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THE COLORIMETRIC DETERMINATION OF CARBOHYDRATES IN PLANTS BY THE PICRIC ACID REDUCTION METHOD II. THE DETERMINATION OF STARCH AND OTHER "RESERVE" POLYSACCHARIDES[1]

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That the determination of starch presents one of the most difficult problems in analytical chemistry is clearly indicated in the literature of the subject. This is due largely to the variable products of hydrolysis formed under the different conditions of conversion.

Of the three chief modes of attacking the problem the method of conversion by acid hydrolysis, even if the products were definitely known, which is not the case, can be applied, in the case of plants, only to the determination of the approximate "reserve" polysaccharides, because other complexes like the hemi-celluloses would also be hydrolyzed. The method, introduced by O'Sullivan,[2] of the conversion by diastase followed by a secondary hydrolysis with hydrochloric acid of the products (the dextrins, glucose and maltose) and the estimation of the glucose formed either by the gravimetric or optical methods is, apart from the difficulties attending the polarization of small amounts of glucose, open to the objection that the accuracy may be affected by the destruction of the sugar by the hydrochloric acid, with the formation of levulinic acid and humin substances.[3] Additional data with respect to the destruction of glucose under these conditions will be given in the experimental part of this paper.

The conversion of starch into glucose and maltose by the mixed enzyme taka-diastase—secreted by *Aspergillus oryzoe*—which possesses very strong converting power has been used by Stone and Wright,[4] Croft Hill,[5] and later more successfully by Davis and Daish,[6] though Horton,[7] with the comparatively large amounts of starch used by him, encountered difficulties which he attributed to the persistence of dextrin, but he conceded that the evidence was not clear.

[1] Read at the Milwaukee meeting of the American Chemical Society, September, 1923.

The cost of this investigation has largely been defrayed by a grant from the Adams Fund for Agricultural Research.

[2] O'Sullivan, *J. Chem. Soc.*, **45**, 1 (1884).

[3] Davis and Daish, *J. Agr. Sci.*, **5**, 454 (1912–13).

[4] Stone and Wright, THIS JOURNAL, **20**, 639 (1898).

[5] Croft Hill, *Proc. Chem. Soc.*, **240**, 184 (1901).

[6] Davis and Daish, *J. Agr. Sci.*, **6**, 152 (1914).

[7] Horton, *ibid.*, **11**, 240 (1921).

It is also to be observed that Tottingham and Gerhardt[8] claim to have obtained higher results with salivary digestion than with taka-diastase.

Inasmuch as the application of the Benedict-Osterberg method has proved so satisfactory for the determination of sugars (see preceding paper), its application to the determination of starch, using taka-diastase, was investigated. The experimental evidence[6] is strongly in favor of the view that glucose and maltose are the sole products of conversion by taka-diastase, although, as already stated, Horton[7] considered the possibility of small amounts of dextrin persisting. The analytical error involved in this assumption would, however, be very small. Accordingly, the picrate-picric reducing ratio of glucose to maltose was determined and found to be considerably greater than the cupric reducing ratio, which fact is of importance since, as we shall see, no great error is introduced by a slight variation in the proportion of glucose to maltose in the products of conversion.

Experimental Part[9]

The maltose hydrate used for the determination of the picrate-picric reducing power was the Pfanstiehl brand; $[\alpha]_D^{25} = +131.0°$; water, 0.15%; ash, 0.02%.

Ten-cc. portions of solutions containing equal concentrations of the standard glucose and of the maltose hydrate (calculated as the anhydride) were taken, and the color developed, in a series of experiments, under the conditions described in the preceding paper (p. 1664). The value obtained in this way was $0.91 \doteq 0.01$. This compares with a cupric reducing ratio of glucose to maltose of 0.61.

The preliminary experiments with the method were made on potato starch. It gave on analysis: protein (N × 6.25), 0.168%; ash, 0.270%; crude fiber, 0.00%; ether extract, 0.370%; moisture, 12.860%. It was freed from moisture by drying under reduced pressure at 120° for eight hours. The starch determinations were all made on this vacuum-dried product.

The Ratio of Glucose to Maltose

The value of the ratio of glucose to maltose was determined by resolving the equations,

$$g + 0.61\,m = R \quad (1)$$
$$g + 0.91\,m = R' \quad (2)$$

where R and R' represent the sum total of the cupric and picrate-picric reducing powers, respectively, of glucose (g) and maltose (m), as indicated by the amounts of the reduction products obtained from equal volumes of the solution containing the sugars from the diastatic conversion of the starch, by the gravimetric and colorimetric methods, respectively.

In a series of 19 experiments[10] using 0.25 g. of the starch and in eight

[8] Tottingham and Gerhardt, *Ind. Eng. Chem.*, **16**, 139 (1924).

[9] For details concerning Benedict and Osterberg's solutions used, the reader is referred to the preceding paper (p. 1664).

[10] These experiments were made using three different lots of the enzyme preparation, one of which was known to be at least a year old, another about three years old. The age of the third lot was not known.

experiments using 0.1 g. starch, the ratio was found to range from 1.9 to 2.3, with a mean value of 2.05; that is, when about 65% has been converted into glucose. Whether or not this represents the equilibrium position at these low concentrations of the direct and reversible reactions between glucose and maltose has not been determined.

The cupric reducing ratio of glucose to maltose varies from 0.52 to 0.69 according to the conditions of reduction. Since, however, the difference between the picrate-picric reducing power of glucose and maltose is not very great and the ratio of glucose to maltose is approximately equal to 2.0 under the conditions to be described, it is not necessary to obtain the cupric reducing value; for by solving the equations

$$g/m = 2.0 \quad (3)$$

$$g + 0.91\ m = R' \quad (4)$$

we can estimate the starch by a colorimetric determination alone without a great sacrifice of accuracy. An example will make this clear.

In one experiment R' was found to be 0.2618 g. in terms of standard glucose.

Hence $g + 0.91\ m = 0.2618$ (5)

and substituting we have $2\ m + 0.91\ m = 0.2618$, whence $m = 0.09$ and $g = 0.18$.

Starch value of $g = 0.18 \times 0.900 = 0.1620$

Starch value of $m = 0.09 \times 0.947 = 0.0852$

Found	0.2472
Calcd.	0.2480

That the error involved by taking the ratio of glucose to maltose as 2.0 is small can be seen by calculating the values obtained in an experiment in which an extreme ratio (2.3) was obtained.

R for the total solution (250 cc.) = 0.2365 g.

R' = 0.2600 g.

Resolving Equations 1 and 2 we find a value for starch of 0.2443 g., and from Equations 3 and 4 of 0.2454. The difference is 0.0011 g.

The Method

The details of the method for determining starch are as follows.

From 1 to 4 g. (according to the quantity of starch present; the absolute amount of starch should not exceed 0.3 g.), of the vacuum-dried residue[11] freed from all traces of sugars is gelatinized by heating to boiling for 40 minutes on a steam-cup or water-bath with 200 cc. of water with continued stirring for at least ten minutes, in order to prevent the accumulation of starch aggregates, and occasional stirring thereafter. It is then cooled to 38° and incubated at this temperature for 24 hours, with 0.1 g. of the enzyme preparation and 2 cc. of toluene. During the incubation it is desirable to stir the solution gently and frequently during the first few hours since the starch gel tends to separate into two phases on standing. Moreover, the toluene[12] which is lost through evaporation is replenished as needed. At the end of this period the solution is

[11] See p. 1666 of the preceding paper.

[12] Toluene was first used by Fischer, *Ber.*, **27**, 2985 (1894); **28**, 1429 (1895).

heated to boiling in a steam-cup or water-bath for 15 minutes, in order to render the enzymes inactive, and afterwards the residue is filtered and washed thoroughly by decantation with water into a carefully calibrated 250cc. flask. An aliquot portion (30 cc. is sufficient) is taken, and 5 cc. of the mercuric nitrate reagent and sodium bicarbonate, are added, followed by filtration and treatment with zinc and hydrochloric acid as described in the preceding paper (p. 1666); 1 to 5 cc. is transferred by means of an Ostwald pipet to a 50cc. Pyrex test-tube, diluted to 10 cc., and followed by 10 cc. of the picrate-picric solution and 2 cc. of 25% sodium carbonate solution. The color of this solution and the standard is developed under the same conditions as already described in the preceding paper (p. 1666).

In calculating, the dilution factor for the mercuric nitrate solution added must, of course, be used, and a blank determination on the same amount of the taka-diastase (0.1 g.) used in the conversion experiment is carried out simultaneously. The blank experiments were found to be remarkably uniform, being 66 ± 0.03 mg. for the total amount of enzyme used. The recovery by this method of 0.25 g. of potato starch added to (1) spurs and (2) leaves of apple trees in six experiments was from 98.4 to 99.4% (calcd. 99.2%).

Experiments on a Secondary Hydrolysis with Hydrochloric Acid

A comparison of the results obtained in this manner was made with those from a secondary hydrolysis with hydrochloric acid, since a number of workers[13] have reported losses of glucose amounting to 1.0–1.5% as a result of hydrolysis for two and a half hours with 2.5% actual hydrochloric acid, due apparently to the formation of levulinic acid and humin substances; while others,[14] using the same concentration of acid, have not had the same experience.

From the results of the determinations of "reserve" polysaccharides, to be described later, it is certain that a concentration of 0.5% of the catalyst, acting at the boiling point for four hours, is sufficient for the conversion of the comparatively small quantities of "reserve" polysaccharides used for these colorimetric determinations. The inference from the results given in Table I is that glucose has been destroyed even at this low concentration.

TABLE I

COMPARISON OF THE RESULTS OBTAINED BY HYDROLYSIS WITH (1) TAKA-DIASTASE ALONE AND (2) TAKA-DIASTASE FOLLOWED BY HYDROCHLORIC ACID

Hydrolysis with taka-diastase only — Starch recovered		Hydrolysis with taka-diastase followed by HCl — Starch recovered	
G.	%	G.	%
0.2475	99.7	0.2430	98.0
...	..	.2430	98.0
.2466	99.4	.2418	97.5
...	..	.2425	97.8
.2475	99.7	.2432	98.1
...	..	.2425	97.8

[13] Noyes, Crawford, Jumper, Flory and Arnold, THIS JOURNAL, **26**, 266 (1904). Ref. 3. Ref. 8.

[14] Olmsted, *J. Biol. Chem.*, **41**, 45 (1920). Walton and Coe, *J. Assoc. Official Agr. Chem.*, **6**, 350 (1923).

The Determination of Other "Reserve" Polysaccharides

Because of the important physiological functions that polysaccharides other than starch may exercise as reserve materials in plants, no special difficulties were found in the extension of the colorimetric method to the determination of materials that are, like the hemi-celluloses for example, hydrolyzed by dil. acids and which are utilized by the plant through transformation into the corresponding sugars by elaborating the specific enzyme. However, the results by this means are to be regarded as only relative; moreover, the possible destruction of glucose may vitiate the results to some extent. Since this work has been carried out, Tottingham and Gerhardt[15] have indicated their preference for hydrolysis with 1% sulfuric acid rather than 2.5% hydrochloric acid, because of the smaller quantity of reducing substances precipitated by the clarifying agent from the former hydrolysates. These authors do not, however, appear to have recognized the work of Englis and Tsang,[16] who have shown that the losses of sugar when basic lead acetate is used as a clarifying agent were due in a large measure to the insoluble salts formed by the *deleading* agents which varied considerably in their effect; their results clearly indicate that disodium phosphate is the most satisfactory deleading agent. This is in conformity with unpublished data obtained by the present writer.

The Method

From 0.5 to 1.0 g. of the vacuum-dried residue from which all traces of sugars have been removed, as already stated in the preceding paper (p. 1666), is treated in a Pyrex Erlenmeyer flask with 200 cc. of water containing 0.5% of actual hydrochloric acid and boiled for four hours under a reflux condenser. After cooling, the solution is exactly neutralized with sodium hydroxide and filtered into a calibrated 250cc. volumetric flask. After washing the filter with water, the combined filtrate and wash waters are made up to the mark and shaken.

The subsequent procedure is exactly as described under the determination of starch, using suitable aliquot portions to keep within the concentration limits.

TABLE II

Material	Actual HCl present in solution %	"Reserve" polysaccharides as Glucose: Gravimetric %, dry basis	"Reserve" polysaccharides as Glucose: Colorimetric %, dry basis
Leaves	0.5	12.00	10.20
	0.5	10.60	10.48
	1.0	10.00	10.00
	1.0	10.50	10.05
Seedling spurs	0.5	12.00	12.00
	0.5	12.60	12.05
	1.0	11.90	11.00
	1.0	10.50	11.15
	1.0	10.50	11.00

[15] Ref. 8, p. 140.

[16] Englis and Tsang, THIS JOURNAL, **44**, 865 (1922).

A comparison of the cupric and picrate-picric reduction methods in these determinations of "reserve" polysaccharides is given in Table II.

These results would indicate that no advantage is offered in the hydrolysis of this class of material by increasing the percentage of hydrochloric acid over 0.5%, for there is clearly a destruction of glucose even with 1% hydrochloric acid.

Summary

A colorimetric method for the determination of starch by means of conversion with taka-diastase without a secondary hydrolysis has been described. Under the conditions of conversion, the ratio of glucose to maltose is remarkably constant and the analytical error introduced by regarding this ratio as 2.0 is very small, since the picrate-picric reducing ratio of glucose to maltose is relatively high. A method also for the determination of other "reserve" polysaccharides has been given.

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PLATINUM OXIDE AS A CATALYST IN THE REDUCTION OF ORGANIC COMPOUNDS. V. THE PREPARATION OF PRIMARY ALCOHOLS BY THE CATALYTIC HYDROGENATION OF ALDEHYDES[1]

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In a previous paper,[3] the writers have described the results of a study of the behavior of the platinum oxide catalyst of Voorhees and Adams[4] in the hydrogenation of benzaldehyde and heptaldehyde. It was shown that these aldehydes are only slowly and very incompletely reduced when certain impurities are rigorously excluded from the aldehyde and the catalyst, but that in the presence of small amounts of iron salts, the reduction is rapid and complete. The study has now been extended to include, on the one hand, the effect of a large number of substances on the rate of hydrogenation of benzaldehyde and, on the other hand, the effect of ferrous chloride on the catalytic hydrogenation of a variety of aldehydes and some other types of compounds. In this communication are described the re-

[1] Part of the chloroplatinic acid used in this investigation was purchased with the aid of a grant from the Bache Fund of the National Academy of Sciences. For this aid the authors are greatly indebted.

[2] This communication is an abstract of part of a thesis submitted by W. H. Carothers in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry at the University of Illinois.

[3] Carothers with Adams, THIS JOURNAL, **45**, 1072 (1923).

[4] (a) Voorhees with Adams, *ibid.*, **44**, 1397 (1922). See also (b) Adams and Shriner, *ibid.*, **45**, 2172 (1923); and (c) Kaufmann and Adams, *ibid.*, **45**, 3029 (1923).