XL. METHODS FOR THE ESTIMATION OF MIXTURES OF FOUR OR MORE CARBOHYDRATES, INVOLVING OXIDATION WITH BROMINE.

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In the course of investigations upon the diurnal changes in the carbohydrate content of green plant tissues, many analytical difficulties were encountered, and it was therefore decided critically to examine existing methods and to devise others if possible. The work was unavoidably delayed and since there is but little prospect of bringing it to completion in the near future, we have decided to publish what has been outlined up to the present. While carrying out this work a series of papers has appeared by Messrs Davis and Daish [1913] and their collaborators, dealing with the same subject and we have been much indebted to them.

THE CARBOHYDRATES OF THE GREEN LEAF.

The five carbohydrates usually found in foliage leaves are starch, sucrose, *d*-glucose, *d*-fructose and maltose, and in addition to these sometimes small quantities of pentoses are known to be present. The estimation of starch has been fully discussed by Davis and Daish [1914] and that of the pentoses by Davis and Sawyer [1914] and by Daish [1914]. In the present work, therefore, only the remaining four sugars will be considered.

REDUCTION METHOD.

Before beginning this work, it was necessary to find a suitable method of quantitatively estimating reducing sugars gravimetrically. As all methods based on the use of Fehling's solution are liable to error, owing to the solvent action of the hot sodium hydroxide on cuprous oxide, it was decided

after careful tests to use Kendall's method [1912], weighing the copper as cupric oxide as advocated by Davis and Daish [1913] and by Zerban and Naquin [1908], the action of reducing gases being avoided by heating the Gooch crucible containing the precipitate inside a larger porcelain or nickel crucible.

It has been stated by some that cupric oxide is hygroscopic and that this constitutes an objection to the method. However we have found no trace of this hygroscopic property throughout the numerous experiments carried out during this research, provided the carbonate used was pure. We have noticed, however, that if there is any iron present as an impurity in the carbonate used for reductions, then hygroscopic properties are observed, apparently in the cupric oxide, in reality due to the presence of red oxide of iron (Fe_2O_3), and moreover in such cases, we observed that the cuprous oxide was precipitated yellow whereas in all other cases it came down red. The blue amphibole asbestos for use in the Gooch crucibles must also be treated as insisted on by Davis and Daish, in order to ensure its constancy in weight when exposed to hot alkaline solutions.

Kendall's procedure, very briefly described, consists in adding to the sugar solution which has been made up to 100 cc. with distilled water, solid salicylic acid, a solution of copper sulphate and a strong solution of potassium carbonate in the order named¹; the mixture having been gently shaken is heated in a rapidly boiling water-bath for 20 minutes. Boiling should begin again within from one to two minutes of the introduction into the bath of the cold solution. Kendall has prepared tables for the reducing powers of glucose, invert sugar, maltose and lactose, but since a table for fructose was not included, one has been prepared by us and used for our analyses [1916].

ANALYSIS OF SUCROSE, MALTOSE, *D*-GLUCOSE AND *D*-FRUCTOSE.

(1) *Sucrose.*

The estimation of sucrose is usually carried out by inversion under Clerget conditions or by some appropriate modification of the method. When, however, maltose is present treatment with a mineral acid results in hydrolysis of a portion of this sugar. To avoid this citric acid in from 2 % to 10 % solution has been employed. Davis and Daish [1913] have studied this point carefully and recommend the use of invertase in preference, or at least

¹ The amounts employed are: salicylic acid 5 g., copper sulphate 15 cc. of a solution containing 133.33 g. of pure crystals per litre, and potassium carbonate 25 cc. of a solution containing 600 g. of anhydrous salt per litre.

as a check upon, hydrolysis with citric acid. The invertase may most conveniently be prepared and preserved according to Hudson's [1914] directions.

The calculation of the amount of sucrose is based upon the readings of the polarimeter before and after inversion or upon the copper reducing power of the solution. The changes in these two values should of course give concordant results; but sucrose itself has a slight reducing power when mixed with reducing sugars, so that a slight discrepancy will usually be observed.

When the amount of sucrose has been determined the rotation due to it may be calculated and hence the rotation of the remaining three reducing sugars in the mixture. One other sugar has now to be determined directly (as a solution of equations containing three unknowns is not satisfactory). The most usual sugar to determine now is maltose, and then by subtracting the rotation due to sucrose and maltose from the initial observed rotation and deducting the amount of copper due to maltose from the observed copper reducing power of the original solution, there remain two equations for the two unknowns, glucose and fructose, which may easily be found.

Now maltose may be estimated either by Brown and Morris's method [1893], of hydrolysis with hydrochloric acid under standard conditions, or by Davis and Daish's method [1913] of fermentation with pure cultures of maltase-free yeasts, which seems to have given very accurate results in the hands of its originators. But as each estimation requires at least twenty-one days and involves the use of a pure culture of a particular yeast it must be conceded that it is cumbersome. Accordingly a method is here suggested whereby fructose and not maltose is determined directly in the remaining mixture of three sugars. This method is outlined in the following pages.

(2) *Oxidation methods in acid or neutral solution.*

In all cases in which it is desired to estimate the separate sugars in a mixture of glucose, fructose and maltose, it is necessary as previously pointed out either to have three equations or to have a means of estimating one sugar by some special method.

At the outset it seemed that use might be made of the differential action of bromine upon the ketonic and aldehydic sugars. The former are scarcely oxidised at all, whereas the latter readily yield aldonic acids.

Bunzel [1909] has carefully studied the mechanism of the oxidation of glucose by bromine. He found that the amounts of acid and of bromide produced, as well as the amounts of glucose and of bromine disappearing, are

equal to those calculated on the hypothesis that one molecule of bromine oxidises one positive glucose ion to gluconic acid. Thus when acidified with hydrochloric acid, 0.1 normal, the equation is as follows:

The correctness of this view is supported by the constancy of the velocity constant, K , in the following equation, even under widely varying conditions of acidity and with different concentrations of bromine and glucose.

$$\frac{dx}{dt} = K \cdot \text{C}_{\text{C}_6\text{H}_{13}\text{O}_6^+} \times \text{C}_{\text{OH}^-} \times \text{C}_{\text{Br}_2 (\text{free})}.$$

From this it is seen that the rate depends upon three factors: (a) the concentration of the positive glucose ion $\text{C}_6\text{H}_{13}\text{O}_6^+$; (b) the concentration of the hydroxyl ions; (c) the concentration of the free bromine in solution.

Bunzel has shown that provided the concentration of the hydrogen ions is over 0.1 normal the hydroxyl ion factor may be entirely neglected. The velocity under these conditions is proportional to two variables, the concentration of the glucose and of the free bromine. Bunzel has also proved that in an acid medium the positive glucose ion goes quantitatively into gluconic acid. This may be expected to give rise to its lactone which is further oxidised by bromine very slowly.

The oxidation of the negative glucose ion $\text{C}_6\text{H}_{11}\text{O}_6^-$ probably yields a large number of acids. These have been studied by Nef [1907, 1910, 1914]. Since glucose is a very weak acid its dissociation in this manner is almost completely prevented by strong acids, with which it forms oxonium salts and ionises to $\text{C}_6\text{H}_{13}\text{O}_6^+$ as previously mentioned.

From these considerations it is evident that in 0.1 normal acid saturated with bromine and maintained in this condition, the rate of oxidation of glucose will be that of a unimolecular reaction. For the other variable, the concentration of bromine has now been reduced to a constant. This being so the same fraction of the glucose present will be oxidised to gluconic acid in a given time, irrespective of the initial concentration, provided the temperature is maintained constant.

The reaction under these conditions is a perfectly definite one and should be available for the estimation of glucose. The standard conditions will be discussed again in relation to the actual procedure adopted.

The action of bromine upon maltose in the cold is quite similar to that upon glucose, maltobionic acid being the product. Fructose is, however, usually considered to be almost unaffected by this halogen. At the outset

it appeared possible to remove the glucose and maltose quantitatively by oxidation with bromine. The reducing power of the solution would then have been due to fructose alone. Direct experiment, however, has shown that this is not rigorously exact, for when sufficient time has elapsed for the oxidation of all the maltose a small proportion of the fructose has in some way been destroyed. Moreover the oxidation of the glucose is never complete, or to be more accurate, prolonged treatment with bromine always leaves a solution which has still some reducing action upon Kendall's solution, corresponding to about 2 % of the glucose originally present. With maltose too the residual reducing power after treatment with bromine, though negligible, is quite perceptible.

This residual reducing power of glucose solutions most probably has its origin in the formation of gluconic lactone from the first formed gluconic acid as its concentration increases. That this is the case is borne out by the fact that all our attempts to isolate the phenylosazone of glucose from such solutions were unsuccessful, giving rise only to the production of gluconic phenylhydrazide. Moreover two experiments performed under the standard conditions and at average temperatures differing by about two degrees showed that oxidation by bromine for 66 hours afforded a solution with a larger residual reducing power than a similarly conducted oxidation for 42 hours. This apparently indicates the formation of the lactone on prolonged oxidation. A similar explanation is most likely applicable to the very slight residual reducing power observed also in maltose solutions.

Details of these analyses are given in the experimental portion of the paper.

The action of bromine upon these three sugars was studied at temperatures between 0° and 35° before all hope of a very exact separation was abandoned.

Since, however, the action may be reduced to the monomolecular type by maintaining the bromine in saturated solution, and by having a suitable excess of mineral acid, it is obvious that constants can be determined for each sugar, representing the fraction of its initial reducing power remaining after treatment with bromine under standard conditions. Accordingly such constants were found for experiments carried out at room temperature, after an attempt to work at 0°; the latter steady temperature was not suitable since the solution became of indefinite composition owing to the separation of very large amounts of bromine hydrate. The solutions were shaken continuously to ensure complete saturation with bromine throughout the period of oxidation. Shaking was effected by rotating the bottles, which

were partly filled with glass beads, about a horizontal axis. To ensure a more uniform temperature they were placed in a tank filled with water, and covered to exclude the light. A small electric motor running at about three thousand revolutions a minute was geared down suitably to rotate the solutions.

The equations available for determining the composition of a mixture of three sugars are then those usually employed for polarisation and reduction, with a third similar to the reduction equation but introducing a constant for residual sugar after halogen oxidation, thus:

$$(1) \quad 100 [\alpha]_D^{20} = x [\alpha]_{D_g}^{20} + y [\alpha]_{D_f}^{20} + z [\alpha]_{D_m}^{20},$$

where x , y and z represent the amounts of glucose, fructose and maltose respectively.

$$(2) \quad w_1 = k_g x + k_f y + k_m z,$$

where k_g represents the weight of cuprous oxide obtainable from Kendall's solution by 100 mg. or other suitable quantity of glucose; k_f and k_m have similar meanings.

$$(3) \quad w_2 = k_g \cdot a \cdot x + k_f \cdot b \cdot y + k_m \cdot c \cdot z,$$

where a , b and c are the percentages of residual sugar after treatment with bromine under the standard conditions.

On substituting the experimental data in the above equations an attempt was made to solve for the three unknowns; but in such methods of solution experimental errors are often largely multiplied and the magnitude of the errors so introduced in the mathematical operations was found in this case to be so great as to render the method useless.

Now, when the time allowed to elapse was 66 hours at room temperature (17° – 18°) the values for the residual sugar constants were $a = 0.033$; $b = 0.970$; $c = 0.006$. It will be noticed that the sum of these constants is 1.009, from which it follows that it is very nearly correct to assume that no fructose has disappeared, but that the other two sugars have been completely oxidised away.

It has been found that part at least of the small loss of fructose is due not to its oxidation by bromine but to its decomposition by the decinormal acid added to the solution, and further reinforced in the case of mixtures of the three sugars by the hydrobromic acid generated in the course of the reaction. Experiments proving this are shown in the experimental portion of this paper.

In order to test by direct experiment the supposition that under certain

standard conditions it is legitimate to assume that all the residual reducing power of the solution is due to fructose when glucose and maltose have also been present at the start, solutions containing these three reducing sugars were made up. They were then examined by the polarimeter, and the reducing power both before and after oxidation with bromine was determined for suitably diluted aliquot portions of the original solutions by Kendall's method¹. The results are shown in Table I. It must be remembered that, of the three, fructose is the only sugar which has been directly determined. The quantities of the other two had to be found by solving the simultaneous equations (1) and (2) for two unknowns. The multiplication of errors is not nearly so serious as with three unknown quantities, yet the accuracy to be expected is not of the same degree as in direct determinations.

TABLE I.

Temperature Time of oxidation	I 13°-16°		II 17°-18°		III 10°-11°	
	42 hours		66 hours		66 hours	
	Taken g.	Found g.	Taken g.	Found g.	Taken g.	Found g.
Fructose	0.7686	0.7650	0.4166	0.4150	0.4975	0.4615
Glucose	1.0260	0.9904	0.6955	0.5886	0.4910	0.4556
Maltose	0.8950	0.9863	0.6615	0.6953	0.4888	0.4778
Total	2.6896	2.7417	1.7736	1.6989	1.4773	1.3949

These results indicate that the method is capable of giving fairly accurate values for fructose in the presence of either or both of the other reducing sugars. The details of the estimation may possibly be improved so as to render the analysis of greater accuracy. In the latter quality this method is not so good as that of Davis and Daish but inasmuch as it can be carried out in three days instead of in three weeks, it may be found to commend itself for certain purposes.

(3) *Oxidation methods in alkaline solution*².

In alkaline solution the sugars are rapidly oxidised by atmospheric oxygen with the production of a brown colour. The relative rates of oxidation under these conditions are the same as in acetic acid, fructose being the most readily attacked. This difference between the reactions in acid and alkaline solution is most simply accounted for by the hypothesis that the sugars are

¹ The solutions for standard oxidation consisted of 50 cc. sugar solution diluted to 70 cc. and then 10 cc. normal sulphuric acid added and finally bromine 5 cc.

² This problem was forced upon our notice through the accidental addition of a slight excess of alkali in neutralising, as explained in the "Experimental" portion of this paper.

weak acids, the ionisation of which is hindered by other acids whereas the sodium salts formed in alkaline solution are readily ionised. This results in the liberation of very unstable and easily oxidisable negatively charged sugar ions. Mathews [1909] noticed that the rate of oxidation of glucose and other sugars in alkaline solution increased as the reaction proceeded. He adduced reasons for the belief that the glucose molecule is broken up into a number of active reducing molecules under these conditions. This process takes place even in the absence of atmospheric oxygen, and so has no connection with any oxidation processes in which gaseous oxygen is concerned, though intra-molecular oxidations are not excluded.

When alkaline solutions of the three reducing sugars glucose, fructose and maltose are exposed to gaseous oxygen they are readily oxidised as has already been mentioned. In consequence one might expect a decrease in their reducing power towards copper solutions. On testing this point directly it was found that the change in reducing power was an increase, followed by a decrease. In one case for example the procedure was as follows: a 2 % solution of each of the three sugars was mixed with an equal volume of normal sodium hydroxide. Before mixing, immediately after mixing and at various intervals of time the reducing powers of the solutions were determined by means of Fehling's solution. In the first case an equal volume of water was added to the sugar solution, in order that the analyses might be exactly comparable with the others of the series. The flasks were allowed to stand at room temperature, about 15°, and were not shaken up with air. At the same time in these preliminary experiments no precautions were taken to avoid oxidation. The results are shown in Table II.

Since there is a definite change in the reducing powers of the sugars when allowed to stand in half-normal sodium hydroxide it is evident that a set of constants can be obtained for the different sugars showing what alteration takes place in each under standard conditions. In obtaining these due regard must be paid to the most suitable times, temperatures and concentrations. Means must also be taken to prevent any appreciable amount of oxidation, for example by filling the flasks with hydrogen. If it so happened that all the reducing sugars were altered in the same proportions the effect would only be to alter the magnitudes of the usual constants for the reducing powers of these substances towards the various alkaline copper solutions in use. But experiment has shown that the change in the reducing powers of the various sugars takes place to very unequal extents, as after an initial increase, the rate of decrease is most rapid for fructose.

The results of these experiments are tabulated below; the concentrations recorded refer in each case to the mixture not to the original strengths of the separate solutions.

TABLE II.

Action of N/2 sodium hydroxide on sugars at room temperature.

Glucose 1 %			Glucose 3 %			Fructose 1 %			Maltose 1 %		
Time hrs. mins.	Reducing power		Time hrs. mins.	Reducing power		Time hrs. mins.	Reducing power		Time hrs. mins.	Reducing power	
0 0	100.0		0 0	100.0		0 0	100.0		0 0	100.0	
0 15	132.0		0 7	103.5		0 8	132.3		0 7	137.4	
0 25	131.5		0 15	104.7		3 37	132.3		3 35	128.8	
0 44	131.5		0 28	104.7		23 45	83.3		23 0	115.3	
21 55	95.7		4 25	102.3		47 0	66.4		46 45	108.9	
22 8	94.6		24 20	94.6		—	—		—	—	
43 40	88.0		—	—		—	—		—	—	

TABLE III.

Action of 2N sodium hydroxide on sugars at room temperature.

Glucose 1 %			Glucose 2 %		
Time hrs. mins.	Reducing power		Time hrs. mins.	Reducing power	
0 0	100		0 0	100	
0 5	113.2		0 7	104.4	
4 50	117.0		5 47	151.1	
24 20	88.5		23 50	95.3	
126 30	34.0		—	—	

The results are graphically shown in the figure.

Considering the N/2 alkali, with the 1 % solutions there is a very rapid rise in reducing power consequent upon the addition of alkali. This rise is identical in magnitude for glucose and for fructose within the limits of accuracy of the method. With maltose a somewhat greater rise is found. It must however be pointed out that the hexoses are present in equimolecular proportions, viz. 10/180 normal (= 0.055 N), whereas the molecular concentration of the maltose is far smaller, 10/342 normal (= 0.029 N).

With 3 % glucose the increase in reducing power is far smaller, and attains its maximum value more slowly.

Mathews [1909] clearly showed that there was an increase in the number of active reducing molecules consequent upon the addition of alkali. This he ascertained by measuring the rate of absorption of oxygen by a glucose

solution which was kept over mercury after being made alkaline. From the preceding tables it is evident that the increase is very considerable and a maximum is quickly reached. However the relationship between the quantity of alkali added and the percentage of sugar undergoing change requires further investigation. It has recently been found by Powell [1915] that sodium hydroxide reacts with glucose and fructose with the formation of sodium salts, each molecule of sugar taking up one sodium ion. This conclusion was deduced from the study of the retardation in the hydrolysis of ethyl acetate by the hydroxide, which follows the addition of a sugar.

Before becoming acquainted with Powell's results an attempt was made by us to obtain evidence concerning the union of sugar and alkali by means of conductivity measurements. The conductivity of 1 % glucose solution was measured—it was found to be about half that of the Dublin water supply—and also the conductivity of seminormal sodium hydroxide. These quantities were chosen as being those employed in obtaining the results shown in Table II, though as the sugar solution is only 0.055 normal it would perhaps have been advisable to use more dilute alkali. Then however a compound, if formed, would be almost completely ionised, whereas with one component in large excess such ionisation would be diminished. The

objection to using a strong sugar solution is that the viscosity changes would then begin to have a serious effect on the conductivities.

The conductivity of a solution composed of alkali and glucose in the same final concentrations was then measured at various intervals in a closed vessel.

TABLE IV.

Solution		Conductivity in mhos at 0°, $\times 10^5$	
Glucose 1 % (0.055 N)	...		2.181
Sodium hydroxide 2 % (0.5 N)			5661.0
Glucose 1 % ...	...	15 minutes after mixing	5272.0
Sodium hydroxide 2 %			
" "			
" "			
" "		55 minutes	" 5124.0
" "		105 minutes	" 5121.0
" "		22.5 hours	" 5170.0
Sodium hydroxide 2 % taken fresh from the corked flask, after standing in it for 23 hrs.			5214.0

Now neglecting the slight effect that the alteration in viscosity due to the glucose will have upon the conductivity, and assuming that the glucose combines in unimolecular proportions with the hydroxide, a conductivity of $C \times 10^5 = 5095$ should result if the conductivity varied directly as the concentration. This value is in reality slightly too low, for the more dilute solutions have higher molecular conductivities, 0.45 N hydroxide conducting better relatively than does 0.50 N. However the increase in viscosity acts in the opposite direction. Thus a comparison of the observed conductivity after 105 minutes, $C \times 10^5 = 5121$, with the calculated conductivity, $C \times 10^5 = 5095$, points to the formation of a compound between the sugar and alkali. This takes place to the extent of 95 % of the theoretical amount, the compound being dissociated to the extent of 5 %, or taking the final conductivity as $C \times 10^5 = 5214$, the dissociation amounts to 21 %. On the whole however it would be very unwise to conclude that the changes in such an unstable reaction-mixture as an alkaline sugar solution could be accurately represented by any one reaction.

In concluding this section it may be remarked that though the study of the oxidation of the sugars in alkaline solution has not lent itself to any quantitative method for their estimation when two or more are present together, yet it has shown what grave errors may result if sugar solutions are allowed to become alkaline in the course of analyses. Such solutions should always be neutral or faintly acid.

(6) *Oxidation by cupric acetate in presence of acetic acid.*

The use of Barfoed's solution is well known as a qualitative test to distinguish glucose and fructose, which reduce this reagent, from maltose and lactose, which under the specified conditions are without this action. All attempts to use the method for quantitative work have failed, since the disaccharides effect some reduction when the boiling is continued sufficiently long to oxidise all the hexoses. The amount of acetic acid present is also an important factor, and during the heating some of it is driven off.

This reaction has been very carefully studied by McGuigan [1907], Mathews and McGuigan [1907] and by Bunzel [1908]. It was found by Bunzel that the reaction is of the fifth order. However it seemed possible that it might be reduced to the first order, by adopting certain standard conditions, and be then available for the analysis of mixtures by differential oxidation of the sugars. With this object sodium acetate, 10 g., was added to 100 cc. of Barfoed's solution¹ to ensure a constant acidity of small magnitude. A large excess of the reagent was also taken, 100 cc. for 25 cc. of a 2 % solution of glucose, so that the change in the concentration of the copper should not be of too considerable magnitude. Furthermore the boiling of the mixture was effected under a reflux condenser. The cuprous oxide was collected in a Gooch crucible and weighed as cupric oxide. The following table shows that the solvent action of the acetic acid upon the oxide is too great to allow of accurate quantitative estimations by the method. Even the addition of the sodium acetate did not suffice to reduce this to negligible proportions.

TABLE V.

Cupric oxide from 100 cc. Barfoed's solution, 10 g. sodium acetate and 25 cc. of 2 % glucose, the latter being added cold to the former already heated to 90° in a water-bath. Solution cooled under water-tap at end of heating.

Time of heating	Cupric oxide
10 min.	1.1296 g.
15	1.2773
20	1.2257

Furthermore it was found that when 1 % glucose was used, and the heating was continued for fifteen minutes, the yield of cupric oxide was 0.8234 g.; indicating a reducing power nearly half as great again as for a 2 % solution.

¹ Copper acetate crystals 66 g. per litre, glacial acetic acid 10 cc. per litre.

A similar attempt using the mono- and di-hydrogen sodium phosphates instead of sodium acetate was also found to be unsatisfactory. However though this attempt at reaching suitable conditions is a failure it is perhaps worth recording to save others from similar waste of time.

EXPERIMENTAL.

The sugars used in the course of this investigation were the following: (a) for preliminary work, the pure anhydrous glucose, fructose (laevulose) and maltose of commerce; (b) for accurate work, determinations of constants, etc., Kahlbaum's sugars were used as a rule, the fructose being the "laevulose from inulin I." In some experiments pure commercial glucose and maltose were employed, after recrystallisation from alcohol three times.

The powdered sugars were kept in desiccators over sulphuric acid for long periods. The residual moisture was determined by heating a small portion, about 2 g., in an exhausted flask connected with drying tubes of phosphoric oxide at air temperature which led to a Geryk pump. The temperature at which the sugars were heated was that of the water-oven. When drying fructose it is advisable to use a lower temperature, about 50°, at first if the sample contains any appreciable amount of moisture. The "laevulose from inulin I" however did not melt and remained perfectly white when heated to 98° at atmospheric pressure in a vessel containing phosphoric oxide. Its purity was further tested by determining its specific rotation, in 5 % solution at 17.4° in a 20 cm. tube, using a Schmidt and Haensch polarimeter. When corrected for temperature it gave $[\alpha]_{20}^D = -92.3^\circ$, which agrees closely with the value chosen for use throughout this work, viz. -92.5° . Later on a polarimeter by Adam Hilger was employed.

Anhydrous maltose was prepared by distillation with alcohol and benzene as recently described by us [Atkins and Wilson, 1915], but owing to its extremely hygroscopic nature it was deemed advisable to employ the hydrate for quantitative work, and to correct for the small excess of moisture beyond the theoretical amount of water of crystallisation.

Action of Bromine on Glucose, Fructose and Maltose.

To study this action solutions of the sugars were saturated with bromine, and allowed to stand in the dark over excess of the liquid halogen. In some experiments the bottles were shaken from time to time to maintain saturation. In determining constants the shaking was continuous.

At the end of the period chosen the liquid bromine was removed by means of a separating funnel and what remained over was converted into hydrobromic acid by passing in sulphur dioxide till the solution was nearly colourless. The last traces were destroyed by the careful addition of a solution of sulphurous acid from a burette. The mixture was then almost neutralised with solid potassium carbonate, taking care to leave the solution faintly acid at the end. A blank experiment showed that this procedure afforded a solution which had no reducing action on alkaline copper solutions.

The reducing power of the sugar solutions after treatment with bromine was determined by Kendall's method, but the cuprous oxide was oxidised by heating in air, and weighed as cupric oxide. The amounts of fructose were found from a table prepared by the authors in exact accordance with Kendall's method of drawing up tables for the other sugars [1916].

The following tables show the results of the preliminary experiments.

TABLE VI.

Oxidation of glucose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of glucose remaining. Initial concentration = 100 %
Room 13°-16°	10	16 days	2.5
"	10	12 "	3.0
33°	2	4 "	8.9
0°	1½	90½ hours	50
0°	1½	91½ "	69.13
0°	1½	90 "	{ 34.77 35.76* 53.03† }
	Same experiment		solution shaken

TABLE VII.

Oxidation of fructose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of fructose remaining. Initial concentration = 100 %
Room	12½	16 days	97
"	11½	12 "	98
40°-45°	11	4 "	92
22°	5	4 "	95
0°	1	90 hours	{ 100.0* 101.6† }
	Same experiment		solution shaken

TABLE VIII.

Oxidation of solution of maltose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of glucose remaining. Initial concentration = 100 %
Room	11	16 days	0.0
"	12	12 "	0.0
33°	1½	4 "	2.3
0°	2	90½ hours	37.4
0°	1	92 "	50.8
0°	1	90 "	{ 19.50* 30.75† }
Same experiment			solution shaken

The figures marked with an * resulted from experiments where the solutions had been allowed to stand for four hours with a slight excess of potassium carbonate. Those marked with a † were given by the same solution after standing for two days. The solid carbonate was added to neutralise, and the slight excess varied in each case. The delay was accidental, only the glucose being estimated immediately after neutralisation in the case of these experiments carried out at 0° during 90 hours.

The action of alkali in bringing about an increase in the reducing power of the sugar solutions was here forced upon our notice for the first time. The phenomenon was studied at some length later on, and has been already mentioned in the earlier portion of this paper.

Inspection of the foregoing results shows that fructose is not appreciably oxidised at 0°, when allowed to stand for nearly four days. Prolonged action at higher temperatures appears to destroy some of this sugar.

It must however be noted that the experiments at 0° were carried out with "laevulose from inulin I," which is quite free from glucose. The others were performed with crystallised laevulose, Kahlbaum's also, and any small quantity of glucose it may have contained would have been oxidised and so cause the loss of fructose to appear too great. We suggest that the purity of samples of laevulose might be tested by allowing their solutions to stand with bromine at air temperature for one day or at 0° for four days. The loss of reducing sugar will then be due almost entirely to the destruction of glucose, maltose or other aldehydic sugar. Our experiments on fructose are in agreement with those of Votoček and Nemecek [1910]. They found that whereas, when exposed to the action of bromine for 24 hours at 21° only 5.10 % of *d*-galactose remained and 22.20 % of *d*-glucose, yet there was 100.00 % of *d*-fructose.

Action of dilute acid (N/8) on fructose.

On considering the results shown in Table I, it was thought that it might be of interest to determine what percentage of fructose apparently lost by oxidation in these experiments might have been really destroyed by the action of dilute acid on this sugar.

Accordingly to test this the following experiments were carried out, the procedure being exactly the same as in the standard experiments (Table I) except that no bromine was added. A certain portion of the sugar solution made by dissolving a weighed quantity of fructose in water was taken and the amount of sugar present determined, in one case by reduction by Kendall's method and in the other by polarimetric observations. Then 50 cc. of the original solution were diluted to 70 cc. and 10 cc. normal sulphuric acid added giving a solution of $\frac{1}{8}$ normal acidity which is the original strength of the acid in the oxidation experiments. This solution having been shaken to mix was allowed to stand for 66 hours after which period it was made up to a known volume, then an aliquot portion removed and the remaining fructose estimated by reduction. The percentage of fructose destroyed by N/8 acid under the above conditions was thus determined. It must be observed however that in experiments where a mixture is being quantitatively oxidised the acidity is greater than $\frac{1}{8}$ normal owing to the production of acid in the usual way when halogen oxidation occurs and moreover owing to aldonic acids produced from the sugars.

The results of these experiments are shown in Table IX where the figures represent the weight of fructose in grams.

TABLE IX.

	Taken	Found	Left	Percentage destroyed
A.	0.4953	0.4715	0.4664	1.061
B.	0.6740	(0.647)	0.6688	0.771

The sugars used in the above experiments were: in (A) Kahlbaum's pure "laevulose cryst" and in (B) Kahlbaum's "laevulose from Inulin II," both having been dried by exposure in finely powdered layers to sulphuric acid in a vacuum desiccator for several weeks. It will be noted that in experiment (A) the percentage destruction is calculated from the fructose found, not from the sugar weighed, since the "laevulose cryst" is not so pure as "laevulose from Inulin II," and so the value for actual fructose taken as found by reduction is used, whereas in experiment (B) where the sugar

was estimated by polarimetric readings, and the instrument available only read accurately to one-tenth of a degree, the percentage destruction is calculated from the actual amount of sugar weighed out.

It will also be apparent that in the case where the "laevulose cryst" is used, i.e. the less pure of the two qualities of sugar used (laevulose from Inulin II being when dried really an extremely pure sample), the loss indicated is greater than in the second case.

SUMMARY.

1. Glucose and maltose are quantitatively oxidised by bromine at room temperature when allowed to stand in a saturated solution. However the liquid still retains a small amount of its original reducing power towards alkaline copper solutions.

2. Fructose is not oxidised at all, or only to a very small extent, by bromine under these conditions.

3. In the presence of deci-normal sulphuric acid there is a slight loss of fructose in 66 hours at room temperature.

4. A mixture of sucrose, maltose, glucose and fructose may be analysed as follows:

(a) The amount of sucrose is determined by polarisation and reduction before and after treatment with invertase.

(b) The resulting mixture of reducing sugars is treated with bromine under standard conditions of acidity and temperature for a given time. The aldehydic sugars, glucose (including the portion derived from sucrose) and maltose are oxidised. The ketonic sugar, fructose (including the portion derived from sucrose), remains behind. The reducing power of the solution may then be taken as due to the fructose only.

(c) The rotations due to the fructose and sucrose are then allowed for in the initially determined rotation. This gives the rotation due to glucose and maltose.

(d) The reduction due to fructose is subtracted from the initial reduction. This gives the reduction due to glucose and maltose. From these data two equations can be constructed which when solved by the usual algebraical method give the amounts of the two remaining sugars. When only glucose and fructose are present the bromine method enables the latter to be directly determined, instead of indirectly by solving an equation.

5. The addition of dilute alkali to a solution of glucose, fructose or

maltose produces a large temporary increase in its reducing power. Consequently in analytical work sugar solutions must be kept neutral or very faintly acid.

6. From a study of the electrical conductivity of a mixture of sodium hydroxide and glucose some indication has been obtained of the formation of a compound between these two substances.

7. An unsuccessful attempt was made to modify Barfoed's solution for quantitative work, by reducing the reaction to the first order instead of the fifth.

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