannot be due to differences in personal technique. A confirmation of this fact is afforded by the agreement as to the value of a for 10 cc. sugar volumes obtained by the authors carrying out independent series of estimations. It might be expected that, using 15 cc. sugar volumes, a higher value for a would be obtained. Actually using such a volume it was impossible to obtain results sufficiently constant to be able to give any definite value to a under these conditions; values between 0·02 and 0·04 were obtained. This may be due to several causes—inequality of the initial stages of boiling, too high dilution of ferricyanide, *etc.*

The existence of factor a may mean (a) that some constant amount of sugar is destroyed before oxidation by ferricyanide, or (b) that a definite amount of re-oxidation of the ferrocyanide takes place during boiling, or (c) that auto-reduction of the ferricyanide occurs in water but not in the presence of sugar. This latter possibility seems hardly feasible in view of the identical water blank values obtained with 5, 10 and 15 cc. of water in the cold or with boiling for the standard time, while varying the volume of sugar solution does vary the value of a. Some evidence in favour of (b) is given by the fact that Hanes found a somewhat low observed thiosulphate value when the sugar solutions were saturated with oxygen.

Thus it is difficult to find the theoretical significance of factor *a*, but from the point of view of actual sugar estimations it hardly warrants further investigation; indeed the factor may be neglected without introducing appreciable error so long as the amount of sugar to be estimated does not fall below 0·75 mg. Where a higher degree of accuracy is essential, and for amounts of sugar below 0·75 mg., the experimentally determined value of 0·05 cc. *N*/100 thiosulphate is quite satisfactory. To avoid error the factor is best applied by adding 0·05 to the water blank value, expressed as *N*/100 thiosulphate, when the requisite *b* factor may be applied directly.

Mixtures of sugars.

From the linearity of the relation between sugar and thiosulphate it is clear that a unit amount of sugar gives a constant reduction of ferricyanide in all parts of the scale investigated. It is essential to ascertain whether, in mixtures of sugars, one unit of each sugar will bring about a constant amount of ferricyanide reduction quite independent of the ferricyanide reduction caused by the other sugars. This was tested by comparing the observed thiosulphate value for mixtures of sugars with the simple sum of the calculated values for the component sugars. The results obtained are summarised in Table IV.

Table IV.

Sugar mixture (mg. sugar)				cc. *N*/100 thiosulphate	
Glucose	Invert sugar	Fructose	Maltose	Observed	Calculated
0·50	—	—	0·50	2·56	2·52
1·00	—	—	1·00	5·12	5·08
0·80	2·00	—	1·00	10·47	10·45
0·64	3·20	—	—	11·30	11·30
—	1·25	—	1·25	6·41	6·40
0·424	—	1·972	—	6·94	6·97
1·060	—	1·233	—	6·68	6·68
0·530	—	1·972	—	7·27	7·29
1·500	—	1·500	—	8·74	8·76

From these results it is clear that each sugar behaves towards ferricyanide as if the others were absent. However, the case of invert sugar considered as a mixture of equal parts of glucose and fructose is an apparent exception to this generalisation since the *b* factor, 0·338, is 0·0025 lower than the mean for glucose and fructose, 0·3405. "Synthetic" invert sugar (equal amounts of glucose and fructose) gives the expected factor as can be seen from the results for such an "invert sugar" solution given in Table IV. This suggests that the reducing power of invert sugar (as prepared by hydrochloric acid inversion of sucrose) with respect to ferricyanide is slightly higher than the expected additive reducing power of glucose and fructose. To suggest a reason for this would be pure speculation, although one obvious but unexpected possibility is that the acid present when the glucose and fructose are being formed from sucrose might in some way slightly affect the reducing power of the two sugars. However, on treatment of a mixture of equal parts of glucose and fructose with 2·5 % hydrochloric acid under the conditions given by Hinton and Macara [1927] for the preparation of invert sugar from sucrose, the ferricyanide reducing power of the glucose and fructose was found not to have been affected in the slightest, the theoretical reducing value being found using the glucose-fructose *b* factors; see Table V.

Table V.

Glucose + fructose (mg.) (equal amounts)	Reducing value in cc. $N/100$ thiosulphate		
	Observed	Calculated for *b* factor for glucose + fructose	Calculated for *b* factor for invert sugar
2·134	6·23	6·22	6·27

Thus the possibility of hydrochloric acid affecting the reducing power of glucose and fructose under the conditions of sucrose inversion employed is ruled out. That the Hinton and Macara method for sucrose hydrolysis gives similar results to the Lane and Eynon method used in the invert sugar standardisation will be seen from Table VII, since the use of the *b* factor obtained for invert sugar prepared by the latter method gives agreement between actual and observed amounts of invert sugar prepared by the former method.

In addition to investigating the effect of one reducing sugar on another, the effect of the presence of sucrose was also investigated, since in copper reduction methods the presence of this sugar affects the reducing value obtained for reducing sugars. As will be seen from the results given in Table VI, the ferricyanide method is not appreciably affected by the presence of sucrose.

Table VI[1].

	Sucrose (mg.)	cc. $N/100$ thiosulphate
Glucose approx. 1·0 mg. (constant)	0·5	2·92
	1·0	2·92
	2·0	2·92
	4·0	2·94
Invert sugar approx. 1·0 mg. (constant)	0·5	2·94
	2·5	2·95

Fructose and maltose results are also unaffected by the presence of sucrose.

The fact that the total reducing value of a mixture of sugars, as estimated by the modified ferricyanide method described in this paper, is the simple sum of the reducing values of the component sugars and is not affected by the concentration of sucrose should give the method a great advantage over copper reduction methods in the estimation of the individual sugars present

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[1] Note added July 29th. The authors have since found that sucrose alone does not cause any appreciable reduction of ferricyanide under the conditions of the modified Hanes estimation; this confirms the advantage of this method over copper reduction methods.

in, for example, leaf extracts. In the simplest case, the estimation of sucrose in a mixture of sugars, the increase in the reducing value following inversion may be converted directly into mg. invert sugar, using the appropriate factor (0·338). The invert sugar used for standardisation was prepared by hydrochloric acid inversion in the cold. In order to test whether the use of other methods of inversion would affect the ferricyanide reducing value, estimations of sucrose were carried out (1) by the citric acid inversion method and (2) by the hydrochloric acid inversion for 12 minutes at 60° as suggested by Hinton and Macara [1927].

(1) Inversion was carried out by 10 % citric acid in a boiling water-bath for 15 minutes, cooling and neutralising the solution with 5*N* NaOH; the requisite amount of NaOH was previously found on a separate amount of sugar-citric acid solution, using bromothymol blue indicator. The solution was then made to a definite volume and an aliquot taken for a reducing value estimation. It was found unsatisfactory to take 10 cc. of the neutralised solution for this estimation, for if this was done, the citrate present interfered with the precipitation of the potassium zinc ferrocyanide causing a shifting, indefinite end point, especially in the case of small amounts of sugar. It was found possible, however, to overcome this difficulty by taking only 5 cc. of inversion liquid and 5 cc. of distilled water, and then adding to the solution after the boiling with ferricyanide and cooling, in addition to the usual solutions, 1 cc. of 15 % zinc sulphate solution. The smaller amount of citrate present combined with the extra excess of zinc sulphate, and allowed of complete precipitation of the potassium zinc ferrocyanide, so that the thiosulphate titration end point was once more fixed and definite. The results obtained are given in Table VII.

(2) By the Hinton and Macara method, inversion was carried out by approximately 2·5 % hydrochloric acid in a water-bath at 60° for 12 minutes, agitating the liquid during the first 3 minutes of inversion. The solution was then neutralised with 5*N* NaOH and made to a definite volume; aliquots were taken for the determination of the reducing value. In both the citric and hydrochloric acid methods of inversion, control experiments were carried out, the sugar solution being replaced by distilled water. These controls were treated in a manner identical with that used for the sugar solutions and

Table VII.

	mg. sugar		% deviation observed from present
	Present	Observed	
Citric acid hydrolysis:			
Sucrose (as invert sugar)	0·263	0·267	1·5
,,	1·315	1·324	0·7
,,	2·632	2·670	1·4
HCl hydrolysis:			
Sucrose (as invert sugar)	1·894	1·894	Nil
,, Glucose, fructose (equal amounts)	1·067 1·067	2·140	0·3

the water blank (W.B.) value used for the reducing value determinations on the invert sugar solutions was obtained from these controls.

In order to confirm the validity of the hydrochloric acid method of inversion in sugar mixture determinations, an inversion was also carried out in the presence of equal amounts of glucose and fructose.

The results of the above determinations are given in Table VII.

Thus for estimation of sucrose as invert sugar by the ferricyanide reduction method inversion by 2·5 % hydrochloric acid for 12 minutes at 60° gives accurate results, while citric acid inversion gives results which are appreciably high. These high results are probably partly due to the effect of the citrate on the potassium zinc ferrocyanide. The presence of hydrochloric acid does not affect the reducing power of the other reducing sugars under the conditions of sucrose inversion employed. The hydrochloric acid method has, therefore, been adopted by the authors, although invertase inversion methods are being investigated to ascertain whether invert sugar prepared by this latter method gives a similar *b* factor to the one obtained on acid inversion.

For the hydrolysis of maltose in the presence of fructose or sucrose no really satisfactory method of acid hydrolysis is as yet available and investigations on this point and on the use of maltase are being carried out, especially in relation to sugar estimations in plant extracts.

For mixtures of glucose and fructose the modified Hanes method combined with iodimetric estimation gives easily calculated and accurate results.

The application of these two methods for the estimation of the individual sugars in simple mixtures of glucose, fructose and maltose and in leaf extracts is under investigation.

SUMMARY.

1. A modification of the Hanes technique for the estimation of reducing sugars by oxidation with ferricyanide is described. This modification is shown to give a linear relation between the amount of sugar and the amount of ferricyanide reduced over a range of 0·2 to 3·0 mg. in the case of glucose, fructose and invert sugar, and of 0·2 to 3·5 mg. in the case of maltose. By statistical treatment the linear relation is found to be accurate to 0·0067 mg. sugar (mean value for the four sugars), *i.e.* 0·5 % of the mean amount of the sugars estimated.

2. Expressing the relation between amount of sugar and the amount of ferricyanide reduced in terms of $N/100$ thiosulphate, the following relation is found:

$$\text{Sugar} = b\,(\text{observed thiosulphate} + a),$$

a having the value 0·05 cc. $N/100$ thiosulphate for all the sugars investigated; factor b is shown to be, for glucose 0·340, for fructose 0·341, for invert sugar (prepared by HCl inversion of sucrose) 0·338 and for maltose 0·455.

3. In a mixture of sugars the ferricyanide reducing power of one sugar is shown to be unaffected by the presence of the others. The presence of

sucrose does not affect the reducing value of the other sugars present in a mixture.

4. Invert sugar, prepared by acid hydrolysis of sucrose, has a slightly higher reducing value than a synthetic mixture composed of equal amounts of glucose and fructose.

5. For the estimation of sucrose, inversion by 2·5 % hydrochloric acid for 12 minutes at 60° (after Hinton and Macara) followed by determination of ferricyanide reducing power is found to give accurate results, while citric acid inversion gives results which are too high.

6. The application of the ferricyanide reduction method to the estimation of individual sugars in plant extracts is discussed.

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