V. THE DETERMINATION OF SMALL QUANTITIES OF STARCH IN VEGETABLE TISSUE.

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IN some work on the reserve foods of the bracken fern, it was found necessary to use an accurate method of estimating starch in small quantities. Methods commonly in use which involve comparison of determinations of the sugar mixture, obtained after hydrolysis, by reduction of copper salts, and by the polarimeter, were not suitable as the quantities were too small for the use of the polarimeter. A method was therefore devised by which the copper-reducing power of the hydrolysed starch in the plant material was related to the reducing power of known quantities of a carefully purified starch preparation. Such a method does not give absolute values as the reducing power of starches of different origins is not the same. It does, however, give values comparable with each other for any one material and this is sufficient for many purposes, such as the comparison of the amounts of starch present at different stages of development.

The standard starch used for comparisons was prepared as follows. Lintner's soluble starch was washed carefully by decantation with six changes of distilled water. The remaining water was removed by washing in a similar way with three changes of absolute alcohol and drying to constant weight at 105°. Amounts of this preparation ranging from 0.1 to 0.8 g. were then weighed into clean dry conical flasks of 125 cc. capacity and 40 cc. of distilled water added. The mixture was then boiled for 30 seconds to emulsify the starch and cooled to room temperature. 5 cc. of a 0.2 % or 0.4 % aqueous solution of takadiastase (Parke Davis and Co., Ltd.) were added together with 0.05 cc. 5 % acetic acid and 0.2 cc. toluene, and the mixture was incubated at 36.8° for 24- or 48-hour periods. The same concentration of enzyme was used throughout; 0.2 % or 0.4 % gave very nearly the same results but smaller concentrations led to variations. Trials showed that after 48 hours the reaction was not quite complete, though even after 24 hours it was sufficiently slow to give consistent results, while shorter periods gave variable results.

The most suitable temperature was found by experiment to be 37°. At lower temperatures hydrolysis was slower and the copper-reducing power was not consistent. The temperature of the incubator was regulated to $\pm 0.3^\circ$ about a mean of 36.8°, whilst that of the hydrolysis liquid varied $\pm 0.1^\circ$ about the same mean. The addition of the acetic acid fixed the p_H at 5.5, at which value it remained constant throughout the reaction.

The copper reducing power was determined by Shaffer and Hartmann's method [1921] after several others, such as those of Pavy, Bertrand and Benedict, had been tried. All determinations were carried out in the following manner. The hydrolysis mixture, after the proteins, tannins and mucilages had been precipitated (see later), was neutralised to litmus and made up to a definite volume. 50 cc. of this solution were then pipetted into a conical 750 cc. flask and 50 cc. of Shaffer and Hartmann's reagent added, together with some pieces of unglazed plate. The mixture was brought to boiling point in four minutes, boiled for exactly five minutes, and cooled for three minutes under running water. As a constant source of heat an electric hot plate or a Bunsen burner, shielded from air currents by enclosure in a suitably ventilated tin, was used. A reflux condenser prevented concentration of the liquid by evaporation. The "cuprous titration" [Shaffer and Hartmann, 1921] was used exclusively.

A series of determinations upon different quantities of prepared starch was thus made. For use, it is convenient to plot the copper values (mg. copper reduced) against the weight of starch employed. The points fall very accurately in a straight line and from this graph, the amount of starch in the sample of plant material corresponding to the amount of copper reduced may be readily obtained. As has been said, this is only relative.

Table I. *Standard starch/copper values after hydrolysis with 0.2 % takadiastase.*

24 hours' incubation		48 hours' incubation	
Wt. of starch (mg.)	Wt. of copper reduced (mg.)	Wt. of starch (mg.)	Wt. of copper reduced (mg.)
31.0	47.4	20.0	36.1
40.0	56.2	30.0	50.0
53.2	67.4	41.0	62.4
60.0	76.8	59.8	82.4
71.4	86.5	69.7	92.9
89.9	106.9	78.5	105.6
100.1	116.5	80.0	108.4
110.0	123.0	88.1	115.9
119.9	135.3	103.5	129.7
140.0	157.9	108.7	134.7
—	—	119.9	152.4
—	—	139.9	171.0

The plant material, in this case bracken rhizome, was washed in water, killed by immersion in a bath of boiling methylated spirit to which sufficient ammonia (sp. gr. 0.8) had been added to make the total concentration of ammonia 1 %, and dried for 24 hours at 105°. The weight was then constant, as far as constant weight is possible with hygroscopic material. The dried material was powdered and could then be stored in well-stoppered bottles without showing any fungus growth even after 3 years.

A weighed amount of the dried tissue powder was placed in a tared Soxhlet extraction thimble and extracted for 24 hours with absolute alcohol in a Soxhlet apparatus, dried for 12 hours at 105° and weighed again. It was

absolutely necessary to conduct these weighings in a stoppered weighing-bottle. This extraction removed the sugars, chlorophyll and some of the proteins, and also rendered the material more easily soakable with water afterwards. 4 to 10 g. of the dry powder were weighed into a dry conical 125 cc. flask, emulsified with 40 cc. of water and hydrolysed in exactly the same manner as the standard starch. The reaction was stopped at the end of 24 or 48 hours by the addition of the reagent used for precipitating the remaining proteins, tannins, and mucilages.

Two such reagents were used with equal success and equally without effect upon the copper-reducing power of the products of hydrolysis.

Fig. 1.

Standard starch/copper values

A. 24-hour period incubation.

B. 48-hour period incubation.

(1) *Lead acetate.* 20 cc. of a saturated solution of neutral lead acetate (A.R.) were added and the lead was almost entirely precipitated by the addition of 20 % potassium hydroxide, excess of the latter being avoided to prevent re-resolution of any protein material. Upon filtration, a practically colourless liquid was obtained which contained no tannin, mucilage or protein. The excess of lead was removed by passing hydrogen sulphide, filtering, and subsequently aerating the filtrate for ten minutes to remove excess of hydrogen sulphide. The liquid was neutralised to litmus by potassium hydroxide and made up to a definite volume.

(2) *Mercury salts.* This was a modification of the method described by Thomas and Dutcher [1924], the mercury solution being prepared according to their formula. 20 cc. of this solution were added to the hydrolysis liquid after incubation. Solid sodium bicarbonate was then added in small quantities until frothing ceased, followed by 5 % sodium bicarbonate solution until

neutral to litmus. After filtration to remove the precipitate, which included the tannins, proteins and mucilages, the filtrate was acidified with dilute hydrochloric acid. The remaining mercury was precipitated by passing hydrogen sulphide and filtering. Excess of hydrogen sulphide was removed by aeration as before, and the liquid neutralised to litmus. The copper-reducing power was then determined in the same way as for the standard starch.

Successive estimations on the same sample of rhizome gave concordant results, *e.g.*

	Sample 1	Sample 2	Sample 3
	(%)	(%)	(%)
	16.0	26.4	14.2
	16.2	26.0	14.4
	16.5	26.6	14.6
Average	16.2	26.3	14.4

A further test of the reliability of the method was given by estimating the amount of starch in a rhizome sample, adding a known weight of the prepared starch to another portion of the same material and making an estimation of the total starch.

The results of two such experiments show a very close agreement.

Wt. of starch in dried rhizome (g.)	Wt. of starch added (g.)	Wt. of starch determined (g.)
0.712	0.105	0.820
0.630	0.050	0.690

SUMMARY.

This paper describes a method for comparative estimation of small amounts of starch in vegetable tissue.

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