XCVIII. A METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF MIXED REDUCING SUGARS AND ITS APPLICATION TO THE ESTIMATION OF THE PRODUCTS OF HYDROLYSIS OF STARCH BY TAKA-DIASTASE.

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Preliminary investigations on the carbohydrates in the developing apple have shown that the concentration of sugars in the fruit is less than 1 % during the first weeks of growth, and further, that the maximum starch concentration reached at any time is only about 2 %. Estimation of these carbohydrates by the usual methods would require the collection of a very large number of apples, especially in the early stages of development, when the fruit is small, with the resultant risk that the crop on any tree would be so seriously depleted that the development of the remaining fruit might be abnormal.

Since starch appears to be most satisfactorily estimated by determination of the glucose and maltose produced by hydrolysis with taka-diastase, a method for the estimation of small quantities of reducing sugars has been investigated. With such a method, determinations of starch could be carried out during the development of the apple, and furthermore, a delicate method of estimation of reducing sugar would enable the size of the samples required for sugar determination on the young and very small fruits to be kept within reasonable limits.

Estimation of small amounts of reducing sugars.

In order to determine two mixed reducing sugars it is necessary to employ two methods of estimation, and to solve the pair of simultaneous equations obtained from the results of the determinations.

Hagedorn and Jensen [1923] have used the method of oxidation by alkaline ferricyanide for the determination of amounts of glucose ranging from 0·02 to 0·36 mg. in 2 cc. Hanes [1929] has applied this method to the estimation of quantities of glucose and maltose up to 3·8 mg. in 5 cc.; also, Macleod and Robison [1929] have shown that the oxidation of reducing sugars by hypoiodite can be satisfactorily carried out in solutions of similar concentration.

The iodimetric method has already proved satisfactory for the determination of sugar in the apple if the oxidation is carried out on solutions containing about 0·06 g. sugar. It was therefore decided to investigate the oxidation of mixtures of glucose and fructose and of glucose and maltose by alkaline ferricyanide and alkaline iodine in concentrations of about 3 mg. per 5 cc.

Preparation of samples of pure sugar.

The glucose and fructose used were taken from the same samples as those employed by Archbold and Widdowson [1931] for an investigation of the iodimetric method.

Their rotatory powers and copper reducing values were determined:

Glucose.

$[\alpha]_D^{20°}$ (10 % solution) observed 52·73°; calculated 52·74°.

Weight of glucose taken	10·000 g. per 100 cc.
Weight of glucose (Cu value) found	9·998 g. per 100 cc.

Ash < 0·01 %.

Fructose.

$[\alpha]_D^{27°}$ (5·59 % solution) observed −88·19°; calculated (using Vosburgh's [1920] formula) −88·15°.

Weight of fructose taken	5·59 g. per 100 cc.
Weight of fructose (Cu value) found	5·52 g. per 100 cc.

Preparation of maltose. A sample of maltose, purchased as pure, was recrystallised twice by dissolving 25 g. in 200 cc. of 80 % alcohol heated in a water-bath under a reflux condenser. The solution was filtered, and sufficient alcohol was added to make 90 % concentration. The maltose was allowed to crystallise, filtered off and recrystallised in the same way. The maltose hydrate so obtained was dried *in vacuo* over sulphuric acid and then over phosphorus pentoxide until constant in weight, and finally dried at 50° until again constant in weight.

Copper reducing power (g. maltose hydrate per 100 cc. solution).

Calculated	Found
0·3771	0·3829

Optical rotation (calculated as anhydrous maltose).

$[\alpha]_D^{28°}$ (4·7146 % solution) Calculated 137·63 [Meissl, 1882]
Found 137·55

Oxidation of sugars by alkaline potassium ferricyanide.

The oxidation of the sugars by alkaline ferricyanide was carried out as described by Hanes, using sodium thiosulphate *N*/75 standardised against potassium iodate[1] solution and the following reagents:

[1] The potassium iodate was dried by heating at 100° for 1 hour. It was found that prolonged heating at 100° caused a slight discoloration of the salt. The thiosulphate was further standardised against potassium permanganate solution, and this gave a result differing only by 0·2 % from that given by the dried iodate.

A.	Potassium ferricyanide	8·25	g. per litre
	Anhydrous sodium carbonate ...	10·6	g. per litre
B.	Potassium iodide	12·5	g. in 500 cc.
	Zinc sulphate	25·0	g. in 500 cc.
	Sodium chloride	125·0	g. in 500 cc.
C.	Acetic acid	7·5 %	

5 cc. sugar solution containing about 3 mg. sugar were measured into a glass specimen tube $1\frac{1}{4}'' \times 4''$ and 5 cc. of solution A were added. The tube was covered with a glass lid and placed in a boiling water-bath for exactly 15 minutes. It was then cooled in water for 1 minute and in ice for 2 minutes, after which 5 cc. of solution B and 2 cc. of solution C were quickly added, and the iodine liberated was titrated with $N/75$ sodium thiosulphate solution. Two drops of a 1 % solution of soluble starch in saturated sodium chloride were used as indicator. The tubes were left immersed in ice till the contents were titrated in order to prevent any loss of iodine. It was found convenient to carry out the estimations in batches of six. Blank determinations were made by substituting 5 cc. of distilled water for the sugar solution, and all determinations were carried out in triplicate.

Hanes used boiling-tubes $1'' \times 7''$ for his estimations, and apparently did not find it necessary to cool the tubes in ice to prevent loss of iodine. Using specimen tubes $1\frac{1}{4}'' \times 4''$ however, a series of blank determinations has shown that a loss of iodine equivalent to 0·06 cc. $N/75$ thiosulphate occurred if the tube containing the liberated iodine was allowed to stand for 10 minutes at room temperature, while the loss was equivalent to 0·33 cc. $N/75$ thiosulphate at the end of an hour. If the estimations are carried out in batches of six, 10 to 15 minutes will elapse between the titration of the liberated iodine in the first tube and the sixth, so that a serious error may be introduced by loss of iodine unless the procedure for cooling described above is adopted. The results of the determinations are shown in Table I.

Table I. *Loss of iodine on standing at room temperature.*

	$N/75$ thiosulphate required cc.
Titrated immediately	9·02
Titrated after 10 min. at room temperature	8·96
,, 30 ,,	8·79
,, 60 ,,	8·69
,, 10 in ice	9·02
,, 60 ,,	8·99

Sobotka and Reiner [1930] have investigated the oxidation of various sugars by alkaline ferricyanide, and obtained the same results as Hanes for glucose and maltose, confirming his observation that the reducing power of glucose increases slightly as the concentration of sugar increases, while that of maltose is practically constant. They also investigated the oxidation of fruc-

tose, and obtained a constant value for the reducing power, but for mixtures of fructose and glucose they found a lower reducing power than that obtained for either sugar alone. Callow [1930] found for invert sugar a simple relation between the thiosulphate equivalent and the invert sugar reduced, and his result differs only slightly from that obtained by Sobotka and Reiner for mixtures of glucose and fructose. Similar determinations were repeated under the conditions described above on a series of glucose, maltose and fructose solutions, containing weights of sugar varying from 0·5 to 3·5 mg. in 5 cc.

The results are shown in Table II.

Table II. *Oxidation of glucose, maltose and fructose by alkaline ferricyanide.*

	N/100 thiosulphate≡ ferricyanide reduced cc.	cc. *N*/100 thiosulphate/mg. sugar
mg. glucose		
0·671	1·99	2·97
1·342	4·04	3·01
2·013	6·09	3·03
2·684	8·23	3·07
3·353	10·36	3·09
mg. maltose (anhydrous)		
0·558	1·43	2·56
1·117	2·80	2·51
1·675	4·25	2·54
2·792	7·02	2·52
3·351	8·46	2·53
	Mean value	2·53
mg. fructose		
0·559	1·58	2·83
1·118	3·25	2·91
1·677	4·88	2·91
2·795	8·24	2·95
3·354	9·93	2·96

The results obtained show an increase in oxidation with concentration for glucose and fructose, and a constant value for maltose. For glucose and maltose the results are slightly higher than those found by Hanes, but repeated determinations, using every precaution to prevent loss of iodine always gave the same result. For fructose a definitely lower series of values is obtained than that observed by Sobotka and Reiner. In order to confirm this result determinations were carried out on invert sugar, obtained by dissolving 0·95 g. of sucrose in 150 cc. of water, boiling with 30 cc. of 0·5 *N* HCl for 1 minute, cooling, neutralising and diluting as required. The values obtained for the reducing power of the invert sugar estimated by oxidation with alkaline ferricyanide agreed very closely with those for mixtures of equal quantities of fructose and glucose and with the "mean value" for the sugars alone. The results are shown in Table III.

In describing the preparation of his sample of glucose, Hanes states that the sugar was dried *in vacuo* over concentrated H_2SO_4 for 48 hours. It has been found, however, that after 4 months *in vacuo* over P_2O_5, glucose still contains

2–3 % of water which can be driven off at 100°. Further, Hanes gives a value for the specific rotation of his sample of glucose $[\alpha]_D^{20°} = +52{\cdot}2°$ in 10 % solution.

Table III. *Oxidation of mixtures of equal quantities of glucose and fructose by alkaline ferricyanide.*

(N/100 thiosulphate≡1 mg. sugar.)

mg. sugar	Mean value for glucose and fructose estimated separately cc.	Mixture of glucose and fructose cc.	Invert sugar cc.
0·5	2·91	—	2·87
1·0	2·95	2·93	2·94
2·0	—	2·96	2·97
3·0	3·02	3·05	3·02

According to Tollens [1884] the theoretical value for a 10 % solution is 52·75°. (In the theoretical value 52·5° given by Hanes there is no correction for the concentration of the solution.) Both these observations suggest that his sample of glucose was not completely dry. Assuming that the glucose still contained 2 % of water his results would agree very closely with those shown in Table II.

Sobotka and Reiner give no details of the preparation of their samples of sugar, and it is difficult to account for the high reducing power obtained by them for fructose.

Oxidation of mixtures of glucose and maltose and of glucose and fructose by alkaline ferricyanide.

A series of determinations was next carried out on solutions containing mixtures of glucose and maltose and of glucose and fructose, the relative proportions of the sugars and the total concentration of the sugar in the solution being varied. For glucose and maltose the total sugar content of the solution was varied from 1 to 3 mg. in 5 cc., while the ratio of glucose to maltose was varied from 3:1 to 1:3. Then from pairs of results, the ferricyanide factors for glucose and maltose in the presence of one another were calculated by the method shown below.

	Wt. maltose (anhydrous) in 5 cc. mg.	Wt. glucose in 5 cc. mg.	Total wt. sugar in 5 cc. mg.	N/100 thiosulphate≡ ferricyanide used by 5 cc. cc.
1	1·117	1·071	2·188	5·95
2	0·558	1·607	2·165	6·21

$$1{\cdot}117M + 1{\cdot}071G = 5{\cdot}95$$
$$0{\cdot}558M + 1{\cdot}607G = 6{\cdot}21$$

$$0{\cdot}653M + 0{\cdot}598G = 3{\cdot}32$$
$$0{\cdot}653M + 1{\cdot}795G = 6{\cdot}94$$
$$1{\cdot}197G = 3{\cdot}62$$
$$G = 3{\cdot}02$$

$$1{\cdot}607G = 4{\cdot}85$$
$$0{\cdot}558M = 1{\cdot}36$$
$$M = 2{\cdot}44$$

G. Glucose factor = 3·02
M. Maltose factor = 2·44

where M and G stand for the maltose and glucose factors respectively, *i.e.* cc. N/100 thiosulphate/mg. sugar.

The values for the series of estimations are shown in Table IV. The calculation shown above is made for experiment 5. It will be seen from the mean values obtained that the oxidation of one sugar is not affected by the presence of the other.

Table IV. *Oxidation of mixtures of glucose and maltose by alkaline ferricyanide.*

(N/100 thiosulphate≡1 mg. sugar.)

	Glucose cc.	Maltose cc.
1.	3·09	2·47
2.	2·93	2·52
3.	3·05	2·55
4.	2·96	2·59
5.	3·02	2·44
Mean	3·01	Mean 2·53

A similar set of determinations was carried out on mixtures of glucose and fructose, the ratio of glucose to fructose being varied from 1:1 to 1:5, whilst the total concentration of sugar in the solution used for estimation was maintained at about 3·0 mg. The factors for the oxidation of glucose and fructose in the presence of one another by alkaline ferricyanide were calculated from pairs of results as for the glucose and maltose, and a series of the results obtained is shown in Table V.

Table V. *Oxidation of mixtures of glucose and fructose by alkaline ferricyanide.*

(N/100 thiosulphate≡1 mg. sugar.)

Glucose cc.	Fructose cc.
3·11	2·99
3·13	2·97
3·15	2·97
Mean 3·13	Mean 2·97

The factors for both sugars are seen to be slightly higher than those obtained for either sugar alone, so that there appears to be a slightly increased oxidation of these sugars in the presence of one another if the ratio of fructose to glucose is greater than 1. It was found however that the use of the factors obtained for the sugars in their mixtures, when combined with the results obtained for the oxidation by hypoiodite, gave a value for the amount of fructose in a given solution which only differed by 1 % from the value for fructose when the factors obtained for the sugars alone were substituted in the calculation, while the calculated amount of glucose remained unaltered.

Oxidation of glucose, maltose and fructose by hypoiodite at 1°.

For the oxidation of glucose, maltose and fructose by hypoiodite the following reagents are required:

Sodium thiosulphate	$N/75$
Iodine in potassium iodide ...	$N/40$
Sodium hydroxide	0·3 %
Sulphuric acid	$N/4$

Samples of 5 cc. of sugar solution, containing about 3 mg. sugar are measured out into 2 oz. stoppered bottles which are left at 1° until the solutions have cooled to this temperature. 5 cc. $N/40$ iodine solution are then added followed by 2 cc. 0·3 % sodium hydroxide, and the bottles are placed in a water-bath maintained at 1° until the oxidation is complete. The stock solutions of iodine and sodium hydroxide are always kept at 1°, so that the temperature of the reaction mixture is not raised by their addition. When the reaction is complete the bottles are removed from the bath, 2 cc. $N/4$ sulphuric acid are added to each and the excess of iodine is titrated with $N/75$ sodium thiosulphate solution. All estimations are carried out in duplicate and blank determinations are made by substituting 5 cc. water for the sugar solution.

The oxidation of glucose to gluconic acid by hypoiodite at 1° has been found to be complete in 2 hours for a concentration of glucose about 0·06 % [Archbold and Widdowson, 1931]. The time of reaction for small quantities of glucose and maltose under the conditions described above was determined by allowing 5 cc. of solutions containing about 3 mg. of the sugars to react with hypoiodite at 1° for varying lengths of time. It was found that the oxidation of both sugars is complete in 2 hours and this period was accordingly used for all subsequent estimations.

The results are shown in Table VI.

Table VI. *Oxidation of glucose and maltose by hypoiodite. Time of reaction at 1°.*

Time of oxidation hours	$N/100$ thiosulphate ≡ I used cc.	cc. $N/100$ thiosulphate/mg. sugar
Glucose 3·353 mg. in 5 cc.		
$0\frac{1}{2}$	3·35	1·00
1	3·61	1·08
$1\frac{1}{2}$	3·71	1·11
2	3·72	1·11
	Theoretical value	1·11
Maltose 3·776 mg. (anhydrous) in 5 cc.		
$0\frac{1}{2}$	2·08	0·55
1	2·15	0·57
$1\frac{1}{2}$	2·21	0·58
2	2·23	0·59
	Theoretical value	0·59

Estimations were also carried out on solutions containing mixtures of glucose and maltose in varying proportions, and from pairs of results the iodine factors of the two sugars in the presence of one another were calculated by a method similar to that used for the calculation of the ferricyanide factors. A summary of the results is shown in Table VII. From the mean results it will be seen that the oxidation of one sugar by hypoiodite is not affected by the presence of the other.

Table VII. *Oxidation of mixtures of glucose and maltose by hypoiodite.*

(N/100 thiosulphate≡1 mg. sugar.)

Glucose cc.	Maltose cc.
1·11	0·59
1·09	0·60
1·11	0·62
1·13	0·56
1·13	0·56
Mean 1·11	Mean 0·59

The oxidation of small quantities of fructose alone by iodine and sodium hydroxide at 1° for 2 hours showed that the mean value of N/100 thiosulphate equivalent to 1 mg. sugar was 0·02 cc. For mixtures of glucose and fructose the oxidation of fructose was too small to be detected when less than 3 mg. of this sugar was present. For quantities of fructose above 4·0 mg. the fructose factor decreased with increasing quantities of fructose. The results are shown in Table VIII.

Table VIII. *Oxidation of fructose by hypoiodite at 1° for 2 hours.*

Fructose mg.	N/100 thiosulphate equivalent to fructose cc.	N/100 thiosulphate≡1 mg. fructose cc.
Oxidation of fructose alone.		
0·559	0·01	0·018
1·118	0·03	0·027
1·677	0·04	0·024
2·795	0·05	0·018
3·354	0·06	0·018
	Mean value	0·021

Fructose mg.	Glucose mg.	N/100 thiosulphate≡glucose (calculated) cc.	N/100 thiosulphate≡glucose + fructose (found) cc.	N/100 thiosulphate≡fructose cc.	N/100 thiosulphate≡1 mg. fructose cc.
Oxidation of fructose in the presence of glucose.					
1·677	1·607	1·78	1·78	—	—
2·236	1·071	1·19	1·19	—	—
2·795	0·536	0·59	0·60	—	—
0·559	0·536	0·59	0·60	—	—
1·118	1·071	1·19	1·18	—	—
0·839	0·161	0·18	0·17	—	—
1·677	0·321	0·36	0·35	—	—
4·974	1·281	1·42	1·56	0·14	0·028
7·461	1·921	2·13	2·29	0·16	0·021
14·922	3·842	4·26	4·46	0·20	0·013

The oxidation of fructose by hypoiodite can therefore be neglected in calculating the quantities of the sugars in solutions containing mixtures of glucose and fructose in which the amount of fructose is less than 3 mg.; the iodine value can therefore be taken as a measure of the glucose present.

Macleod and Robison [1929] applied the iodimetric method to small quantities of reducing sugars and obtained a considerable oxidation of fructose (9 % in 20 min. at 21°) when sodium hydroxide was used as alkali. If sodium carbonate were used instead of the sodium hydroxide, however, fructose was oxidised only to a very small extent. The small oxidation of fructose (less than 2 %) here obtained when sodium hydroxide was used as alkali, as compared with the higher oxidation found by Macleod and Robison is probably due to the much lower temperature employed. Since satisfactory results could be obtained with sodium hydroxide at 1° its replacement by sodium carbonate was thought to be unnecessary.

The fructose-glucose ratio in the apple rarely exceeds 4:1, and in the young fruit for which this method was to be used the fructose and glucose are in approximately equal proportions, so a dilution of the cleared apple extract to bring it to a concentration of 3 mg. sugar per 5 cc. necessarily reduces the quantity of fructose present to a level at which its presence may be neglected.

The oxidation of solutions containing known weights of glucose and fructose was then carried out by both alkaline ferricyanide and hypoiodite, and by means of simultaneous equations the weights of glucose and fructose were calculated

$$1{\cdot}11x = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{iodine reduced by 5 cc. sugar solution}$$
$$3{\cdot}13x + 2{\cdot}97y = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{ferricyanide reduced by 5 cc. sugar solution}$$

where x and y are the weights of glucose and fructose in 5 cc. of solution.

The results given in Table IX show a maximum error of 2 % on the weight of fructose present, while the recovery of glucose is quantitative.

Table IX. *Oxidation of mixtures of glucose and fructose by alkaline ferricyanide and hypoiodite.*

	Glucose taken mg.	Glucose found mg.	Fructose taken mg.	Fructose found mg.
1	1·458	1·459	1·809	1·845
2	0·972	0·973	1·809	1·822
3	0·486	0·486	1·809	1·801
4	0·486	0·486	3·015	3·050

In sugar solutions containing sucrose estimations of the total sugar present in the solutions can be carried out by hydrolysing the sucrose with hydrochloric acid and re-estimating the reducing sugars by both methods of oxidation. Citric acid must not be used for the inversion since it is oxidised both by hypoiodite and by alkaline ferricyanide.

The combination of the two methods of oxidation, by alkaline ferricyanide and by hypoiodite, thus appears to give satisfactory results for the estimation of small quantities of glucose and fructose and glucose and maltose, and could probably be applied to mixtures of any two sugars provided that the difference

between the ratio of the factors of the two sugars obtained from each method of estimation is sufficiently large. The method is convenient since the same thiosulphate solution is used for both sets of titrations.

Hinton and Macara [1924] have shown that unreliable results are obtained for iodimetric determinations of quantities of glucose of about 0·06 g. unless there is added more than twice the quantity of iodine required to oxidise the sugar. Macleod and Robison [1929] state that they obtain satisfactory results for about 1 mg. sugar if 3–4 times the theoretical quantity of iodine is present.

Since the ferricyanide factor for glucose is about 3 times the iodine factor for quantities of sugar of about 3 mg., and for maltose the ferricyanide factor is 5 times the iodine factor, if aliquot samples of the same solution are used for oxidation by both methods under the conditions described, the quantity of iodine present is always more than three times the theoretical quantity required to oxidise the sugar present, so this condition is automatically fulfilled.

Investigation of the use of taka-diastase for the estimation of small quantities of starch.

The hydrolysis of starch in plant materials by taka-diastase instead of by mineral acid was investigated by Davis and Daish [1914], and it is now generally recognised that the older method of starch hydrolysis by boiling with dilute hydrochloric acid is quite valueless, because plant material contains a considerable quantity of pentose polymerides and other substances which yield reducing sugars when boiled with dilute acid. In addition, Davis and Daish pointed out that a destruction of glucose occurs on prolonged boiling with acid. This would introduce an error even if the preliminary hydrolysis were carried out with malt diastase since a subsequent acid hydrolysis is necessary to convert the dextrins which are produced to reducing sugars.

Davis and Daish showed that under suitable conditions, starch is converted quantitatively into glucose and maltose by taka-diastase, and that therefore no subsequent acid hydrolysis is necessary. The glucose and maltose present in the hydrolysis mixture were estimated by a copper reduction method and polarimetrically, and by means of simultaneous equations the quantities of glucose and maltose present were calculated, and hence the amount of starch. They found that the optimum conditions for the hydrolysis were 24 hours at 38°.

Horton [1921] repeated the work of Davis and Daish both on pure starch and on starch in the grain of wheat. His first result on pure potato starch was satisfactory, but he was apparently unable to repeat this, and the results he obtained in all later experiments gave a recovery of starch which was too low. He suggests that the conversion of starch to glucose and maltose is not quantitative but that a small quantity of dextrin still persists. He also finds a large variation in the glucose-maltose ratio, and concludes that different preparations of the enzyme vary in their maltase content.

Tottingham and Gerhardt [1924] obtained a smaller recovery of starch

with taka-diastase than with ptyalin from woody tissues and their difficulties appear to be similar to those encountered by Horton.

Thomas [1924] has stated that the ratio of glucose to maltose obtained by hydrolysis of starch with taka-diastase is constant, and therefore an estimation of the sugars in the hydrolysate by the picric acid reduction method gives a measure of the starch present.

Bish [1929] used taka-diastase in the determination of small quantities of starch in bracken rhizome, and he estimated the resulting mixture of sugars by Shaffer and Hartmann's [1921] copper reduction method. He claims that the results so obtained give comparative values for the starch content of the tissue. This method is only reliable if there is no variation in the glucose-maltose ratio.

In view of the differences of opinion with regard to the hydrolysis of starch by taka-diastase, the hydrolysis of pure starch by this enzyme has been investigated, and the application of the methods of oxidation by alkaline ferricyanide and by hypoiodite to the products of hydrolysis has been studied.

Purification of starch. A sample of maize starch was washed with cold water, alcohol and ether, and was dried at 100°. Duplicate weighed samples were then dried at 120°, one *in vacuo* and the other under atmospheric pressure, for 16 hours. It was found that more than 99·9 % of the water remaining after drying at 100° was removed at 120° at atmospheric pressure, and that after drying at 100° to constant weight the starch contained 3·575 % of water. Quantities of 0·3, 0·2 and 0·1 g. of purified starch, dried at 100°, were weighed out into 200 cc. conical flasks, and 100 cc. distilled water were added to each. The flasks were then heated in a boiling water-bath for half an hour, cooled to room temperature and 10 cc. of freshly prepared 1 % taka-diastase solution, 0·05 cc. of 5 % acetic acid and a little toluene were added to each. It had previously been found that 5 cc. of a 0·3 % solution of taka-diastase was sufficient for the complete hydrolysis of 0·3 g. of purified starch, but a preliminary trial showed that the minimum quantity of the enzyme required for the hydrolysis of a corresponding weight of starch in apple tissue was 0·1 g., so this quantity was adopted for the investigation of the hydrolysis of the purified starch.

The flasks were plugged with cotton wool and placed in an incubator at 38° for 24 hours. After removal from the incubator the solutions were heated to boiling to destroy the enzyme and to remove the toluene. They were then cooled and diluted to 500 cc. and the sugars in 5 cc. of the solution estimated as already described. A blank determination was carried out omitting the starch. The thiosulphate titration for the hydrolysed starch solution was corrected for the titration for the blank determination for the taka-diastase before calculation of results. Then by means of simultaneous equations the glucose and maltose present were calculated

$1{\cdot}11x + 0{\cdot}59y =$ cc. $N/100$ thiosulphate $\equiv$ iodine reduced by 5 cc. hydrolysate
$3{\cdot}01x + 2{\cdot}53y =$ cc. $N/100$ thiosulphate $\equiv$ ferricyanide reduced by 5 cc. hydrolysate

where x and y are mg. of glucose and maltose present in 5 cc. of the hydrolysis mixture. The amount of starch originally present was calculated by multiplying the estimated glucose by 0·9 and the maltose by 0·9479, and adding the products. An example of the calculation of results is shown below.

Calculation of results.

Wt. of starch dried at 100° = 0·1880 g.
Wt. of dry starch = 0·1813 g.
Diluted hydrolysate to 500 cc. Estimated sugar in 5 cc.

Thiosulphate (0·01303 N) ≡ ferricyanide reduced by 5 cc. of solution =	5·94 cc.
Thiosulphate (0·01303 N) ≡ ferricyanide reduced by 5 cc. of blank determination on taka-diastase =	1·51 cc.
Difference	4·43 cc.
Thiosulphate (0·01303 N) ≡ iodine reduced by 5 cc. of solution =	2·05 cc.
Thiosulphate (0·01303 N) ≡ 5 cc. of blank determination on taka-diastase =	0·52 cc.
Difference	1·53 cc.
$N/100$ thiosulphate ≡ ferricyanide reduced by hydrolysed starch in 5 cc. solution =	5·77 cc.
$N/100$ thiosulphate ≡ iodine reduced by hydrolysed starch in 5 cc. solution =	1·99 cc.

Then if x and y are the mg. glucose and maltose present in 5 cc. solution

$$1{\cdot}11x + 0{\cdot}59y = 1{\cdot}99$$
$$3{\cdot}01x + 2{\cdot}53y = 5{\cdot}77$$

$$3{\cdot}341x + 1{\cdot}776y = 5{\cdot}990$$
$$3{\cdot}341x + 2{\cdot}808y = 6{\cdot}405$$
$$1{\cdot}032y = 0{\cdot}415$$
$$y = 0{\cdot}402$$
$$0{\cdot}59y = 0{\cdot}24$$
$$1{\cdot}11x = 1{\cdot}75$$
$$x = 1{\cdot}577$$

$$\text{Starch originally present in 500 cc. solution} = \frac{(1{\cdot}577 \times 0{\cdot}9) + (0{\cdot}402 \times 0{\cdot}9479)}{10}\ \text{g.}$$
$$= 0{\cdot}1800\ \text{g.}$$

It was found that under these conditions more than 99 % of the starch could be accounted for; a series of results is shown in Table X.

Table X. *Hydrolysis of purified maize starch with taka-diastase.*

	Starch taken g.	Starch found g.	Starch found %	Glucose/ maltose
1	0·3184	0·3158	99·18	4·69
2	0·1813	0·1800	99·28	3·92
3	0·1431	0·1426	99·65	2·74
4	0·1386	0·1376	99·28	4·96

The glucose/maltose ratio obtained was higher than that found by Davis and Daish and was not constant even with two samples of the same preparation of enzyme. This bears out the observations made by Horton in this regard, though the variation obtained is not so great as that recorded by him. No indication of the persistence of dextrin in the hydrolysate as suggested by Horton was obtained, and it appears that the only products of hydrolysis are glucose and maltose.

The effect of clearing glucose-maltose and hydrolysed starch solutions with basic lead acetate and sodium phosphate.

Since this method for the estimation of starch was intended to be applied to apple tissue, a preliminary investigation was carried out on the effect of clearing the solution containing hydrolysed starch derived from this material. This work showed that satisfactory results could not be obtained unless the solutions were cleared before estimation of the sugars, so the effect of clearing on glucose-maltose solutions was investigated.

A solution containing about 0·3 g. glucose and 0·3 g. maltose was diluted to 1000 cc. and three aliquots of 200 cc. were further diluted to 250 cc. To one was added 0·5 cc. of basic lead acetate solution, followed by 1·5 cc. of saturated sodium phosphate, and to another was added 1·0 cc. of basic lead acetate and 3·0 cc. sodium phosphate before diluting to 250 cc. The third was diluted to 250 cc. without clearing. The solutions were filtered, and the sugar in 5 cc. of each was estimated by oxidation with alkaline ferricyanide. It was found that no loss of sugar occurred when clearing was carried out with these reagents. This agrees with results obtained with glucose-fructose solutions on the macro-scale [Archbold and Widdowson, 1931]. The results are shown in Table XI.

Table XI. *Effect of clearing glucose-maltose solutions with basic lead acetate and sodium phosphate.*

	$N/75$ thiosulphate ≡ ferricyanide reduced cc.
Uncleared	6·25
Cleared 0·5 cc. basic lead acetate	6·24
Cleared 1·0 cc. basic lead acetate	6·26

The effect of clearing on solutions obtained by hydrolysis of starch with taka-diastase was next investigated. 0·3 g. starch was hydrolysed as already described, and the solution diluted to 500 cc.; 200 cc. were cleared with basic lead acetate and sodium phosphate, filtered, and diluted to 250 cc. A second 200 cc. were diluted to 250 cc. without clearing. A similar pair of experiments was carried out on taka-diastase and 100 cc. of water, with no starch added. The reducing power of the sugar in 5 cc. of all four solutions was estimated by oxidation with alkaline ferricyanide and hypoiodite. The results are shown in Table XII.

Table XII. *Effect of clearing solutions of starch hydrolysed by taka-diastase.*

Results expressed as $N/75$ thiosulphate ≡ ferricyanide or iodine reduced by 5 cc. solution.

	Ferricyanide		Iodine	
	Cleared cc.	Uncleared cc.	Cleared cc.	Uncleared cc.
Taka-diastase blank	0·58	0·60	0·22	0·22
Starch	4·44	4·45	1·52	1·52

It was found that there was no precipitate when the lead acetate was added in either case although the solution became slightly turbid, and also, there was little or no difference between the values for the cleared and uncleared solutions. Hence it is unnecessary to clear solutions obtained by hydrolysing samples of pure starch before estimating the sugars. The presence of oxidisable material other than sugar in the aqueous extract from alcohol-insoluble apple residue after enzyme hydrolysis, however, necessitates clearing, but no loss of sugar occurs during the process.

It is thus evident that satisfactory results for starch can be obtained provided that no other polysaccharide present in the tissue under consideration is attacked by the enzyme. This point will be dealt with in a later paper.

Effect of boiling coloured apple extracts with "Suchar" on the ferricyanide and iodine oxidations.

It has previously been stated [Archbold and Widdowson, 1931] that the solutions from the evaporated alcoholic extracts from apples, after clearing with basic lead acetate and sodium phosphate, are colourless when first prepared, but gradually develop a brown colour on standing. They have also been shown to contain some material, other than sugar, which is oxidised by hypoiodite, but which has no effect on the copper reduction value of the solution. This oxidisable material can be removed by boiling the solution with charcoal before the iodine estimation is carried out. Solutions prepared from the alcoholic extracts of young apples are coloured immediately after clearing and become considerably darker on standing than those prepared from the more mature fruit. Accordingly, before proceeding to a series of estimations of the fructose and glucose in the extracts from the young apples, the effect on the ferricyanide and iodine oxidations of decolorising the brown solutions by boiling with charcoal was investigated. Coloured solutions were boiled for different numbers of times with "Suchar," a preparation of charcoal, and the sugars in the coloured and colourless solutions were estimated by both methods of oxidation. A difference of 0·66 cc. was obtained between the thiosulphate titrations for the ferricyanide oxidation of the coloured and the decolorised solutions, while the difference in the titrations for the iodine estimation was 0·24 cc. Decoloration is therefore necessary for both estimations. From the results obtained the apparent glucose and fructose present were calculated, and the results for one solution are shown in Table XIII.

Table XIII. *Effect of boiling with "Suchar" on a coloured apple extract.*

	% in apple	
	Glucose	Fructose
Unboiled	2·81	6·19
Boiled three times with "Suchar" (colourless)	2·37	6·22
„ five „ „	2·37	6·20

The effect of boiling with "Suchar" on solutions of fructose and glucose was next investigated to see if a measurable loss of sugar occurred during the process. Solutions of glucose and of glucose-fructose in the ratio 1:4, were left unboiled, boiled once, and boiled a number of times with "Suchar" and estimated by both ferricyanide and iodine methods.

The thiosulphate equivalent to the iodine reduced by 5 cc. of the solution decreased from 3·45 cc. for the unboiled solution to 3·35 cc. for the solution after boiling six times with "Suchar." The ferricyanide value also decreased from 9·71 cc. to 9·42 cc. This decrease in titration was equivalent to a 3 % loss of sugar. The results are shown in Table XIV.

Table XIV. *Effect of boiling with "Suchar."*

	mg. in 5 cc. solution	
	Glucose	Fructose
Glucose		
Unboiled	3·102	—
Boiled once	3·064	—
Boiled six times	3·009	—
Glucose + fructose		
Unboiled	0·838	3·071
Boiled once	0·838	3·030
Boiled four times	0·838	3·003

In the case of the glucose-fructose solution, in which the glucose only comprises about $\frac{1}{5}$ of the total weight of sugar present, there is no apparent loss of glucose after boiling the solution four times with "Suchar," but a loss of fructose of about 5 % occurs. Hence after boiling the solution four times with "Suchar" the ferricyanide value shows a decrease of 0·20 cc. while there is no change in the iodine value, since the oxidation of fructose by hypoiodite is negligible. In this case the effect of the adsorption of glucose by the "Suchar" is too small to be detected in the iodine titration, and the loss of reducing sugar appears to be entirely due to adsorption of fructose. This result is similar to that found for more concentrated sugar solutions, when the iodine value decreases 0·8 % after six boilings with "Suchar", while the reducing power estimated by copper reduction shows a loss of 5 % [Archbold and Widdowson, 1931].

The decrease in both the iodine and the ferricyanide values found after boiling the coloured apple extract with "Suchar" is much larger than can be accounted for by adsorption of sugar by the charcoal. From these results it was concluded that coloured solutions must be decolorised by boiling with charcoal before the sugars in them are estimated by alkaline ferricyanide or hypoiodite.

Determinations were carried out on a number of cleared solutions from evaporated alcoholic extracts of apples after boiling with "Suchar" till colourless, and diluting suitably for estimation. The values obtained for the percentage of glucose and fructose in the apple, determined by oxidation with alkaline ferricyanide and hypoiodite, as compared with the results for the same

samples estimated on a larger scale by copper reducing power and iodimetrically are given in Table XV.

Table XV. *Determination of glucose and fructose in apple extracts.*

	% sugar in apple tissue (wet weight).					
	Estimated by copper reduction and oxidation by hypoiodite (sugar solution 0·2 %)			Estimated by oxidation with alkaline ferricyanide and hypoiodite (3 mg. sugar per estimation)		
	Glucose	Fructose	Reducing sugar	Glucose	Fructose	Reducing sugar
1	1·28	2·70	3·98	1·23	2·77	4·00
2	1·19	3·32	4·51	1·17	3·32	4·49
3	1·28	3·77	5·05	1·22	3·74	4·96
4	1·26	3·98	5·24	1·21	4·08	5·29
5	1·46	4·25	5·71	1·39	4·30	5·69
6	1·58	4·36	5·94	1·51	4·38	5·89

Although with pure sugar solutions a loss of fructose and glucose occurs on boiling the solutions with charcoal, it will be observed that the results obtained for the total reducing sugar determined by copper reduction on the coloured unboiled apple extracts agree with those obtained on the decolorised solution by oxidation with ferricyanide, suggesting that the coloured substance is preferentially adsorbed by the charcoal. It does not therefore appear necessary to make a correction for any loss of sugar which occurs during decoloration. The percentage of glucose calculated from the small scale estimations is always slightly lower than that obtained from the more concentrated solutions. The differences are in no case more than 5 %.

Summary.

The Hanes modification of the Hagedorn and Jensen method for the determination of reducing sugars by oxidation with alkaline ferricyanide, when combined with the iodimetric method, has been satisfactorily applied to the estimation of small quantities of mixtures of glucose and fructose and of glucose and maltose.

These methods have also been applied to the determination of the glucose and maltose obtained by the hydrolysis of starch by taka-diastase. Tests with pure starch showed that the method was accurate to within 1 %.

It has been shown that there is no loss of sugar when glucose-maltose solutions are cleared with basic lead acetate and sodium phosphate.

It has been shown that cleared, coloured solutions obtained from alcoholic extracts of young apples should be boiled with a preparation of charcoal such as "Suchar" before estimating the fructose and glucose by oxidation with alkaline ferricyanide and hypoiodite.

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