2. A fast red dye, flavone-(4′)-azo-β-naphthol, and two fast orange dyes, flavone-(2′)-azo-β-naphthol, and flavone-(3′)-azo-β-naphthol have been synthesized.

3. 2-Nitroflavanone has been prepared by a rapid method, although in poor yield.

4. Other compounds which have been synthesized for the first time are: 2′-hydroxyflavone, 2′-acetoxyflavone; 2′-diacetylaminoflavone; 3′-diacetylaminoflavone; 4′-diacetylaminoflavone; β-phenoxyhydrocinnamic acid, the barium salt of a disulfo derivative of β-phenoxyhydrocinnamic acid; and methyl-β-bromohydrocinnamate.

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QUANTITATIVE DETERMINATION OF SOLUBLE STARCH IN THE PRESENCE OF STARCH AND ITS HYDROLYTIC CLEAVAGE PRODUCTS.

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Introduction.

Solutions of alcohol, barium hydroxide, ammoniacal lead acetate and ammonium sulfate have been employed in the fractioning of dextrins and the separation of soluble starch from the dextrins. These reagents precipitate not only soluble starch, but also some of the products of more advanced starch hydrolysis, the action in any case depending upon the concentration of the precipitant in the hydrolysis mixture. In effecting a quantitative precipitation of soluble starch, some of the higher dextrins are precipitated as well. Because of this fact, and because of the difficulty in handling such precipitates as well as their constant tendency to undergo further hydrolysis on manipulation, it has been difficult to obtain a precipitate of soluble starch sufficiently pure to warrant its quantitative determination.

Young[1] first called attention to the separation of soluble starch from the dextrins and lower carbohydrates by means of half-saturated ammonium sulfate. This reaction is slow and not dependable for quantitative results. The precipitate can be washed free from the lower carbohydrates only.

Our ignorance of the number and nature of the products of the hydrolysis of starch, adds to the difficulties encountered in a study of the soluble starch fraction of hydrolysis mixture. Much confusion exists in regard to the mode of progress of the hydrolysis reaction and as to the number and the properties of the products. If starch be regarded as a

[1] *J. Physiol.*, **22**, 401 (1898).

polysaccharide, the very large molecule of which might theoretically be built up by the condensation of dextrose molecules with the loss of water, then the nature of its hydrolysis can be regarded as a reversal of this process where each addition of a molecule of water implies a splitting and a simplification of the complex starch molecule. Each step in the process of simplification forms a new compound. The number of these compounds serving as stepping stones downward is not known, nor is the size of the original starch molecule known. We know that soluble starch results as an early product in the reaction. Whether it arises as a simple hydrate of starch or as product of hydration plus an asymmetrical splitting of the starch molecule, are disputed points. Soluble starch is followed by an undetermined number of dextrins. Each successive product shows less tendency to produce colored compounds with iodine and a nearer approach toward the properties of the simpler reducing carbohydrates. The dextrins are followed by isomaltose and maltose which in turn split symmetrically on hydration, yielding two molecules of dextrose.

If each stage in the hydrolysis reaction were completed before the next began, the study of the cleavage products would be much simplified. But each cleavage product, as fast as it is formed in solution or in suspension, is itself a participant in further reaction. In fact, it appears that each step in the hydrolysis, after the soluble starch stage, yields a product which in turn is more easily hydrolyzed than its predecessor. From this it follows that maltose and dextrose must result very early in the reaction.

The isolation and identification of the various products of hydrolysis are rendered difficult by the transitional character of the properties of these products. The earlier products retain many of the properties of starch. The later ones, losing these, take on more of the properties of the simpler carbohydrates. The earlier products are colloidal and for differential study are of difficult manipulation. The later products are crystalloidal and more easily handled.

Quantitative studies of the hydrolysis of starch as it proceeds step by step have been limited and inexact. The amount of reducing carbohydrate present in any given product has been the criterion for determining the rate of hydrolysis. The iodine color compounds have served as a means of following the progress of the reaction. These methods give little idea of the early changes in the reaction. To gain an idea of these early changes, it appeared necessary to determine quantitatively the early products of hydrolysis. Soluble starch being the initial product of starch hydrolysis, the first step was to find a method for its rapid quantitative precipitation in a form which could be washed free from the other carbohydrates present in a hydrolysis mixture. The iodide of soluble starch

was found to be precipitated readily from its solution in the presence of dextrins and lower carbohydrates, by ammonium sulfate. This precipitate could be washed free from the other carbohydrates by means of solutions of ammonium sulfate. On heating the precipitate in water suspension, its iodine could be driven off and the soluble starch could then be hydrolyzed by acids and estimated by the usual methods for determining dextrose.

Experimental.

Potato starch was used in this study. Carefully washed and peeled potatoes were grated to a pulp; the starch was separated from the bulk of the fiber by leaching it out through several layers of cheese-cloth, using large volumes of water and actively manipulating the starch-bearing pulp within the cheese-cloth bag.

The starch was separated from the small amount of fiber passing through the bag, by means of sedimentation. Continued washing by decantation freed the starch granules from soluble impurities.

The washed starch was then dried in air at room temperature and put through a 100-mesh sieve which removed the last traces of fiber. From this product as a stock supply, samples containing soluble starch were prepared as needed by acid hydrolysis in 95% alcohol under conditions chosen so as to yield hydrolysis mixtures of varied composition. The acid was neutralized with sodium hydrogen carbonate, the residue washed with water and dried at room temperature. These mixtures were prepared in bulk.

Four 3-g. samples of each of the preparations containing soluble starch were then weighed out and transferred to 350 cc. Erlenmeyer flasks. Two of these were run as duplicates. To each of the other two, 3 g. of a commercial erythrodextrin was added and these were then run in duplicate to show the separation of such a large amount of dextrin from the soluble starch of the particular sample used.

Each of the 4 samples was then suspended in about 200 cc. of water and agitated continuously while the temperature was raised to the boiling point. Thus everything except the unchanged starch was put in solution.

The samples were then cooled as quickly as possible by allowing the flasks to stand in cold water. The solutions were next transferred to 250 cc. volumetric flasks and made up to volume with water. The insoluble starch was separated at this point by filtration. Where considerable unchanged starch appeared as starch paste which would have rendered filtration tediously slow, preliminary centrifuging was employed to sediment most of this and the supernatant solution was drawn off and filtered.

Aliquot portions of this filtrate (200 cc. in most cases, 150 cc. in a few cases) were taken and transferred to 500 cc. centrifuge bottles, fitted with

rubber stoppers. To each was then added 10 cc. of 4% iodine solution in 6% potassium iodide solution. This furnished the excess of iodine necessary for immediate precipitation of the iodine compound of soluble starch when the ammonium sulfate solution was subsequently added. An equal volume of saturated ammonium sulfate solution was then added to each aliquot sample. The bottles were stoppered and the contents thoroughly mixed. The soluble starch iodine compound separated immediately in the form of a flocculent precipitate. The bottles were now filled and balanced with half-saturated ammonium sulfate solution and centrifuged at moderate speed for 5 to 7 minutes.

The precipitate was completely thrown down so as to occupy on an average about the lowest tenth of the bottle. The supernatant liquid, which appeared reddish brown in color, was siphoned off into a 2-liter flask. This solution was subsequently poured through a filter to collect any particles of the precipitate which might have been drawn off with the liquid. Distilled water was added to the precipitate up to half the capacity of the bottle, the bottle tightly stoppered and shaken vigorously. This effected solution of a minor portion of the precipitate and suspension of the major portion. An equal volume of saturated ammonium sulfate solution was added in each case. The bottles were then balanced and centrifuged as before. Five such washings were made. The samples which contained the soluble starch product alone were easily rendered free from the red dextrin coloration, while those containing an equal weight of commercial dextrin were rendered free with more difficulty. As the washings became colorless, tests were made with iodine solution prior to filtration, to make certain that no soluble starch was in solution. This was found to be an unnecessary precaution. Occasionally the supernatant solution would have a homogeneous blue color due to the suspension of finely divided starch iodide particles. Filtration invariably removed these suspensions, quantitatively. The blue precipitate on the filter should be kept at a minimum in order to assure rapid filtration of the rather large volumes of solution used in the washing process.

After the last washing, the precipitate in the bottle was drained as free as possible from the washing solution by inverting the bottle over the filter. When the filters had drained completely, the precipitate on them was washed back with hot water into the respective bottle containing the bulk of the precipitate. Great excess of water must be avoided, for when the precipitate is finally quantitatively transferred from the bottle into a 500 cc. Erlenmeyer flask, the volume should not exceed 300 to 400 cc. After transferal, 5 cc. of hydrochloric acid (1.125 sp. gr.) was added and the contents of the flasks boiled over a direct flame for 2.5 hrs. and the boiling regulated so as to reduce the volume to about 200 cc.

This boiling breaks up the starch iodine combination and drives off

the free iodine so that there results a clear, colorless solution of the starch, containing some ammonium sulfate carried over by the precipitate.

Twenty cc. of hydrochloric acid (1.125 sp. gr.) was then added to the flasks containing the 200 cc. of solution. They were connected with reflux condenser and heated in a boiling water bath for 4 hours.

On removal and cooling, the solutions were made up to 250 cc., then filtered and the amount of dextrose determined by the aid of the polariscope, using a Fric instrument, a 20-cm. tube, white electric light through a chrome eyepiece and reading the Ventzke scale (1° Ventzke = 0.3466° angular).

From the readings the amount of dextrose in each sample was then calculated. These figures were reduced to soluble starch content by multiplying by the factor 0.9.

TABLE I.[1]

Sample No.	Starch, 3 g.	Starch, 3 g.	Dextrin, 3 g. Starch, 3 g.	Dextrin, 3 g. Starch, 3 g.	Av. excess.
I (Lintner's Starch).....	2.359	2.340	.....	2.460	0.110
II.....	2.340	2.358	2.525	2.543	0.185
III.....	2.192	2.173	2.404	2.422	0.230
IV.....	2.312	2.312	2.469	2.469	0.157
V.....	2.574	2.558	2.728	2.709	0.152
VI.....	2.496	2.496	2.515	2.543	0.033

Discussion.

These results indicated a rather constant excess in the mixed samples of starch and dextrin in spite of the fact that the dextrin-red had been washed out. Determinations on 3-g. samples of the dextrin used were made in the same manner as upon the above samples. A small precipitate resulted in half-saturated ammonium sulfate with excess of iodine. This was washed free of dextrin-red. On transferal of the washed precipitate from the centrifuge bottle to a flask, it went into solution readily and gave a violet or purplish solution much different from the blue of soluble starch. Since no soluble starch could be identified in the dextrin used, the disturbing factor was recognized as amylodextrin, or the erythrodextrin No. 1 of some authors. This compound was found to be readily precipitated from a half-saturated solution of ammonium sulfate on addition of an excess of iodine. In subsequent determinations no additions of iodine were made in the consecutive washings of the starch and amylodextrin precipitate and the latter factor was easily washed out. The determinations on the duplicate 3-g. samples of dextrin further confirmed amylodextrin as the disturbing factor by yielding 0.133 g. and 0.138 g. results, when calculated as in the above table.

[1] In this series an excess of iodine was kept up by the addition of 2.5 cc. of the 4% iodine in potassium iodide with each washing.

TABLE II.[1]

Sample No.	Starch, 3 g.	Starch, 3 g.	Starch, 3 g. Dextrin, 3 g.	Starch, 3 g. Dextrin, 3 g.	Av. excess.
I	2.312	2.312	2.312	2.312	0.000
II	2.312	2.312	2.312	2.312	0.000
III	1.603	1.603	1.618	1.664	0.038
IV	2.018	2.018	2.050	2.035	0.029
V	1.706	1.726	1.706	...	(—0.010)
VI	2.266	2.281	2.312	2.312	0.038
VII	1.600	...	1.618	...	0.018
VIII	2.052	...	2.035	...	(—0.017)
IX	1.705	...	1.705	...	0.000
X	2.176	...	2.190	...	0.014

A study of Table II shows in an indirect manner that amylodextrin has been washed out since the constant excess of the mixed samples had disappeared.

It has been possible to precipitate the red erythrodextrin iodine compound in the presence of saturated solution of ammonium sulfate when the iodine solution used in these experiments was added to the point of incipient precipitation of free iodine. This fact, however, need not be considered as a possible disturbing factor in the methods outlined here, but it deserves further consideration in methods for the quantitative study of the simpler hydrolytic fractions in the conversion of starch.

Acknowledgment is made of the assistance and helpful suggestions of Dr. Welker throughout the course of this work.

Conclusions.

The experiments outlined here seem to warrant the following conclusions:

1. Soluble starch can be estimated quantitatively in the presence of starch or of any or all of its hydrolytic cleavage products.

2. The soluble starch and amylodextrin fraction of a hydrolytic cleavage complex can be estimated with consistent accuracy.

3. The amylodextrin fraction in any similar complex may be estimated either by direct or indirect methods.

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[1] Samples of dextrin alone yielded a small precipitate which disappeared on washing in the absence of excess iodine, showing in a direct manner that amylodextrin, while precipitated along with the soluble starch, may be washed out as the initial excess of iodine disappears.