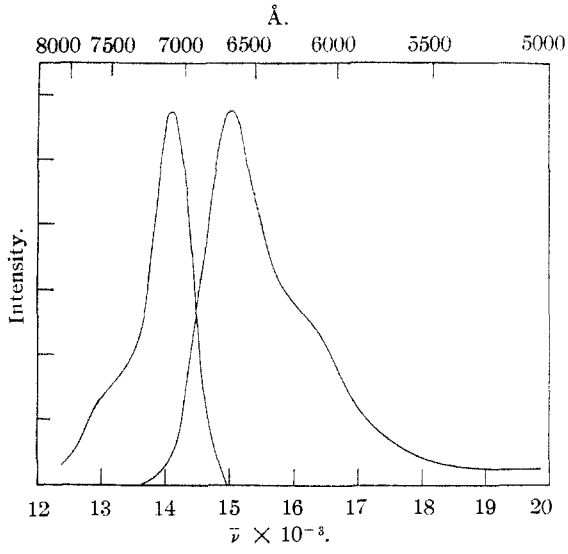

Fig. 4.—The absorption and fluorescence of methylene blue: right, absorption curve in glycerol at room temperature; left, emission curve in glycerol at −25°; the ordinates are direct microphotometer readings, no corrections having been made for plate character; nor has correction been made for self absorption, as shown by the rapid falling off on the high frequency side of the fluorescence curve.

methylene blue is shown in Fig. 4 which gives the absorption and emission curves of methylene blue in glycerol. Finer structure could doubtless be obtained, for example in 95% ethanol at 110°K., but this figure will suffice to show that the subsidiary bands of methylene blue are vibrational.

Summary

The dimeric methylene blue ion which Rabinowitch and Epstein demonstrated in aqueous solution also occurs in 95% ethanol at low temperature and at high concentration. The absorption curve of the pure monomeric ion is obtained in ethanol at room temperature and, by extrapolation, in 95% ethanol at 110°K. The latter curve is identical with one obtained by illuminating leuco methylene blue at low temperature in a rigid solvent.

In addition to the ionic dimers in solvents of high dielectric constant, molecular dimers and polymers are found in solvents of low dielectric constant. Their absorption curves are given.

There is a colorless form of the ion obtained by hydrolytic addition, in which a hydroxide ion goes to the central nitrogen and a hydrogen ion to one of the amino nitrogens. It is shown that in pure water, and especially in a molal salt solution, a considerable part of the dye is in the colorless form. At infinite dilution in water the absorption of methylene blue at its main peak diminishes with increasing temperature, in contrast with the increase found by Rabinowitch and Epstein in concentrated solutions. Except at infinite dilution the colorless ion itself polymerizes. In order to explain the effect of salts, the concept of an ion of a *distributed charge* is introduced.

By comparing the fluorescence and absorption spectra it is shown that the subsidiary absorption bands are due to the vibrational resolution of a single electronic band.

Berkeley, Calif. Received February 23, 1943

[Contribution from the Division of Plant Nutrition, College of Agriculture, University of California]

The Separation and Quantitative Estimation of Amylose and Amylopectin in Potato Starch

By R. M. McCready[1] and W. Z. Hassid

It has long been recognized that starch can be separated into two fractions of widely different physical properties. Maquenne and Roux[2] designated these fractions as amylose and amylopectin. The former is easily soluble in water and forms a slightly viscous solution; the latter is more insoluble and gives relatively opalescent and highly viscous solutions. Various methods have been employed to isolate the two starch fractions, but no quantitative separation has yet been effected. Different workers reported the amylose fraction to vary from 17 to 60%.[3] It now appears that the fractions designated as amylose and amylopectin were by no means identical, and did not represent the same portions of the starch.

When starch grains are swollen in water at 60 to 80°, they are not ruptured, but the crude

(1) Present address, Western Regional Research Laboratory, Albany, California.

(2) L. Maquenne and E. Roux, *Compt. rend.*, **137**, 88 (1903).

(3) Z. Gatin-Gruszewska, *ibid.*, **146**, 540 (1908); H. C. Sherman and J. C. Baker, This Journal, **38**, 1885 (1916); A. R. Ling and D. R. Nanji, *J. Chem. Soc.*, **123**, 2666 (1923); T. J. Schoch, *Cereal Chem.*, **18**, 121 (1941).

amylose diffuses out of them and can be separated from the swollen grains by settling or centrifuging. Although initially the amylose seems more soluble than the amylopectin, it becomes more insoluble than the latter after it has been precipitated and dried. The small amount that dissolves, rapidly retrogrades from solution. It appears that the more completely the amylose is separated from the amylopectin, the more insoluble the amylose becomes. Samec and Mayer[4] isolated a fraction (amyloamylose) from potato starch by autoclaving and electrodialysis, which was later shown to be completely hydrolyzed with β-amylase to maltose.[5] With unfractionated starch the enzymic hydrolysis ceases when approximately 60% has been converted into maltose. This behavior of the amylose toward β-amylase assumes a position of theoretical interest with regard to the molecular constitution of starch. Synthetic starch prepared by Hanes[6] through the action of potato phosphorylase on the Cori ester is similar to amylose in being completely hydrolyzed with β-amylase to maltose. Furthermore, the synthetic starch closely resembles the amylose in solubility, tendency to retrograde from solution, and in the intensity of the blue color resulting from the addition of iodine. Hassid and McCready[7] and Haworth and collaborators[8] showed that the synthetic starch was made up of very long chains of glucopyranose units with little or no branching. It is known that natural starches possess a branched structure, consisting of a multiple of relatively short chains of 24 to 30 glucose units. Meyer and collaborators[9] presented evidence that the amylose fraction of starch also consists of long non-branched chains. Synthetic starch, therefore, resembles the amylose fraction of starch not only in its physical properties but also in its molecular constitution. The vague relationship that hitherto existed between the two starch fractions, amylose and amylopectin, now becomes clarified. Thus, the starch fraction (amylose) which can be completely hydrolyzed with β-amylase to maltose is identified with a non-branched long chain structure, while the fraction (amylopectin), which is incompletely hydrolyzed, is recognized by its branched and relatively short chain structure.

The present work deals with the correlation between the degree of β-amylase hydrolysis of starch and its fractions, and their color intensity with iodine. The authors describe a simple method for preparing pure amylose and amylopectin, and a quantitative method for their determination, based on the color reaction with iodine. By examining the intensity of the blue color with iodine, of mixtures of amylose and amylopectin, their relative proportion can be estimated.

Experimental

Preparation of Amylose and Amylopectin.—A mixture of 25 g. of air-dried potato starch and 25 cc. of cold water was added slowly with stirring to 150 cc. of water at 80°. The temperature soon dropped to 70° and was so maintained for five minutes. The starch paste was then poured into 1200 cc. of water at 60° and stirred very slowly at this temperature for four hours. This treatment dissolved out the amylose from the granules. Slow stirring and adherence to this temperature are essential in order to avoid rupture of the swollen starch granules. At the end of this period, the starch solution was cooled, centrifuged, and the clear supernatant liquid carefully decanted from the gelatinous deposit of crude amylopectin. This operation separated the major part of amylose from the amylopectin. The centrifuged solution was filtered with suction on a no. 1 filter paper precoated with diatomaceous silica, "Hyflo-Super-Cell." Upon the addition of enough methanol to the clear centrifugate to make up a 20% alcoholic solution, a flocculent precipitate was formed. The latter was allowed to settle for several hours and then collected by centrifugation. It was ground in a mortar with 95% ethanol, filtered and dried in a vacuum oven. The product, as will later be shown, was pure amylose and weighed (A) 2.20 g.

The turbid 20% methyl alcoholic solution, left after separation of the amylose fraction (A), was precipitated by the addition of more methanol to a concentration of 50%. This fraction, representing a mixture of amylose and amylopectin, weighed (B) 0.70 g.

The gelatinous deposit left after the first centrifugation was removed from the centrifuge tubes, ground in a mortar with 95% ethanol, filtered and dried. The yield of the crude amylopectin (contaminated with 7% amylose) was (C) 17.6 g. (see Table I).

Further purification of amylopectin can be accomplished by making approximately a 1% solution of the crude gelatinous product as soon as it is removed from the centrifuge tubes, and placing in an electrodialysis apparatus for twenty-four hours with a little toluene. At the end of this period the solution containing the small amount of amylose is siphoned off, the gelatinous precipitate dissolved again and dialyzed for another twenty-four hours. The precipitate deposited on the membrane nearest the anode is collected, dehydrated with alcohol and dried as before. A

(4) M. Samec and A. Mayer, *Kolloidchem. Beihefte*, **13**, 272 (1921).

(5) M. Samec and E. Waldschmidt-Leitz, *Z. physiol. Chem.*, **203**, 16 (1931); G. G. Freeman and R. H. Hopkins, *Biochem. J.*, **30**, 446 (1936).

(6) C. S. Hanes, *Proc. Roy. Soc.* (London), **B129**, 174 (1940).

(7) W. Z. Hassid and R. M. McCready, THIS JOURNAL, **63**, 2171 (1941).

(8) W. N. Haworth, R. L. Heath and S. Peat, *J. Chem. Soc.*, 55 (1942).

(9) K. H. Meyer, M. Wertheim and P. Bernfeld, *Helv. Chim. Acta*, **23**, 865 (1940); **24**, 378 (1941).

preparation thus treated, using the criteria described later, consists of pure amylopectin.

Table I represents a summary of the yields of amylose and amylopectin, and also shows the purity of the various fractions.

Table I

Yields and Purity of Amylose and Amylopectin Obtained from 21.5 G. of Dried Potato Starch

Sample	Dry wt., g.	Iodine color intensity, Klett–Summerson scale reading	Amylose, %	Wt. amylose, g.
25 g. potato starch (air-dried)	21.5	100	19.0	4.09
(A) Amylose	2.20	310	100	2.20
(B) Amylose–amylopectin (mixture)	0.70	210	65	0.45
(C) Amylopectin	17.60	68	7	1.23
(A), (B) and (C)	20.50		18.9	3.88

The 18.9% total recovery of amylose obtained by analyzing the separate fractions agrees well with the value of 19% found by direct determination of starch.

Separation of the Two Fractions by the Samec and Mayer Method.—Samec and Mayer[4] separated starch into two fractions, which they named amyloamylose and erythroamylose, by autoclaving and electrodialysis. The amyloamylose fraction was later found[5] to be completely hydrolyzed with β-amylase to maltose, a property characteristic of amylose prepared by our method. In view of this similarity, it was of interest to isolate the two fractions by the Samec and Mayer method and to compare their properties with those of amylose and amylopectin which we prepared. Half a gram of amyloamylose and 3 g. of erythroamylose were thus prepared.

The amyloamylose, similar to the amylose, was soluble to the extent of about 0.1% in water, completely hydrolyzed with β-amylase to maltose and produced a brilliant blue color with iodine. The erythroamylose formed a viscous opalescent solution in water, was only partially hydrolyzed with β-amylase and treatment with iodine produced a much less intense color. These results are summarized in Table II. The data indicate that the properties of amyloamylose and erythroamylose, prepared by the method of Samec and Mayer, are practically identical with those of amylose and amylopectin prepared in our Laboratory.

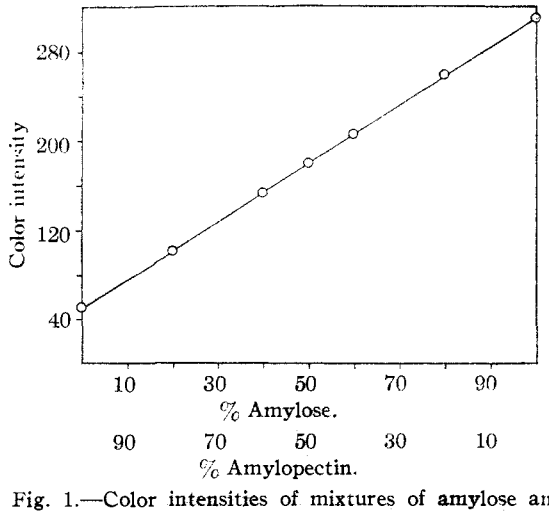

Fig. 1.—Color intensities of mixtures of amylose and amylopectin in concentrations of 0.001%, obtained from direct scale readings on the Klett–Summerson photocolorimeter with K_{66} filter.

Table II

Degree of β-Amylase Hydrolyses of Starch Fractions and their Color Intensities with Iodine

Sample	Solubility in water	β-Amylase hydrolysis, %	Iodine color intensity, Klett–Summerson scale reading
Amylose	Insoluble	100	310
Amyloamylose (Samec and Mayer)	Insoluble	100	310
Amylopectin	Colloidal soln.	54	50
Erythroamylose (Samec and Mayer)	Colloidal soln.	54	50
Synthetic potato starch	Insoluble	98	305
Natural potato starch	Colloidal solution	64	100

Preparation of Synthetic Starch.—Starch was synthesized by the action of potato phosphorylase on Cori ester by the method of Hanes.[6] Its properties were similar to those of the amylose fraction from potato starch (see Table II).

Procedure for Determination of Amylose and Amylopectin.—The method is based on the fact that pure amylose, when greatly diluted, turns a brilliant deep blue with iodine, while amylopectin gives a much less intense color. Natural potato starch, containing about 20% amylose, produces a color which is intermediate between that of amylose and amylopectin. By quantitatively determining the color intensity of a starch sample, the proportion of amylose and amylopectin can be estimated.

The starch samples were prepared for analysis as follows: one hundred mg. of the powdered dry sample was introduced into a 100-cc. volumetric flask, wetted with 1 cc. of ethanol and 10 cc. of water. The sample was dissolved by adding 2 cc. of 10% sodium hydroxide and heating on a water-bath until a clear solution formed. The flask with its contents was cooled and diluted to the mark. (Amylose or synthetic starch does not retrograde from an alkaline solution.)

A 5-cc. portion (equivalent to 5 mg.) of the alkaline starch solution was introduced into a 500-cc. volumetric flask, about 100 cc. of water was added and slightly acidified with 3 drops of 6 *N* hydrochloric acid. The contents were well mixed by shaking the flask, 5 cc. of the iodine solution was added and diluted to the mark. (The solution was prepared by making a 0.2% iodine solution in 2% potassium iodide.) The color developed immediately to its full intensity and remained stable for many days. The intensity of the blue color was estimated, using a Klett–Summerson photoelectric colorimeter. The 20-mm. glass cell was used in conjunction with the red K_{66} filter furnished with the instrument. 85% of the light transmitted through this filter has a wave length of 640 to 700 milli-

microns, which is near Simerl and Browning's[10] optimum wave length values for the estimation of this blue color. The photocolorimeter was adjusted so that the blank which had a light yellow color, due to the iodine, gave a reading of 0.

The readings on the logarithmic scale of the colorimeter are directly proportional to the concentration, if Beer's law holds. In Fig. 1 direct scale readings are plotted against percentage concentrations of mixtures of amylose and amylopectin. The total starch concentration, whether a pure sample or a mixture, is kept constant at 1.0 mg. per 100 cc. of solution. As evident from Fig. 1, artificial mixtures of amylose and amylopectin (20, 40, 50, 60 and 80% amylose) fall on a straight line connecting pure amylopectin and pure amylose.

Hydrolysis of Starch Fractions with β-Amylase.—A criterion for purity of the starch fractions was based on 100% hydrolysis with β-amylase to maltose. The degree of hydrolysis of unfractionated potato starch by this enzyme is from 60 to 64%. Complete hydrolysis of amylose (also Samec and Mayer's amyloamylose) with β-amylase coincided with a reading of 310 on the photocolorimeter. Enzymatically synthesized potato starch hydrolyzed to the extent of 98% and its color intensity corresponded to a reading of 305.

The hydrolyses were carried out with β-amylase prepared from ungerminated barley, according to Hanes and Cattle.[11] The correlation between the degree of hydrolysis with the enzyme and the intensity of the blue color with iodine of starch and its different fractions is summarized in Table II.

(10) L. E. Simerl and B. L. Browning, *Ind. Eng. Chem., Anal. Ed.*, **11**, 125 (1939).

(11) C. S. Hanes and M. Cattle, *Proc. Roy. Soc.* (London), **B125**, 387 (1938).

The authors express their appreciation to Dr. C. S. Hanes, Cambridge University, for his helpful suggestions on the separation of the two starch fractions.

Summary

1. A simple method for separation of potato starch into two fractions, amylose and amylopectin, is described.

2. The amylose is almost insoluble in water, completely hydrolyzed with β-amylase to maltose and gives a brilliant blue color when treated with iodine.

3. The amyloamylose fraction of starch prepared by Samec and Mayer's method possessed properties similar to amylose and is therefore considered to be identical with the latter.

4. Enzymatically synthesized potato starch also resembles the amylose fraction of starch in all its properties.

5. A quantitative method based on the color reaction with iodine, for estimation of the relative proportion of amylose and amylopectin in mixtures, or in unfractionated potato starch is described.

BERKELEY, CALIFORNIA RECEIVED OCTOBER 6, 1942

[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

The Molecular Constitution of Amylose and Amylopectin of Potato Starch

BY W. Z. HASSID AND R. M. MCCREADY[1]

The molecular structure of the two starch components, amylose and amylopectin, known to possess different physical properties, was first studied by Hirst, Plant and Wilkinson.[2] These authors concluded that both fractions are made up of chains consisting of 24 to 30 glucopyranose units and that their molecular constitution is essentially the same. They attributed the differences in physical properties of the two starch components to the difference in state of hydration and degree of aggregation, or interlocking of the chains to form large colloidal micelles. Recently Meyer and collaborators[3] presented evidence, based on methylation experiments, showing that the two starch fractions possess a different molecular structure: the amylose, consisting of long unbranched chains of glucopyranose units, and the amylopectin of branched chains of about 27 units. Haworth, Heath and Peat,[4] however, appear to doubt the accuracy of their experimental results. They state that Meyer and collaborators "have attempted a very rough end-group estimation of an incompletely methylated amylose from potato starch . . . ."

The question as to whether starch is a uniformly structural compound, or whether it exists as a mixture of two chemical entities, therefore, requires further study. In view of the importance of this question, the present writers undertook a reinvestigation of the problem.

Amylose and amylopectin were prepared by the

(1) Present address, Western Regional Research Laboratory, Albany, California.

(2) E. L. Hirst, M. M. T. Plant and M. D. Wilkinson, *J. Chem. Soc.*, 2375 (1932).

(3) K. H. Meyer, M. Wertheim and P. Bernfeld, *Helv. Chim. Acta*, **23** 865 (1940); **24**, 378 (1941).

(4) W. N. Haworth, R. L. Heath and S. Peat, *J. Chem. Soc.*, 55 (1942).