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CHARACTERIZATION OF SOME PRODUCTS OF STARCH-ENZYME DIGESTION

by

Vera Dawson Martin

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Enzyme Chemistry

Approved:

Signature was redacted for privacy.
In charge of Major Work

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INTRODUCTION

In enzyme-substrate studies two factors are important, the nature of the substrate and the nature of the enzyme. The question of the mechanism of amylase action on the naturally occurring substance called "starch" is complicated by uncertainty regarding both factors. The great mass of conflicting data recorded in the literature shows a definite need for clarification of the terms used to designate the exact substrate as well as the source and characterization of the enzyme.

The enzymes which hydrolyze starches have been variously classified into two or three types. Kuhn (33) first demonstrated that there were two types of amylases which he designated alpha and beta depending on the mutarotation of the products. This work has been duplicated by Ohlsson and Edfeldt (46) and Freeman and Hopkins (17). Sherman and coworkers (61, 63, 64, 65) considered two types of amylases depending on whether the products of hydrolysis were mainly maltose or dextrins. The enzyme which produced maltose predominantly was called a "saccharogenic" amylase. The other type was called "amyloclastic" because of the formation of dextrins. Measurement of the saccharogenic power depended upon the determination

of reducing values after one half hour of digestion. The reducing power is assumed to be due entirely to the maltose formed during the digestion. The Wohlgemuth (86) method was used to determine the "amyloclastic power". The disappearance of the blue iodine color in the digestion mixture serves as a basis for this method. However, as pointed out by Samec (57), the iodine color does not definitely distinguish starch from dextrins.

Sherman and coworkers considered the decrease in viscosity of the starch paste during digestion by an enzyme to be associated with the formation of dextrins and therefore a property of the "amyloclastic" amylase. That the liquefaction of starch pastes might be due to a separate enzyme was proposed by Waldschmidt-Leitz and Mayer (79). They claimed to have isolated this particular enzyme, and reported that the decrease in viscosity appeared to be associated with the liberation of phosphorus from the starch. This seemed to indicate that this enzyme was an esterase and not an amylase at all. Taylor and Keresztesy (71) found that dry grinding of corn starch in a ball mill greatly lowered the viscosity of the pastes made from it. It seems probable that the viscosity changes during enzyme digestion of starch are in part merely changes in the colloidal nature of the starch. The existence of a separate enzyme to bring about a physical change is doubtful. Furthermore, the decrease in viscosity of a starch paste could be a

natural accompaniment of the change in the state of division of the starch granule as well as of the decrease in molecular size during hydrolysis.

Of the methods of classifying enzymes the method of Kuhn (33) appears to be the most definite and reproducible. For this reason it offers the best means of characterizing amylases, although designation of an amylase as alpha or beta from mutarotation studies does not give a complete picture of all the properties that might be observed.

Much of the uncertainty in the classification of enzymes may be due to an even greater uncertainty as to the exact nature of the substrate. Any modification of natural starch could conceivably affect the action of an amylase upon it. Furthermore, a modification of natural starch introduces still greater uncertainty because the changes brought about by modification are not known. The treatment of natural starch with alcoholic hydrochloric acid, as in the preparation of "soluble starch", produces changes in the physical properties. The exact nature of these changes is not known. Kuhn (33) used a substrate which was prepared from Lintner soluble starch that had been electrodialyzed. He was using a fraction of modified starch. Sherman and Baker (60) eliminated the less soluble portion from their substrates by centrifuging a gelatinized suspension of natural starch. The more soluble portion they called "amylose" to distinguish it from the original starch.

in the work of Caldwell and Hildebrand (8) the terms "amylose" and "starch" are used as synonyms for a carbohydrate material completely dispersing in water but insoluble in 55 percent alcohol.

The use of an arbitrary fraction of natural starch as a substrate involves certain assumptions. In the first place, the methods used for separating starch into fractions are assumed to give clearly defined products. However, the preparation of two products showing identical physical properties is very difficult, if not impossible at the present stage of our knowledge. In the second place, one cannot be sure that in taking a certain portion of the starch for the substrate he has not eliminated fractions which would have an important effect upon the manner and rate of enzyme digestion. An extreme point of view is presented by Haworth and Percival (28) who present chemical evidence which they interpret as meaning that potato starch is a chemical entity composed of long chain molecules made up of alpha-glucopyranose units.

The only way to account for the various ideas regarding the starch-enzyme problem is to consider that the investigators were using different fractions or modifications of starches from different sources. Unless a definite well-defined fraction of natural starch could be prepared, it should be better to use the natural starch as substrates in starch-amylase studies. The fact that natural starch consists of a

mixture of substances must be recognized.

None of the workers mentioned takes cognizance of the phosphorus and fatty acid residues occurring in native starch. Table 1 shows the phosphorus content of several starches as found by different investigators.

Table 1. The phosphorus content of various starches

Kind of starch	Samec (58)	Glock (20)	Posternak (49)	Taylor (70, 74)
Potato	0.051	0.052	0.06 - 0.09	
Wheat	0.047	0.062	0.06 - 0.07	0.059
Corn	0.015	0.024	0.015 - 0.02	
Arrowroot	0.014		0.015 - 0.02	
Sago			0.01 - 0.015	
Tapioca			0.01	0.03
Rice	0.017	0.029		

While the individual results vary somewhat, all of the investigators mentioned found that potato and wheat starches are higher in phosphorus content than the others of the series. Moreover, the phosphorus content of these two starches is about three times as great as that of the other starches which all contain between 0.01 and 0.02 percent phosphorus. Samec (56) considers that the phosphorus present in potato starch inhibits or blocks the action of the enzyme. On the other hand Pringsheim and Ginsberg (52) report that complete hydrolysis of starch was obtained without liberating any free phosphoric acid.

Taylor and his coworkers were the first to prove that

there were fatty acids attached to the starch molecule. Taylor and Sherman (73) identified palmitic, oleic and linoleic acids in the mixture obtained by treating corn starch with alcoholic ammonia. The percentage of fatty acids in starches as reported by different investigators is given in Table 2.

Table 2. The percentage fatty acids present in various starches

Kind of starch	Taylor et al.	Lehrman
Potato	0.04 (72)	0.00 (36)
Tapioca	0.10 (74)	
	0.12 (72)	
Corn	0.61 (72)	
	0.66 (73)	
Wheat	0.58 (74)	0.95 (34)
Rice	0.83 (72)	0.65 (35)
Sago	0.11 (72)	

Here again the order is in agreement while the absolute values are not. Corn, wheat, and rice starches contain from 0.6 - 0.9 percent combined fatty acids, while potato starch contains very little, if any at all. Tapioca and sago starches are intermediate with 0.1 percent fatty acid content.

The role of esterified fatty acids in native starches in the digestion by amylase was investigated by Taylor and Sherman (73). They concluded that a lipase free amylase did attack the linkage of the fatty acids to the starch molecule. Myrback (42) suggested that both the fatty acids and the phosphorus in starch might be the cause of stopping enzyme action on

starches. Studies on the rate of enzyme attack on the various starches containing widely different amounts of phosphorus and fatty acids should offer a means of establishing the effects of these groups on enzyme action.

The action of various amylases on starches of different origin has been studied by several investigators. O'Sullivan (47) and Ford (16) reported the cereal starches more rapidly attacked by amylases than potato starch. On the other hand, Sherman, Walker, and Caldwell (66) and Stone (67) found that potato starch was more rapidly digested than the cereal starches. Glock (20) pointed out that the results of several investigators were contradictory and suggested that each investigator might have been measuring a different effect of amylase action.

Day (13) studied the effect of cooking different starches upon their digestibility by amylase. Potato and arrowroot starch pastes which had been made with hot water and not boiled were found to be digested as well as those which had been boiled three hours. Corn and wheat starches were made somewhat more digestible by long cooking. Nagai (43), using pancreatin and potato starch, found that first heating the starch with water at different temperatures for the same time caused variations in the rapidity of enzyme digestion. The work of Day and Nagai suggested that the discrepancies in the reported rates of digestion of different starches might be due

to differences in the methods of preparing the substrates. This question needed to be settled before an investigation of the products of amylase action on different starches could be undertaken.

The fact that a flocculent material appears in enzyme digestion of starch was first noticed by Baker (2) in 1902. He described the "breaking" of the substrate, but merely filtered off and discarded the precipitate. Ling and Nanji (37) also described the appearance of this material in a digestion of potato starch. Fernbach and Wolff (15) ascribed this coagulation to the presence of a specific enzyme which they named "amylocoagulase". However, they stated that the presence of the liquefying enzyme was necessary for this coagulation to take place. Sallinger (54) pointed out that the phenomenon might be due to digestion of the smaller starch particles while the larger ones were precipitated. Sherman and Punnet (62) while attempting a rigorous analysis of the products of potato starch digestion by different amylases, filtered out the precipitated material and weighed it. They found that this material amounted to about 1.08 - 1.4 percent of the starch, and that there were no significant differences in amounts depending on the enzyme used. Starch determinations using the malt method are complicated by the appearance of this insoluble substance (9).

Clayson and Schruyver (11) and Schruyver and Thomas (59)

separated the flocculent material from takadiastase digestions by supercentrifuging. They measured the optical activity of this material which they called "hemicellulose." Mallock (41) and Hermano and Rask (29) also noted the appearance of the so-called "hemicellulose" in enzyme digestions of different starches.

Taylor and coworkers (71, 73, 74) found that the fatty acid content of corn starch was largely present in a less soluble portion. From this, it seemed that the material which flocculated from corn starch substrates might be this insoluble fraction that was more difficult for the amylase to attack. This same phenomenon occurs in potato starch substrates, however, where the fatty acid content is very small (72, 36). The flocculation is apparently not due to the fatty acid content, at least in all the different starches. The appearance of this material must be in some way dependent on the nature of the starch.

The most important contributions in starch-amylase studies have been the results of investigations of the independent action of alpha and beta amylases. Numerous experiments on the products of hydrolysis of starches by amylases have been reported. Only those experiments in which the use of either alpha or beta amylase was clearly indicated are summarized here.

The mode of attack of the alpha amylase has been in-

vestigated chiefly by numerous attempts to characterize the dextrins formed. The modern ideas on the manner of alpha amylase action have been summarized by Hanes (24, 25). This enzyme presumably is able to split the starch molecule at any point, the nature of the products being dependent entirely upon experimental conditions. The limit of digestion occurs when the reducing power of the digestion mixture approaches 50 percent of the theoretically possible value, assuming the reducing power to be due entirely to maltose. This assumption is very poor because Freeman and Hopkins (17) were able to isolate only a very small amount of maltose from alpha amylase digestions. They concluded that the reducing action and downward mutarotation were due to alpha dextrins formed.

Hanes (24) has prepared a summary of the properties of the various dextrins separated from the alpha amylase digestion of potato starch by several workers. The great variation in the properties found indicates that these preparations are not definite substances. Myrback (42) stated that the nature of these so-called dextrins varies with the enzyme and starch used.

Much of the variation in results with alpha amylases might be due to the presence of the beta form of the enzyme. Simultaneous concentration of the two forms in different fractions of the same extract has been accomplished by Caldwell and Doebbeling (7), but the result was in no way a complete

separation. Inhibition of one form or the other by heat or acid according to the methods of Ohlsson and Edfeldt (46) is not very satisfactory, particularly for the preparation of alpha amylases. The beta amylase appears to occur free from the alpha form in ungerminated barley and wheat (27, 76) and in soy beans (1). The alpha amylase has not been found to occur in the absence of the beta form.

The modern theory regarding the method of attack of the beta amylase has also been summarized by Hanes (24). This amylase supposedly hydrolyzes maltose units successively from the non-aldehyde end of the starch molecules. The formation of beta maltose by this enzyme was taken by Kuhn (33) to mean that beta linkages were present in the starch molecules. However, Haworth and Percival (28) have proved that there are no beta linkages in the starch molecule. Beta maltose must be formed by a Walden inversion of the fragment containing two glucose units after it has been split off of the starch molecule.

Baker (2), Syniewski (68), Hanes (23), Freeman and Hopkins (17), and Blom, Bak and Braae (6) have confirmed the fact that the increase in reducing action during the beta amylase digestion of starch is due almost entirely to the formation of maltose. The limit of the production of maltose is 60 - 67 percent from potato starch as reported by Hanes (24), van Klinkenberg (77, 78) and Samec (55). Hanes reported that this

limit was the same as in the digestion of various other modified starches. van Klinkenberg stated that the limit of beta amylase action was independent of the enzyme concentration, but that the initial velocity was dependent upon the enzyme concentration.

Throughout the hydrolysis of starch by beta amylase residual starch-like substances are present which can be precipitated by 50 - 60 percent alcohol. The material that is left at the end of the reaction, which should amount to 30 - 35 percent of the starch, has been separated by Wijsman (83) who named it "erythrogranulose". Baker (2) and Haworth, Hirst, and Waine (27) prepared a fraction in about the same way and named it "alpha amylodextrin". A summary of the properties of this material as obtained by different investigators is given by Hanes (24). The results are not at all in agreement. The properties most often measured are optical activity and reducing action. The results of both measurements would be affected by the presence of maltose or unchanged starch in the material. The properties reported would, therefore, depend upon the degree of purification.

Most investigators agree that the material left at the end of beta amylase action is a part of the substrate which is resistant to the enzyme rather than that this material is a result of a secondary reaction. Hanes reported that repeated additions of fresh enzyme would give further hydrolysis up to

15 - 25 percent. Pringsheim and Beiser (51) concluded that the 60 percent alcohol precipitate from beta amylase action was an intact part of the original starch. It is possible that the original starch might contain a fraction which was resistant to the action of beta amylase. Myrback (42) considers the residual material to be fragments of the original starch molecules upon which, for some reason, the beta amylase cannot act. His idea was that the beta amylase could split maltose from all the starch molecules, but to varying degrees. He suggested that the enzyme would split off maltose units successively until blocked by some anomaly in the molecule. The anomalies that he suggested were:

1. The presence of esterified phosphoric and fatty acids.
2. Linkages other than 1-4 between glucose units in the starch molecule.
3. Branched chains of glucose units in the starch molecule.

From this point of view the variations in the properties of the residual material from the digestion of starch by beta amylase could be due to differences in the starch. Contamination of the beta amylase with the alpha form would also cause variations in the nature of this material. Ling and Nanji (37) pointed out that any preliminary treatment given the enzyme would influence the nature of the product. In a study of this product of enzyme action on starches particular attention must

be paid to the enzyme, the substrate, and the purification of the product.

From the foregoing discussion, three fairly definite materials appear to be the result of amylase action on starches. The insoluble material which flocculates from the digestion has never been separated out and studied. The formation of this material during beta amylase action has not been reported in the literature. The formation of maltose to the extent of 60-67 percent in the digestion of starches by beta amylase is assumed to be the result of hydrolysis of similar parts of the starch molecules. The "residual dextrin" or "alpha amylodextrin" forms the third product. For the present, this fraction can be considered as being composed of various fragments of the starch molecules and possibly containing the anomalies which cause differences in the properties of starches from different sources.

STATEMENT OF THE PROBLEM

The purpose of this investigation was two-fold:

1. To prepare and study the material which flocculates during enzyme digestion of a series of starches.
2. To prepare and study the residual material from the action of beta amylase on a series of starches.

In this work the natural unmodified starches were used as eliminating a source of uncertainty regarding the substrates. The natural starches are recognized as being mixtures of substances. The word "starch" will be used to indicate this heterogeneous material and will be prefixed by the origin of the starch.

The investigation divides itself into the following parts:

1. Study of experimental methods of following enzyme action.
2. Investigation of the effect of gelatinization temperature on the rates of amylase action on starches of different origin.
3. Development of methods of separating the flocculent material and the residual portion from beta amylase digestion of potato, tapioca, corn, wheat, and rice starches.

4. Characterization of these preparations as to the following:

   a. The extent of hydrolysis by fresh portions of beta amylase.

   b. Phosphorus and fatty acid content.

   c. Reducing action.

   d. Recovery in the starch determination of Denny (14).

MATERIALS USED

A. Starches. The starches were furnished through the courtesy of the following companies:

Corn starch - Penick and Ford, Cedar Rapids, Iowa.

Wheat and rice starches - Keever Starch Co., Columbus, Ohio.

Potato and tapioca starches - Stein-Hall Company, Chicago, Illinois.

B. Enzymes.

1. The oat enzyme used in the preliminary experiments was prepared by the method of Naylor and Dawson (44).

2. The wheat enzyme was prepared from germinated wheat according to the method of Creighton and Naylor (12).

3. Soy bean amylase was used in the preparation of the flocculent material and residual substance. The amylase was prepared from ether extracted soy bean meal by extraction with 50 percent alcohol and precipitation by adding absolute alcohol to make the concentration 70 percent. By mutarotation studies (45) according to Kuhn the enzyme was classified as a beta amylase.

EXPERIMENTAL

Methods of Following Enzyme Action

The methods, viscosity and "residual starch"

Viscosity. The decrease in viscosity of a starch paste during diastatic hydrolysis has been studied by several investigators. Chrzaszcz and Janicki (10) have reviewed the methods of measuring viscosity of starch pastes as a measure of the starch liquefying power of amylase. Lüers and Löther (40) and Jozsa and Johnston (32) have also studied viscosity changes in starch-enzyme reactions. Wies and McGarvey (82) and Thompson and McGarvey (75) studied the effect of the method of preparing the substrate on viscosity determinations. Willaman, Clark and Hager (84) used a 22 millimeter capillary in an Ostwald viscometer in following the liquefaction of starch paste by diastase. This method was unsatisfactory because of the difficulties encountered in cleaning the pipette and in temperature control.

The design of the pipette used in these experiments is shown in Figure 1.

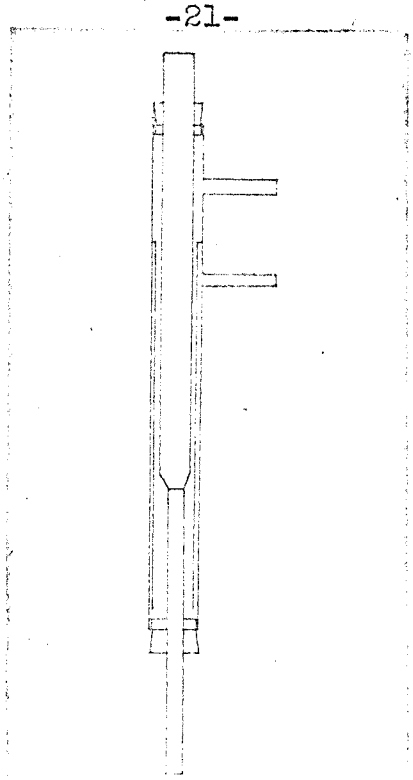

Figure 1. Water-jacketed viscosity pipette.

In use the pipette was clamped in a vertical position, with the lower end of the capillary extending one-fourth inch below the surface of the liquid in the digestion flask. Water from the thermostat was circulated through the condenser surrounding the pipette by a combination of siphoning and directing the water from the stirrer up through a glass tube and into the condenser. Direction of the water up the tube was accomplished by placing a brick in the bottom of the thermostat, beneath the blades of the stirrer. In this way viscosity measurements were taken at the temperature of the digestion mixture. The pipette was not moved during the course of an enzyme digestion. Measurements were made as often as desired

merely by drawing the digestion liquid up into the pipette and timing the flow back into the digestion flask. The ratio between the time of drainage for the material and for water at 40° (21.9 sec. for this pipette) gives the relative viscosity at 40°. Different volumes of liquid in the flask did not produce measurable variations in the viscosity. If the pipette was not moved during an experiment, it was possible to duplicate results on two experiments to within one percent.

Figure 2 shows the results of viscosity studies during the digestion of soluble starch with varying amounts of enzyme. The substrate was two percent soluble starch gelatinized by boiling and buffered to pH 5.0 with phosphate buffers. The enzyme was prepared from oats and had saccharogenic activity 250 and dextrinogenic activity 73,000 (46).

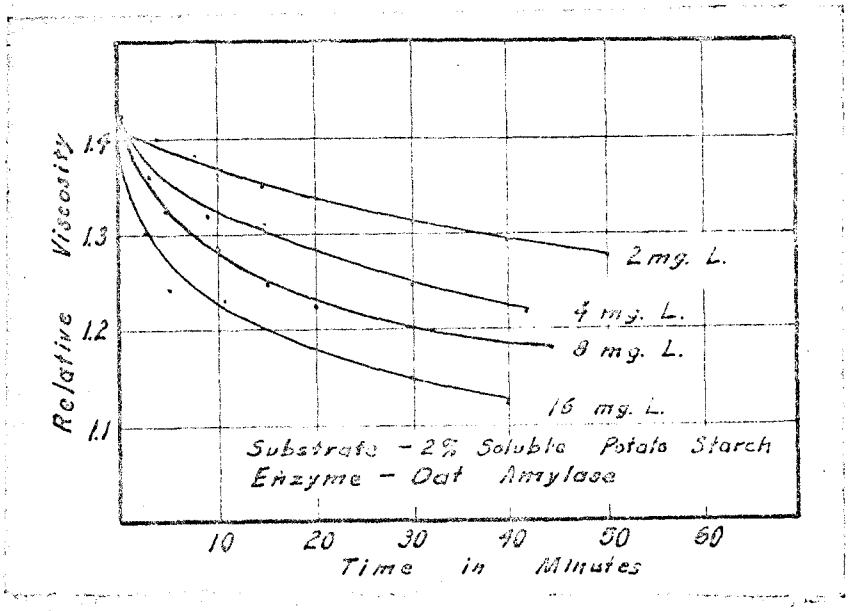

Figure 2. Viscosity studies during digestion of soluble starch.

The rate of liquefaction was markedly influenced by the amount of enzyme present. The rate curves show less initial curvature as the amount of enzyme is reduced. The effect of variation in the amounts of enzyme on the sugars formed in the same experiments is shown in Table 3. The modified Fehling method as described by Naylor and Dawson (44) was used in the determinations of reducing values as maltose. These measurements were made after 30 minutes digestion at 40°.

Table 3. Effect of varying amounts of enzyme on sugar formation.

Amount of enzyme	Wt. Cu_2O from 100 cc.	Mg. maltose in 100 cc.
2 mg. per liter	44.0	30.0
4 mg. per liter	93.5	75.7
8 mg. per liter	161.5	145.5
16 mg. per liter	353.1	291.0

The reducing value as maltose after 30 minutes of digestion with varying amounts of enzyme does not increase regularly. Since the reaction proceeds very rapidly in the initial stages, characterization of an enzyme by measurements during this period is not definite. Furthermore, as shown by Table 3 the ratio of enzyme to substrate markedly affects the results. For a definite characterization of the enzyme the course of hydrolysis should be followed for a period of time.

"Residual starch" determination. The feasiblity of de-

termining the decrease in "starch" during the digestion was investigated. The fact that "starch" is largely insoluble in alcohol solutions has been known for a long time. Witte (85) in 1904, worked on the determination of "starch" and similar substances by alcohol precipitation. He experienced difficulty in separating lower molecular weight carbohydrates, "dextrins", from the "starch". Baumert (3), in 1909, found that it was necessary to raise the alcohol concentration to 80 percent to recover all of a known sample of potato starch. Blake (4), in 1916, in attempting to fractionate the products of starch-amylase action, noticed that a substance he called "erythrodextrin" began to precipitate when the alcohol concentration reached 50 percent. Caldwell and Hildebrand (8), in 1935, devised a method for determining the "residual starch" during the hydrolysis of soluble potato starch by different amylases. The method consisted in removing aliquots and precipitating the "residual amylose" by making the samples to 55 percent alcohol by volume.

In attempting to use the method of Caldwell and Hildebrand for "residual starch" determinations a decrease in the weight of the precipitate was noted as the starch-enzyme digestion proceeded. In view of the earlier work on this method there was some doubt as to whether this precipitate was identical with the original starch. The percentage recovery in the 55 percent alcohol precipitate of some known samples of starch

are given in Table 4. The samples were gelatinized by boiling and precipitated according to the procedure of Caldwell and Hildebrand.

Table 4. The recovery of starch by the method of Caldwell and Hildebrand.

Character of the sample	Percent recovery
2 percent soluble starch	79.0
5 percent soluble starch	98.6
2 percent suspension of 55 percent alcohol precipitated starch	79.6

The data in Table 4 show that the starch in two percent suspensions is not all precipitated in 55 percent alcohol. The material precipitated in this way from samples taken during enzyme digestion does not contain all of the unchanged substrate.

If these 55 percent alcohol precipitates from samples taken during enzyme digestion of starches are identical with the original starch, the optical properties should agree with those of the original starch. Large samples of this material were prepared exactly as in the quantitative procedure by removing portions at intervals during the course of a starch-enzyme digestion. The dried material was suspended in 10 percent glycerol as follows. A paste of 2 g. of the dried precipitate in cold water was poured into a boiling mixture of 70 cc. of water and 10 cc. of glycerol. The mixture was boiled two minutes, cooled, and diluted to 100 cc. with distilled

water. The suspensions were clear and the optical activity could be read through a two decimeter polariscope tube. In order to determine the effect of the glycerol, the rotation of C.P. glucose in 10 percent glycerol solution was measured. The rotation was the same as in water solution.

Table 5 shows the specific rotations obtained for these precipitates from digestion of Baker and Adamson soluble potato starch. Oat enzyme having saccharogenic activity 230 and dextrinogenic activity 73,000 was used in the first experiment. Wheat enzyme prepared by the method of Creighton and Naylor (12) was used in the other experiment. The saccharogenic activity was 550 and the dextrinogenic activity was 2000.

Table 5. Rotatory power of 55 percent alcohol precipitates.

Enzyme used	Specific rotations			
	Original starch	Ppt. from 30 min. digestion	60 minute digestion	90 minute digestion
Oat enzyme	195	175	162	
Wheat enzyme	190	150	128	54

The data in Table 5 show that the optical properties of these precipitates are not the same as the original starch. The specific rotations are lower when the samples were prepared after longer digestion. The more rapid decrease in the specific rotation of this material from the digestion by the wheat enzyme is probably due to a larger amount of enzyme present. The material precipitated by 55 percent alcohol is not the same as the original starch. While this method does

not give a measure of the unchanged substrate it does indicate the progress of the reaction.

Comparison of different methods of following enzyme action. The purpose of these studies was to compare the various methods of following the course of starch-enzyme digestions. The data plotted in Figure 3 were obtained from the digestion of two percent soluble starch buffered to pH 5.0 with phosphate buffers. 16 mg. of oat enzyme per liter of substrate was used.

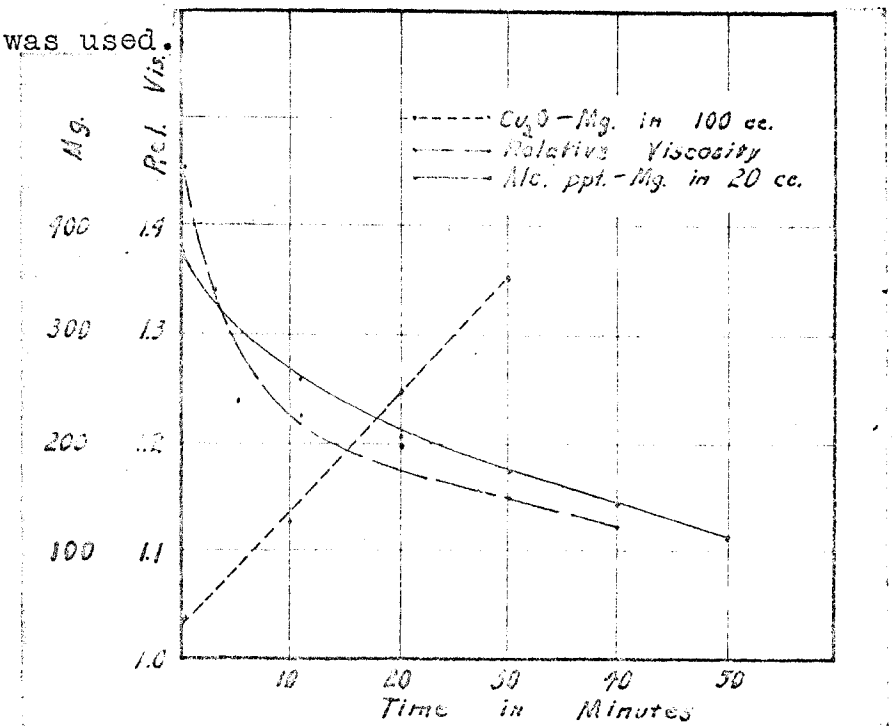

Figure 3. Comparison of viscosity, "residual starch", and Fehling's methods.

The viscosity had reached a constant value after 30 minutes of digestion, but the amount of material precipitated by 55 percent alcohol was still decreasing after one hour. These two methods measure different effects of the amylase action. Nei-

ther method is strictly a measure of the decrease in molecular size of the substrate as digestion proceeds. The reducing action against alkaline copper is a measure of still another effect of enzyme action. The amount of Cu_2O precipitated is still increasing after 50 minutes of digestion.

Comparative rates of hydrolysis of corn and potato starches. The substrates used were prepared from unmodified corn and potato starches which had been gelatinized by boiling two minutes with mechanical stirring. The substrates contained four percent starch and were adjusted to pH 5 with phosphate buffers. 11 mg. of wheat enzyme was used for each liter of four percent starch paste. This study was incomplete because of inadequate methods. It was impossible to obtain a series of values for reducing action using the modified Fehling's method during the digestion of natural starches. After the digestion had proceeded four hours values could be obtained. The values are given in Table 6.

Table 6. Reducing values after four hours digestion of corn and potato starches.

Kind of starch	Mg. of Cu_2O in 100 cc.	Mg. maltose
Unmodified corn	282	231.9
Unmodified potato	280	231.1

The data on viscosity and 55 percent alcohol precipitates are given in Figure 4.

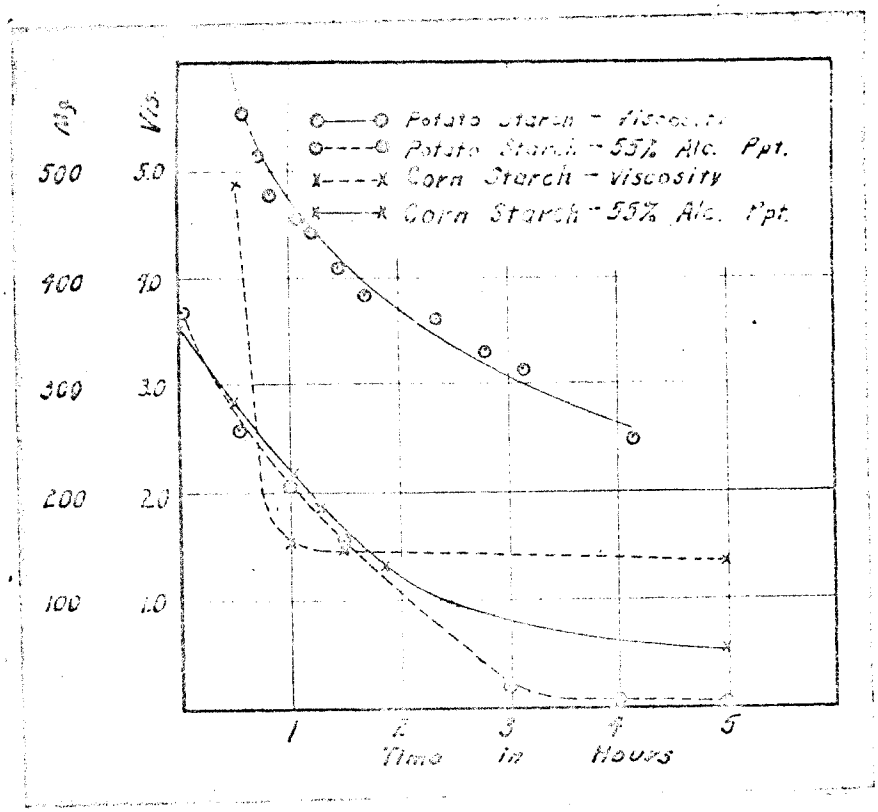

Figure 4. Digestion of four percent corn and potato starches with wheat amylase.

These data show the viscosity of corn starch being decreased more rapidly than that of potato starch. On the other hand the decrease in amount of 55 percent alcohol precipitates is much more rapid in the digestion of potato starch. The presence of the flocculent material in the digestion made the removal of representative samples for 55 percent alcohol precipitates very difficult.

While these measurements show differences in the rates of digestion of corn and potato starches, the results are open to question. The comparisons of established methods of following enzyme action have been of value in pointing the need for

better experimental methods.

Modified Hagedorn and Jensen method of determining reducing power.

Method. The gravimetric Fehling method was impracticable for following the course of hydrolysis of natural starches. The method was too laborious and the results were in error because part of the starch was filtered out and weighed as Cu_2O. The modified Hagedorn and Jensen method (22) was first used as offering the advantages of a volumetric method and because it required a smaller sample than the Fehling method. The method used was adapted from the methods of Blish and Sandstedt (5), Gore and Steele (21) and Widdowson (81).

The procedure was as follows. 25 cc. portions of 0.1 N potassium ferricyanide reagent[1] were put into 250 cc. Erlenmeyer flasks. The samples, which should not be larger than 5.0 cc. were added, and the flasks placed in a boiling water bath for 15 minutes and then cooled two to three minutes in

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[1]Reagents for H. and J. sugar method.

1. Potassium ferricyanide reagent.
   0.1 N potassium ferricyanide in five percent sodium carbonate solution.
2. Acetic acid reagent.
   80 g. $ZnSO_4 \cdot 7H_2O$, 70 g. KCl, 20 cc. of glacial acetic acid, and distilled water to make one liter.
3. KI solution.
   50 percent KI with one drop of conc. NaOH per 100 cc.
4. 0.1 N sodium thiosulfate.
   Standardized against KIO_3 as a primary standard.

running water. After cooling, 25 cc. of acetic acid reagent[1] were added immediately from a graduate. 5 cc. of KI solution[1] were added just before titrating with standard sodium thiosulfate solution[1]. The starch which was present served as the indicator.

The milligrams of maltose were read directly from a curve obtained by plotting mg. of maltose against cc. of 0.1 N potassium ferricyanide reduced by the sample. The data for this curve are given in Table 7 and were the result of determinations on samples of pure maltose.

Table 7. Data for the standard reference curve for modified H. and J. method for determining reducing value as maltose.

Mg. maltose in 5.0 cc.	cc. 0.1034 N $Na_2S_2O_3$	Blank minus titration (Ave.)	cc. 0.1 N $K_3Fe(CN)_6$ used
Blank	25.1 25.1		
5 mg	23.88 23.78	1.26	1.30
10 mg.	22.27 22.25	2.84	2.94
15 mg.	20.66 20.75	4.44	4.58
20 mg.	18.40 18.90	6.45	6.67
30 mg.	15.20 15.30	9.9	10.22
40 mg.	12.40 12.10	12.8	13.25
60 mg.	5.52	19.58	22.5

Hydrolysis of corn and potato starches (natural and modified). The modified Hagedorn and Jensen sugar method offered

a means of comparing the rates of hydrolysis of natural and "soluble" starches. Soluble starches were prepared from corn and potato starches as follows. 1500 g. of the unmodified starch was stirred into 2 l. of absolute alcohol to which had been added 400 cc. of 1:1 HCl. This was allowed to stand for four hours with occasional stirring and then filtered on a Buchner funnel. The starch was washed three times by stirring up with water and filtering, and dried by washing with alcohol and ether. It was then put through a 100 mesh screen to remove the lumps. The effect of this treatment upon enzyme hydrolysis of corn and potato starches was studied.

One liter of two percent substrate gelatinized by boiling was prepared for each experiment. The pH of the substrate was adjusted to 5.0 with phosphate buffers as measured with the glass electrode. 250 cc. portions were put into four 500 cc. Erlenmeyer flasks. One portion was used for viscosity measurements; two portions were used as duplicates for the sugar determinations; and one without enzyme served as the control. The enzyme used in these experiments was the beta-amylase from soy beans (42). 20 cc. of a suspension containing 40 mg. per 100 cc. was added to each 250 cc. of starch, making an enzyme-starch ratio approximately 1:300. Figure 5 shows the results of these experiments.

Figure 5 shows that the treatment of the starches has very little effect upon the rate of sugar formation by beta-

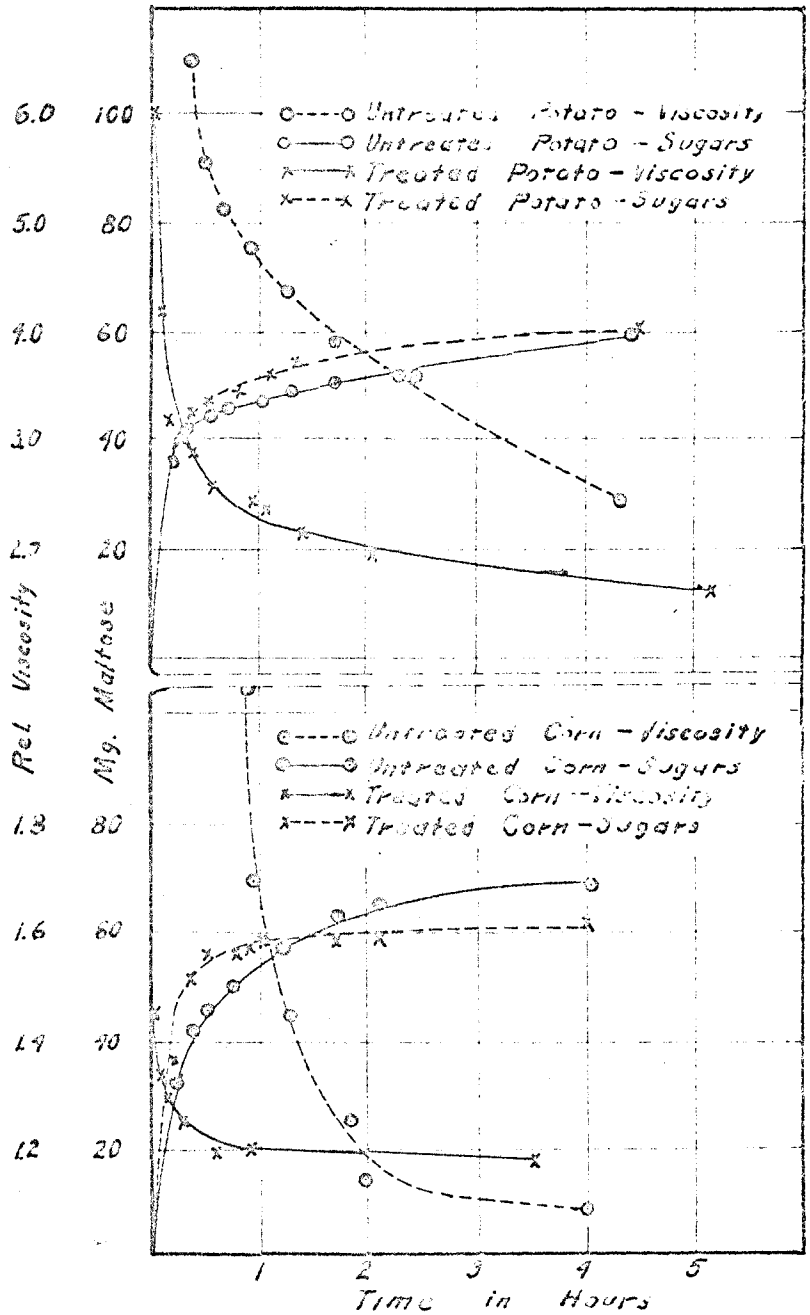

Figure 5. Comparison of natural and treated corn and potato starches.

amylase. Sugar production proceeds more rapidly during the digestion of corn starch than either natural or treated potato starch. The values for the sugar production after 24 hours are given in Table 8. The percentage reducing value as maltose was calculated as milligrams of maltose formed per 100 mg. of original starch.

Table 8. Reducing power after 24 hours digestion with soy bean amylase.

Kind of starch	Percent reducing value as maltose
Unmodified corn	68
Treated corn	61
Unmodified potato	66
Treated potato	60.6

The unmodified starches were digested to about 6 - 8 percent farther as shown by the reducing value. It is possible that the treatment of the starches has washed out some more soluble portion of the starch which is digestible by beta-amylase. This would account for a lower final value on the treated starches.

The decrease in viscosity on hydrolysis is very markedly affected by the alcoholic HCl treatment of the starch. This is to be expected because the treatment is used to make so-called "soluble" starch. Liquefaction of the corn starches proceeds more rapidly than that of potato starch, either treated or natural.

Flocculation of the substrates in these experiments was

very interesting, because this has never been reported in a beta-amylase digestion. In the case of corn starch, the material appeared in 15 - 30 minutes and had settled to the bottom half of the flasks in one and one-half hours. The supernatant liquid was clear and gave a blue color with iodine, while the liquid containing the precipitate gave a red-violet color. This material was noted in the digestions of potato starch. However, the appearance of the material in this case was different. It was clearly visible in a thin column of the digestion liquid, but did not flocculate and settle to the bottom of the flask.

Electrometric sugar method.

The modified Hagedorn and Jensen method for determining reducing value was found to be inadequate for following digestions of starches that had been gelatinized below the boiling point. Unswollen starch particles were present and held the iodine so tenaciously that no definite end-point could be obtained. The method of Hassid (26) was modified to a macro determination as was done recently by Hildebrand and McClellan (30). However, in the present work the titration of the ferrocyanide with ceric sulfate was followed electrometrically[1]. The procedure was as follows.

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[1]The voltages were measured on an experimental model of an instrument operating directly off the 110 volt AC. line, which can also be used to measure pH with glass or quinhydrone electrodes. The instrument is being manufactured for sale by Precision Scientific Company of Chicago, Ill.

5.0 cc. of sample containing from 5 to 60 mg. of maltose was pipetted into 25 cc. of alkaline ferricyanide reagent[2]. This mixture was placed in a boiling water bath for 15 minutes, then cooled in running water 2 or 3 minutes. 25 cc. of a 1-4 solution of HCl was added immediately, and the contents of the flask poured into a 250 cc. beaker for titrating. The flask was rinsed into the beaker with two 10 cc. portions of distilled water. The final volume of the solution was 80-85 cc., and the acid concentration 1.0-1.5 N. According to Furman and Evans (18) this is the optimum acid concentration for the following reaction to proceed rapidly and quantitatively.

$$Ce^{+4} + Fe(CN)_6^{-4} \longrightarrow Ce^{+3} + Fe(CN)_6^{-3}$$

The solution was titrated potentiometrically with 0.1 N ceric sulfate[2] solution (18) using a platinum-saturated calomel electrode system and a KCl-agar bridge. These electrodes were found to give an increase of 350 millivolts at the endpoint in 1.0-1.5 N acid. This voltage jump was much greater than could be obtained with a platinum-tungsten bimetallic

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[2]The reagents were prepared as follows:
Alkaline ferricyanide reagent - 0.1 N potassium ferricyanide (by weighing) in five percent sodium carbonate solution.
0.1 N ceric sulfate - 53 g. of C.P. ceric sulfate (1.6 times the theoretical) was added to 900 cc. of a solution containing 100 cc. of concentrated sulfuric acid; This was digested on the hot plate until all the solid had dissolved. It was then filtered and made up to 1 liter. The solution was 1.0 N in sulfuric acid. The ceric sulfate solution was standardized potentiometrically against a standard ferrous iron solution.

electrode (19). Before titrating the solutions were green, and the color changed abruptly to yellow about 0.05 cc. before the voltage change.

The results of these determinations were calculated to milligrams of maltose by converting the titration value to cc. of 0.1 N ceric sulfate and reading the value for maltose directly from a graph prepared from data obtained by titrating solutions of known concentrations of C.P. maltose hydrate. The purity of the maltose was checked by the standard Munsen-Walker method. The data for the standard reference curve are given in Table 9. The results of the sugar determinations run on portions of simultaneous duplicate digestions checked within 0.5 mg. The results on a repetition of the experiment showed a variation which was never more than one percent on a single determination. The time-maltose curves could be duplicated, therefore, with occasional points off the curve.

Effect of Temperature of Preparing the Substrate on the Rate of Beta Amylase Action

Method

The starches used were potato, corn, tapioca, rice, and wheat. Substrates were made from these starches by heating at 60°, 70°, 80°, 90°, 100°, and 120°. The rates of hydrolysis of the starch substrates by soy bean amylase were measured by

Table 9. Data for standard reference curve for potentiometric sugar determination.

Mg. maltose in 5.0 cc.	cc. ceric sulfate 0.1016 N	cc. ceric sulfate 0.1 N
5.0	1.9	1.93
10.0	3.5	3.55
15.0	5.25	5.33
20.0	6.80	6.90
25.0	8.55	8.68
30.0	10.10	10.25
35.0	12.05	12.20
40.0	13.50	13.70
45.0	15.1	15.32
50.0	16.60	16.80
55.0	18.55	18.85
60.0	20.05	20.35

the potentiometric determination of the sugars formed.

All substrates contained two percent starch and were at pH 5.0 as measured with the glass electrode. 10 g. of the untreated starch were stirred with 100 cc. of cold water. This paste was poured into 350 cc. of a solution containing distilled water and 49 cc. of 0.2 M NaH_2PO_4 solution and 1 cc. of 0.2 M Na_2HPO_4, which had been brought to the desired temperature in a water bath. The mixture was kept at the desired temperature 30 minutes, then cooled to 40° and made up to 500 cc. The substrates heated at 120° were prepared the same as those heated at 100° except that instead of boiling 30 minutes, they were boiled two minutes and then heated under 15 pounds of steam pressure in an autoclave for 30 minutes. The substrate was divided into two 250 cc. portions and the same amount of enzyme added to each. The digestions were carried out at 40°. 5.0 cc. portions were removed simultaneously from each digestion for the sugar determinations, giving two values for every point on the curves.

For each 250 cc. of the two percent starch paste, 20 cc. of a suspension in water containing 40 mg. of soy bean amylase per 100 cc. was used. The ratio of enzyme to starch was 1 to 625, and the ratio of enzyme to maltose at 70 percent digestion was about 1 to 400. There was an excess of enzyme present at all times during the digestions.

Results.

There was very little increase in the reducing power during the digestion of the various starches that had been heated at 60° for 30 minutes, except in the case of potato starch. Soy bean amylase does not digest potato starch heated at 50°. Figure 6 shows the results of the sugar determinations plotted against time of amylase action for starches which had been heated at 70° and 100° as previously described.

When the starches were prepared by heating at 70° for 30 minutes, potato starch was digested most rapidly by soy bean amylase. As shown in Figure 6, the tapioca starch is next in order and wheat, corn and rice are slower than either potato or tapioca. The limit of hydrolysis after 24 hours digestion of the potato and tapioca starches heated at 70° was between 66 and 68 percent of the oven dry starch. The limit for the cereal starches heated at 70° was 49 - 50 percent.

When the substrates were prepared by heating at 100° for 30 minutes the order of rapidity of enzyme digestion is reversed. Corn starch was digested most rapidly, with wheat, rice, potato and tapioca less rapidly in order. The limit of digestion of wheat, rice and tapioca starches heated at 100° was 57 - 60 percent, for corn starch 70 percent and for potato starch 65 percent after 24 hours digestion. Heating the

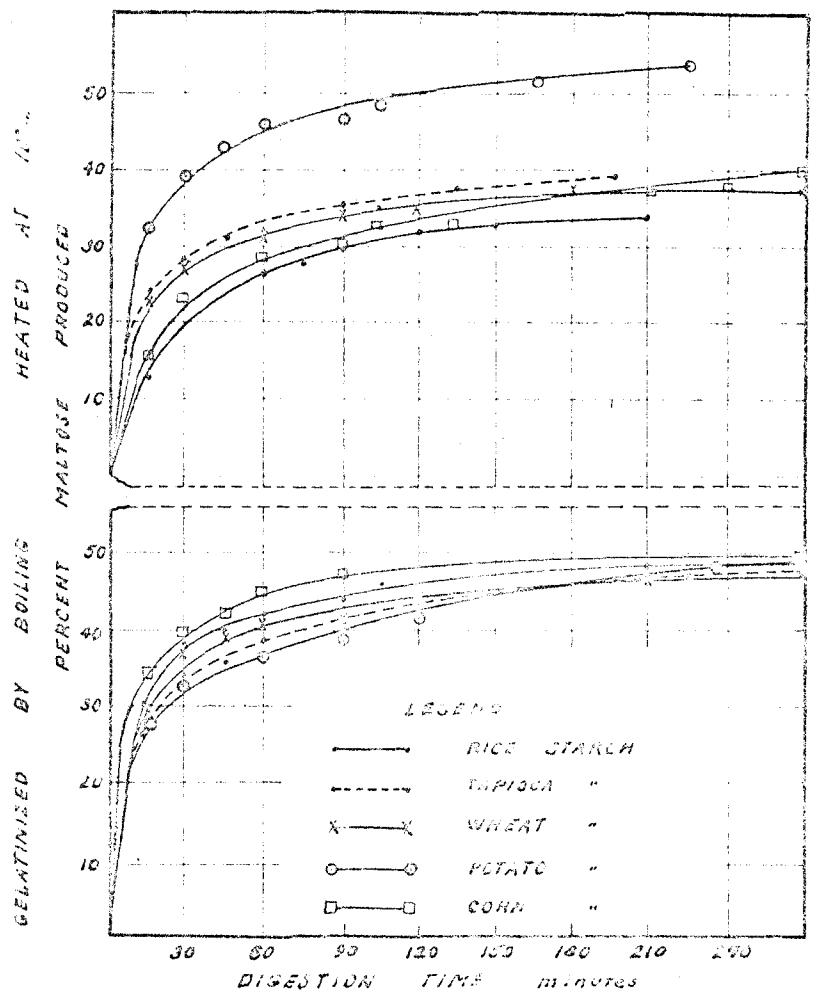

Figure 6. Rates of hydolysis by soybean amylase of different starches prepared at 70° and 100° C.

starches at a higher temperature slowed the enzyme action on potato and tapioca starches so much that the cereal starches were hydrolyzed most rapidly.

The effect of heating the starches is shown more clearly by Figure 7 in which maltose formed in 240 minutes of enzyme action is plotted against the temperature at which the starches were prepared.

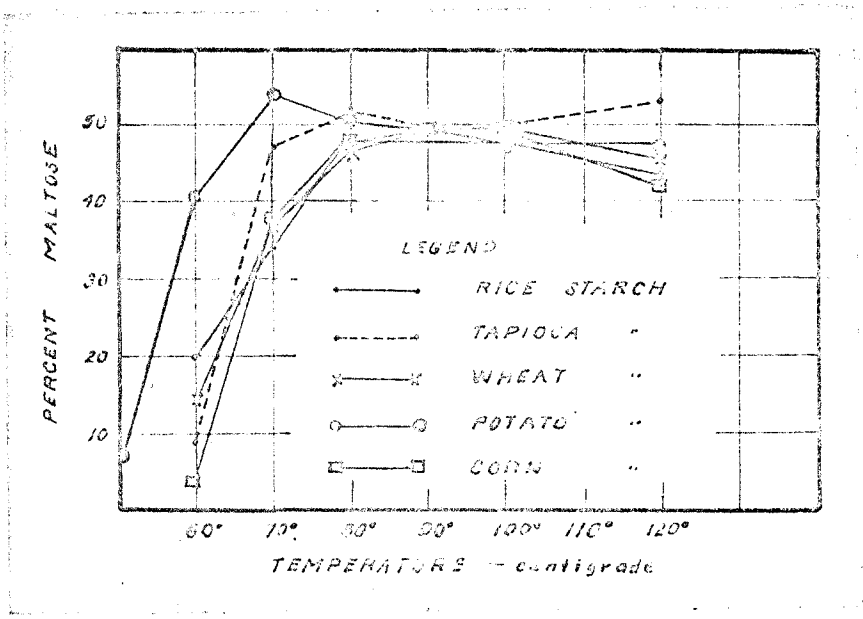

Figure 7. Maltose formed after four hours digestion of starches heated at different temperatures.

In every case except rice starch an optimum temperature of preparing the starch for soy bean amylase action is indicated--70° for potato, 80° for tapioca, and 90° for wheat and corn starches. Temperatures of 80° or above for preparing rice starch substrates will give the maximum rate of soy bean enzyme hydrolysis.

The decrease in rate of soy bean amylase action on corn,

wheat, and potato starches which have been heated above the optimum temperatures is an interesting phenomenon. However, after 24 hours digestion the different starches prepared at these higher temperatures approach about the same limits of maltose formed as the same starches heated at their optimum temperatures. These results suggest that there is some effect on the starches when heated, other than swelling and rupture of the granules. It is possible that heating causes agglutination of the particles in the gelatinized starch paste, so that a change in the degree of dispersion occurs. The individual starch molecules would then be less accessible to the attack of the soy bean amylase. The result would be a retarding effect, but eventually the same degree of hydrolysis would be accomplished.

Preparation of Certain Products of Beta Amylase Digestion

The general procedure followed in separating these products from the digestion of different starches can be represented by Figure 8.

Precipitates A and B were prepared from corn, wheat, rice, potato and tapioca starches. Precipitate A has never been characterized. Precipitate B has been named "alpha amylodextrin" (2, 27), "residual dextrin" (38), and "erythrogranulose" (83). Since none of these names disignates a definite sub-

stance, the material will be referred to as precipitate B in the following discussion.

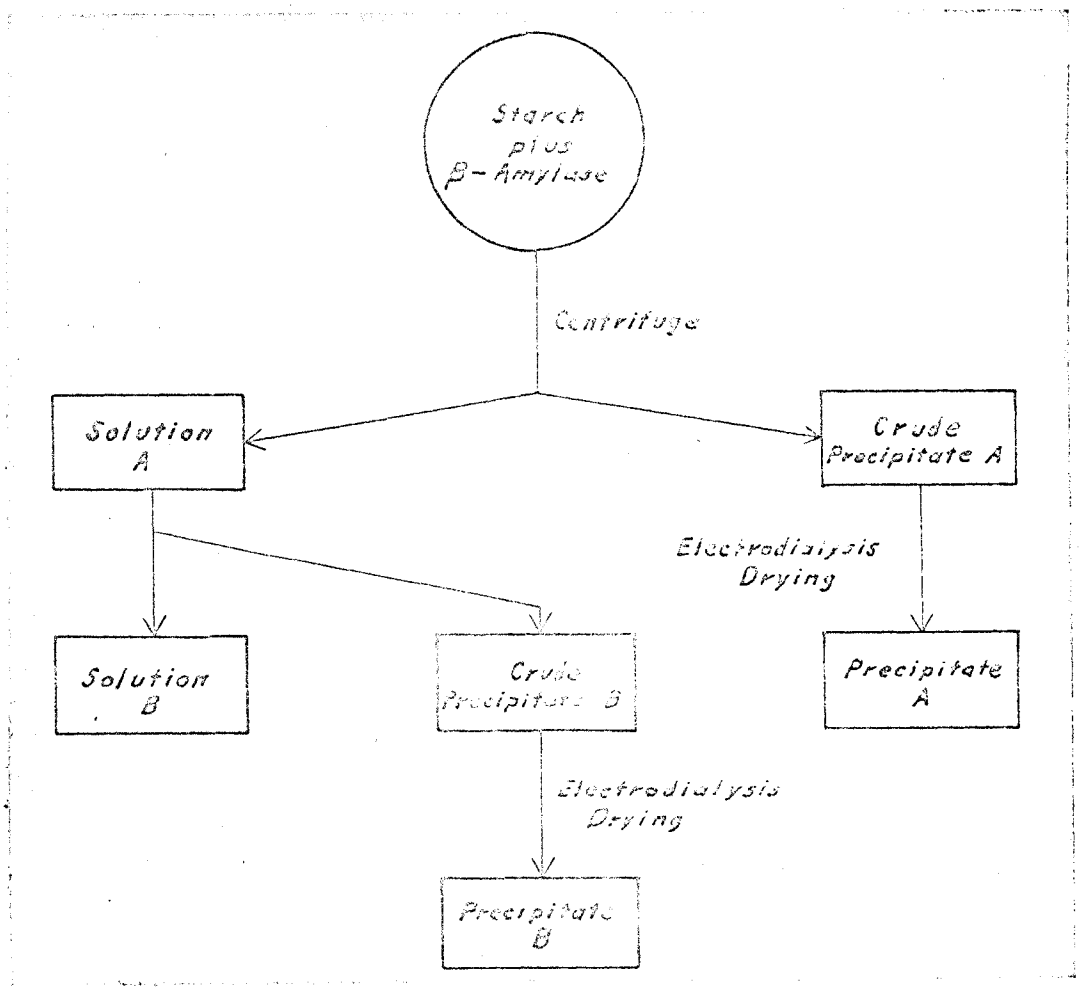

Figure 8. The products of beta amylase digestion.

Electrodialysis.

The process of electrodialysis was used in this work in the purification of the materials which were not hydrolyzed to maltose by the action of soy bean amylase. To accomplish this it was desirable to remove all ions and at the same time to cause a coagulation of the carbohydrate material in order

to facilitate its recovery from the liquor in the inner chamber. Various forms of apparatus have been used for electrodialysis. The apparatus of Taylor and Kerecztesy (71) was not satisfactory for this purpose because the electrodes were too small and too far apart. The Löddesöl (39) modification of Pauli's (48) apparatus was better suited to this purpose because it was a three chambered apparatus used in a horizontal position, with the electrodes fairly close together. The disadvantages of this apparatus were that the inner cell was too small and the electrode chambers too large. This type of cell was modified to remove these difficulties.

The center cell was constructed of a glass cylinder with parchment membranes stretched over the ends. The whole was clamped to two pieces of plate glass by means of four inch bolts with rubber gaskets between the ends of the cylinder and the glass plates. The positive electrode was of platinum foil and the negative electrode was of copper foil as recommended by Humfield and Alben (31). These were held in place as shown in Figure 9 by rectangles of glass tubing held in place by rubber bands. The glass rectangles also serve the purpose of supporting the membranes. Electrical connections are secured by short wires leading through holes in the top of the glass plates and held in place beneath the foil by the same rubber band arrangement.

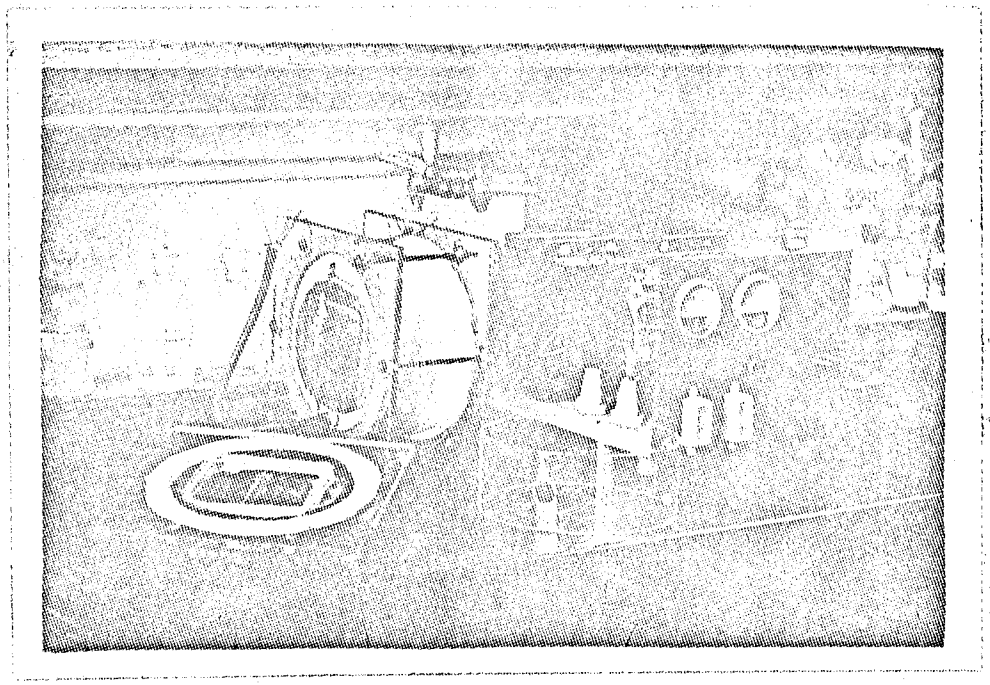

Figure 9. Apparatus for electrodialysis.

The advantages of this type of cell over those previously mentioned were: a large inner cell with capacity about 1500 cc., electrodes relatively close together, and large electrode surfaces. There is provision for draining and refilling the electrode chambers, as shown in Figure 9, by means of rubber tubes carrying funnels which can be raised or lowered. The central chamber can be cooled by allowing cold water to flow over the sides when the whole cell is supported over a pneumatic trough.

Any source of D.C. electricity can be used with this type of apparatus. In the work on electrodialysis of carbohydrates it was convenient to use a vacuum tube rectifier with either

resistance or transformer steps in order to vary the voltage applied to the electrodes of the dialyzer. This was necessary because at the beginning of dialysis if the voltage was too high, heating of the colloidal material in the inner cell was noted. After the conductance due to ions decreased, the dialysis could be speeded up by increasing the voltage applied.

The course of the electrodialysis was followed by the voltage and milliamperes of current going through the cell, and also by titrating the liquid from the anode and cathode chambers with 0.1 N acid and base, respectively. It was found that the cations were removed most rapidly, a fact noted by Watson (80). Migration of the carbohydrate material toward the anode confirmed the work of Taylor and Beckman (58, 69).

Preparation of precipitates A and B. (Fig. 8)

From the studies on gelatinization temperature it is evident that starch substrates need not be boiled. This fact was of value in handling large quantities. The starches were prepared by heating to their optimum temperatures as reported in Figure 7.

A large galvanized can served as the water bath. Water in the bath was heated to the desired temperature by passing superheated steam into it. 8 l. of a solution containing 980 cc. of 0.2 M NaH_2PO_4 and 20 cc. of 0.2 M Na_2HPO_4 was placed in a 16 l. balloon flask in the water bath and allowed to come to

temperature. 600 g. of untreated starch, suspended in 2 l. of distilled water, was then poured into the flask with stirring. After 30 minutes the flask was removed from the bath and cooled in running water. When the starch had cooled to 40° degrees, it was placed in a thermostat at 40°.

100 cc. of a suspension containing 700 mg. of soy bean amylase was added. The mixture was stirred vigorously and let stand five hours. At the end of this time the flask was placed in the refrigerator for 16 hours. This procedure seemed to facilitate the removal of precipitate A. The digestion mixture was then run through a Sharples supercentrifuge to remove precipitate A. 1.0 cc. samples were removed at this time and placed in 25 cc. of alkaline ferricyanide reagent for the potentiometric sugar determination.

The solid material (precipitate A) which collected in the bowl of the centrifuge was shaken up in 1 l. of water and electrodialyzed. Electrodialysis was necessary because the material could not be recovered by centrifuging. The electrode chambers were drained periodically and titrated with acid and base. When the material had settled in the dialyzer (after about 12 hours) the supernatant liquor was siphoned off. More water was added, the solids shaken up again, and dialysis continued. Dialysis was repeated two or three times until the liquid from the anode chamber gave no more test for phosphate.

Attempts to dry the precipitate in air at this point re-

sulted in a dark colored hard mass which could not be ground. After dialysis the thick suspension from the bottom of the electrodialyzer was put into about twice its volume of absolute alcohol and allowed to stand overnight. The supernatant liquid was siphoned off and another portion of absolute alcohol added. The solid material was again allowed to settle. This process was continued until the precipitate was sufficiently granular to filter with suction. Dehydration was completed by repeatedly grinding under absolute alcohol and filtering. The material was then dried with ether and placed in a vacuum desiccator for two or three days. When dry, precipitate A was ground to a white powder in an agate mortar.

The fraction called precipitate B (Fig. 8) was prepared from five different starches. The precipitate was prepared by adding 2200 cc. of absolute alcohol to 1500 cc. of the centrifugate from the preparation of precipitate A. The mixture was allowed to settle and then centrifuged or the supernatant liquid siphoned off, according to the nature of the precipitate. The appearance and nature of precipitate B were quite different in the cereal and root starches. When prepared from cereal starches it was curdy and settled out nicely, while if prepared from potato and tapioca starches, it was formed as a transparent sticky mass. In the latter case the supernatant liquid remained turbid but only a very little more of the transparent sticky material could be collected in the super-

centrifuge. When the precipitate was flocculent, it was washed once by stirring up with 60 percent alcohol, then dried by grinding under absolute alcohol. The gummy precipitates were repeatedly ground under absolute alcohol and dried to white powders.

These crude products were later redissolved in water and electrodialyzed until free from phosphates. The material was recovered as before. However, in the case of the material from potato and tapioca starches electrodialysis was necessary to separate it from the alcohol mixtures. In this purification process from 50 - 75 percent of the precipitate B was lost.

The yields of precipitates A and B from a series of starches are given in Table 10. The yields of precipitate B are given as the amount of the crude products. The percentage maltose formed is also given.

Table 10. Yields of some products of beta amylase action.

Kind of starch	Maltose equiv. (percent)	Precipitate A (percent)	Precipitate B (percent)	Total yields (percent)
Corn	51.4	1.64	32.2	88.24
Rice	38.5	1.92	34.4	74.82
Wheat	51.8	1.0	38.8	91.6
Potato	55.1	0.84	30.0	85.94
Tapioca	55.6	0.05	33.5	89.15

Characterization of Precipitates A and B (Fig. 8)

Hydrolysis by fresh beta amylase.

The substrates were made up to be one percent and were not buffered. The calculated amount of air dry material to make 1.0 g. of oven dry was weighed into a 250 cc. Erlenmeyer flask. 100 cc. of distilled water was added, and the mixture brought to a boil with shaking. The suspension was cooled to 40° and placed in a thermostat at 40°. 5.0 cc. of an enzyme suspension containing 40 mg. of soy bean amylase in 50 cc. were added. 5.0 cc. samples were removed at intervals for the potentiometric sugar determination. The original starches were tested in exactly the same way as the precipitates A and B.

Table 11 shows the percentage increase in reducing value as maltose after 24 hours of digestion.

Table 11. Percentage hydrolysis of preparations by beta amylase.

Kind of starch:	Unmodified	Precipitate A	Precipitate B
Corn	58.5	14.1	15.3
Wheat	60.0	23.9	26.2
Rice	65.7	30.0	34.0
Potato	59.3	9.65	9.02
Tapioca	77.2	6.3	9.8

Both precipitates A and B from beta amylase digestion of potato and tapioca starches are more resistant to further action of the beta amylase than the preparations from corn,

wheat, and rice starches. There is very little difference in the resistance to further enzyme action between precipitate A and precipitate B from any one kind of starch.

Phosphorus and fatty acid content.

Since the phosphorus content of these materials was very low, micro technique was used in analyzing for phosphorus. The volumetric method of Pregl (50) was modified somewhat, in that the yellow precipitate was washed with three percent potassium nitrate solution instead of ammonium nitrate and alcohol. The solutions used were the same as in the Pregl method.

The procedure was as follows. 100 - 200 mg. samples were weighed on an ordinary analytical balance. The samples were transferred to Pyrex test tubes and 1.0 cc. of concentrated H_2SO_4 and six drops of concentrated HNO_3 added. The mixture was heated over a small gas flame until SO_3 fumes appeared. Six drops of nitric acid were added again, the mixture was then again heated until SO_3 fumes appeared. This process was repeated until the solutions were clear on cooling. The contents of the test tubes were rinsed into 50 cc. beakers with 5.0 cc. of 1:1 nitric and a little distilled water. Two cc. of nitric containing sulfuric were added. The samples, which were now in about 15 - 20 cc., were heated to around 60° on a hot plate. In the meantime the molybdate reagent was filtered. Fifteen cc. of molybdate was then added dropwise from a pipette,

with stirring.

The precipitate was allowed to stand 24 hours. The supernatant liquid was drawn off through a filter stick. The precipitate was washed with three percent potassium nitrate solution, the filter stick being used to draw off the liquid, until five cc. of the washings remained pink when one drop of phenolphthalein and one drop of 0.1 N NaOH were added. The precipitate was dissolved in 4.0 cc. of standard 0.1 N NaOH from a micro burette. This solution was drawn through the filter stick in order to dissolve the precipitate which remained on the filter stick. The solution and washings were collected in a clean receiver. Three portions of boiled distilled water were drawn through the filter stick to rinse it. The solution was transferred back into the beaker in which the precipitation was carried out. This solution was boiled gently almost to dryness, five cc. of less. After cooling, 5.0 cc. of standard 0.1 N HCl and one drop of phenolphthalein were added. The solution was again boiled 30 seconds, cooled and titrated immediately with standard 0.1 N NaOH to a permanent faint pink color.

The calculation of the percentage phosphorus was based on the factor 0.1107 (50) for converting cc. of 0.1 N NaOH used to milligrams of phosphorus. This factor is based on the formula for the yellow precipitate which contains two molecules of nitric acid of crystallization instead of water.

The phosphorus contained in the various samples is shown in Table 12.

Table 12. Phosphorus content of preparations from beta amylase digestion.

Kind of starch	Unmodified starch: Percent P	Precipitate A: percent P	Precipitate A: percent recovery	Precipitate B: percent P	Precipitate B: percent recovery
Corn	0.015	0.031	3.58	0.033	70.8
Rice	0.035	0.041	2.25	0.033	32.8
Wheat	0.051	0.041	1.80	0.152	103.
Potato	0.050	0.112	1.88	0.222	102.5
Tapioca	0.010	0.020	1.0	0.020	67.0

The fatty acid content of the original starches and precipitates A and B was determined by the method of Taylor and Nelson (72). The sample was hydrolyzed with strong HCl, the sludge filtered out and washed free from acid. The fatty acids were extracted from the sludge with ethyl ether.

Table 13 shows the results of these analyses, along with the approximate percentage recovery of the fatty material from the original starch.

Table 13. Fatty acid content of the preparations.

Kind of starch	Original starch	Precipitate A: Percent fatty acids	Precipitate A: Percent recovery	Precipitate B: Percent fatty acids	Precipitate B: Percent recovery
Corn	0.66	1.31	3.26	0.71	34.5
Rice	0.62	0.95	2.90	0.56	31.0
Wheat	0.57	0.95	1.5	0.91	51.4
Potato	0.076	0.17	1.8	0.18	71.0
Tapioca	0.174	0.51	1.46	0.22	42.3

The data on fatty acid and phosphorus content seem to indicate that the role of these groups in starch has been greatly overemphasized in enzyme studies. The phosphorus content of the starches studied varied from 0.015 to 0.05 percent and yet the yields of precipitate B were all between 30 and 38 percent (Table 10). There were no significant variations in the yields depending on the phosphorus content. Although the fatty acid content of precipitate A is higher in all cases than the original starches, precipitate B accounts for a much larger amount of the total fatty acids in the starch. The yield of precipitate A from potato and tapioca starches (Table 10) is quite low, and these materials have the least amounts of fatty acids present. There is no apparent correlation between the yields of precipitate B and the fatty acid content of the precipitate or the original starch.

Furthermore, the differences in precipitate B obtained from cereal starches and root starches (page 49) cannot be explained on the basis of different phosphorus and fatty acid contents. Precipitate B from wheat and potato starches has a very high phosphorus content (Table 12). However, the material from wheat starch formed a curdy white precipitate, while that from potato starch formed a sticky mass. Precipitate B from the cereal starches is higher in fatty acid content than the material from potato and tapioca starches. At first glance this fact might seem to explain the differences in the nature

of precipitate B. However, the fatty acid content of these materials is only slightly higher than the original starches, yet the differences between cereal and root starches is much more apparent in precipitate B.

Reducing value.

The reducing value as maltose was measured on a 5.0 cc. sample of a one percent suspension which had been boiled. Determinations were made according to the procedure given on page 36. The reducing values given in Table 14 are calculated as maltose per gram of sample.

Table 14. Reducing values by potentiometric sugar method.

Kind of starch	Unmodified	Precipitate A	Precipitate B
Corn	16.8	27.3	27.3
Rice	37.8	48.3	48.3
Wheat	25.2	46.2	46.2
Potato	16.8	63.0	63.0
Tapioca	33.6	52.5	33.6

Since this method involved titrations which were between 0.5 and 1.5 cc., the accuracy of the results is questionable. The R_{Cu} values of Farrow (53) were therefore obtained as a check on these results. The R_{Cu} values are summarized in Table 15.

Table 15. R_{Cu} values on the precipitates and starches.

Kind of starch	Unmodified	Precipitate A	Precipitate B
Corn	5.5	9.6	8.2
Rice	7.8	11.2	13.0
Wheat	10.4	11.3	13.0
Potato	4.3	36.5	13.8
Tapioca	4.3	19.0	14.6

Both Tables 14 and 15 show that the reducing action of precipitates A and B is greater than that of the original starches. These precipitates are all apparently quite different from the original starches. The R_{Cu} values of precipitate A and precipitate B from the cereal starches are almost the same and not so very much higher than the original starches. The R_{Cu} values on precipitate A from potato and tapioca starches are very high in comparison to precipitate B from these starches. Precipitate A from the digestions of all the starches studied was apparently partially degraded and not an intact portion of each original starch.

Recovery of precipitates A and B in the starch determination of Denny (14).

The absorbed iodine method of determining starch was used to compare precipitates A and B with the original starches. In this method the starch-iodine complex was precipitated from half-saturated calcium chloride solution. The precipitate was washed free from excess iodine, and digested in an excess

of standard sodium thiosulfate solution. The solution was back-titrated with standard iodine solution, with the starch present serving as the indicator. The difference between the amount of sodium thiosulfate and the amount of iodine for back-titration gave the amount of iodine on the starch precipitates. The mg. of iodine taken up by 40 mg. of sample are given in Table 16. The calculated percentage recovery of these samples is based on Denny's factor $\frac{\text{g. iodine}}{\text{g. starch}}$ equals 0.11 (14). This factor was determined on soluble potato starch.

Table 16. Recovery in the starch determination of Denny.

Kind of starch	Unmodified		Precipitate A		Precipitate B	
	mg. I	percent	mg. I	percent	mg. I	percent
Corn	2.9	65.6	3.25	69.0	0.86	19.6
Wheat	3.15	71.6	3.2	76.7	4.0	90.5
Rice	3.12	71.0	3.9	89.2	4.05	92.0
Potato	3.5	79.5	-	--	--	--
Tapioca	2.95	67.0	-	--	--	--

In the case of the cereal starches very little difference between the original starch and precipitates A and B is shown, except that only about 20 percent of precipitate B from corn starch is precipitated by iodine. Precipitates A and B from potato and tapioca starches were not precipitated at all by iodine. The customary deep violet-black color was noted, but no precipitate could be centrifuged or filtered out. The results of this experiment definitely show a difference in precipitates A and B from the cereal and root starches.

DISCUSSION AND CONCLUSIONS

The preliminary experiments on starch-enzyme digestions were carried out in order to establish, as nearly as possible, uniform conditions for the preparation of the residual products of beta amylase digestion. The course of enzyme action was studied to determine the length of time to allow the enzyme to act and to determine any differences in beta amylase action on starches of different origin. The effects of the enzyme on viscosity, "residual starch", and reducing action of the substrates were measured. However, the results of these determinations on the same digestion could not be correlated. Figure 3 shows a comparison of these effects. The viscosity of the substrate decreased very rapidly, while the amount of 55 percent alcohol precipitates decreased more slowly. The formation of reducing substances was still proceeding after both the viscosity and amounts of 55 percent alcohol precipitates had reached a constant value.

The significance of viscosity and "residual starch" determinations during the digestion of unmodified starches was questioned. The viscosity of the pastes was so high that five or six minutes was required for one measurement. Since changes in the substrate are proceeding rapidly at the same time, a measurement which takes such a long time is meaningless.

"Residual starch" determinations were complicated by the appearance of precipitate A in the digestions. Viscosity and "residual starch" determinations during digestions of starches that had been heated below the boiling point for 30 minutes, without stirring, were not significant because the substrates were not uniform.

The gravimetric Fehling method could not be used to determine the reducing action during digestion of unmodified starches. The results were high because part of the starch was filtered out and weighed as cuprous oxide. The volumetric Hagedorn and Jensen method gave satisfactory results during digestions of starches that had been boiled. However, when the substrates had been heated below the boiling point the results with this method were in error due to unswollen starch particles which held the iodine. The potentiometric sugar method was found to be generally the most suitable method. It was the only one that could be used when the starches had not been boiled. These methods of determining reducing value are empirical, that is, a set of reference data is necessary to calculate the results as maltose.

The meaning of the reducing values as maltose determined during beta amylase digestions might be questioned, since Table 14 shows that the reducing action of precipitates A and B is considerable. These materials in the digestion should contribute their reducing action also. That this effect is

small is shown by the following considerations. The concentration of precipitate A is very small in an enzyme digestion--not more than 0.1 percent, and so its contribution to the reducing action of the solution would be small. Actually, there was no difference in the titration values when the samples were removed from the clear supernatant liquid and when the samples were removed from the bottom of the flasks after this material had settled. The presence of precipitate B in solution would have more effect. After five hours digestion of six percent corn starch by beta amylase, 51 mg. of maltose and 30 mg. of precipitate B are formed per 100 mg. of the dry starch. From Table 14 0.88 mg. of maltose is calculated as due to 30 mg. of precipitate B. In this case 0.88 percent is the reducing value due to precipitate B in solution. Similar calculations on the other starches show a larger effect of precipitate B, a maximum value of 1.9 percent being shown in the case of potato starch digestions.

That the difference between natural and "soluble" starch is very great is brought out in the experiments on the digestion of natural and alcoholic HCl treated corn and potato starches. The viscosity of the pastes were much lower in the case of the "soluble" starches. While the rate of sugar formation was not changed appreciably, the final value for maltose was higher in the digestions of the natural starches. The alcoholic HCl treatment had produced unknown changes in the

starch. Therefore, the natural starches were used in preparing precipitates A and B (Fig. 8) from beta amylase digestions as eliminating a source of uncertainty in the substrates.

Due to the fact that precipitates A and B apparently carried down soluble material and electrolytes, methods of purification were studied. Precipitate A could be resuspended in water but could not then be recovered by centrifuging. Electrodialysis of the water suspension served to remove the electrolytes and to cause the material to migrate toward the positive electrode and settle out. It could then be recovered by centrifuging. Precipitate B was also electrodialyzed in order to remove electrolytes. When the materials were dried by direct evaporation of the water the products were hard, dark-colored masses that could not be ground. After the dialyzed precipitates had been dehydrated by grinding under absolute alcohol, they dried to white powders.

When dry, the powdered precipitates were rather resistant to wetting. One percent suspensions could be prepared as described on page 51. The suspensions of precipitate A and B from cereal starches were turbid but not viscous. Suspensions of precipitate A from potato and tapioca starches were clear and quite viscous. Precipitate B from root starches formed clear limpid suspensions.

Table 17 presents a summary of all the properties measured on precipitates A and B from five different starches. A

Table 17. Tabulation of data on products of beta amylase digestion.

Substance	Percent yield	Percent enzyme hydrolysis	Reducing value: Mg. maltose/g.	R_{Cu}	Fat: Percent	Fat: Percent Recovery	Phosphorus: Percent	Phosphorus: Percent Recovery	Starch: Mg. I	Starch: Percent Recovery
Corn starch		58.5	16.8	5.5	0.66		0.015		2.9	65.6
Ppt. A	1.64	14.1	27.3	9.6	1.31	3.26	0.031	3.38	3.25	69.0
Ppt. B	32.2	15.3	27.3	8.2	0.71	34.5	0.033	70.8	0.86	19.6
Rice starch		65.7	37.8	7.8	0.62		0.035		3.12	71.0
Ppt. A	1.92	30.0	48.3	11.2	0.95	2.90	0.041	2.25	3.9	89.2
Ppt. B	34.4	34.0	48.3	13.0	0.56	31.0	0.033	32.8	4.05	92.0
Wheat starch		60.0	25.2	10.4	0.57		0.051		3.15	71.6
Ppt. A	1.0	23.9	46.2	11.3	0.90	1.5	0.041	1.80	3.2	76.7
Ppt. B	38.8	26.2	46.2	13.0	0.91	51.4	0.152	103.0	4.0	90.5
Potato starch		59.3	16.8	4.3	0.076		0.050		3.5	79.5
Ppt. A	0.84	9.65	63.0	36.5	0.17	1.8	0.112	1.88	-	--
Ppt. B	30.0	9.02	63.0	13.8	0.18	71.0	0.222	102.5	-	--
Tapioca starch		77.2	33.6	4.3	0.174		0.010		2.95	67.0
Ppt. A	0.05	6.3	52.5	19.0	0.51	1.46	0.020	1.0	-	--
Ppt. B	33.5	9.8	33.6	14.6	0.22	42.3	0.020	67.0	-	--

natural division occurs between the cereal and root starches. The optimum gelatinization temperatures for the root starches were much lower than those of the cereal starches. The optimum temperatures can be correlated fairly well with the fat content of the original starches. However, the rates of soy bean amylase action on the different substrates prepared at their optimum temperatures were all about the same. The final reducing values were about 60 - 70 percent of the original starch. When the substrates were prepared at their optimum temperatures, soy bean amylase action on corn, potato, wheat, rice, and tapioca starches was very similar. This series contained starches of very high and very low phosphorus and fatty acid content. If these groups are important in enzyme action, some differences should have been noted. However, since the starches were all digested at about the same rate and stopped at about the same reducing value, the effect of fatty acid and phosphorus on beta amylase action must be negligible.

The difference between cereal and root starches cannot be explained on the basis of phosphorus and fatty acid content. Precipitate A from the cereal starches was formed in larger amounts and settled out of the digestions. Precipitate B from the root starches was a transparent sticky mass before dehydration. There is no apparent explanation of these differences in the data of Table 17.

Precipitate A seems to be a portion of precipitate B

which for some reason is thrown out of the suspension. Precipitate B can be considered as a composite residue after the amylase has split off successive maltose units until blocked by some unknown agent or group. It is reasonable then that portions of this residue should have a concentration of factors which make for less solubility. This portion would settle out of the digestion mixture and appear as precipitate A. The data in Table 17 support this hypothesis in the following ways.

1. Precipitates A and B from any one kind of starch are hydrolyzed by beta amylase to about the same degree. Here again a difference between cereal and root starches is noticeable. These materials from root starches are hydrolyzed to a much less degree than precipitates A and B from cereal starches.

2. The reducing values of precipitates A and B are about the same. This is confirmed by both potentiometric determinations as on page 36 and by the R_{Cu} values. Precipitate A from potato starch has an exceptionally high R_{Cu} value, which may be the result of experimental difficulties encountered due to the gummy viscous nature of the material. There is another exception in that the reducing value as maltose of precipitate A from tapioca is very high.

3. Precipitates A and B from any one kind of starch behave similarly in the starch determination of Denny (14), with the exception of precipitate B from corn starch. There

is a very clear differentiation between cereal and root starches here. The residual materials from the cereal starches react in about the same manner as the original starches. Precipitates A and B from root starches do not react at all in this determination.

4. The data on fat and phosphorus content of precipitates A and B indicate that the decreased solubility of precipitate A may be due to either fat or phosphorus. Precipitate A from corn starch is much higher in fatty acids than precipitate B, but the phosphorus content of the two is essentially the same. On the other hand, the phosphorus content of precipitate B from wheat starch is much higher than that of precipitate A, but the fatty acid contents of the two are the same. Precipitate A then may be formed because the presence of high content of fatty acids makes this fraction less soluble as in corn starch. In wheat starch the formation of precipitate A may be due to a lower phosphorus content than in precipitate B which remains in solution.

The residual material from beta amylase action seems to be present in about the same amounts in digestions of different starches. This fact would exclude the possibility that the phosphorus or fatty acid groups block the action of the enzyme, since these groups are present in varying amounts in the different starches. Further work is necessary before the exact reason for beta amylase action stopping at 60 - 70 percent in

different starches is explained.

SUMMARY

1. A water-jacketed viscosimeter for measuring the rate of liquefaction of starch pastes by diastase has been described.
2. A macro volumetric modification of Hagedorn and Jensen's sugar method, applicable to following the course of amylase action has been developed.
3. A potentiometric method of determining reducing values during amylase action has been developed.
4. The temperature at which the substrate is prepared has been found to affect the rate of beta amylase action. Optimum temperatures for preparation of potato starch for amylase action is 70°, for tapioca 80°, for rice 80° or above, and for corn and wheat starches 85° - 90°.
5. Methods of separating the flocculent material (precipitate A) and 60 percent alcohol insoluble residue (precipitate B) from beta amylase digestions have been described.
6. The preparations were characterized as to further enzyme hydrolysis, phosphorus and fatty acid content, reducing action against ferricyanide and against copper, and recovery in the starch determination of

Denny (14).

7. The measured properties of precipitates A and B tend to show that precipitate A is a portion of precipitate B which is less soluble.
8. Precipitates A and B from cereal and root starches show marked differences. These differences have not as yet been explained.

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