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# THE CONCENTRATION, CHARACTERIZATION AND PROPERTIES OF SOYBEAN AMYLASE

by

J. Marshall Newton

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Enzyme Chemistry

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## TABLE OF CONTENTS


| | Page |
|---|---|
| INTRODUCTION | 5 |
| PART I. THE CONCENTRATION, CHARACTERIZATION AND PROPERTIES OF SOYBEAN AMYLASE | |
| Review of Literature | 7 |
| Materials Used | 14 |
| Statement of Problems | 15 |
| Varietal Differences in the Amylase Content of Soybeans | 16 |
| Effect of Germination | 19 |
| Concentration of Soybean Amylase | 21 |
| Characterization of the Starch Hydrolyzing Enzyme in Soybeans | 34 |
| Isolation and characterization of the starch degradation products | 34 |
| Mutarotation of degradation products | 37 |
| Characterization by the diffusion technique of Wijsman | 40 |
| Determination of the dextrinogenic or amyloclastic activity | 42 |
| Viscosity changes during digestion | 44 |
| Deterioration of Soybean β-amylase | 46 |
| Discussion | 54 |
| Summary | 60 |


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Page

PART II. THE DEGRADATION PRODUCTS FORMED FROM STARCH BY THE ACTION OF ALPHA- AND BETA-AMYLASE

Review of Literature........................................ 61

Materials Used............................................ 81

Statement of Problem...................................... 82

Procedures for the Isolation of the Degradation Products Formed from Starch by the Action of α-amylase..................................... 83

Corn starch degradation products. Experiment I.................................. 83

Primary procedure........................... 83

Fractionation of Ppt. II.................... 88

Fractionation of Ppt. III................... 89

Fractionation of Ppt. V..................... 91

Corn starch degradation products. Experiment II................................. 93

Waxy maize starch degradation products......... 100

Primary procedure........................... 100

Fractionation of Ppt. IIb................... 106

Fractionation of waxy maize dextrins with $[\alpha]_d$, +164-173° and Rm 22-27................ 107

Discussion of the Corn and Waxy Maize Dextrins.... 110

Chemical and Physical Properties of the Dextrins.. 114

Molecular weight from freezing-point depressions.................................. 114

Dextrin acetates.............................. 115

Oxidation of dextrins......................... 118

Procedure................................... 118

Page

Isolation of the potassium salt of the dextrinic acid formed by the oxidation of corn dextrin V.................................... 118

Melting points of dextrins and dextrin acetates........................................ 122

The reactions of dextrins with phenylhydrazine. 124

Discussion........................................ 126

Summary........................................ 135

ACKNOWLEDGMENTS........................................ 137

LITERATURE CITED........................................ 138

# INTRODUCTION

Most studies of the starch-amylase problem are complicated by the absence of adequate knowledge of the nature of the substrate and the nature of the enzyme. The responsibility for this lack of knowledge probably lies in the fact that the investigators are either carbohydrate chemists interested in the starch angle with subsequent acceptance of the enzyme as an entity, or are biologists interested primarily in the enzyme with subsequent acceptance of the starch as a chemical entity. Because few investigators have been interested in the starch-amylase problem as a whole, there are available few comprehensive studies including purification, characterization and properties of the enzyme, rate and degree of the amylase action on various starch substrates, and isolation of the starch degradation products, followed by an examination of the physical and chemical properties of these products. In this investigation an attempt has been made to study the problem from both the starch viewpoint and the enzyme viewpoint. Because the literature survey and the experimental work in each case assumes different points of attack, this thesis is divided into two sections:

1. The concentration, characterization and properties of soybean amylase.

## 2. Investigations of the degradation products formed from starch by the action of α- and β-amylase.

# PART I

## THE CONCENTRATION, CHARACTERIZATION AND PROPERTIES OF SOYBEAN AMYLASE

### Review of Literature

The use of soybeans as a source of amylase is limited. Orestano and Zummo (1) in a study of the amylase in soybeans report after heat-treatment studies, that their results do not indicate the existence of two different amylases, one causing liquefaction and the other saccharification of the starch substrate. Gore and Jóźsa (2) record marked saccharifying action in soybeans. In further substantiation of the saccharifying action of soybeans, Orestano (3) reports that only one component, probably β-amylase, is present. Teller (4) reports the presence and relative activity of various plant amylases. Soybean amylase proved to be a stronger saccharifying agent than any of the enzymes tested except for the amylase from dry sweet potato. The work of Newton and Naylor (5), based on mutarotation, diffusion, iodine color and degree of hydrolysis studies, has definitely established that the starch hydrolyzing enzyme present in soybeans is β-amylase.

Some information as to the rate of reaction of the soybean amylase is given by Artom and Orestano (6). These

authors present the equations for the rate of liquefaction and saccharification of starch by soybean amylase. That the rate of reaction of soybean amylase can be effected by the presence of maltose, which is one of the end-products, is shown by the data of Schultz and Landis (7). These authors show that removal of the fermentable sugars by a yeast fermentation increased the production of fermentable material by about 15% at the end of a six hour digestion. In cases where the maltose was removed, the diastatic activity of soybeans was found to be a linear function of time and enzyme concentration throughout large variations in substrate concentration. If the maltose is not removed, it acts as an inhibiting agent causing a marked reduction in the rate of hydrolysis of the starch.

The β-amylase preparations from soybeans prepared according to Newton and Naylor (5) have been utilized in studies of the chemical and physical nature of starches. The action of the enzyme on starch from different species of plants is reported by Martin and Newton (8). Their digestions of rice, tapioca, wheat, potato and corn starch by soybean amylase show marked variations in the rate of digestion of the starches, especially in the earlier phases of the reaction. For example, after a 60 minute digestion of gelatinized samples, 36% of the theoretical maltose was produced from potato starch while corn starch gave 45% of the theoretical maltose. However, if

instead of gelatinizing the starch the substrate was prepared by heating the aqueous suspension of the raw starch at a lower temperature, the difference in the rate of digestion was much greater. A sample of potato starch heated in aqueous suspension at 70° C. and then subjected to the action of soybean β-amylase gave 46% maltose after 60 minutes, while a sample of corn starch prepared under identical conditions gave only 29% theoretical maltose. Their subsequent studies of the effect of temperature of preparation of starch pastes on the extent of hydrolysis by soybean amylase suggest the use of this enzyme as a tool for differentiating starches and for following starch modification. The use of the soybean β-amylase to follow the modification of starch was investigated by Newton, Farley and Naylor (9). In addition to the starches studied by Martin and Newton, these authors have extended the investigations to include waxy maize, waxy sorghum and sweet potato starch, several varieties of corn starch, mechanically modified starches, oxidized corn starches, corn flour, brewer's flakes, dextrins and acid-hydrolyzed corn starches. Characteristic digestion curves of these starches show the variance in susceptibility to enzyme action. Some of the starches tested have but slight variations in physical and chemical properties, yet the action of β-amylase emphasizes the marked differences which actually occur.

Martin (10) used soybean β-amylase in the preparation of the products of starch-amylase digestion. The dextrins produced by the action of β-amylase on various starches were isolated and purified. They were then studied as to further hydrolysis by β-amylase, reducing value, fat content, phosphorus content and the starch content according to the adsorbed iodine method of Denny (11). The principal results of these studies were published by Martin, Naylor and Hixon (12). The dextrins were isolated by alcohol precipitation, collected by centrifugation and purified by electrodialysis. The dextrins were observed to be quite different from the original starch, the reducing power and phosphorus and fatty acid content in general being somewhat higher than in the original starches. This is in accord with the theory that the dextrins are fragments of the original starch molecule and should contain a higher concentration of the phosphorus and fatty-acid groups. However, the residual dextrin formed by the action of β-amylase is obtained in about the same yield in digestions of different starches irrespective of the phosphorus and fatty-acid content of the starch. This fact casts considerable doubt on the theory that the phosphorus or fatty-acid groups act to block the action of the amylase at a certain stage of hydrolysis. The only direct correlation between the fatty-acid or phosphorus content and the properties of the starch was obtained between the optimum

temperature for preparing the substrates and the fatty-acid content. A starch with a high fatty-acid content apparently has a lower solubility, thus requiring a higher optimum temperature for preparing the substrate. Caldwell (13), in a study of the fractionation of starch, has utilized soybean β-amylase in the preparation of the limit dextrins for characterization by chemical means. It is from these studies that further evidence for the heterogenity of the β-amylase limit dextrin is obtained. The heterogeneous nature of the residual dextrin was previously demonstrated by Beckmann and Landis (14) in their studies based on ultracentrifugation of dextrins produced from potato starch by the action of the β-amylase of soft-wheat flour.

Another use of soybean amylase is suggested by the work of Schoene, Fulmer and Underkofler (15). In studies of the saccharification of starchy grain mashes for the alcoholic fermentation industry they have found that the addition of small amounts of soybean meal to some of their fermentations gave a considerable increase in the alcohol yield.

The concentration of amylases has been accomplished by numerous procedures. One of the earliest efforts toward concentration of an amylase was reported by Payen and Persoz (16) in which alcohol was used as the precipitating agent. Dubrunfaut (17) found that better results were obtained by precipitating the amylase from aqueous extracts of malt with

sumac or tanning liquid. An early use of glycerine as an extracting agent is reported by Gorup-Besanez (18). The alcohol and ether precipitate obtained from the glycerin extraction was analyzed and reported to contain sulphur and nitrogen. One of the first reports of the use of ammonium sulphate in the preparation of amylase is given by Osborne (19). The amylase was precipitated by the addition of ammonium sulphate, the salt removed by dialysis and the amylase finally precipitated by dialysis against alcohol. Another early method (20) for concentration of amylase utilized lead acetate as the enzyme precipitating agent, followed by precipitation of the lead and fractionation of the liberated amylase by alcohol precipitation. Although the use of adsorption methods was occasionally reported in the nineteenth century, their use was very limited until after 1900. Michaelis and Ehrenreich (21) and Peters (22) almost simultaneously reported the use of adsorbing agents in the purification of amylases. In many of the modern techniques for the concentration of enzymes adsorption serves as one phase of the process. If the enzyme is adsorbed, it is then eluted by some means and is finally precipitated or further purified by other methods, such as precipitation by an organic solvent. Numerous materials have been used as adsorbing agents, for example, starch grains (23), alumina and aluminum hydroxide (24, 25, 26, 27), kaolin (28, 29), $Ca_3(PO_4)_2$ (30, 26) and bauxite (31, 32).

The following is a summary of the fundamental methods used, individually or in combinations, in attempts to concentrate the amylase from various sources:

1. Extraction of the amylase with water.
2. Extraction of the amylase with glycerol-water solutions.
3. Precipitation by organic solvents (alcohol, ether, acetone, etc.).
4. Precipitation by organic precipitants (tannin, etc.).
5. Precipitation by heavy metals (Hg, Pb, etc.).
6. Precipitation by salting out (ammonium sulphate).
7. Precipitation by change in pH (dialysis and electrodialysis).
8. Adsorption of the enzyme.
9. Adsorption of the impurities.

From the foregoing discussion it is evident that the studies involving the use of soybean β-amylase are very limited. However, the investigations indicate some of the valuable uses of a pure β-amylase. Such a convenient source of β-amylase concentrates in appreciable quantities is unavailable from any other source with the possible exception of the sweet potato. This suggests the utilization of the soybean as a primary source of the enzyme, the primary concentrates then being further purified by any of the above methods.

## Materials Used

### A. Amylase

All soybean amylase preparations used in this investigation were prepared by the author in this laboratory. The soybeans were furnished through the courtesy of the Agronomy Department of Iowa State College and the Agronomy Section of the Iowa Agricultural Experiment Station.

### B. Starches

The starch used in all quantitative enzyme measurements was a commercial soluble starch prepared by the J. T. Baker Company. A large quantity of this starch was obtained at the beginning of the investigation and utilized throughout the work. The starches used in rate studies were regular commercial starches.

## Statement of Problems

The purpose of this study was to investigate:

1. The concentration and characterization of the amylolytic enzyme in soybeans.

2. A study of the properties of the amylase in soybeans.

## Varietal Differences in the Amylase Content of Soybeans

Before any attempt was made to concentrate the amylase, a study of the amylase content of different varieties of soybeans was undertaken. Samples of three varieties of soybeans, Mukden, Variety No. 65424 and Variety No. 54818, were obtained. The beans were carefully hand-sorted to remove all broken or discolored beans and then ground in a Wiley mill to pass a 40 mesh screen. The procedure for extraction of the enzyme, preparation of the substrate and determination of the reducing equivalent, expressed as maltose, has been previously published (5). Expressed as milligrams of maltose produced per milligram of bean extracted, the average values for these three varieties of soybeans were 19.7, 22.3 and 21.2, respectively. The experimental error in individual determinations of the same variety was as great as the difference between the varieties. However, Variety No. 65424 was consistently higher in saccharogenic power than either of the other varieties. A supply of this sample was therefore obtained and used as the source of amylase for all of the preliminary studies.

At a later date fifteen varieties of soybeans were obtained through the cooperation of the Agronomy Section of the Iowa Agricultural Experiment Station. The beans were grown in Iowa in the summer of 1938. These samples were tested for saccharogenic power. The amylase content of the

soybeans, as shown in Table I, varied from 7.14 to 15.80.

TABLE I

Variation in the amylase content of soybeans.

| Variety No. | Saccharogenic Power | Variety No. | Saccharogenic Power |
|---|---|---|---|
| 101 | 11.22 | 325 | 9.69 |
| 107 | 11.72 | 409 | 7.65 |
| 114 | 12.75 | 411 | 7.39 |
| 211 | 7.39 | 412 | 7.14 |
| 213 | 11.72 | 506 | 11.47 |
| 215 | 15.80 | 518 | 9.69 |
| 301 | 11.72 | 525 | 11.22 |
| 314 | 14.80 | | |

Three samples grown in a different section of the state in the previous growing season had saccharogenic powers of about 20. Thus it is evident that there is a marked difference in the amylase content from variety to variety and from year to year. The number of varieties tested was not sufficient to draw any definite conclusions. However, marked varietal differences in the amylase content of other plant seeds have been observed by numerous investigators (33, 34, 35, 36). Therefore, even though a large number of soybean samples were not investigated, these data, in collaboration with data pertaining to other seeds, indicate a marked

variation in the amylase content of different strains of soybeans and in the same strain from season to season.

## Effect of Germination

Bray and Naylor (37) have suggested that the amount of extractable amylase in soybeans does not appear to increase during germination. Germination of Mukden and Variety No. 65424 was carried out under laboratory conditions. Preliminary germination of one sample indicated that off-colored and broken beans have a marked tendency to mold in the early stages of germination, even when fumigated with carbon disulphide according to the method of Naylor and Dawson (38). Much of this molding was eliminated by careful hand-sorting before germination, all broken or discolored beans being discarded. In addition, any beans which began to mold during the germination were removed. After sorting, the beans were fumigated with carbon disulphide (38). They were spread in a single layer on moistened blotting paper in enameled pans and placed in a small glass hood to prevent excess dust-contamination and sharp changes in temperature. The temperature of the hood varied from 20-25° C. The samples were removed at 24 hour intervals, crushed on a glass plate, dried for 12 hours in a stream of air maintained at 40-45° C. and stored in tightly stoppered bottles. Crushing the beans was essential to rapid and adequate drying. The crushed samples were ground in a Wiley mill to pass a 40-mesh screen and the saccharogenic power determined by the method described in

the previous section.

Three individual germinations of Variety No. 65434 were run for a six day period. For comparative purposes the saccharogenic power (milligrams of maltose produced per milligram of bean used in the extraction) has been calculated. Figure I presents the results of these determinations.

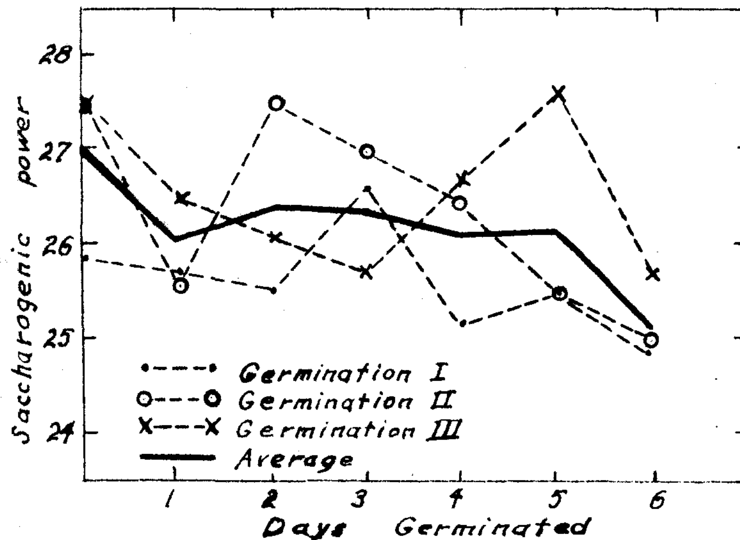



Figure I. Variation in saccharogenic power of soybeans during germination.

In every case the saccharogenic power of the sample after six days germination was slightly less than that of the ungerminated beans. The average of the samples shows a slight but definite decrease as the germination proceeds. This decrease is in sharp contrast to the marked increase in the saccharogenic power shown during the germination of other grains.

## Concentration of Soybean Amylase

In a previous publication (Newton and Naylor (5)), a method is given for the preparation of amylase concentrates from soybeans in which diethyl ether was utilized as the extractant in the removal of the oil from the beans. Because of the difficulties involved in the use of ether in extractions, other solvents have been investigated. Trichloroethylene has been used as an oil extractant in pilot-plant tests and possibly on a commercial scale. A sample of soybeans extracted in a continuous extractor using trichloroethylene was obtained. The amylase content of the beans was determined and found to be markedly reduced, the extracted beans containing less than 30% of the amylase present in the original beans. This marked reduction of the amylase is due either to the trichloroethylene or to the heat treatment of the soybean flakes in removal of the solvent, or perhaps to a combination of these factors. The use of a petroleum ether (b.p. 68-70° C.) was then investigated. Because the petroleum ether was less expensive, had no injurious effect on the enzyme, satisfactorily removed the oil, presented less of a fire hazard and was more easily recovered it was used in place of diethyl ether in the later preparations. Another slight change, suggested in the publication mentioned above (5), was adopted as general procedure. This involves precipitation of the active enzyme concentrates by 70% alcohol rather than 65% alcohol.

In Table II, data on a number of typical amylase concentrates are presented. All of these concentrates were prepared from beans extracted by diethyl ether, and in most cases the concentrates were precipitated by 70% alcohol.

TABLE II

Data on amylase concentrates prepared from soybeans.

| Date of prepn. | Gms. extd. bean used | Gms. conc. Obtained | Alc. concn. for pptn. | Saccharo. power[1] | Amylase units[2] | Yield (%)[3] |
|---|---|---|---|---|---|---|
| 12-31-36 | 500 | 0.72 | 65% | 745 | 536 | 5.4 |
| 1-20-37 | 220 | 1.05 | 65 | 990 | 1040 | 23.6 |
| 4-23-37 | 480 | 2.45 | 70 | 1000 | 2450 | 24.5 |
| 8- 7-37 | 500 | 2.28 | 70 | 1209 | 2750 | 27.5 |
| 8-10-37 | 500 | 2.60 | 70 | 653 | 1700 | 17.0 |
| 8-11-37 | 500 | 3.91 | 70 | 512 | 2000 | 20.0 |
| 8-14-37 | 500 | 12.45 | 72 | 219 | 2730 | 27.3 |
| 11-10-38 | 600 | 2.13 | 70 | 1072 | Data | |
| 11-11-38 | 600 | 4.24 | 70 | 528 | not | |
| 11-17-38 | 600 | 3.74 | 70 | 455 | available | |
| 11-18-38 | 600 | 4.25 | 70 | 399 | | |
| 11-22-38 | 600 | 1.20 | 58 | 753 | | |

(1) Saccharogenic power is defined as the milligrams of maltose produced per milligram of amylase concentrate under the conditions specified for the determination of amylase activity.
(2) Amylase units are obtained by multiplying the saccharogenic activity of the concentrate by the yield in grams.
(3) The saccharogenic power of the extracted beans was 20.

$$\text{Yield }(\%) = \frac{(\text{gm. extd. bean used})\ (20)}{(\text{gms. conc. obtained})\ (\text{saccharogenic power of conc.})} 100$$

All concentrates were dried in a vacuum desiccator over calcium chloride and were stored in an ice-box at 5° C. Three general statements apply to these amylase concentrates:

1. With increased alcohol concentration the yield of precipitate increases.

2. The saccharogenic power of the amylase concentrate usually varies inversely with the yield of the enzyme material.

3. Those concentrates precipitated in greater yields and at higher alcohol concentrations were more soluble in water than the concentrates obtained in lower yields and at lower alcohol concentrations.

After removal of the active concentrate the residual solutions were evaporated under reduced pressure to a light syrup to recover the alcohol for other extractions. Upon further slow evaporation of this residual syrup at room temperature a small quantity of white crystalline material was observed in the thick syrup. After numerous attempts it was found that by mechanically removing the larger crystals from the syrup, repeatedly washing the crystals with anhydrous ether, then with absolute EtOH and again with ether, the crystalline material was freed of all coloring matter and residual syrup. The crystals were then recrystallized from hot 99% EtOH. The yield was 0.664 grams from 250 gms. of extracted beans. The material possessed a sweet taste, was non-reducing to Fehling's solution, melted at 185-187° C. and

with $[\alpha]_d^{25°} = +65.5°$. Treatment with hot dilute HCl gave a strongly reducing solution. With sucrose melting at 185-187° C., $[\alpha]_d^{25°} = +66.5°$ and possessing the other properties given above, it is evident that the white crystalline solid isolated from soybeans is sucrose. This substantiates the isolation of sucrose from soybeans as reported by Iwasa (39).

From preliminary experiments on the purification of the crude amylase concentrates it was evident that dilute alcohol at room temperature and higher alcohol concentrations at lower temperatures caused a marked decrease in the activity of the amylase concentrates. This is in agreement with the quantitative measurements of the effect of alcohol and temperature on the amylases of wheat as reported by Blish, Sandstedt and Mecham (40). This suggests two possibilities for the improvement of the concentration procedure, namely, working at as low a temperature as possible and the substitution of some other organic solvent for the alcohol. After numerous experiments with various organic solvents the most satisfactory results were obtained by using acetone in place of ethyl alcohol for the precipitation of the enzyme. However, alcohol gave better results in the preparation of the crude amylase concentrates.

During drying a portion of the total activity is destroyed, this destruction perhaps being due to denaturation of the proteins in the drying process. By elimination of this process whenever possible better yields of more active concentrates were obtained.

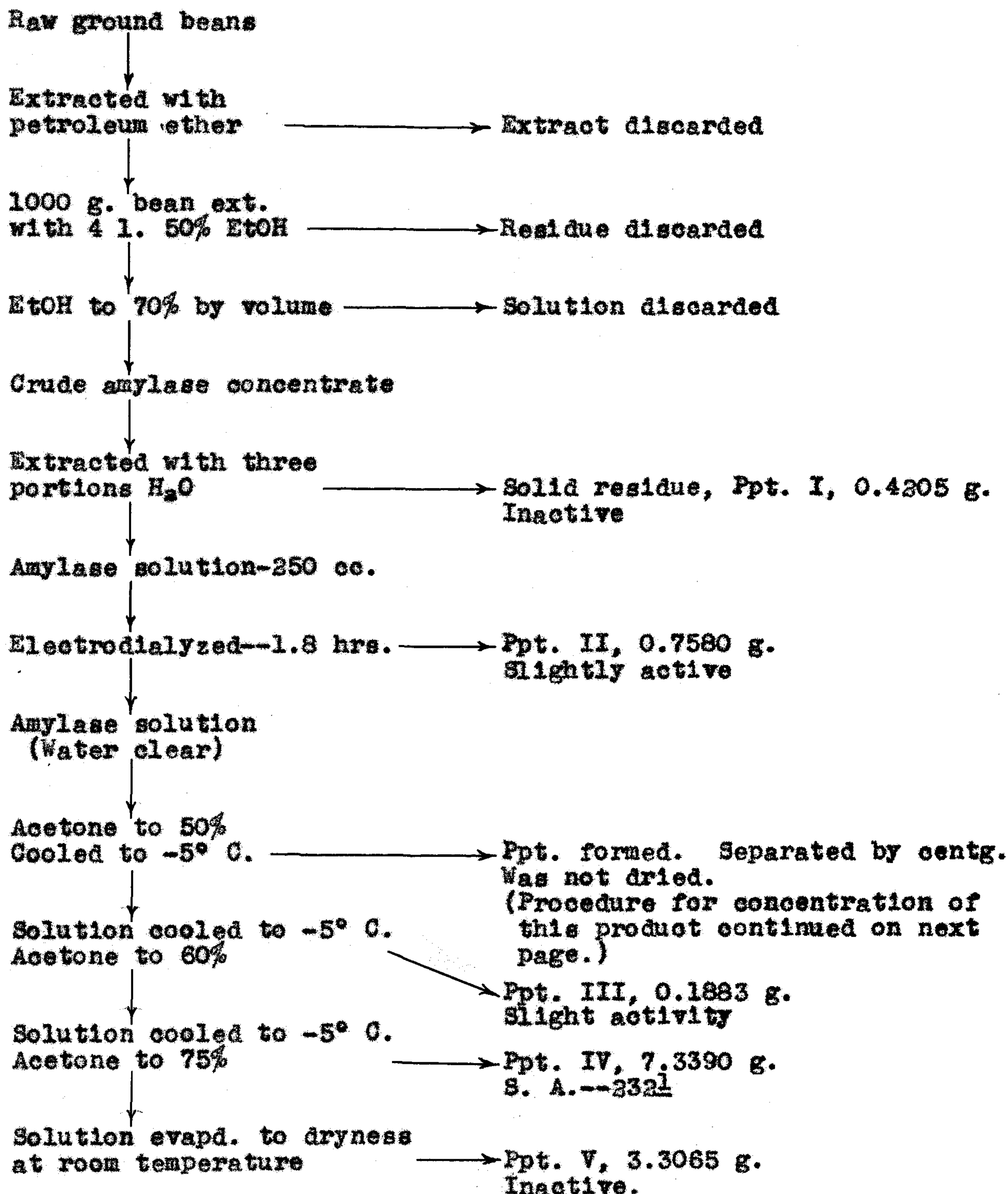
Electrodialysis was introduced into the purification procedure only after its effect on the enzyme was determined. The apparatus used was similar to that described by Hixon and Martin (41), except that the center cell had a capacity of about 300 cc. and both electrodes were of platinum. The electrodes were oval-shaped sheets of platinum foil measuring about 14.3 x 16.2 x 0.0025 cm. Long dialysis (24 hours) at 10-15 volts with a current of 120 milliamperes resulted in the formation of a heavy precipitate and an appreciable loss in the total amylase content. Observations during dialysis indicated that the precipitation occurred after about one hour of dialysis, but that no marked decrease in the amylase content of the liquid occurred until after several hours dialysis. Therefore, by electrodialyzing for a short period of time (less than 2 hours) considerable purification was obtained by the precipitation of an inactive material and by the removal of inorganic ions without any appreciable destruction of the enzyme.

The original procedure for the preparation of soybean β-amylase concentrates was therefore revised. Acetone was substituted for alcohol whenever possible, temperatures were maintained at as low a temperature as convenient, precipitates were not dried until purification was completed and electrodialysis was used as a means of removing inactive organic material and inorganic ions. The flow sheet for a typical

preparation is shown in Figure II. The yields of amylase by the concentration procedure given in Figure II are presented in Table III. The heading of the last column Amylase units is defined as the grams of maltose produced per gram of the enzyme concentrate under the standardized experimental conditions. It is only on this basis that any idea of the percentage recovery of the enzyme can be obtained.

As shown in Table III the total yield of amylase for this particular experiment is 10.7%. This is somewhat lower than the percentage yields of 17-24.5% given in Table II for a series of soybean amylase preparations. The lower yield in the experiment presented in Figure II is due to several causes, the exact contribution of each being undeterminable. Some of the contributing causes are: mechanical loss in the manipulation of small quantities of the highly active concentrates; loss due to slight inactivation during electrodialysis, fractional solution and fractional precipitation; inactivation by the organic solvents; the general inactivation of amylase solutions which occurred during the long concentration procedure.

From Table III it is also evident that a large portion of the total amylase recovered remains in Ppt. IV which has an activity of only 232. An attempt was therefore made to further concentrate this material and to follow the yields of the enzyme. Three different procedures were used in the


Raw ground beans
Extracted with petroleum ether
Extract discarded
1000 g. bean ext. with 4 l. 50% EtOH
Residue discarded
EtOH to 70% by volume
Solution discarded
Crude amylase concentrate
Extracted with three portions $H_2O$
Solid residue, Ppt. I, 0.4205 g. Inactive
Amylase solution-250 cc.
Electrodialyzed--1.8 hrs.
Ppt. II, 0.7580 g. Slightly active
Amylase solution (Water clear)
Acetone to 50% Cooled to -5° C.
Ppt. formed. Separated by centg. Was not dried. (Procedure for concentration of this product continued on next page.)
Solution cooled to -5° C. Acetone to 60%
Ppt. III, 0.1883 g. Slight activity
Solution cooled to -5° C. Acetone to 75%
Ppt. IV, 7.3390 g. S. A.--332[1]
Solution evapd. to dryness at room temperature
Ppt. V, 3.3065 g. Inactive.

Ppt. formed. Separated by centg.
Was not dried.
(Continued from preceding page)

↓

Extracted 6 times with 30 cc. portions 40% acetone → Residue, Ppt. VI, 0.2021 g. S. A.--251

↓

180 cc. ext. evapd. at 25°C. to 20.5 cc.

↓

Stored at 5° C. for 5 days. Slight ppt.[2] → Ppt. VII, 0.0095 g. Activity not determined.

↓

Centrifugate cooled to -5° C. Acetone to 50% → Ppt. VIII, 0.1066 g. S. A.--2260

↓

Centrifugate cooled to -5° C. Acetone to 60% → Ppt. IX, 0.0661 g. S. A.--4580

↓

Centrifugate cooled to -5° C. Acetone to 80% → Ppt. X, 0.0815 g. S. A.--2155

↓

Evapd. to dryness at room temperature. → Ppt. XI, 0.0837 Slight activity

(1) Saccharogenic activity or milligrams of maltose produced per milligram of enzyme concentrate.
(2) This storage period is not necessary in preparation of β-amylase concentrates. It was allowed to stand in this case to determine whether any material would crystallize from the concentrated solution.

Figure II. Flow sheet for the preparation of β-amylase concentrates from soybeans.

TABLE III

Total yield of amylase as amylase concentrate by the procedure given in Figure II

| Precipitate number | Yield of amylase (gms.) | Saccharogenic activity | Amylase units |
|---|---|---|---|
| Original bean | 1000 | 23.1 | 23,100 |
| I | 0.4205 | Inactive | ------ |
| II | 0.7580 | Inactive | ------ |
| III | 0.1883 | | |
| IV | 7.3390 | 232 | 1,702 |
| V | 3.3065 | Inactive | ------ |
| VI | 0.2021 | 251 | 50.9 |
| VII | 0.0095 | Not tested | ------ |
| VIII | 0.1066 | 2260 | 240.8 |
| IX | 0.0661 | 4580 | 303.0 |
| X | 0.0815 | 2155 | 175.9 |
| XI | 0.0837 | Inactive | ------ |
| Total | 12.5618 | | 2,472.5 |
| Total yield (as %) | | | 10.7 |

concentration studies. They were as follows:

Experiment I. Two grams of Ppt. IV (See Table III) were placed in solution by shaking with 240 cc. of 40% acetone. This solution was then cooled to -5° C. in an ice-salt bath and cold absolute acetone added until the acetone concentration was 55% by volume. The white precipitate so obtained

was settled by centrifugation, dried at room temperature in a vacuum desiccator over $CaCl_2$ and preserved as Ppt. A. The centrifugate was again cooled to -5° C. and cold absolute acetone added to a final concentration of 60% acetone by volume, the precipitate settled by centrifugation, dried as above and preserved as Ppt. B. Centrifugate B was again cooled, acetone added to 80%, the precipitate removed, dried and designated as Ppt. C. The residual solution was then evaporated to dryness at room temperature to give Ppt. D.

Experiment II. Two grams of Ppt. IV (Table III) were dissolved in the least possible amount of 40% acetone. Given sufficient time, the entire sample of the enzyme concentrate dissolved in 11.0 cc. of the 40% acetone, giving a thick syrupy solution. The solution was cooled to -15° C. and cold absolute acetone added until a definite precipitate formed. This required 2.00 cc. of acetone. The precipitate was collected by centrifugation, dried and preserved as Ppt. A. To the centrifugate at -15° C., 1.40 cc. of acetone was added to give Ppt. B which was collected and dried. The centrifugate from B was then cooled to -15° C. and 1.65 cc. acetone added to give a further precipitate. This material was collected, dried and preserved as Ppt. C. Final precipitation was accomplished by the addition of 9.95 cc. of acetone to centrifugate C giving Ppt. D. Evaporation of the centrifugate from Ppt. D gave only a trace of solid material.

The activity of this residue was not determined.

Experiment III. One gram of Ppt. IV (Table III) was dissolved in 50 cc. of 50% acetone at room temperature. Upon cooling in an ice bath a small amount of solid material precipitated. This material was collected by centrifugation, dried and preserved as Ppt. A. The acetone concentration was then raised to 55.5% to give Ppt. B, to 65.6% to give Ppt. C and then to 75% to give Ppt. D. The centrifugate from D was then evaporated to dryness at room temperature to give Ppt. E. All precipitations were run at 0° C.

Table IV presents the results of the three purification procedures just given. Experiment I and II gave slightly lower yields of the amylase than Experiment III, with I yielding a concentrate of high activity and all of the other concentrates in Experiments I and II showing little improvement over the original concentrate. With Experiment III the concentration of the amylase in Ppts. A and B was 5- and 10-fold, respectively, with 68% recovery of the total amylase. This method thus gives greater concentration of the amylase as well as more efficient recovery of the total amylase.

The method employed in Experiment III was also applied to a commercial takadiastase preparation. All precipitates obtained were inactive.

The yields of this method are much better than other data in the literature. For example, Sherman and Neun (42)

TABLE IV

Results of attempts to further purify an amylase concentrate with saccharogenic activity of 232.

| Precipitate number | Yield (gms.) | Saccharogenic activity | Amylase units |
|---|---|---|---|
| **Experiment I** | | | |
| Original | 2.0000 | 232 | 464.0 |
| A | 0.1610 | 1304 | 210.0 |
| B | 0.3259 | 251 | 82.7 |
| C | 0.9599 | Inactive | ----- |
| D | 0.0578 | Inactive | ----- |
| Total | 1.5082 | | 292.7 |
| Yield | 75.4% | | 63.2% |
| **Experiment II** | | | |
| Original | 2.0000 | 232 | 464.0 |
| A | 0.3090 | 402 | 124.0 |
| B | 0.0676 | 334 | 22.6 |
| C | 0.4064 | 234 | 95.0 |
| D | 0.6446 | 67 | 43.0 |
| Total | 1.3276 | | 284.6 |
| Yield | 66.3% | | 60.8% |
| **Experiment III** | | | |
| Original | 1.0000 | 232 | 232.0 |
| A | 0.0184 | 1248 | 25.0 |
| B | 0.0383 | 2310 | 88.5 |
| C | 0.2987 | 148 | 44.2 |
| D | 0.2635 | Inactive | ------ |
| E | 0.2188 | Inactive | ------ |
| Total | 0.8377 | | 157.7 |
| Yield | 83.77% | | 68.0% |

in studies on the purification of amylase were able to recover 13.5, 19.76, 24.63, 24.95 and 27.81 per cent of the amylase in five separate experiments. This compares to 60.8, 63.2 and 68.0 per cent in the three experiments presented in Table IV.

## Characterization of the Starch Hydrolyzing Enzyme in Soybeans

### Isolation and characterization of the starch degradation products.

The enzyme concentrate prepared from soybeans was known to rapidly hydrolyze gelatinized starch with the formation of products having a high reducing value. In order to characterize the enzyme, a study of these products and of the rate at which they were formed was made. The digestion rate was followed by determination of the reducing power, relative viscosity and iodine color as the reaction proceeded. Figure III presents the reducing value-time curves and the relative viscosity-time curves for two typical digestions.

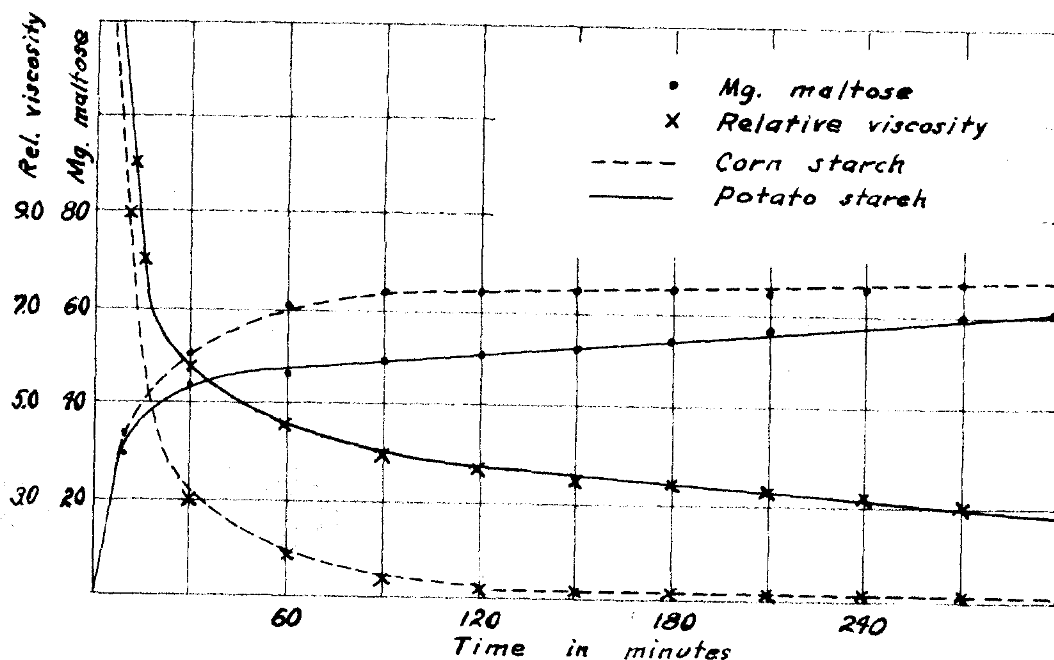



Figure III. Reducing value-time curves and relative viscosity-time curves for the digestion of corn and potato starch with soybean amylase.

A large excess of enzyme was present at all times during the reactions. These curves are typical for the digestion of starches by β-amylase (for similar curves for the digestion of various starches by β-amylase preparations see Klinkenberg (43), Hanes (44) and Freeman and Hopkins (45).

The isolation of the products was accomplished by alcohol fractionation of the digestion mixture. The dextrin isolated from cornstarch by precipitation with 60% alcohol was obtained in yields of about 30%, was soluble in boiling water to give a cloudy solution, and with small amounts of iodine gave a deep blue color which changed to a reddish-purple on increasing the iodine concentration. The value for $[\alpha]_D^{25°}$ in 2% NaOH varied slightly with the average value being about 156°. The alcohol solution from the precipitation of the dextrin was evaporated to a thick syrup and poured into sufficient absolute alcohol to make the final alcohol concentration about 85%. The precipitate which formed was dehydrated with absolute alcohol to give a fine white granular mass (Fraction A). The alcoholic filtrate was placed in an ice-box at 5° C. for two days. The small amount of precipitate which had formed was collected by centrifugation and the centrifugate returned to the ice-box. After standing 22 days at 5° C. considerable precipitate had formed as a thin, hard layer on the bottom and sides of the flask. This material was collected, washed twice with absolute alcohol, twice with anhydrous ether and

dried under reduced pressure over $CaCl_2$ (Fraction B). Both fraction A and B were sweet tasting solids, very soluble in water and were strongly reducing against Fehling's solution. Fraction A and B were acetylated according to the method of Liebermann (46). The results were as follows:

Melting point of known maltose octaacetate-----159-160°C.
Melting point of acetate of Fraction A---------158-160°C.
Melting point of acetate of Fraction B---------158-160°C.
Mixed melting point of A with known sample-----158-160°C.
Mixed melting point of B with known sample-----158-160°C.
Melting point of maltose octaacetate (47, 48)--159-160°C.

The osazones of these fractions were also prepared. The precipitation from the reaction solution and the crystal structures of the osazones were identical with known samples of maltosazone. After recrystallization, the melting point of the osazone of fraction A was 155-157° C. and for fraction B 188-190° C. The melting point of the osazone of fraction A checks with the melting point of fraction D1 reported by Brown and Morris (49) in their studies of the osazone of "isomaltose". Therefore fraction A must be contaminated by a small amount of the dextrin which causes the production of the low melting osazones of maltose. Sample B, on the other hand, is much more nearly pure maltose. The isolation of the maltose octaacetate, however, definitely establishes the presence of maltose in the digestion products formed from starch

by the action of soybean amylase, even though the osazones from these same fractions melt at a temperature below that of pure maltosazone.

Mutarotation of degradation products.

Kuhn (50) proposed the classification of the amylases into the α- and β-types according to the negative or positive mutarotation of the degradation products formed in the early stages of the hydrolysis. Ohlsson (51) then observed that the isolated components of malt amylase gave starch degradation products of opposite mutarotation, the dextrinogenic amylase giving products which mutarotate downward and the saccharogenic amylase giving products which mutarotate upward.

The substrate for the mutarotation studies of soybean amylase was prepared according to the directions given by Kuhn (50). A 2% solution of the substrate was prepared by adding the required amount of the solid material to boiling water, stirring until dissolved, filtering to remove any insoluble material, cooling to room temperature and making to the required volume. The solution so prepared was quite clear, offering no special difficulties in the rotation measurements. The digestion mixture was maintained at 26-28° C. during the digestion. Before adding the amylase the rotation of the substrate was checked by removing two 20 cc. portions, one portion being added to exactly 5.0 cc. of 2 N $Na_2CO_3$. The samples were placed in two decimeter polarimeter tubes, the

rotation of the untreated sample being run immediately while the other sample was allowed to mutarotate for exactly 30 minutes before reading. The solid enzyme was then added (60 mg. of a dry concentrate with saccharogenic power of 219), this amount being slightly greater than the theoretical amount necessary to completely hydrolyze the substrate, assuming that the substrate is digested at as rapid a rate as the same quantity of starch under identical conditions. The digestion was allowed to proceed at room temperature (25-26° C.). Immediately after the addition of the enzyme a polarimeter tube was filled with the digestion mixture and was then used as the control throughout the entire digestion period. The rotation of the control was observed as rapidly as possible and immediately after, a sample of 20 cc. of the digestion mixture was added to 5.0 cc. of 2 N $Na_2CO_3$. The latter sample was allowed to mutarotate for 30 minutes and the rotation then observed. By comparing the rotation of the control and the rotation of the sample to which $Na_2CO_3$ had been added the mutarotation was obtained. The rotation of the samples mutarotated by the use of $Na_2CO_3$ were corrected for the dilution. Table V presents the comparative values obtained by Ohlsson (51) for the dextrinogenic and saccharogenic amylase of wheat as well as that obtained for soybean amylase. These data characterize the soybean amylase as the saccharogenic or β-type.

TABLE V

Mutarotation of products of the two malt amylases and soybean amylase. Data for malt amylase are taken from Ohlsson (51).

| Malt amylase | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Saccharogenic component | | | | Dextrinogenic component | | | | Soybean amylase | | | |
| | Rotation | | | | Rotation | | | | Rotation | | |
| Time min. | Without $Na_2CO_3$ | With $Na_2CO_3$ | Muta-rotation | Time min. | Without $Na_2CO_3$ | With $Na_2CO_3$ | Muta-rotation | Time min. | Without $Na_2CO_3$ | With $Na_2CO_3$ | Muta-rotation |
| 8 | 2.63 | 2.68 | +0.05 | 5 | 3.46 | 3.42 | -0.04 | 10 | 6.80 | 6.90 | +0.10 |
| 25 | 2.38 | 2.46 | +0.08 | 15 | 3.47 | 3.35 | -0.12 | 20 | 6.58 | 6.70 | +0.12 |
| 40 | 2.26 | 2.37 | +0.11 | 30 | 3.43 | 3.27 | -0.16 | 31 | 6.34 | 6.53 | +0.19 |
| 90 | 2.19 | 2.27 | +0.08 | 45 | 3.39 | 3.25 | -0.14 | 60 | 6.09 | 6.35 | +0.26 |
| 120 | 2.20 | 2.27 | +0.07 | 90 | 3.33 | 3.22 | -0.11 | 80 | 6.01 | 6.18 | +0.17 |
| 300 | 2.25 | 2.25 | 0.00 | 210 | 3.21 | 3.18 | -0.03 | 120 | 6.01 | 6.10 | +0.09 |

Characterization by the diffusion technique of Wijsman.

Klinkenberg (52) reported the confirmation of the observations of Wijsman, which were obscurely published in 1890 (53). Wijsman demonstrated that the saccharogenic amylase diffuses more rapidly through a stiff gelatin gel than does the dextrinogenic amylase. Giri (54) also has employed this technique in an investigation of the pancreatic and *Aspergillus* amylase, modifying the procedure to the extent of substituting agar for the gelatin. A 0.5% solution of soluble starch in 1% agar gel was prepared and allowed to solidify in petri dishes. Two amylase suspensions were prepared in approximately equivalent concentrations, and one drop of each placed on an agar-starch plate. The dishes were covered and placed in an ice-box. At the end of 24 hours the plates were flooded with a solution of 0.01 N $I_2$ in KI and then rinsed with distilled water to remove excess $I_2$ and KI. The amylase preparations tested were a solution of a germinated wheat concentrate ($\alpha + \beta$) and a soybean amylase concentrate. The wheat concentrate gave a large colorless area surrounded by a mauve-staining ring. This characterizes mixtures of α- and β-amylase. The α- and β-amylase act together to give the colorless centrum, while the β-amylase diffuses more rapidly than the α-amylase and acts alone in the outer zone to give the characteristic mauve-staining products. With the soybean amylase, the mauve-staining area

extended over a large area radiating from the point of application of the enzyme solution. No colorless area was obtained on the β-amylase plate. This reaction toward iodine coloration is typical of β-amylase.

Determination of the dextrinogenic or amyloclastic activity.

Numerous methods based on the color reaction of the starch degradation products with iodine have been utilized in the quantitative measurement of α-amylase activity. Sameo's investigations (55) indicate that the quantitative value of the starch-iodine color reaction may be questioned. Many other investigators have also questioned the accuracy of the various modifications of the original Wohlgemuth method (56) for the quantitative measurement of amylolytic activity. We have, however, applied a modification of the Wohlegemuth method (57) to numerous preparations formed at various stages in the isolation of the soybean amylase concentrates. These samples include aqueous extracts of germinated and ungerminated soybeans, samples precipitated from aqueous extracts by 50% alcohol, concentrates of high β-amylase activity precipitated from aqueous extracts by 65% alcohol and samples of material precipitated at higher alcohol concentrations. The iodine method showed that all of these samples had an amyloclastic power much below that reported by Creighton and Naylor (57) for their preparations containing the least amount of the amyloclastic enzyme. The amyloclastic power is expressed as the milligrams of starch in a 1% solution which are hydrolyzed by one milligram of the enzyme to products giving no blue color with iodine. On this basis no soybean preparation had an amyloclastic power greater than 10. This

can be compared to the wheat amylase concentrate of Creighton and Naylor (57) with an amyloclastic power of 830, the oat amylase preparation of Naylor and Dawson (38) with a power of 833, and the purified malt amylase concentrates of Sherman and Schlesinger (58) with amyloclastic powers varying from 500 to 1670. These data are further indications that the amylase of soybeans is entirely of the β-type.

Viscosity changes during digestion.

The digestion of various starches by soybean amylase also has been followed by viscometric methods. The starch substrates were prepared by suspending sufficient starch to make a 2% solution in cold water and pouring this suspension into boiling water. Boiling was continued for ten minutes, the solution was cooled, then buffered at pH 5.0 with phosphate buffers, mixed thoroughly and placed in the water bath maintained at 40° C. After reaching the exact temperature of the water bath two 250 cc. portions were transferred to Erlenmeyer flasks. To one of the flasks 20 cc. of the enzyme solution, containing 40 mg. of a concentrate with saccharogenic activity of 1000 in 100 cc. of solution, was added and thoroughly mixed. An equal quantity of distilled water was added to the other flask to serve as a control on the action of the buffer. At regular intervals the viscosity was measured in a water-jacketed pipette maintained at the same temperature as the digestion mixture. The time of outflow required by the digestion mixture was then compared to the outflow time of an equal volume of water at the same temperature. The values obtained for corn, potato and treated potato starch are presented in Figure IV. The curves so obtained show that the liquefaction of the various starches proceeds at a rapid rate for the first 30 minutes of the digestion; at the same time the typical starch-iodine color

persists for 19 hours in the corn starch and up to 48 hours for the potato starch. The marked liquefaction as measured by the viscosity tends to indicate that a specific liquefying enzyme was present in the enzyme concentrates used in the digestions. On the other hand, the persistence of the starch-iodine color in the degradation products shows that the starch has not been broken down into the smaller non-coloring molecules which are typical of the action of the liquefying amylase.

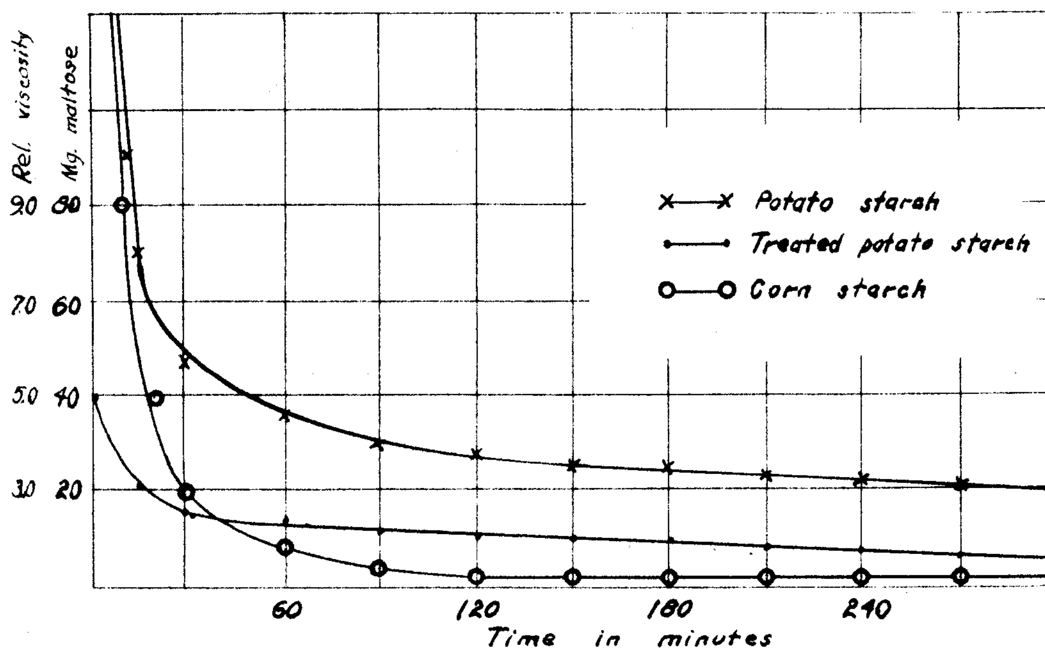



Figure IV. Viscosity-time curves for the digestion of various starches by soybean amylase.

## Deterioration of Soybean β-Amylase

Several amylase preparations were combined to give 16 grams of a standard concentrate for this series of studies. On the date of combination the saccharogenic activity of the composite was 586. The material was stored at 5° C. in small screw top vials each containing about two grams of the enzyme concentrate. The small vials were then placed in a larger sealed container to reduce the possibility of breakage. After standing for five months the saccharogenic activity was 600. After two years' storage the saccharogenic activity of this concentrate remained at 600. These values show that the amylase in the dry crude concentrates is relatively stable at low temperatures.

Even at higher temperatures the enzyme is quite stable. Heating the ground whole soybeans for 10 days at 100° C. did not completely inactivate the amylase. However, soybeans which were roasted commercially for sale as a confection contained no traces of an active amylase. In another experiment a portion of the soybean amylase composite was heated for 30 minutes at 200° C. This treatment completely destroyed the amylase. This inactivation at higher temperatures appears to be due to coagulation of the accompanying proteins. A similar explanation has been suggested by Oparin and Manskaja (59) and Oparin, Manskaja and Magaram (60) for the inactiva-

tion of malt amylase when heated in solution

The combined effect of organic solvent and heat was also investigated in two cases. A sample of soybeans which had been extracted in a continuous extractor on a pilot plant scale using trichloroethylene as a solvent and the excess solvent removed by heating at 100-130° C. for 60 minutes was tested for amylolytic activity. About 70% of the activity was destroyed. Another sample of soybeans, extracted commercially with hexane, had been heated at 95° C. for 20 minutes to remove most of the solvent and then with superheated steam to remove the last traces of the hexane. Tests on this material showed that the amylase was completely destroyed.

In the Ohlsson method (61) for the preparation of α-amylase the β-amylase is destroyed by heating an aqueous solution at pH 6-7 for 10 minutes at 70° C. That the β-amylase of soybeans is not destroyed at this temperature in the presence of starch is shown by the following experiment. Ten grams of corn starch and 100 cc. of water was heated to 78° C. in a water bath. A warm-water extract of the desired quantity of ground soybeans was then added to the heavy starch paste, mixed thoroughly and the digestion allowed to proceed for thirty minutes at 76-78° C. At the end of the digestion the mixture was diluted with water, made to volume and an aliquot used for the reducing equivalent determination. Table VI shows the results of three experiments using different

quantities of soybeans in the extraction.

TABLE VI

The maltose equivalent of the products obtained by the digestion of corn starch with soybean amylase at 76-78° C.

| Sample No. | Starch used (gms.) | Soybeans used (gms.) | Maltose equiv. (gms.) | % Theoretical maltose |
|---|---|---|---|---|
| I | 10 | 0.160 | 2.28 | 22.8 |
| II | 10 | 0.32 | 3.33 | 33.3 |
| III | 10 | 0.48 | 3.40 | 34.0 |

It is evident that the β-amylase of soybeans, when exposed to temperature generally regarded as destructive to β-amylase, retains a major portion of its activity. For example, in Sample I the 22.8% maltose equivalent represents over 70% of the maltose equivalent obtainable if the weight of beans used in the extraction were extracted with a large excess of water and allowed to act on a gelatinized starch under ideal conditions. It should be explained that this experiment was designed for purposes other than a study of the effect of temperature, but since it so definitely shows the resistance of the enzyme to heat-inactivation the data have been presented.

Numerous reports in the literature show the marked inactivation of aqueous solutions of amylase held at or slightly above room temperature. For example, Sherman and Tanberg (63)

present data which show that solutions of the amylase of *Aspergillus oryzae*, obtained as a commercial takadiastase preparation of low activity, is not markedly inactivated when kept at 32° C. for several days. A laboratory preparation which was several times as active lost less than 10% of its activity in 17 days at ice temperature. Sherman and Schlesinger (63) report that in all cases aqueous solutions of pancreatic amylase lost all of its activity within 24 hours. One preparation with saccharogenic power of 8700 lost 45.5% of its activity by standing in pure water for 20 minutes. In further studies (64), the same authors substantiated the rapid deterioration of pancreatic amylase in aqueous solutions. However, concentrates of very low activity retained about 50% of their activity after 72 hours. In an extension of the studies to malt amylase little or no deterioration was observed in aqueous solutions maintained at room temperature for 13 days. One aqueous solution of a malt amylase preparation had as great an activity after 27 days at room temperature as at the beginning, while two other samples lost 33% and 53% of their activity in 27 days. Furthermore, the greater the dilution of the amylase solution the more rapidly the enzyme deteriorated. Exposure to 50% alcohol also increased the rate of deterioration. The increase in rate of deterioration with increased dilution is also reported by Ono (65) in studies of yeast amylase. In aqueous

solution yeast amylase lost approximately 50% of its activity when heated at 37-38° C. for one hour. In similar studies, Yamagishi (66) reports that the detrinizing amylase of rice loses one-half of its activity when kept at 71.5° C. for one hour, while the saccharogenic amylase lost 50% of its activity when kept at 66.5° C. for one hour. This indicates the greater sensitivity of the saccharogenic enzyme to temperature. In a study of maize amylase, Patwardhan (67) found that the enzyme is not completely destroyed by heating at 70° C. as is the amylase of barley and cholam. This observation of Patwardhan is in agreement with the conclusions of Bulbrook (68) that corn amylase is of the α-type and is more thermostabile than the β-amylase. The destruction of β-amylase at 70° C. is substantiated and utilized in the method of Ohlsson (61) for the preparation of α-amylase concentrates by differential inactivation of the β-amylase. Blish, Sandstedt and Mecham (40) discuss the combined effect of alcohol and temperature on wheat amylase. Their studies show that deterioration of amylase in alcohol solution occurs at a much greater rate at 40° C. than at 0° C. For example, one preparation which was allowed to stand in contact with alcohol at 0° C. for 24 hours converted 51% of a starch while an identical sample standing at 40° C. under the same conditions converted only 8% of the same starch.

From our previous work there is a definite indication

that soybean β-amylase is more resistant to deterioration and heat inactivation than is usually the case with amylase concentrates. To determine the rate of deterioration of soybean β-amylase concentrates six samples were dissolved in distilled water and allowed to stand at room temperature in loosely stoppered volumetric flasks. No effort was made to prevent contamination from the air. Figure V presents results of this study.

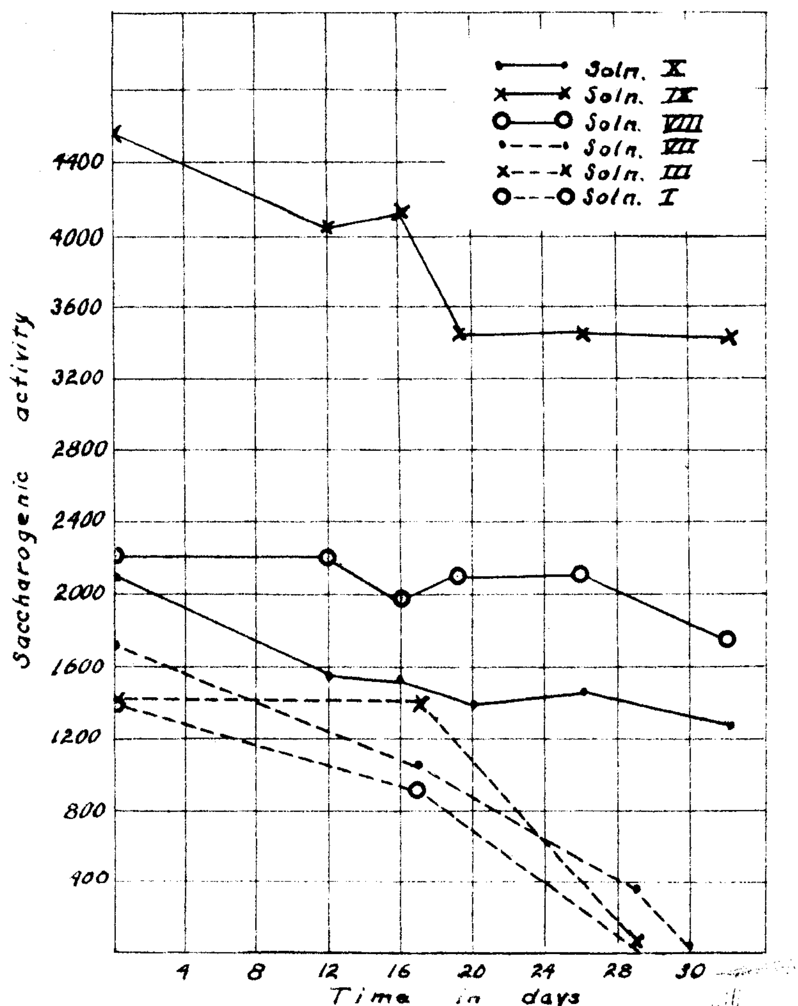



Figure V. Deterioration of soybean β-amylase in aqueous solutions at room temperature (23-28° C.). Concentration of solutions was: I and III - 40 mg. in 50 cc.; VII - 9.2 mg. in 10 cc.; VIII - 16.4 mg. in 50 cc.; IX - 16.3 mg. in 50 cc.; X - 15.6 mg. in 50 cc.

The results vary markedly from sample to sample. Enzyme solution I was completely inactive after 29 days, solution III was slightly active at this time, while VII still retained 19.5% of the original activity. Sample VII had only slight activity after 32 days. This marked deterioration was not observed in samples VIII, IX and X. These latter concentrates were some of the most highly purified concentrates and were in greater dilution than the other three solutions which became inactive. From observations of other investigators the most highly purified samples and the samples in greatest dilution deteriorate most rapidly. This fact was not observed with the soybean β-amylase concentrates. For example, samples VIII, IX and X retained 76%, 87% and 62% of their respective activities after standing 34 days at room temperature. The reason for the greater rate of deterioration of samples I, II and VII as compared to VIII, IX and X is not definitely known. It should be mentioned, however, that the first three samples were prepared about two weeks previous to the other preparations and had been stored in an ice box as dry concentrates. This storage has no observed effect on the enzymic activity of freshly prepared solutions of these concentrates. Whether this storage could effect the enzyme complex in some manner to make it more susceptible to deterioration is highly problematical. A more satisfactory explanation probably lies in variations of the preparative

technique, the more highly purified concentrates being freed of substances which are more subject to bacterial decomposition, this bacterial action producing substances which inactivate the enzyme. An interesting observation in this respect is that after 8-10 days a marked putrefactive odor existed in all solutions except Sample IX. At no stage was any marked odor observed in this solution. Furthermore, considerable growth of organisms was observed in solutions I, III and VII. A slight but distinct cloudiness was observed in solutions VIII and X, while solution IX remained water clear throughout the experiment. There are thus some indications that the removal of certain impurities may reduce bacterial growth and thus reduce the rate of deterioration.

The argument may be presented at this stage that the enzyme solutions should be protected from bacterial action by the addition of some preservative, such as thymol or toluene. The tenability of such an argument was realized at the time the experiment was undertaken. However, because preservatives may in themselves have a marked action on the enzyme it was deemed more expeditious to eliminate the preservatives and subject the enzyme to the action of pure water. Since the amylase concentrates were known to contain various micro-organisms no exact effort was made to prevent contamination from the air.

## Discussion

The determination of the varietal differences of the amylase content of soybeans suggests that such a determination might be of some value to the plant breeder. Whether the amylase content can be correlated with a desired characteristic in the soybean lies beyond the scope of this investigation. However, if the amylase content can be correlated with the specific characteristic desired by the plant breeder, then the determination of the amylase content could serve as a quantitative measurement of that characteristic. With this possibility in mind, it is necessary to note that the method utilized for the determination of amylase content in seeds is subject to large errors. The major difficulty lies in inadequate means for the quantitative extraction of the enzyme from the bean. This obstacle must be removed from the method before small variations in the amylase content can be accurately measured.

The lack of increase in total amylase content by germination of the soybeans is very unusual. All other seeds show a marked increase in the total available amylase during germination. The ungerminated seeds have a larger proportion of β-amylase than α-amylase. After germination the α-amylase is predominant. Thus the increase in total amylase activity must be due to the formation or to the liberation of α-amylase during germination, the α-amylase acting in conjunc-

tion with the original $\beta$-amylase. For some reason the soybean lacks this ability to produce or liberate $\alpha$-amylase during the process of germination.

The preparation of highly active $\beta$-amylase concentrates was greatly assisted by the fact that soybeans contain only $\beta$-amylase. Methods for the differential inactivation or selective adsorption of $\alpha$-amylase were unnecessary. The problem was thus resolved into the separation of the active $\beta$-amylase from the accompanying impurities. The final procedure as presented, scarcely resembles the original attempts made to concentrate the enzyme. In the first attempts at concentration much trouble resulted from the oil-water emulsions which formed when the soybeans were shaken with water to extract the enzyme. These emulsions were eliminated by first extracting the beans with diethyl ether or hexane, preferably the latter. A study of the effect of these solvents on the enzyme showed that they caused little or no decrease in enzyme content of the beans either by extraction or by inactivation.

Substitution of 50% alcohol for water in the extraction eliminated one precipitation and caused an increase in the yield of enzyme. The enzyme was then precipitated from the alcoholic extract by the addition of absolute alcohol. The precipitates so obtained were gelatinous materials. Collection of these precipitates by filtration was a tedious, nearly

impossible task, and resulted in products highly contaminated with filter paper. Only by adopting a technique based on centrifugation in a Sharple's supercentrifuge was it possible to obtain uncontaminated products in satisfactory yields.

Little or no success was observed in attempts to further purify the crude concentrates when alcohol was used as the precipitating agent. The deleterious effect of the alcohol persisted even at low temperatures. By the substitution of acetone for alcohol and by working at as low temperatures as convenient considerable purification was obtained. Even then the recovery of active enzyme and the degree of purification were not as great as desired.

Improvement in the concentration procedure was observed when the drying of concentrates was eliminated whenever possible. Purification was further increased by the incorporation of dialysis into the procedure. This process removed considerable quantities of organic impurities as well as the inorganic salts.

The final procedure yielded pure white products of high saccharogenic activity. The most highly active concentrate was capable of producing 4580 times its weight of maltose in 30 minutes at 40° C. When allowed to act on soluble starch, 0.033 milligrams of this concentrate converted 10 grams of the gelatinized starch to maltose (60%) and limit dextrin (40%) in 36 hours at 40° C. This corresponds to a saccharo-

genic power of 181,000.

The application of the concentration procedure to β-amylase concentrates of low activity gave amylase concentrates of considerably higher activity. The recovery of the amylase in three experiments was 63.2%, 60.8% and 68.0%. By reworking such low-activity concentrates the amylase concentration has been increased as much as ten-fold in one operation. Since a large percentage of the total amylase extracted from the soybeans remains in the low-activity concentrates a method for their further concentration is of much value.

The requirements for classification of an amylase as being of the α- or β-type are not rigidly defined. As a result the classification of soybean amylase as of the β-type rests on a series of measurements and observations which are considered indicative of the amylase type. Characterization of the enzyme by the diffusion method of Wijsman, determination of the amyloclastic power of the concentrates, the mutarotation of the starch degradation products, the isolation of maltose and a residual dextrin as the starch degradation products, the viscosity changes of the starch substrate during digestion and the iodine-starch reaction of the degradation products characterize the starch-hydrolyzing enzyme of soybeans as β-amylase. The only evidence which points toward the presence of α-amylase in the soybean concentrates is the marked ability of the enzyme to reduce the relative viscosity

of the starch pastes in the early stages of the hydrolysis. However, since soybean amylase is one of the most powerful sugar-forming enzymes, this sugar coming from the hydrolysis of the starch substrate, it is not disturbing to observe a marked decrease in the relative viscosity of the substrate during digestion with soybean β-amylase.

A quantitative method for the determination of β-amylase in the presence of α-amylase would have aided and simplified the characterization problem. Such a method has been a vital need of the chemist studying the amylases. Very recently a method has been devised for the accurate determination of the relative roles of α- and β-amylase in saccharification (69). The author regrets that time prevented the application of this quantitative procedure to the amylase of soybeans.

From the experiments on rates of deterioration of soybean amylase there is a definite indication that the more highly purified β-amylase concentrates have a marked stability, even in the presence of bacterial action. This leads to the conclusion that the chemical nature of the soybean β-amylase molecule is such as to resist the action of the micro-organisms from the air and in the amylase concentrates. Since putrefaction was observed in five of the six solutions studied, yet did not destroy the amylase in the two most highly purified concentrates, and since the solution in which no putrefaction was observed contained the greatest concentra-

tion of amylase and was exposed to identical possibilities of contamination by the micro-organisms which brought about putrefaction of protein materials in the other solutions, there exists some question as to whether this most highly purified amylase concentrate contains any large proportion of protein material. The limit of time and material prevented further investigation of the most highly purified concentrates. This preliminary work, however, suggests most interesting possibilities for future studies.

## Summary

1. The amylase content of soybeans differs from variety to variety. A difference in the amylase content of the same variety from year to year is also indicated.
2. The total amylase in soybeans decreases slightly during germination.
3. A method is given for the preparation of amylase concentrates from soybeans. A maximum concentration of approximately 225-fold has been obtained. The most highly purified concentrate was capable of producing 4580 times its weight of maltose in 30 minutes at 40° C.
4. Isolation and characterization of the starch degradation products, mutarotation of these products, characterization of the enzyme by the diffusion method of Wijsman, determination of the dextrinogenic or amyloclastic activity, viscosity changes during digestion and the iodine-color reaction of the products characterize the starch-hydrolyzing enzyme of soybeans as β-amylase.
5. The purified soybean β-amylase concentrates were remarkably stable in aqueous solutions at room temperature. Increasing the purity increased the stability of the aqueous solution. A concentrate with a saccharogenic activity of 4580 retained 87% of its activity after 34 days in an aqueous solution maintained at 24-28° C.

# PART II

## THE DEGRADATION PRODUCTS FORMED FROM STARCH BY THE ACTION OF ALPHA- AND BETA-AMYLASE

### Review of Literature

The three most important achievements of starch-amylase studies in the last half-century are:

1. The general substantiation of the fact that diastatic ferments consist of mixtures of separate enzymes which induce distinct types of starch breakdown.

2. The isolation and characterization of the Schardinger dextrins.

3. The isolation of the phosphorylase systems which catalyze the breakdown of starch to glucose-1-phosphate and the synthesis of starch from the same compound.

The following literature survey is concerned only with the first of these three important achievements. The earliest demonstration of the separation of malt amylase into two components is given in the work of Wijsman (70). The obscure publication of these studies prevented general recognition until they were reviewed and confirmed by Klinkenberg (52). In the meantime Ohlsson's demonstration of the differential inactivation of the two component amylases (61) definitely established the existence of the two types of amylase. How-

ever, even as late as 1939 certain investigators (71, 72) have regarded the existence of two distinct amylases as unproven or even doubtful. The objections of these authors are based on the possibility that the relatively drastic treatments employed in selective inactivation might substantially alter a single enzyme to give the results observed. Accordingly, by a variety of such treatments, there might be derived several forms of a single enzyme exhibiting distinctive properties.

In general two types of amylase are accepted. They are most commonly referred to as dextrinogenic or α-amylase and saccharogenic or β-amylase.

The three major products formed by the action of β-amylase on starch are as follows:

1. Alcohol soluble sugars (primarily maltose).

2. A water-insoluble material which flocculates from the digestion mixture and which, after complete digestion, can be recovered by centrifugation of the resulting liquors.

3. A water-soluble dextrin which is precipitated from the digestion mixture by the addition of alcohol to a concentration of 50-60% by volume.

The identity of maltose as the major product of low molecular weight has been substantiated by numerous investigators. Baker (73) found that the reducing products soluble in 60% alcohol agreed in reducing power and optical rotation with

maltose and that crystalline maltose could be obtained in good yield from these liquors. These observations have been confirmed by Syniewski (74), Hanes (44), Freeman and Hopkins (45), and Blom, Bak and Braae (75). Baker and Day (76) and Harding (77) have suggested using β-amylase for the preparation of pure maltose free of dextrins. Hanes (44), using a differential yeast fermentation method, concludes that, except in cases of long hydrolysis, maltose is the sole product of low molecular weight. In the long digestions traces of glucose were found. Freeman and Hopkins (45) examined starch digestions in the early stages of conversion and found that the reducing power/rotation ratio of the alcohol-soluble fraction agreed with that of maltose, and that the rates of fermentation by brewer's yeast and the total $CO_2$ evolved were not significantly different from the values found for pure maltose.

The method of attack by the enzyme is indicated by a number of lines of evidence which point to the conclusion that beta-amylase causes successive terminal disaccharide fragments (maltose) to be split off from one end of the molecular chain structure of the starch substrate. The most important evidence in support of this is the fact that maltose constitutes almost the sole product of low molecular weight produced by the action of β-amylase on starch. Only on the basis of an orderly end-wise attack is it possible to explain the production of maltose as the one low molecular weight product;

on any alternative basis, the production of other short chain reducing fragments would seem inevitable.

Ohlsson (61), on the basis of his observations on the osmotic behavior of the products formed when soluble starch was degraded by β-amylase, advanced the view that by the action of the enzyme, successive maltose molecules are detached from each starch molecule in such a manner as to leave at each stage a single residual molecule of dextrin. At no stage in the particular experiment was there observed any increase in the total number of undialyzable particles. This can be explained only by the assumption that small dialyzable fragments were being split from the end of the starch molecule.

The question then arises as to which end of the molecule the β-amylase attacks. The existing evidence suggests that the degradation by β-amylase must proceed from the non-aldehydic end of the starch chain. The fact that the action of β-amylase on starch produces normally non-reducing dextrins suggests that the liberation of maltose proceeds without disturbing the configurational features which render non-reactive the aldehydic end-group of starch itself. Further evidence is given by the work of Brown and Millar (78). They were able to prepare a maltodextrinic acid by the oxidation of a maltodextrin with mercuric oxide and barium hydroxide. The maltodextrin had been isolated from a kiln-dried-malt digestion of potato starch. Further digestion of this malto-

dextrinic acid with malt amylase gave a 40% yield of maltose and 60% of a smaller maltodextrinic acid. This digestion by the amylase indicates that the reducing group has no effect on the action of the enzyme. Therefore, it is evident that the action of the amylase must occur at the other end of the starch and dextrin molecules, that is the non-aldehydic end. Experiments were also conducted by Myrbäck (79) to determine the effect of oxidizing the reducing end-groups to carboxyl groups on the splitting of starches by α- and β-amylase. The data indicate that the reducing groups are of no significance for the hydrolysis of the starch. More recently, Örtenblad and Myrbäck (80) have found that all amylases studied by them are independent of the existence of reducing groups in the substrate, since starch and dextrins when oxidized with hypoiodite remain unaltered in their behavior towards the amylases.

The production of glucose as a primary product from the digestion of starch by amylase only recently has been reaffirmed by the work of Somogyi (81). He concluded from his experimental evidence that, besides non-fermenting reducing dextrins and maltose, glucose is one of the normal products of diastatic digestions. By using a yeast fermentation method described earlier by the same author (82), the quantities of the three constituent groups were determined separately. In one portion of the reaction mixture the total reducing

power was determined (a); another portion was fermented with washed brewer's yeast at an alkaline reaction (pH 8.0 to 8.4) for 30 minutes before determination of the reducing power (b); a third portion was fermented at unmodified reaction for 2 hours and the residual reduction (c) was then determined. The following calculations were made: a - b = copper reduced by glucose; b - c = copper reduced by maltose; c = copper reduced by non-fermentable dextrins. Using this technique on the degradation products formed by the diastase of human urine acting on an acid-washed corn starch, Somogyi (81) concludes that glucose is a normal product of diastatic reactions. That the production of glucose was not caused by the action of maltase on the maltose formed in the diastatic reaction was shown by the observation that the urine amylase preparation acting on pure maltose caused no increase in reducing power. This production of glucose as a primary product of amylolytic action is in accord with the earlier contentions of Sherman and Walker (83), Pringsheim and Schapiro (84), Gottschalk (85) and Lohmann (86).

Somogyi's data indicate that the same diastase preparation, depending upon experimental conditions, may react with the same substrate to form (1) no other sugar than non-fermentable polysaccharides, or (2) non-fermentable reducing dextrins plus maltose, where the latter appears as the end-product of the reaction, or (3), in addition to reducing dex-

trins and maltose, appreciable amounts of glucose. The experimental conditions which are varied to give these results are concentration of the enzyme, concentration of the substrate and the reaction time. The effect of the increased dilution seems to be a decreased rate of enzyme-substrate combination with a resulting decrease in rate of hydrolysis. The fact that diastase preferertially combines with polysaccharides of colloidal structure has been reported by Blitz (87). This serves to explain the fact that glucose is not produced by diastase so long as substantial amounts of starch and erythrodextrins are present in the reaction mixture. The enzyme molecules become available for reactions with dextrins of smaller molecular size only after far-reaching degradation of the colloidal particles has been effected. This is in accord with the observation that the formation of appreciable quantities of glucose occurs only after long digestion or in solutions having a relatively high concentration of the enzyme.

The appearance of a flocculent, water-insoluble precipitate in the early stages of the digestion of starch by β-amylase was first reported by Baker (73). The flocculent material was removed from the digestion liquors by filtration and discarded, the investigator believing that this material was a "reversal" product of the original starch. Fernbach and Wolff (88) observed this early formation of a precipitate

and ascribed it to the action of a specific enzyme which they called "amylocoagulase". They state that the liquefying amylase must be present for this coagulation to take place. Sallinger (89) suggested that the phenomenon might be due to the digestion of the smaller starch granules while the larger ones were precipitated. Ling and Nanji (90) also have described the appearance of a water insoluble precipitate early in the enzymic digestion of starch. That this flocculation interferes with the malt method for the determination of starch has been reported by Chrzaszcz (91).

The first attempt to isolate and characterize this material was reported by Sherman and Punnet (92). They found no significant differences using commercial takadiastase or malt extract, the yield of material varying only from 1.13-1.44%. Clayson and Schruyver (93) and Schruyver and Thomas (94) separated the flocculent material from takadiastase digestions and measured its optical activity. They referred to the insoluble material as "hemicellulose". Malloch (95) and Hermano and Rask (96) also noted the appearance of the so-called "hemicellulose" in enzyme digestions of different starches.

Taylor and co-workers (97, 98, 99) present data which show that the fatty-acid content of the flocculent material is higher than in the original starch, thus suggesting that the fatty-acid may be responsible for the flocculation.

However, this same flocculation occurs when potato starch, which has a very small fatty-acid content, is used as a substrate (100, 101). It thus seems that the fatty-acid content fails to explain the reason for the formation of this water-insoluble precipitate. The formation of this material must in some way be dependent on the nature of the starch molecule.

A more complete study of this flocculent precipitate has been reported by Martin (10) and Martin, Naylor and Hixon (12). Different species of starch were digested under standardized conditions by a soybean β-amylase preparation. The flocculent material was collected from the digestion mixture by centrifugation, resuspended in water, dialyzed until reprecipitated, and the wash water decanted. This process was repeated two or three times until the liquid from the anode chamber gave no test for phosphate. The material was then dehydrated by trituration with absolute alcohol, collected by filtration, washed with ether to facilitate drying and placed in a vacuum desiccator for two or three days. When completely dry the material was ground to a white powder in an agate mortar. The precipitates obtained from corn, rice, wheat, potato and tapioca starch were characterized as to further hydrolysis by β-amylase, by phosphorus and fatty-acid content and by reducing power. Table VII presents a summary of the data of these characterizations.

TABLE VII

Properties of the water insoluble dextrin formed by the action of β-amylase on starches[1].

| Substance | Per cent yield | Enzymatic digestion % maltose | Reducing Rm[2] | power Rcu[3] | Per cent P | Per cent fat |
|---|---|---|---|---|---|---|
| Corn starch | ----- | 58.5 | 1.68 | 5.5 | 0.015 | 0.66 |
| Dextrin | 1.64 | 14.1 | 2.73 | 9.6 | 0.031 | 1.31 |
| Rice starch | ----- | 65.7 | 3.78 | 7.8 | 0.035 | 0.62 |
| Dextrin | 1.92 | 30.0 | 4.83 | 11.2 | 0.041 | 0.95 |
| Wheat starch | ----- | 60.0 | 2.52 | 10.4 | 0.051 | 0.57 |
| Dextrin | 1.0 | 23.9 | 4.62 | 11.3 | 0.041 | 0.90 |
| Potato starch | ----- | 59.3 | 1.68 | 4.3 | 0.050 | 0.076 |
| Dextrin | 0.84 | 9.65 | 6.30 | 36.5 | 0.112 | 0.17 |
| Tapioca starch | ----- | 77.2 | 3.36 | 4.3 | 0.010 | 0.174 |
| Dextrin | 0.05 | 6.3 | 5.25 | 19.0 | 0.020 | 0.51 |

1. Data from Martin, Naylor and Hixon (12).
2. On Rm scale maltose equals 100.
3. On Rcu scale maltose equals 2000.

It is interesting to note that in every case the fatty-acid content of the precipitate is higher than the fatty-acid content of the original starch. With the exception of the precipitate from wheat starch the phosphorus content of the dextrin is always higher than the phosphorus content of the original starch. In the case of the wheat starch there is a slight decrease. However, there is no correlation between the fatty-acid and the phosphorus content of the original starches and the yield of dextrinous products derived therefrom. From these studies Martin, Naylor and Hixon conclude that the

phosphorus and fatty-acid groups do not appear to be the agents which block the action of the β-amylase at 60-70% conversion of starch to maltose.

At all times during the hydrolysis of starch by β-amylase, residual starch-like substances can be precipitated by the action of 50-60% alcohol. If the β-amylase is allowed to act for a long period of time (24-48 hours) and the residual dextrin precipitated by 50-60% alcohol the starch-like material can be isolated in yields of 30-40%. At the same time the remaining 60-70% of the original starch can be accounted for as alcohol-soluble reducing sugars, nearly all of which is maltose.

Probably the earliest report of the isolation of the water-soluble dextrin by precipitation was made by Wijsman in 1890 (70). He named the dextrinous material "erythrogranulose". Baker (73) prepared a fraction in about the same manner and named it "α-amylodextrin". This material had a specific rotation slightly lower than that of starch and a reducing power slightly higher. Ling and Nanji (102) isolated a similar α-amylodextrin from the action of alcohol-precipitated barley diastase on amylopectin. These authors emphasized that the preliminary treatment of the β-amylase preparation has a marked influence on the properties of the residual material left after the action on amylopectin is completed. An amylase preparation thoroughly dehydrated in strong

alcohol yields a highly polymerized form of α-amylodextrin ($[\alpha]_d$, +221°), while an untreated or freshly prepared preparation yields a disaggregated form of α-amylodextrin which exhibits a lower specific rotation ($[\alpha]_d$, +193°). The dextrin of lower rotation is considered by Ling and Nanji to represent a depolymerized derivative of the first product. Another residual dextrin is reported by Klinkenberg (43) to have a specific rotation of $[\alpha]_d$, + 194°. The residual dextrin was prepared by using a β-amylase concentrate from ungerminated barley. Freeman and Hopkins(45) prepared residual dextrins with a specific rotation of $[\alpha]_d$, +188° from the digestion of starch by β-malt-amylase or ungerminated barley amylase. All of these residual dextrins are reported as having low reducing power (less than 2% maltose equivalent) as measured against copper.

A variance in the reducing power of the residual dextrins is indicated by the work of Samec (103). The dextrin described in this report had a specific rotation $[\alpha]_d$, +193°, but instead of the usual low reducing power this residual dextrin had a maltose equivalent of 16%.

Haworth, Hirst and Waine (104) were able to isolate from the digestion of soluble potato amylose with barley β-amylase a residual dextrin with a specific rotation of $[\alpha]_d$, +200° in $H_2O$ and $[\alpha]_d$, +144° in 5% NaOH, and a reducing equivalent of 3% maltose as measured against hypoiodite.

Haworth, Hirst, Kitchen and Peat (105), using the β-amylase of wheat, report that the residual dextrin obtained from potato starch has a specific rotation of $[\alpha]_d$, + 167° in $H_2O$ and $[\alpha]_d$, +133° in 5% aqueous NaOH. The residual dextrin of the last two investigations was identical in respect to solubility, color with iodine, reducing power, phosphorus content and to the further action of β-amylase. Nevertheless, the marked difference in optical activity is observed. Moreover, this difference persists in the methylated dextrins. Haworth, Hirst and Waine record for their methylated dextrin $[\alpha]_d$, +222°, whereas Haworth, Hirst, Kitchen and Peat record $[\alpha]_d$, +197.2° (maximum value) for their methylated dextrins.

The production of residual dextrins of lower specific rotation is also indicated by the work of Martin (10). A dextrin precipitated by 55% alcohol from an oat β-amylase digestion of solubilized potato starch has a specific rotation of only $[\alpha]_d$, + 162°.

Haworth, Hirst, Kitchen and Peat (105), Haworth, Hirst and Waine (104) and most of the early investigators report that the residual dextrins which they have obtained from the action of β-amylase are soluble in cold water. On the other hand, Caldwell (13) has used alcohol precipitation to fractionate the residual dextrins from soybean β-amylase action on corn starch into hot-water-soluble and hot-water-insoluble fractions. Even the hot-water-soluble dextrins did not give solutions sufficiently clear to allow measurement of the

optical activity. In 2.5% NaOH the hot-water-insoluble fraction had a specific rotation of $[\alpha]_d$, +126.8° and the hot-water-soluble fraction $[\alpha]_d$, + 154.6°. In addition to these two fractions a cold-water-soluble fraction was obtained with a specific rotation of $[\alpha]_d$, +144° (in 2.5% NaOH). Thus Caldwell demonstrates by direct fractionation that the limit dextrin from the action of soybean β-amylase on corn starch is not a homogeneous product, but that such a dextrin can be fractionated. This investigator was unable to obtain a similar fractionation of the limit dextrin from the action of the same amylase on waxy maize starch.

Caldwell (13) also has demonstrated that these various dextrins are further degraded by soybean β-amylase. After isolation the residual dextrins were resuspended in boiling water, cooled to 40° C. and redigested by soybean β-amylase. The observed increase in reducing power corresponded to from 11.9-15.0% of the theoretical maltose producible from the quantity of corn dextrin used. The waxy maize dextrins were dissolved in water by warming to 80° C. After cooling to 40° C. the soybean amylase was added and the digestion allowed to proceed for 24 hours. The increase in reducing power corresponded to from 10.5-18.0% of the theoretical maltose. Thus, dextrins from soybean β-amylase digestions of corn and waxy maize starch are definitely susceptible to further degradation by β-amylase. This susceptibility was

previously demonstrated by Hopkins, Cope and Green (106) in cases where autoclaving was used to redisperse the limit dextrin (107, p. 198).

The demonstration by Caldwell (13) that these limit dextrins are non-homogeneous and that they are further degraded by the action of fresh soybean β-amylase is contrary to the reports of Haworth, Hirst and Waine (104), Haworth, Hirst, Kitchen and Peat (105) and Hanes (44). The last three papers report that the α-amylodextrin is homogeneous and is not degraded by β-amylase.

Support of Caldwell's (13) contention that the residual dextrins from β-amylase action are heterogeneous is given by the work of Beckmann and Landis (14). By use of the β-amylase from soft wheat they prepared limit dextrins from gelatinized potato starch, soluble potato starch (Lintner) and from dry-ground potato starch and investigated them by means of the ultracentrifuge. The gelatinized potato starch and the solubilized potato starch were found to be quite heterogeneous in nature and with both starches limit dextrins were found which could be resolved into two distinct components. The dry-ground starch, on the other hand, was heterogeneous and so was its limit dextrin.

Investigations dealing with the products formed by the action of α-amylase on starch show great differences in the experimental data presented as well as considerable variance

in the interpretation of this experimental evidence. The modern ideas on the manner of α-amylase action have been summarized by Hanes (107) and Sameo (108). This enzyme presumably is able to split the starch molecule at any point, the nature of the products being dependent primarily upon experimental conditions. When proper amounts of enzyme are present, the curve given by plotting the reducing equivalent against the time elapsed exhibits two distinct stages: an initial phase in which the reducing value of the digestion mixture rises rapidly to a value equivalent to 30-40% maltose, followed by a second prolonged stage of extremely slow increase in reducing power.

The expression of the degree of conversion as per cent maltose is confusing in that it assumes the reducing power to be due entirely to maltose. This assumption is erroneous because Freeman and Hopkins (109) have shown that the major part of the reducing material produced by α-amylase digestions has a higher specific rotation than pure maltose and thus must contain larger carbohydrate molecules. Only a small amount of maltose was isolated from such digestions. Stamberg and Bailey (110) have prepared purified α-amylase concentrates which they have used to digest starches. Data obtained from a yeast-manometric method indicate that α-amylase produced non-fermentable reducing dextrins together with some fermentable reducing sugars. Previously Ohlsson

(51, 61) had demonstrated, from osmotic behavior of the degradation products of α-amylase action on starch, that there was a large increase in the number of non-dialyzable (or slowly dialyzable) particles and that these relatively complex products accounted for the bulk of the reducing power. Blom, Bak and Braae (111) present evidence, based on optical activity and Bertrand reduction methods before and after fermentation, which shows that true maltose accounts for 23% of the total digestion products. This value for true maltose must be accepted as a maximum value since numerous investigators (112, 113, 114, 115) have demonstrated that some of the lower molecular weight dextrins are also fermentable.

The most typical feature of the reaction of α-amylase is the more or less abrupt termination of the initial hydrolysis stage at a reducing value equivalent to 30-40% of the theoretical maltose value (see Hanes (107), p. 205, for several typical rate curves). The maximum limit of degradation by α-amylase appears to be dependent on numerous factors such as source of the α-amylase, concentration of the enzyme with respect to the starch, removal of degradation products, etc. Numerous investigators report various values for the maximum hydrolysis. Using saliva and pancreatic amylase in 18 hour digestions, Vonk and Braak (116) found 80% and 54% maltose equivalent, respectively. Hanes (44) reports maximum hydrolysis as ranging from 50-53% maltose equivalent. Using an α-

amylase concentrate prepared from malt by selective inactivation of the β-amylase, Freeman and Hopkins (109) observed values corresponding to 77-84% conversion. Stamberg and Bailey (110) have prepared α-amylase concentrates from germinated wheat by a combination of Ohlsson's (61) and Klinkenberg's (117) methods. These carefully prepared α-amylase concentrates hydrolyze starch to only about 40% after 12-28 hours digestion. Stamberg and Bailey (110) also prepared β-amylase concentrates from normal wheat. The combined action of these two carefully purified amylases acting together gave a combined conversion of about 75% after 24 hours digestion. This is in agreement with the value for the combined α-amylase and β-amylase reported by Blom, Bak and Braae (75). Most values for maximum reducing value attainable in α-amylase digestions of starch vary from 50 to 80% maltose equivalent, thus they approach or equal the value for the combined action of α- plus β-amylase. Since most of the α-amylase preparations have not been carefully purified it is possible that these preparations are more or less contaminated by β-amylase. Therefore, the more highly purified α-amylase concentrates of Stamberg and Bailey give results which more nearly approach the true value for pure α-amylase.

Hanes states (107, p. 206): "a consideration of the available information suggests that with pure α-maltamylase the termination of the initial rapid phase would occur

at about 28-30 per cent apparent conversion, and that the limit of hydrolysis would not exceed a value of approximately 50 per cent maltose. That this degree of purification is obtainable by the Holmberg method, and for certain malt extracts, by the Ohlsson method, is indicated by unpublished observations of the writer." This statement is also substantiated by the work of Stamberg and Bailey (110) in which germinated-wheat α-amylase was purified by a combination of the methods of Klinkenberg and Ohlsson.

Isolation of the products from the action of α-amylase on starches can be accomplished by fractional precipitation of the digestion products by organic solvents. Many investigators have utilized a yeast fermentation to remove the fermentable sugars previous to precipitation of the dextrins. Because there are many indications (112, 113, 114, 115) that the small dextrin molecules are fermentable by yeast, the use of fermentation methods may involve partial loss of the smaller degradation products.

Ahlborg and Myrbäck (118) have isolated the dextrins from the hydrolysis of corn starch by takadiastase α-amylase. A major portion of the dextrins could be dialyzed through ordinary membranes, thus indicating that they are relatively small molecules. The original dextrin was isolated in 20% yields. Sixty per cent of the dextrin had a molecular weight of 950; the remaining 40% had a molecular weight of 650.

Studies of optical rotatory power under various conditions corroborates the assumption that the dextrins are not homogeneous. These dextrins are also susceptible to further hydrolysis by α-amylase, but at a rate of about 1/1000 that of the original starch. Myrbäck (119) reports several isolations of dextrins with molecular weight of less than 660. One dextrin fraction from a takadiastase digestion of corn starch appeared to contain a pure trisaccharide. Örtenblad and Myrbäck (120) have found that several fractions of the dextrins from a malt-amylase digestion of corn starch give practically the same molecular weight of about 500 and consist principally of trisaccharides. It is of particular interest to note that the molecular weights determined from the reducing value and from diffusion measurements are in good agreement. On the basis of these data they conclude that each dextrin molecule contains a free aldehyde group.

The isolation of a crystalline hexaose from pancreatic amylase digestion of erythrogranulose is reported by Waldschmidt-Leitz and Reichel (121). The dextrin has the formula $C_{36}H_{62}O_{31}$, $[\alpha]_d$, +183°, m. 258-63° (decomposition) and gives no color with iodine. It is hydrolyzed by amylase but not by maltase. The amylase splits the dextrin into 3 molecules of maltose which are then hydrolyzed by maltase into 6 glucose molecules. Mutarotation of the maltose formed is in opposite directions according to whether the cleavage is affected by α- or β-amylase.

## Materials Used

### A. α-amylase

The α-amylase preparation used in this investigation was a concentrate high in α-amylase activity and resistant to inactivation at elevated temperatures. The enzyme was furnished through the courtesy of the Wallerstein Laboratories.

### B. Starches

The corn starch was a commercial pearl starch. The waxy maize starch was furnished by H. D. Kaslow.

## Statement of Problem

It is evident from the literature survey that the major portion of the publications on soybean β-amylase has originated from these laboratories. The preliminary studies were undertaken by Bray and Naylor (37). This was followed by various publications dealing with the enzyme (5, 8, 9, 10, 12, 13). The investigations have included studies on methods of preparation of soybean β-amylase, utilization of the enzyme in following the modifications of starches and investigations of the degradation products formed by the action of the enzyme on starch.

In order to compare the action of the β-amylase with the action of α-amylase, it was necessary to have some idea of the products formed by the action of the latter enzyme on starch. With this objective in mind, the fractionation of the degradation products formed by the action of α-amylase on starch and the characterization of the products by physical and chemical methods were carried out.

## Procedure for the Isolation of the Degradation Products Formed from Starch by the Action of α-amylase

Corn starch degradation products. Experiment I.

Primary procedure. One hundred grams of commercial pearl starch were suspended in a small amount of cold water and slowly added to 3 liters of boiling water. This gelatinization was carried out in a 4 liter beaker, the solution being stirred vigorously during addition of the cold-water-suspension of the starch and during the following period of boiling. Boiling was continued for five minutes. The starch paste was very viscous even when hot. The paste was cooled rapidly to 55° C., 0.1 gram α-amylase added, the solution stirred thoroughly to dissolve the enzyme, the whole transferred to a 4 liter Erlenmeyer flask, stoppered with a cotton plug and placed in a water bath maintained at 40° C.

Viscosity measurements were made in a water-jacketed pipette maintained at 40° C. After 8 minutes the relative viscosity had fallen from an unmeasureable level to 2.08, in 60 minutes it had fallen to 1.52 and after 92 minutes the relative viscosity was 1.38. At this stage the digestion mixture was removed from the water bath and heated to boiling. Another 100-gram portion of pearl starch suspended in cold water was then added. Boiling was continued for 5 minutes, the starch suspension then cooled to 70° C., 0.12 gram of α-amylase added, the digestion mixture placed in the

water bath at 40° C. and the digestion allowed to proceed for 26 hours. The relative viscosity of the mixture then stood at 1.82.

At the completion of the digestion period a considerable quantity of flocculent material had begun to settle out. This material was collected by centrifugation in the Sharples supercentrifuge at 21,000 r.p.m. The gelatinous mass so collected was resuspended in about 300 cc. of water, 0.1 gram of α-amylase added, placed in the water bath at 40° C. and allowed to digest for 19 hours. The heavy, viscous suspension was then transferred to glass centrifuge tubes and centrifuged at 3000 r.p.m. for twenty minutes. After centrifugation about 50% of the total volume was removed by decantation of the clear supernatant liquid. Further centrifugation for 10 minutes produced no appreciable sedimentation. The few drops of supernatant liquor so obtained were decanted and the residue treated with an equal quantity of absolute alcohol. After 15 minutes centrifuging at 3000 r.p.m. no visible settling had occurred. The suspension was then transferred to a Whatman No. 40 filter paper in a Buchner funnel and filtration accomplished with suction. The process was slow and tedious, each filtration requiring several hours. The semi-solid so obtained was dehydrated by repeated suspension in absolute ethyl alcohol. This material did not dehydrate to a granular mass, but remained as a gelatinous

material which would not settle out of absolute alcohol. Upon removing the final traces of alcohol by evaporation at room temperature, the material remained as a gray horny solid. The solid was very hard but could be ground in an agate mortar, the finely powdered material being only slightly soluble in boiling water. Yield of Ppt. I -- 12.91 grams.

The centrifugate from the original separation of Ppt. I was evaporated to a volume of 815 cc. under reduced pressure in a water bath maintained at 50° C. A trace of haze was present at this stage. Centrifugation did not clarify the solution. An equal quantity of absolute alcohol was then added. The water-clear syrup so obtained was collected by centrifugation, redissolved in a small amount of water, reprecipitated by the addition of alcohol and finally dehydrated by trituration with absolute alcohol. This yielded a white amorphous material which dissolved in cold water to give a slightly turbid solution. The iodine color was a reddish-purple. Yield of Ppt. II -- 60.72 grams.

The alcohol concentration of the centrifugate from the first precipitation of II was increased to 75%. The clear, viscous syrup so obtained was redissolved in a slight excess of water, reprecipitated, and dehydrated with absolute alcohol. Yield of Ppt. III -- 22.52 grams.

The centrifugate from the first precipitation of III was evaporated under reduced pressure to a volume of 152 cc.

Absolute alcohol was added until a definite precipitate formed (243 cc. alcohol necessary). After standing at room temperature for 48 hours the precipitate had settled into a thick syrup. The supernatant liquor was decanted, the residual syrup redissolved in a small amount of water, reprecipitated by the addition of absolute alcohol and dehydrated in the usual manner with absolute alcohol. Yield of Ppt. IV -- 17.99 grams.

The supernatant liquid decanted from Ppt. IV had a volume of 345 cc. Four hundred cubic centimeters of absolute alcohol was added to give a heavy syrup. This was redissolved, reprecipitated and dehydrated in the usual manner. Yield of Ppt. V -- 33.09 grams.

Centrifugate V was evaporated under reduced pressure to a volume of 16 cc., giving a thick yellow syrup. This syrup was added to 300 cc. of absolute alcohol dropwise and with vigorous stirring to insure rapid distribution of the syrup. The precipitate so formed was dehydrated in the usual manner and preserved as Ppt. VI. This material was a light, fluffy solid having a slight tan tinge. Yield of Ppt. VI -- 9.63 grams.

The original pearl starch used in this preparation contained 10.23% moisture. On this basis, 179.5 grams of starch was used. From the yields of materials given in the above procedure the overall yield as solids was 87.4%.

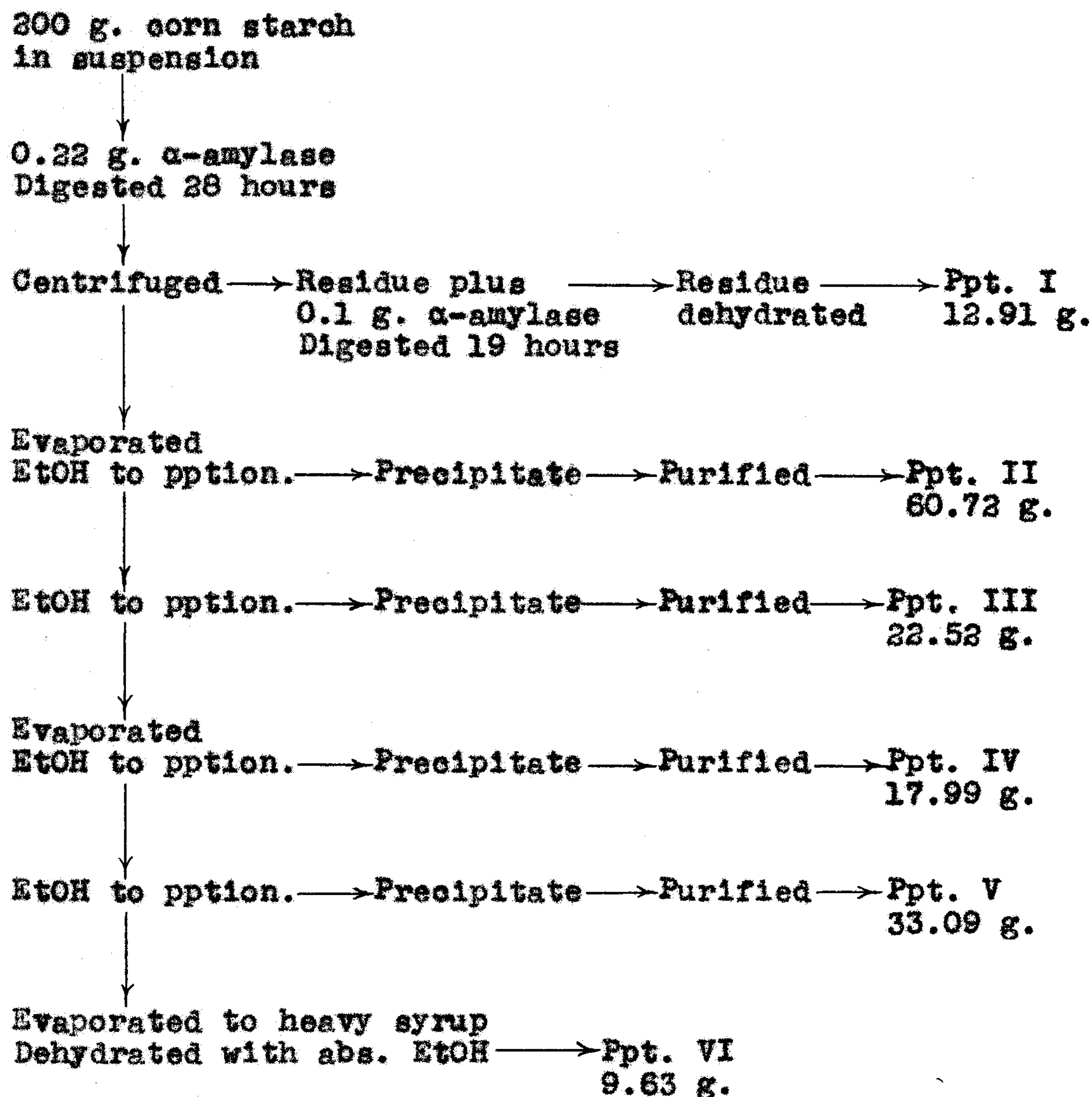



Figure VI. Flow sheet for the fractionation of the degradation products formed by the action of α-amylase on corn starch. Experiment I.

<u>Fractionation of Ppt. II</u>. Five grams of Ppt. II was dissolved in 50 cc. of water, 10 cc. of 0.051 N $I_2$ in KI added and the solution allowed to stand for 18 hours (later work indicated that this standing was not necessary). The iodine-starch solution was extracted with diethyl ether until the ethereal layer was only slightly colored. The solution was then extracted with a small amount of chloroform to remove the last traces of iodine. The extracted aqueous layer was then aerated to remove the major portion of the residual ether and chloroform, the volume of the solution after aeration being 63 cc. Absolute alcohol was added slowly from a pipette and with continuous stirring until a definite black precipitate formed (50 cc. alcohol required). Further addition of a few drops of alcohol caused a white flocculent precipitate to form. The precipitate settled almost instantaneously leaving a light yellow-colored solution. After centrifugation at 1000 r.p.m. for a few minutes the solution was a clear canary-yellow while the precipitate had collected as a gelatinous black mass. After decantation of the supernatant liquid the precipitate was dissolved in a small amount of $H_2O$, reprecipitated with alcohol, collected by centrifugation and dehydrated by comminution with absolute alcohol. Yield of Ppt. IIb -- 1.965 grams.

To the centrifugate from the first precipitation of Ppt. IIb 50 cc. of absolute alcohol was added. A white gummy

material, which collected as a mass in the bottom of the beaker, was obtained. The yellow color of the supernatant solution was destroyed by the addition of one drop of 0.096 N $Na_2S_2O_3$. The precipitate was collected by centrifugation and treated with sufficient water to give a turbid suspension. The material causing the turbidity was collected by centrifuging at 1000 r.p.m. for 30 minutes. The gelatinous mass was dehydrated with absolute alcohol and preserved as Ppt. $IIa_1$. Yield -- 0.211 grams.

Alcohol was added to the centrifugate from the first precipitation of Ppt. II $a_1$ until the precipitate which formed coagulated. The material was collected by centrifugation and dehydrated with absolute alcohol. Yield of Ppt. $IIb_2$ -- 0.820 grams.

The color-reaction of these fractions with iodine are as follows: II -- purple; $IIa_1$ -- a dirty purple; $IIa_2$ -- light orange-yellow; IIb -- crystal-clear deep-blue. These iodine-color reactions indicate that a definite fractionation of Ppt. II has been obtained.

<u>Fractionation of Ppt. III</u>. Five grams of Ppt. III was dissolved in 25 cc. $H_2O$, 5.0 cc. of 0.051 N $I_2$ in KI added and allowed to stand overnight. The excess iodine was extracted with diethyl ether. The extracted iodine required 2.56 cc. of 0.0957 N $Na_2S_2O_3$ for titration, which is exactly equivalent to the iodine added. The absence of iodine con-

sumption indicates that the iodine treatment is not essential and may be eliminated.

The ether was then removed from the aqueous solution by aeration giving a final volume of 28 cc. Absolute alcohol was added slowly and with stirring until a definite precipitate formed. The precipitation occurred sharply after the addition of 25 cc. of alcohol, the total volume added being 28 cc. or exactly 50% alcohol by volume. The precipitate collected as a heavy, slightly yellow syrup. It was placed in an ice box at 5° C. for 20 hours. Because no precipitation had occurred the syrup was therefore dehydrated with absolute alcohol. Yield of Ppt. IIIa -- 1.605 grams.

The centrifugate from the precipitation of Ppt. III a had a volume of 43 cc. The addition of 25 cc. absolute alcohol gave a further precipitate which was collected by centrifugation. After dehydration the solid appeared to consist of fine, sparkling crystals. Microscopic examination showed this material to be made up of irregular, transparent, noncrystalline platelets. Many of the platelets were so large as to be readily picked from the mass of the sample with a small spatula. Yield Ppt. IIIb -- 1.434 grams.

One hundred fifty cc. of absolute alcohol was added to 56 cc. of the centrifugate from the precipitation of IIIb. A milky solution resulted. After standing 48 hours the supernatant liquid was decanted from the semi-granular solid which

had collected on the sides and the bottom of the vessel. Grinding with 25 cc. absolute alcohol for several minutes completed the dehydration. Yield of Ppt. IIIc --- 0.287 grams.

<u>Fractionation of Ppt. V</u>. Five grams of Ppt. V was dissolved in 26 cc. $H_2O$ and the resulting solution filtered to remove the few strands of filter paper present. Absolute alcohol was added until a definite precipitate formed. This required 75 cc. alcohol. After standing overnight the clear supernatant liquor was decanted, the precipitate dehydrated with absolute alcohol and preserved as Ppt. Va. Yield -- 1.385 grams.

The solution decanted from Va was stirred continuously during the addition of 25 cc. absolute alcohol. The precipitate was settled by centrifugation and dehydrated with alcohol. Yield of Ppt. Vb -- 0.787 grams.

The centrifugate from Vb was increased in alcohol concentration by the addition of 100 cc. of absolute alcohol. The precipitate was collected and dehydrated. Yield of Ppt. Vc -- 0.636 grams.

The centrifugate from Ppt. Vc was evaporated in a stream of air at room temperature to give Ppt. Vd. Yield -- 0.320 grams.

The specific rotation and the reducing power of these dextrinous products were determined. The data from these determinations are presented in Table VIII.

TABLE VIII

Summary of the properties of the products obtained from the digestion of corn starch by α-amylase. Experiment I.

| Primary Products | | | | Fractions of Primary Products | | |
|---|---|---|---|---|---|---|
| Ppt. No. | $[\alpha]_d$ in $H_2O$ | $[\alpha]_d$ 2% NaOH | Rm (as % maltose) | Ppt. No. | $[\alpha]_d$ in $H_2O$ | Rm (as % maltose) |
| I | ------ | + | 8.35 | | | |
| II' | +171.2° | +147.5° | 6.00 | $IIa_2$' | +168.7° | 3.20 |
| | | | | IIb' | +167.5° | 3.70 |
| III | +172.5° | +150.0° | 15.6 | IIIa | +169.5° | 7.5 |
| | | | | IIIb | +170.0° | 10.0 |
| | | | | IIIc | +169.0° | ---- |
| IV | +157.3° | +133.8° | 43.2 | | | |
| V | +155.0° | +131.2° | 46.3 | Va | +159.0° | 37.1 |
| | | | | Vb | +159.5° | 40.9 |
| | | | | Vc | +165.5° | 41.3 |
| | | | | Vd | +160.8° | 44.2 |
| VI | ------ | +167.5° | 54.3 | | | |
| VII | +146.3° | ------ | 64.4 | | | |
| VIII | ------- | +122.5°? | 72.1 | | | |

1. Fraction II gave a dirty purple color-reaction with iodine. Fractionation gave $IIa_2$ which had a clear deep-blue color reaction with iodine and IIb which gave a canary yellow. The color-reaction of combined $IIa_2$ and IIb was identical to the original Fraction II.

Corn starch degradation products. Experiment II.

Nine hundred and forty grams of corn starch was suspended in 2 liters of cold water and heated in a water bath until the paste became so thick as to make stirring impossible. The suspension was then cooled to 70° C. and 0.4 gram of α-amylase added in an aqueous solution. The enzyme solution was stirred into the heavy paste, the mixture placed in the 40° C. water bath and digestion allowed to proceed for two hours. At the end of this time another 0.5 gram portion of α-amylase was added, the heavy paste transferred to an Erlenmeyer flask, 25 cc. of toluene added as a preservative and the digestion allowed to continue at 40° C. for 16 hours.

At the end of the first 18 hours of digestion the paste was liquefied to the extent that it would pour. Some undigested lumps were present. To insure complete digestion of the substrate the suspension was heated to 85° C. and maintained at that temperature for 30 minutes. The heavy suspension so formed was cooled to 65° C., passed through a homogenizer, 0.6 gram of α-amylase added in an aqueous suspension, enough toluene added to form a layer over the surface of the digestion mixture and the whole placed in a 40° C. water bath.

After 26 hours the suspension was so heavy as to prevent centrifugation. Dilution to 6 liters with distilled water gave a suspension which could be centrifuged. Repeated

centrifugation at 21,000 r.p.m. gave a clear centrifugate and about 1000 cc. of a semi-solid product. The semi-solid material was transferred to a large Buchner funnel in a suction flask and allowed to filter overnight. The clear filtrate obtained was added to the original centrifugate, 50 cc. of toluene added and the mixture placed in an ice box in a stoppered flask.

The semi-solid material remaining in the funnel was resuspended in 2 liters of water, run through the homogenizer, heated to boiling, cooled to 40° C., 0.5 gram of α-amylase and enough toluene to form a thin layer over the surface added, and the digestion continued for 96 hours. The large quantity of undigested material was collected by centrifugation, suspended in 2 liters of absolute alcohol by passing through the homogenizer and allowed to stand for several hours. The heavy alcoholic suspension was placed in a large funnel and allowed to filter by gravity for twelve hours. The moist gum-like product was then transferred to a large glass plate and allowed to dry under atmospheric conditions. Stirring and crumbling as the drying proceeded helped reduce the size of the particles. After drying for 48 hours the hard, horny material was ground to pass a 100 mesh screen, allowed to dry at 50° C. for two days and preserved as Ppt. I. Yield -- 55.5 grams.

The combined centrifugate and filtrate from the first digestion showed a slight amount of sediment after standing

overnight in the ice box. This was removed by centrifugation. The clear centrifugate was evaporated under reduced pressure (15-20 mm.) in a water bath maintained at 50-55° C. until a heavy syrup was obtained. The syrup was transferred to a large beaker, warmed to 60° C. and absolute alcohol added until the precipitate settled out as a heavy syrup. Upon first precipitating, the material appears to be made up of fine particles of solid, but in the warm solution this formed a syrup which did not solidify upon cooling. After cooling for several hours at 0° C. the clear supernatant liquor was decanted, the residual syrup redissolved in a small amount of water, reprecipitated at 60° C., cooled, allowed to settle, the supernatant liquid decanted, the syrup dehydrated by comminution with absolute alcohol, collected by filtration and dried in the air. Yield of Ppt. II -- 334.7 grams.

The liquid decanted from the first precipitation of II was warmed to 60° C. and absolute alcohol added until a layer of heavy syrup formed. The solution was cooled to 0° C., the supernatant liquor decanted and the syrup redissolved in a small amount of water. The material was then reprecipitated at 60° C. by the addition of alcohol, dehydrated with absolute alcohol, collected by filtration and dried in the air. Yield of Ppt. III -- 89.4 grams.

The supernatant solution from the first precipitation of III (total volume about 2500 cc.) was evaporated to a

syrup, absolute alcohol added at 60° C. until a definite separation of material was evident, cooled to 0° C. and allowed to stand for a short time. The supernatant liquor was then decanted, the syrup redissolved in water, reprecipitated at 60° C., dehydrated with absolute alcohol, collected and dried at room temperature. Yield of Ppt. IV -- 32.6 grams.

The liquid decanted from the first precipitation of IV was evaporated under reduced pressure to a thick syrup, transferred to a small beaker and the evaporation continued in a stream of air until a gummy mass formed. This gum was then dehydrated by treatment with absolute alcohol. Yield of Ppt. V -- 24.0 grams.

The liquors from the reprecipitation and dehydration of Ppt. III also were combined. The resulting precipitate was dehydrated with absolute alcohol, dried and preserved as Ppt. IIIa. Yield -- 39.1 gram.

The combined centrifugate and filtrate from the redigestion of Ppt. I possessed a dull gray appearance. Only a trace of material was removed by centrifugation. The suspension was then evaporated under reduced pressure at about 50° C. The syrup obtained is brown-colored but has none of the murky-grey color typical of the original solution. However, if a small amount of the syrup is diluted with $H_2O$ it again acquires the dull gray color typical of the original solution. The syrup was then warmed to 60° C., alcohol

added until a precipitate formed, the mixture cooled to 0° C. and allowed to stand for a short time. The supernatant liquid was decanted, the residual syrup redissolved in water, then reprecipitated at 60° C. and the resulting syrup dehydrated with absolute alcohol. Yield of Ppt. Ia -- 47.45 grams.

The solution from the first precipitation of Ia was increased in alcohol concentration until a precipitate formed. This was allowed to settle, the supernatant solution decanted and the residual syrup dehydrated to give Ppt. Ib. Yield -- 17.1 grams.

The solution from Ib was evaporated to a volume of about 30 cc. and absolute alcohol added until precipitation occurred. The syrup was dehydrated with absolute alcohol to give Ppt. Ic. Yield -- 9.85 grams.

The solution from the precipitation of Ic was evaporated in a stream of air to give a nearly solid gum. This was dehydrated with absolute alcohol to give Ppt. Id. Yield -- 13.0 grams.

The solution from the reprecipitation of Ppt. Ia and the dehydration liquors from the same product were combined to give a further precipitate. This was redissolved in water, reprecipitated and finally dehydrated with absolute alcohol. Yield of Ppt. Ia (1) -- 14.80 grams.

The liquors from the reprecipitation and dehydration of Ppt. II were combined. The resulting precipitate was collected, dehydrated with absolute alcohol and dried at room temperature. Yield of Ppt. IIa -- 30.9 grams.

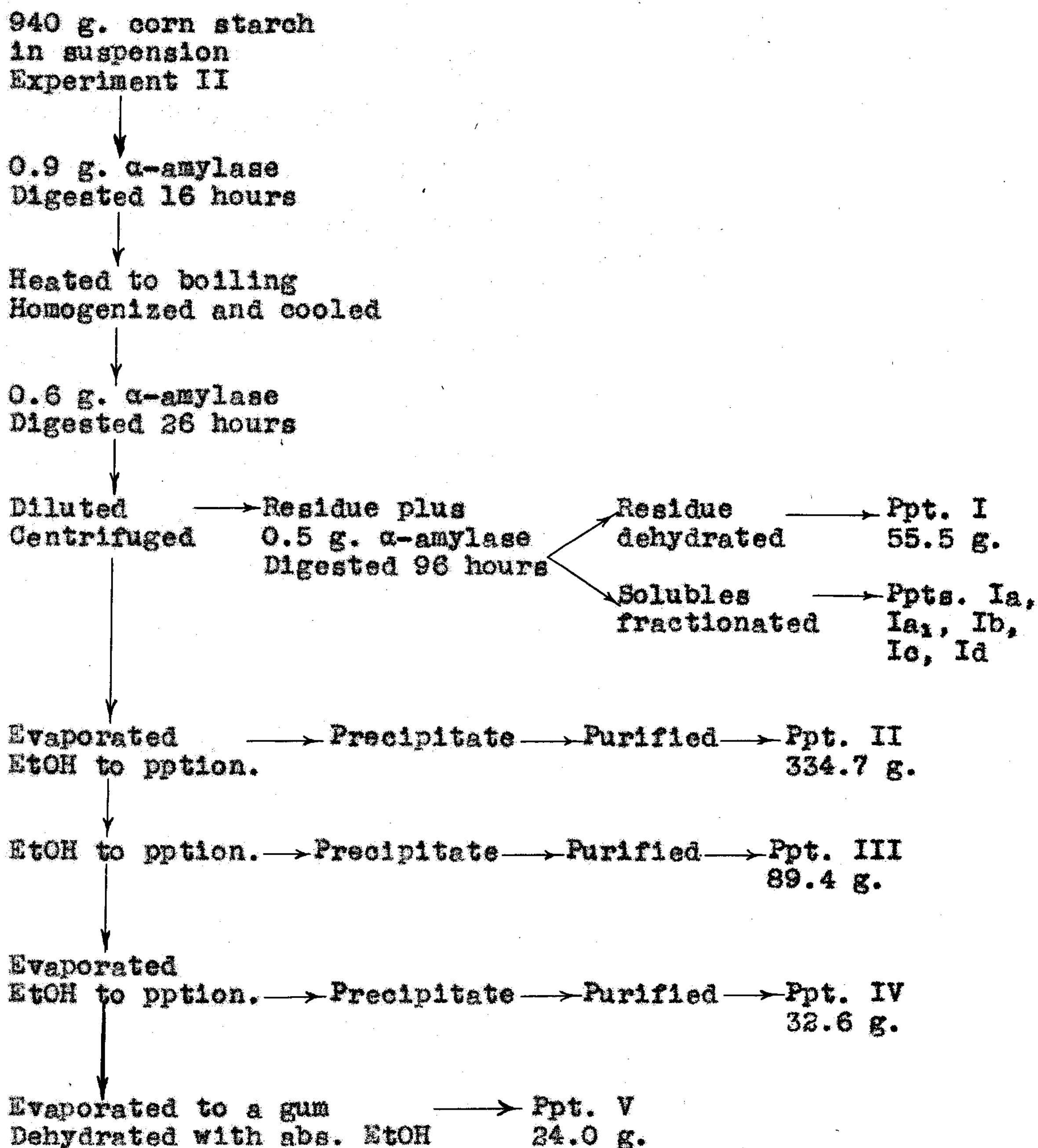



Figure VII. Flow sheet for the fractionation of the degradation products formed by the action of α-amylase on corn starch. Experiment I.

TABLE IX

Summary of the properties of the fractions obtained from the digestion of corn starch by α-amylase. Experiment II.

| Primary products | | | Fractions from primary products | | |
|---|---|---|---|---|---|
| Ppt. No. | $[\alpha]_d$ in $H_2O$ | Rm (as % maltose) | Ppt. No. | $[\alpha]_d$ in $H_2O$ | Rm (as % maltose) |
| I | ------ | 15.2 | Ia | +159.2° | 42.2 |
| | | | Ia(1) | +157.5° | 58.2 |
| | | | Ib | +148.7° | 67.2 |
| | | | Ic | +116.2° | 96.6 |
| | | | Id | +138.2° | 80.8 |
| II | +163.2° | 35.2 | IIa | +159.2° | 56.6 |
| III | +153.2° | 59.0 | IIIa | +142.0° | 91.0 |
| IV | +142.8° | 76.0 | | | |
| V | +123.1° | 98.6 | | | |

The specific rotation and reducing equivalent of the various subfractions are given in Table IX.

The original starch used in the preparation contained 10.28% moisture. On this basis the overall recovery of solids was 84%.

<u>Waxy maize starch degradation products</u>.

<u>Primary procedure</u>. One hundred grams of waxy maize starch was made to a paste in cold water and poured into 3.5 liters of boiling water. The suspension so formed was boiled for five minutes, cooled to about 70° C. and 0.1 gram α-amylase added. In less than one minute the paste was liquefied to an easy flowing liquid. The digestion was allowed to proceed for 30 minutes at 40° C. The digestion mixture was then heated to boiling and 100 grams of waxy maize starch in cold water was added. Boiling was continued for five minutes. After cooling to 70° C., 0.2 gram of α-amylase was added, the digestion mixture was placed in a water bath at 40° C. and the digestion allowed to proceed for 18 hours. At this time the mixture was centrifuged at 21,000 r.p.m. while still warm. The small amount of sediment removed was allowed to stand under absolute alcohol for 24 hours. The dehydrated material was then collected by filtration. Yield of Ppt. I -- 0.608 grams.

After the centrifuged solution was allowed to cool to room temperature a small amount of flocculent material had settled out. This was removed by centrifugation. The amount was so small as to make recovery from the large centrifuge bowl impossible. The four liters of clear solution were then evaporated to a volume of 350 cc. under reduced pressure

in a water bath maintained at 50° C. This concentration period must be considered as part of the digestion time because the amylase continues to act at the temperature of the vacuum distillation (about 40° C.). Thus, the total digestion time was 47 hours.

The heavy syrup left after vacuum distillation of the water and alcohol was cloudy and had considerable color. The syrup was clarified by the addition of 10 grams of animal charcoal, heated to 40° C. for a few minutes and then filtered. This treatment yielded a crystal clear, light yellow syrup. The residual charcoal was washed with two fifty cubic centimeter portions of distilled water to remove the occluded syrup. The combined washings and syrup had a total volume of 500 cc. Seven hundred and fifty cubic centimeters of absolute alcohol was then added to give a heavy precipitate. The mixture was allowed to stand at 5° C. for five hours and the syrupy precipitate collected by centrifugation. This syrup was dissolved in a slight excess of water, filtered through Whatman No. 50 filter paper to remove a small amount of sediment and then reprecipitated by the addition of absolute alcohol. The syrup was again dissolved in water. Upon addition of alcohol, slowly and with constant stirring, a primary precipitate separated as a brownish syrup, followed at a definite stage by a white milky precipitate. The addition of alcohol was stopped at the first appearance of this

white precipitate, traces of the precipitate redissolving upon stirring. The solution was then cooled in an ice bath, the heavy syrup collected by centrifugation and dehydrated with absolute alcohol to give Ppt. IIa. Yield -- 7.545 grams.

Addition of alcohol to the centrifugate of IIa at 0° C. gave a white milky suspension which coagulated upon the addition of a further amount of alcohol. The precipitate was collected as a syrup by centrifugation and dehydrated in the usual manner. Yield of Ppt. IIb -- 39.81 grams.

The centrifugate from the original precipitation of II was stirred continuously during the addition of 500 cc. of absolute alcohol. After standing overnight at 5° C. the precipitate was removed by centrifugation, redissolved in a small excess of water, filtered to remove a few insoluble particles, reprecipitated by the addition of alcohol, again redissolved in water, reprecipitated by the addition of alcohol and finally dehydrated by trituration with absolute alcohol. Yield of Ppt. III -- 34.10 grams.

The centrifugate from the initial precipitation of III was evaporated under reduced pressure to a volume of 140 cc. Four hundred cubic centimeters of absolute alcohol was then added. The precipitate was collected by centrifugation, redissolved in water, reprecipitated and finally dehydrated with alcohol. Yield of Ppt. IV -- 16.98 grams.

The centrifugate from the first precipitation of IV was evaporated to a thick syrup. Addition of absolute alcohol

did not cause granulation of this syrup. Upon boiling with absolute alcohol a small amount of the material dissolved. The hot alcohol solution was decanted from the syrupy residue, cooled and the granular precipitate collected on a filter. The material was dried in a vacuum desiccator over $CaCl_2$. Yield of Ppt. V -- 1.3407 grams.

Upon further extraction with boiling absolute alcohol another small quantity of material was dissolved. The solution was decanted and cooled to yield Ppt. VI. This was also dried in a vacuum desiccator over $CaCl_2$. Yield of Ppt. VI -- 0.252 grams.

The alcohol extracted material became hard and brittle upon cooling. It was ground to a fine white powder in an agate mortar and preserved as Ppt. VII. Yield -- 23.17 grams.

In an effort to make the recovery of solids as quantitative as possible all mother liquors and dehydration liquors were reworked. The following is a summary of these manipulations:

1. All mother liquors from the reprecipitation of IIa and IIb were combined and evaporated to a volume of 50 cc. The addition of 250 cc. of absolute alcohol gave a precipitate which was collected by centrifugation and dehydrated by absolute alcohol. Yield of $IIab_1$ -- 23.10 grams.

2. The mother and dehydration liquors from $IIab_1$ were combined and evaporated to dryness at room temperature.

Yield of Ppt. $IIab_2$ -- 1.006 grams.

3. The mother liquors from the reprecipitation of IV were evaporated to a thick syrup. These were further allowed to evaporate at 40° C. for six days. No solids had formed. The material was then dehydrated with absolute alcohol. Yield of Ppt. IVa -- 11.63 grams.

4. The dehydration liquors of IIa were evaporated to dryness. Yield $IIa_1$ -- 0.0303 grams.

5. The dehydration liquors of IIb were evaporated to dryness. Yield of $IIb_1$ -- 0.2354 grams.

6. The mother liquors from the reprecipitation of III were combined and evaporated to a volume of about 50 cc. Two hundred cubic centimeters of absolute alcohol was added to give Ppt. IIIa. Yield -- 12.21 grams.

7. The dehydration liquors of III were evaporated to dryness. Yield of $III_1$ -- 0.5000 grams.

8. The dehydration liquors of IV were evaporated to dryness. Yield of $IV_1$ -- 0.0791 grams.

9. The mother liquor of V was evaporated to dryness. Yield of $V_1$ -- not recorded.

10. The mother liquor of VI was evaporated to dryness. Yield of VI -- 1.012 grams.

The original waxy maize starch contained 6.7% moisture. On this basis 71.6% of the total solids were recovered.

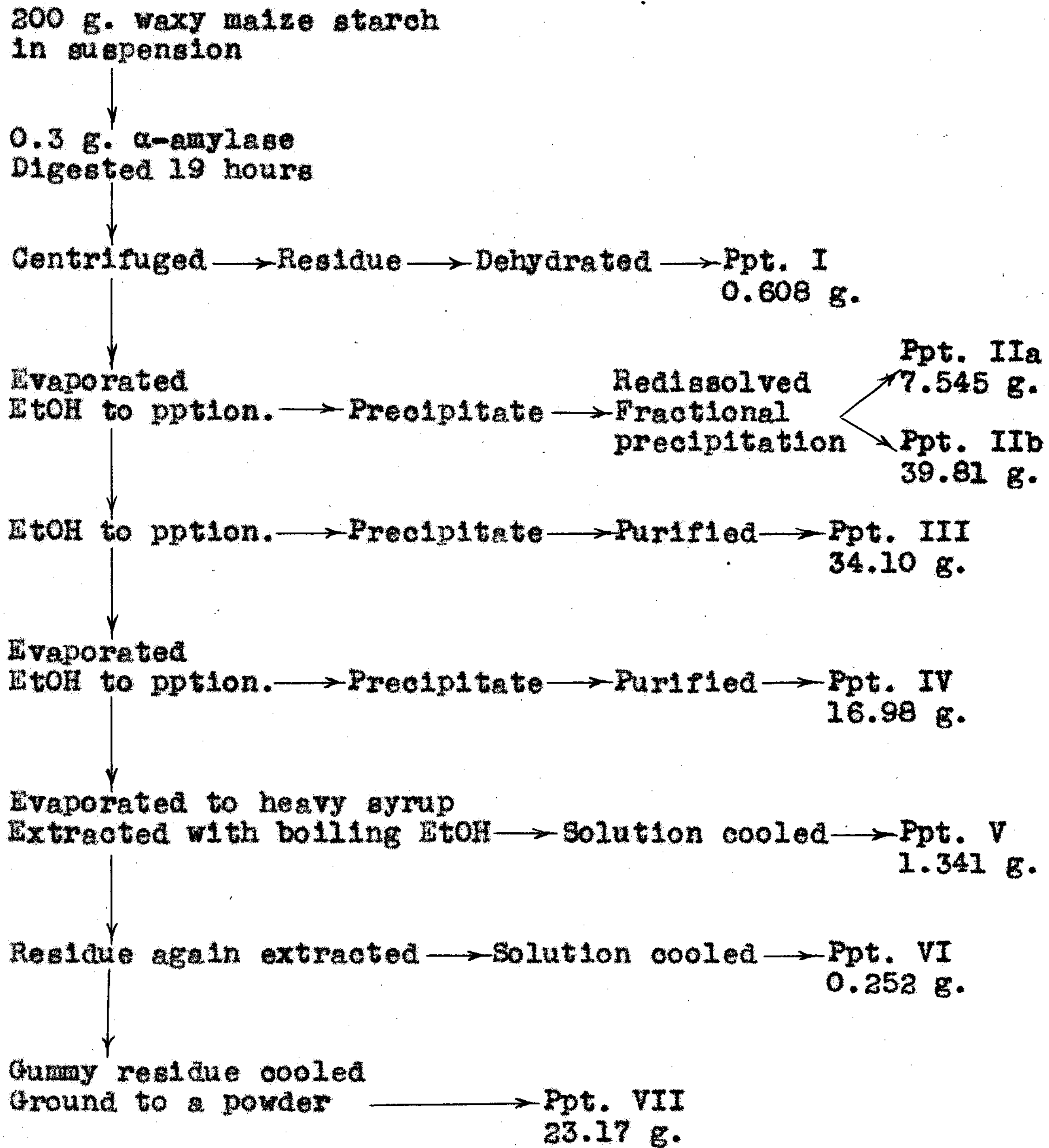



Figure VIII. Flow sheet for fractionation of the degradation products formed by the action of α-amylase on waxy maize starch.

TABLE X

Summary of the properties of the fractions obtained from the digestion of waxy maize starch by α-amylase.

| Primary Products | | | Fractions from Primary Products | | |
|---|---|---|---|---|---|
| Ppt. No. | $[\alpha]_d$ in $H_2O$ | Rm (as % maltose) | Ppt. No. | $[\alpha]_d$ in $H_2O$ | Rm (as % maltose) |
| I | ----- | 29.4 | | | |
| IIa | +174.3° | 13.1 | | | |
| IIb | +172.9° | 22.5 | $IIb_1$ | +163.4° | 61.5 |
| | | | $IIab_1$ | +153.2° | 54.3 |
| | | | $IIab_2$ | +108.9° | 92.0 |
| III | +165.4° | 36.6 | IIIa | +147.4° | 52.0 |
| | | | $III_1$ | +123.0 | 86.2 |
| IV | +160.5° | 48.5 | IVa | +151.9° | 76.0 |
| | | | $IV_1$ | +141.7° | 59.1 |
| V | + 63.8° | 82.3 | $V_1$ | + 43.3° | 53.1 |
| VI | +148.2° | 66.0 | $VI_1$ | + 94.1° | 197.5 |
| VII | +133.2° | 76.0 | | | |

Fractionation of Ppt. IIb. Five grams of Ppt. IIb was dissolved in 10 cc. of water. The addition of 12 cc. absolute alcohol at room temperature caused a slight precipitation. Cooling in an ice bath for a short time gave a brown-colored

precipitate. Centrifugation yielded a water clear centrifugate and a heavy syrup. The syrup was redissolved in 10 cc. water and reprecipitated by the addition of 30 cc. absolute alcohol at 0° C. The precipitate was a slightly colored, viscous gum. Dehydration with absolute alcohol gave a white, amorphous powder. Yield of Ppt. $II_1$ -- 2.55 grams.

To the clear centrifugate from the first precipitation of $II_1$ 20 cc. of absolute alcohol was added and the mixture cooled. The near-solid material was redissolved, reprecipitated and dehydrated. Yield of Ppt. $II_2$ -- 1.53 grams.

One hundred cubic centimeters of absolute alcohol was added to the centrifugate from the first precipitation of $II_2$ to give a white flocculent precipitate. After cooling to 0° C. the precipitate was removed by centrifugation and dehydrated by repeated suspensions in absolute alcohol. Yield of Ppt. $II_3$ -- 0.197 grams.

The addition of 100 cc. absolute alcohol to the mother liquors from the reprecipitation of $II_1$ gave a fine white precipitate which was collected by centrifugation, dehydrated and preserved as Ppt. $II_4$. Yield -- 0.220 grams.

Fractionation of waxy maize dextrins with $[\alpha]_d$,+164-173° and Rm 22-27. A composite was made of three dextrin fractions with $[\alpha]_d$, 164-173° and Rm 22-27. The 55 gram sample so obtained was dissolved in water, filtered and made to a volume of 250 cc. The addition of 250 cc. alcohol at 0° C.

gave a permanent precipitate. The material was collected by centrifugation, twice redissolved and reprecipitated, and finally dehydrated with absolute alcohol. Yield of Ppt. $VIII_1$ -- 15.84 grams.

The addition of 190 cc. of alcohol to the centrifugate from the first precipitation of $VIII_1$ gave a further precipitation. The material was collected, twice redissolved and reprecipitated, and then dehydrated with alcohol. Yield of Ppt. $VIII_2$ -- 14.53 grams.

Four hundred cubic centimeters of absolute alcohol was added to the centrifugate from the first precipitation of $VIII_2$ to give a material which was treated in exactly the same manner as $VIII_2$. Yield of Ppt. $VIII_3$ -- 4.94 grams.

The centrifugate from the precipitation of $VIII_3$ as evaporated to a heavy gum. Dehydration with absolute alcohol gave Ppt. $VIII_4$. Yield -- 7.00 grams.

Measurement of the specific rotation and reducing equivalent indicated that Ppt. $VIII_2$ consisted of a mixture of $VIII_1$ and $VIII_3$. An 11.0 gram sample of Ppt. $VIII_2$ was therefore fractionated in exactly the same manner as given above for fractionation of the composite. The four fractions obtained were numbered $VIII_2$a, $VIII_2$b, $VIII_2$c and $VIII_2$d. The yields obtained from the 11.0 gram sample were 6.069, 3.136, 0.998 and 0.416 grams, respectively.

TABLE XI

Properties of the dextrins obtained by further fractionation of the primary degradation products from waxy maize starch by the action of α-amylase.

| Primary products | | | Fractions from primary products | | |
|---|---|---|---|---|---|
| Ppt. No. | in $H_2O$ | Rm (as % maltose) | Ppt. No. | in $H_2O$ | Rm (as % maltose) |
| IIb | +172.9° | 22.5 | $II_1$ | +167.1° | 12.2 |
| | | | $II_4$ | +165.0° | 20.0 |
| | | | $II_2$ | +171.0° | 20.1 |
| | | | $II_3$ | +166.3° | 32.5 |
| VIII (composite) | +169.5° | 23.4 | $VIII_1$ | +168.8° | 11.4 |
| | | | $VIII_2$ | +168.8° | 17.3 |
| | | | $VIII_3$ | +170.5° | 23.6 |
| | | | $VIII_4$ | +161.2° | 50.0 |
| $VIII_2$ | +168.8° | 17.3 | $VIII_2a$ | +166.0° | 12.8 |
| | | | $VIII_2b$ | +168.3° | 15.6 |
| | | | $VIII_2c$ | +166.3° | 20.7 |
| | | | $VIII_2d$ | +170.0° | 29.8 |

Discussion of the Corn and Waxy Maize Dextrins.

The procedures used for the isolation of the degradation products from the action of α-amylase on corn starch vary in three major elements:

1. Concentration of starch.
2. Concentration of enzyme.
3. Time of digestion.

In Experiment I the final digestion was run on a gelatinized starch suspension containing 200 grams of starch in a total volume of approximately 4 liters. The total quantity of α-amylase added was 0.22 grams. The total time during which the enzyme acted was 48 hours.

In Experiment II the digestion was run on a very heavy paste containing 940 grams of corn starch in a total volume of 2.5 liters. The paste was first digested by 0.9 gram α-amylase for 34 hours. The paste was then heated to boiling to insure complete gelatinization, 0.6 gram α-amylase added and digestion continued for 26 hours. The digest was then diluted to 6 liters and the water-insoluble precipitate removed. During the evaporation of the centrifugate, which contained the water-soluble dextrins and the enzyme, the temperature of the solution was maintained at 35-40° C. for about 20 hours. Thus the enzyme continued to act during the entire evaporation. The overall digestion time was approximately 80 hours and the total α-amylase added was

1.5 grams.

On the basis of total quantity used the ratio of the enzyme to starch was 1/910 in Experiment I and 1/625 in Experiment II. The effect of the greater concentration of enzyme in Experiment II was augmented by the additional digestion time of 32 hours. The combined result of the higher enzyme concentration and longer digestion time was the production of dextrins with lower specific rotations and higher reducing equivalents. This is best shown by the fact that 58% of the water-soluble dextrins isolated in Experiment I had higher specific rotations and lower reducing powers than any of the fractions obtained in Experiment II.

The most disconcerting factor in the studies of the corn dextrins was their failure to give fractions with comparable and somewhat constant properties at any stage during the many fractionation attempts. In order to continue with the study of the chemical and physical properties of the dextrins four fractions were selected from the corn dextrins. Selection was based on constancy of specific rotation and reducing equivalent, relative position in the range of these properties and the quantity of the material available. The four fractions chosen were Ppt. I, Ppt. II, Ppt. V and Ppt. VI of Experiment I. Further studies of the properties of these dextrins will be reported later.

In contrast to the dextrins from corn starch the dextrins

formed by the action of α-amylase on waxy maize starch show considerable fractionability. The properties of several dextrins which have been isolated by comparable procedures are given in Table XII. The members of each of the three types of dextrins have similar specific rotations and reducing equivalents. The fact that a representative of each type of dextrin exists in all fractionations and that further fractionation has little effect on the physical properties of each type indicates that definite limit dextrins may be present.

Three dextrin samples, one of each type, were chosen on the basis of their properties and the quantities of material available. The samples were Ppt. $VIII_1$, Ppt. $VIII_3$ and Ppt. $VIII_4$. They were then used in all physical and chemical studies.

TABLE XII

Types of waxy maize dextrins.

| Type | Sample No. | $[\alpha]_d$ in $H_2O$ | Rm (as % maltose) |
|---|---|---|---|
| A | IIa | +174.3° | 13.1 |
| | II | +167.1° | 12.2 |
| | VIII | +168.8° | 11.4 |
| | $VIII_2a$ | +166.0° | 12.8 |
| B | $II_2$ | +171.0° | 20.1 |
| | $VIII_3$ | +170.5° | 23.6 |
| | $VIII_2c$ | +166.3° | 20.7 |
| | X[1] | +168.7° | 22.0 |
| C | IV | +160.5° | 48.5 |
| | $VIII_4$ | +161.2° | 50.0 |
| | Y[1] | +157.5° | 52.9 |

1. Samples X and Y were isolated from a digestion which is not described in the procedure.

## Chemical and Physical Properties of the Dextrins.

### Molecular weight from freezing-point depressions.

The apparatus used was similar to that devised by Beckmann (122). The concentration of solutions, the depression observed and the calculated molecular weights for the dextrins are presented in Table XIII.

TABLE XIII

Molecular weights of dextrins calculated from freezing-point depressions.

| Dextrin no. | Weight sample | cc. $H_2O$ used | $\Delta Tf$ | Calc'd. mol. wt. |
|---|---|---|---|---|
| Corn | | | | |
| II | 0.4052 | 15 | 0.070 | 718 |
| | | 25 | 0.030 | 1003 |
| V | 0.4030 | 15 | 0.154 | 324 |
| | | 25 | 0.090 | 333 |
| VI | 0.4006 | 15 | 0.342 | 145 |
| | | 25 | 0.199 | 249 |
| Waxy Maize | | | | |
| $VIII_1$ | 0.4121 | 15 | 0.099 | 515 |
| | | 25 | 0.050 | 612 |
| $VIII_2$ | 0.4007 | 15 | 0.070 | 707 |
| | | 25 | 0.060 | 824 |
| $VIII_4$ | 0.4044 | 15 | 0.091 | 548 |
| | | 25 | 0.060 | 500 |

Dextrin acetates.

One to 2.5 grams of the dextrins were weighed into 250 cc. iodine flasks, 80 cc. of pyridine and 60 cc. of acetic anhydride added, shaken at intervals to aid solution and finally placed in a 55° C. air bath. The solution of VI and $VIII_4$ was completed within two hours, V and $VIII_3$ within 5 hours and $VIII_1$ within 90 hours. At the end of 90 hours part of I remained undissolved.

After 19 hours at 55° C. samples V, VI, $VIII_3$ and $VIII_4$ were filtered into 400 cc. of ice-water and allowed to stand for one hour. The precipitates which formed were all white flocculent materials except VI, which was somewhat gummy. By grinding under distilled water in a mortar this precipitate became granular. The acetates were collected on a filter, washed with water and sucked dry. The dry product was dissolved in a small amount (about 15 cc.) of $CHCl_3$ and reprecipitated by pouring into 100 cc. of petroleum ether (b. p. 68-70° C.). The granular precipitate was collected by filtration, washed with petroleum ether and dried at 50° C.

The acetylation of I and $VIII_1$ was allowed to run for 90 hours. They were then isolated by the same procedure as used for the other samples.

The freezing-point depressions as measured in a benzene solution were determined. Table XIV presents these data and the molecular weights calculated from them.

TABLE XIV

Molecular weights of dextrin acetates as calculated from the freezing point depressions.

| Dextrin acetate | Weight sample | cc. $C_6H_6$ used | Tf | Calc'd mol. wt. |
|---|---|---|---|---|
| <u>Corn</u> | | | | |
| II | 0.3259 | 15 | 0.017 | 7430 |
| | | 25 | 0.010 | 7580 |
| V | 0.3021 | 15 | 0.075 | 1563 |
| | | 25 | 0.054 | 1302 |
| VI | 0.3016 | 15 | 0.142 | 824 |
| | | 25 | 0.096 | 732 |
| <u>Waxy maize</u> | | | | |
| $VIII_1$ | 0.3039 | 15 | 0.052 | 2270 |
| | | 25 | 0.028 | 2530 |
| | 0.3373 | 15 | 0.060 | 2180 |
| | | 25 | 0.031 | 2610 |
| $VIII_3$ | 0.3111 | 15 | 0.068 | 1775 |
| | | 25 | 0.048 | 1515 |
| | 0.3095 | 15 | 0.070 | 1718 |
| | | 25 | 0.042 | 1718 |
| $VIII_4$ | 0.3133 | 15 | 0.095 | 1280 |
| | | 25 | 0.058 | 1255 |

In addition to the corn dextrins of Experiment I, the five major fractions of the corn dextrins from Experiment II were also acetylated. The acetylation was carried out in pyridine as previously described except that the reaction was allowed to run for 22 hours. At the end of this time only a small portion of Ppt. I was acetylated. The unacetylated residue from I was returned to the flask, 100 cc.

pyridine and 75 cc. acetic anhydride added and acetylation allowed to continue for 6 weeks. The acetylated products were purified by the method as previously given. Table XV reports the results of these determinations as well as the other properties of these dextrins.

TABLE XV

Properties of the dextrins of corn starch prepared in Experiment II and of the acetates derived from these dextrins.

| No. Dextrin | Rm of dextrin | % $CH_3CO$ in acetate | $[\alpha]_d$ of acetate | Glucose units calculated From Rm | From $CH_3CO$ |
|---|---|---|---|---|---|
| I | 15.2 | ---- | 127.5 | 13.1 | --- |
| II | 35.2 | 47.6 | 138.9 | 5.7 | 4.5 |
| III | 59.0 | 47.6 | 120.8 | 3.4 | 4.5 |
| IV | 76.0 | 52.6 | 118.1 | 2.6 | 1.6 |
| V | 98.6 | 54.5 | 101.0 | 2.0 | 1.2 |

Oxidation of dextrins

Procedure. In an effort to obtain chemical evidence as to the constitution and molecular size of these dextrins the method of Kline and Acree (123) for the estimation of aldose sugars by titration with standard iodine and alkali was applied to these dextrins. The dextrin was dissolved in 50 or 100 cc. water and the standard iodine or alkali added alternately in three to five cubic centimeter portions. When the ratio of cubic centimeters of 0.1 N KOH to cubic centimeters of 0.1 N $I_2$ changed from 1.5/1 to 1/1 the addition of KOH and $I_2$ was stopped. The oxidation was allowed to stand at room temperature for one hour. An excess of 0.1 N HCl was added, the excess $I_2$ liberated was titrated with standard $Na_2S_2O_3$, and the surplus HCl then neutralized with 0.1 N KOH using phenolphthalein as the indicator.

Table XVI presents the results of these oxidation studies. The molecular weight of the dextrins from the consumption of $I_2$ and KOH was made on the basis of one and only one aldehyde group per dextrin molecule. From the equation for the oxidation, $RCHO + I_2 + 3KOH \quad RCOOK + 2KI + 2H_2O$, one mole of $I_2$ is equivalent to one mole of dextrin.

Isolation of the potassium salt of the dextrinic acid formed by the oxidation of corn dextrin V of Experiment II. Ten grams of corn dextrin V was dissolved in 25 cc. of

water. This quantity of dextrin required a total of 11.347 cc. of N $I_2$ or 0.011347 moles. Thus the ten grams of dextrin used in the oxidation was equivalent to 0.011347 moles and the corresponding molecular weight for the dextrin was 880.

TABLE XVI

Oxidation of dextrins $I_2$-KOH method.

| Dextrin No. | Weight sample | cc. 0.1 N $I_2$ | cc. 0.1 N KOH | Mol. wt. from $I_2$ | Mol. wt. from KOH |
|---|---|---|---|---|---|
| Corn | | | | | |
| I | 0.5 | 2.37 | ---- | 4220 | ---- |
| | 0.5 | 2.24 | ---- | 4460 | ---- |
| II | 1.0 | 3.67 | 5.08 | 5730 | 5910 |
| | 1.0 | 3.38 | 5.69 | 5920 | 5280 |
| | 1.0 | 4.26 | 5.64 | 4690 | 5320 |
| | 1.0 | 3.63 | 4.83 | 5502 | 6220 |
| | 1.0 | 3.49 | 4.59 | 5730 | 6530 |
| V | 0.3 | 6.19 | 8.86 | 970 | 1001 |
| | 0.3 | 5.95 | 8.73 | 1010 | 1032 |
| VI | 0.3 | 7.80 | 11.52 | 769 | 782 |
| | 0.3 | 7.76 | 11.38 | 773 | 790 |
| Waxy maize | | | | | |
| $VIII_1$ | 0.6 | 3.48 | 4.69 | 3450 | 3840 |
| | 0.6 | 3.53 | 4.68 | 3400 | 3843 |
| $VIII_2$ | 0.3 | 5.25 | 4.47 | 1144 | 2007 |
| | 0.3 | 5.40 | 4.60 | 1110 | 1955 |
| | 0.3 | 5.19 | 4.50 | 1155 | 2000 |
| $VIII_4$ | 0.2 | 4.62 | 6.78 | 866 | 885 |
| | 0.2 | 4.60 | 6.65 | 869 | 902 |

The solution from the oxidation was evaporated to a volume of 200 cc., filtered and enough alcohol added to raise the concentration to 80% by volume. The precipitate was collected by centrifugation, dissolved in 25 cc. of water, treated twice with absorbent charcoal at boiling temperature and filtered. The filtrate retained a slight yellow color. The volume of the filtrate was reduced to 10 cc. by evaporation, 90 cc. absolute alcohol added and the resulting brown precipitate collected. The material was redissolved, reprecipitated and dehydrated by trituration with absolute alcohol to give a granular grayish-white precipitate. To insure dehydration the salt was resuspended in absolute alcohol and allowed to stand for 48 hours. The salt was then collected by filtration and washed with anhydrous ether. Yield of salt I -- 1.9405 grams.

The further addition of alcohol gave salts II and III. Each was twice redissolved and reprecipitated, and finally dehydrated with absolute alcohol. Yields: I -- 2.118 grams; III -- 0.407 grams.

Evaporation of the residual solution to dryness gave several grams of a mixture of KI, $KIO_3$ and organic matter.

The salts were tested for the presence of iodide, iodate and organic matter. All salts contained organic material. Salt I was free of iodide and iodate, salt II gave weak tests for both, while salt III gave good tests for iodide and iodate.

The potassium content of salt I was determined by oxidation with sulfuric acid, volatilization of the excess acid and weighing of the residual $K_2SO_4$. Table XVII presents the results of the analysis and in addition the data for the analysis of two known salts by the same procedure. Calculation of the molecular weight of the original dextrin from the potassium content of the potassium salt of the corresponding dextrinic acid gives values of 875 and 863.

TABLE XVII

Determination of K in salt of dextrinic acid.

| Salt identity | Weight sample | Weight $K_2SO_4$ | % K | % K (theor.) |
|---|---|---|---|---|
| I | 0.3023 | 0.0302 | 4.47 | |
| | 0.3097 | 0.0313 | 4.53 | |
| Potassium salicylate | 0.1293 | 0.0623 | 21.6 | 22.18 |
| | 0.1178 | 0.0573 | 21.8 | |
| Potassium oxalate | 0.1460 | 0.1380 | 42.4 | 42.45 |
| | 0.1415 | 0.1334 | 42.3 | |

Melting points of dextrins and dextrin acetates

In order to obtain an additional physical measurement of the properties of the dextrins and dextrin acetates the melting points were determined by the microscopic method. Measurements were not exact but were reproducible within a range smaller than the variance between the different dextrins and dextrin acetates. Table XVIII presents the data obtained from determination of the temperature at which the materials soften slightly and the range of temperature at which they melt.

TABLE XVIII

Temperatures at which the corn dextrins of Experiment II and the corresponding acetates soften and melt.

| Sample number | Original dextrins Softens | Original dextrins Melts | Original acetates Softens | Original acetates Melts |
|---|---|---|---|---|
| II | 220° | 235-40° | 132° | 138-40° |
| III | 170° | 200-25° | 110°<br>115° | 120-23°<br>118-23° |
| IV | 150° | 195-215° | 95° | 103-08° |
| V | 140° | 185-205° | 80° | 87-89° |
| β-glucose | ---- | 85-90° | ---- | ------- |
| β-glucose pentaacetate | ---- | ------ | ---- | 130-131° |
| β-maltose | ---- | decomposes | ---- | ------- |
| β-maltose octaacetate | ---- | ------ | ---- | 159-60° |

That the acetates can be fractionated by a simple process of fractional precipitation is easily demonstrated by following the melting points of the resulting products. For example, the acetate of corn dextrin II of Experiment II was dissolved in boiling benzene and diethyl ether added until precipitation began at the boiling temperature of the solution. The solution was then cooled, the resulting precipitate removed by filtration, washed with diethyl ether, dried and preserved as $IIR_1$. The addition of petroleum ether to the filtrate gave a second precipitate which was treated in the same manner. This was designated as $IIR_2$. Table XIX presents the data which show that the acetate was fractionated by the process of fractional precipitation.

TABLE XIX

Properties of the fractions obtained from the acetate of corn dextrin II, Experiment II.

| Sample number | Softens | Melts |
|---|---|---|
| Original dextrin | 220° | 235-40° |
| Acetate II | 132° | 138-40° |
| Acetate $IIR_1$ | 145° | 150-55° |
| Acetate $IIR_2$ | 120° | 125-30° |

The reaction of dextrins with phenylhydrazine.

The preparation of phenylhydrazine derivatives of the acetylated cellulose dextrins (124) suggested that the highly reducing dextrins from starches should give a similar reaction. If chemical reaction occurs on the reducing group such a method should aid materially in the separation of reducing and non-reducing dextrins.

Five grams of waxy maize dextrin III was heated with 25 cc. phenylhydrazine at 125-130° C. for two hours. Solution of the dextrin was complete after two or three minutes. The reaction mixture was cooled and then poured into 300 cc. anhydrous ether. The bright yellow precipitate was collected on a filter, washed repeatedly with ether and then washed by suspension in boiling ethylacetate. The resulting product was dissolved in boiling 90% acetic acid and reprecipitated by the addition of ether. The bright yellow solid was collected on a filter and washed several times with ether. After drying at room temperature for 12 hours the material possessed a sharp acetic-acid odor. The sample was then transferred to a Soxhlet extractor and extracted with diethyl ether for 72 hours. The acetic acid extracted required 7.23 cc. of 0.1 N KOH for neutralization. The extracted solid was then dried at 100° C. for one hour.

In a second preparation, five grams of corn dextrin II (Experiment I) was treated in a similar manner. The reaction

time at 130° C. was extended to ten hours. The product was treated in exactly the same manner, the solid obtained having a bright yellow color.

The rather drastic treatment of the phenylhydrazine products in the purification process, without perceptibly altering their color or general physical properties, indicated that the materials were of a definite composition. However, it was observed that all of the color could be removed from the phenylhydrazine derivatives of similar dextrins by treatment with absorbent charcoal. Samples of the two phenylhydrazine derivatives described above were therefore treated with absorbent charcoal. In both cases charcoal removed all of the color from aqueous solutions of the sample.

Recovery of the residue after treatment with charcoal gives a product containing only a trace of nitrogen. The physical properties and the specific rotation of the recovered material are in good agreement with those of the original dextrin.

The removal of the color, the absence of nitrogen in the products recovered after treatment with charcoal, and the similarity between the properties of the dextrin-like material recovered and the original dextrin indicate that the phenylhydrazine is only adsorbed in the dextrin. Thus the phenylhydrazine reaction does not offer any means for the separation of reducing and non-reducing dextrins from amylolytic digestions.

## Discussion

The physical and chemical properties of the various fractions of the degradation products isolated from the digestion of corn starch by α-amylase indicate that the materials range from highly polymerized insoluble polysaccharides to very soluble di- and trisaccharides. Attempts to separate these digestion products into definite chemical entities have not been successful. In the various purification procedures fractions with similar properties are obtained but further fractionation gives a series of dextrins with a wide range of properties. In some cases, for example in the fractionation of Ppt. V of Experiment I, the products vary but slightly from the original material. Using specific rotation and reducing equivalent as the criterion of purity it would be possible to conclude that Ppt. V consists principally of one chemical entity. However, in the purification of Ppt. II of the same experiment, the specific rotation and reducing equivalent of the two products are in excellent agreement; yet, one product gives a blue iodine-color-reaction while the other gives a yellow color-reaction. This example serves to indicate the unreliability of using specific rotations and reducing equivalents of dextrinous products as the criterion of purity. In most cases the rotation and reducing equivalent are the only properties of the dextrins which can be

conveniently measured. It is thus necessary to base many preliminary conclusions on the determination of these two properties of the dextrins, taking cognizance of the possible unreliability of the measurements.

The production of a considerable quantity of a highly insoluble polysaccharide as one of the products of digestion of corn starch by α-amylase was not expected. However, in all corn starch digestions this material was obtained in about five per cent yields. The isolation and dehydration was difficult, the material tending to retain large quantities of the suspending medium. For example, in Experiment II the water-insoluble residue (55.5 grams) formed a heavy paste with two liters of absolute alcohol. After filtering for 12 hours the residue remained as a gummy mass. At no stage did the material fail to retain a large quantity of solvent. This ability to retain the liquid was no longer evident after the material was dried under atmospheric conditions. The finely ground product appears to be insoluble in cold and hot water, and only slightly soluble in boiling 2% NaOH. In one experiment 0.2 gram of the material was boiled for 30 minutes in 2% NaOH. No perceptible amount of the solid was dissolved by this treatment. After cooling, the suspension was filtered and the optical rotation of the solution measured. A slight positive rotation was observed. Because of the erratic behavior of individual solutions

and the entire lack of reproducibility of the solutions no calculations of specific rotation are recorded for the water-insoluble material.

The reducing equivalent of the water-insoluble fractions varied from preparation to preparation, but good checks were obtained in the determinations from any one preparation. The major portion of the reducing power in these insoluble materials probably was due to small amounts of the water-soluble dextrins which contaminated the preparations.

The water-insoluble fraction isolated from the α-amylase digestions of corn starch resembles the insoluble material which forms in β-amylase digestions. However, the α-amylase product does not flocculate, is more insoluble and is obtained in higher yields than is the β-amylase product. The water-insoluble product isolated from β-amylase digestions of various starches has been investigated by Martin (10), Martin, Naylor and Hixon (12) and Caldwell (13). Similar investigations of the α-amylase product are needed to point out the similarities and differences between the two water-insoluble materials.

The digestion of waxy maize starch by α-amylase yields only a very small quantity of water-insoluble material. The quantity of the material and its physical appearance are such as to indicate that it is entirely extraneous material which was present in the original starch. This absence of a

water-insoluble product in waxy maize digestions indicates a constitutional difference between the waxy maize and the corn starch. A further difference between the two starches is indicated by the greater fractionability of the waxy maize dextrins. The fact that a representative of each of the three types of waxy maize dextrins was isolated from all digestions and that further fractionation had little effect on the physical properties of each type indicates that definite limit dextrins may be present. This suggests that waxy maize starch should be utilized as a substrate for α-amylase digestions in further studies of the amylolytic cleavage products.

The four corn and the three waxy maize dextrins selected as typical fractions were subjected to physical and chemical measurements. Table XX presents a summary of the molecular weights and chain lengths of the dextrins as calculated from the reducing equivalent, the iodine and potassium hydroxide consumed in oxidation by the Kline and Acree method for aldose sugars, freezing point depressions of the dextrins and freezing point depressions of the dextrin acetates.

With the exception of waxy maize dextrin $VIII_3$ rather good agreement was obtained between the molecular weights calculated from the reducing equivalent, and iodine and potassium hydroxide consumption. Although the two methods are quite dissimilar they both involve a reaction of the reducing groups present in the dextrin molecule. The reducing

TABLE XX

Molecular weights and chain length of dextrins as calculated from physical and chemical measurements.

| Dextrin No. | Mol. wt. calculated from Red. equiv. | $I_2$ | KOH | Fr. pt. | Acetate[1] | Glucose units calculated from Red. equiv. | $I_2$ | KOH | Fr. pt. | Acetate[1] | Ratios of $I_2$/ red. | KOH/ red. | KOH/ $I_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| <u>Corn</u> | | | | | | | | | | | | | |
| I | 4100 | 4340 | ---- | ---- | ---- | 25.2 | 26.7 | --- | --- | --- | 1.06 | --- | --- |
| II | 5710 | 5725 | 5985 | 1003 | 7505 | 35.2 | 35.1 | 36.8 | 6.2 | 25.9 | 1.00 | 1.05 | 1.36 |
| V | 738 | 990 | 1002 | 333 | 1563 | 4.5 | 6.0 | 6.1 | 2.0 | 5.0 | 1.34 | 1.35 | 1.44 |
| VI | 630 | 776 | 786 | 249 | 824 | 3.8 | 4.6 | 4.7 | 1.5 | 2.5 | 1.22 | 1.25 | 1.47 |
| <u>Waxy maize</u> | | | | | | | | | | | | | |
| $VIII_1$ | 3000 | 3425 | 3842 | 612 | 2570 | 18.4 | 21.0 | 23.6 | 3.8 | 8.6 | 1.14 | 1.28 | 1.34 |
| $VIII_3$ | 1450 | 1136 | 1993 | 824 | 1737 | 8.8 | 6.9 | 12.2 | 5.1 | 5.7 | 0.78 | 1.37 | 0.86 |
| $VIII_4$ | 683 | 868 | 888 | 548 | 1280 | 4.1 | 5.2 | 5.3 | 3.4 | 4.1 | 1.27 | 1.28 | 1.46 |

1. The molecular weights were calculated from the freezing point depressions. The $CH_3CO$ content of the acetates were: II, 43.1%; V, 45.7%; VI, 46.8%; $VIII_1$, 50.0%; $VIII_3$, 44.4%; $VIII_4$, 45.8%. The molecular weights presented are for the acetate and not the dextrins.

equivalent method measures all reducing groups present while the $I_2$-KOH titration is specific for the aldehyde radical. The method specific for aldose sugars always gives molecular weights equal to or slightly greater than the molecular weights calculated from the method which measures the total reducing groups present. Even though the accuracy of the method may be questioned on theoretical grounds, this fact indicates that at least a portion of the groups sensitive to oxidation by the alkaline-ferricyanide method are not oxidized by the $I_2$-KOH method which is specific for aldose sugars.

The low molecular weight calculated for dextrin $VIII_2$ from the iodine consumption in the oxidation procedure indicates that the dextrin has some anomalous characteristic. This is further indicated by the ratios between the molecular weights calculated from the iodine consumption and the reducing equivalent, the potassium hydroxide consumption and the reducing equivalent, and the potassium hydroxide and iodine consumption which are given in Table XX. These calculations indicate the relationship between the methods and show that the variance in the molecular weight of dextrin $VIII_2$ is due to an abnormal consumption of iodine. For this dextrin the potassium hydroxide/reducing equivalent ratio is in order while the two ratios involving iodine consumption are out of line. The three values reported in Table XVI for this oxidation were obtained

at different times to determine whether the variance was due to errors in manipulative technique or to some characteristic of the dextrin. The excellent agreement of the results shows that the variance is due to some property of the dextrin and not to manipulative errors.

The potassium salt of the dextrinic acid isolated from the oxidation of corn dextrin V was analyzed for potassium content. From the potassium analysis the calculated values for the molecular weight of the salt were 913 and 901, which give corresponding values of 875 and 863 for the molecular weights of the original dextrin. This is in agreement with the value of 880 calculated from the iodine consumed in the oxidation. This indicates that the aldehyde groups in the dextrin were oxidized to the corresponding acid, the acid then being neutralized by the potassium hydroxide to give the potassium salt.

The molecular weights of the dextrins as calculated from the freezing-point depression of the dextrins in aqueous solution are much below the values obtained from the other data. The abnormal freezing-point depression of dextrins and similar polyhydroxy compounds are the rule rather than the exception. An adequate explanation of this abnormality has not been promulgated. However, the values have been determined for the corn and waxy maize dextrins and are presented in Table XX.

To give further evidence as to the molecular size of the dextrins the various samples were acetylated and the freezing-point depression of the acetates in benzene determined. The molecular weights of the acetates calculated from these data are presented in Table XX. The corresponding chain lengths are intermediate between the chain lengths calculated from the freezing-point depressions of the dextrins, and from the reducing equivalent and $I_2$-KOH oxidation of the dextrins.

The corn dextrins from Experiment II were also acetylated and the molecular weight calculated from the acetyl content. (Table XV). This method of calculation is applicable only with molecules containing six or less glucose units. With longer chains the experimental error in the determination of acetyl content corresponds to such a large change in the theoretical chain length that the method becomes worthless. With the shorter-chain dextrins of this experiment good agreement is obtained between the chain length calculated from the reducing equivalent and from the acetyl content. In conjunction with the specific rotations of these fractions (Table IX), these data show that the corn dextrins obtained in Experiment II consist of smaller molecules than the corn dextrins of Experiment I.

The yield of corn dextrin V of Experiment II was about 2.5%. The optical rotation and reducing power of the dextrin are very similar to those recorded for maltose. In addition,

the acetyl content of the acetate corresponds to a molecule between a mono- and disaccharide. However, attempts to crystallize the dextrin and the dextrin acetates were unsuccessful. The melting point of the crude acetate is recorded in Table XVIII as 87-89° C. Reprecipitation of the acetate from diethyl ether by the addition of hexane gave a final product melting at 94-96° C. This melting point corresponds to 159-160° C. as the melting point of maltose-octaacetate. These observations show that although some of the properties of this dextrin fraction indicate that it is similar to maltose other properties definitely establish that it is not maltose.

## Summary

1. Two procedures for the isolation of the degradation products formed from corn starch by the action of α-amylase are described. The combined effect of the higher enzyme concentration and longer digestion time used in the second procedure was the production of dextrins with lower specific rotation and higher reducing equivalents. The yield, specific rotation and reducing power of all fractions are given.

2. The procedure for the isolation of the degradation products of waxy maize starch is given. The yields and properties of all fractions are recorded.

3. The waxy maize dextrins were found to be more easily fractionated than the corn dextrins.

4. The molecular weightsof the dextrins were calculated from the freezing-point depressions of the dextrins, the freezing-point depressions of the acetates, the reducing equivalent of the dextrins and the Kline and Acree method for the determination of aldose sugars as applied to the dextrins.

5. The potassium salt of the acid formed by the $I_2$-KOH oxidation of one of the dextrins was isolated and analyzed.

6. The melting-points of the dextrins and the dextrin acetates were determined. Such determinations were found to be of value in identification of the acetates.

7. The reaction of phenylhydrazine with the reducing-group of the dextrin molecules was investigated. Chemical combination was not obtained.

## ACKNOWLEDGMENTS

The author wishes to thank Prof. Nellie M. Naylor and Prof. R. M. Hixon for their suggestions and friendly criticisms throughout this investigation.

He also wishes to express his appreciation to Dr. Vera D. Martin and Mr. F. F. Farley for their cooperation in some phases of these studies.

Acknowledgment is also made for grants from the Industrial Science Research funds of Iowa State College and from the Iowa Agricultural Experiment Station.

## LITERATURE CITED


1. Orestano and Zummo, Boll. soc. ital. biol. sper. 5, 246 (1930)
2. Gore and Józsa, Ind. Eng. Chem. 24, 102 (1932)
3. Orestano, Arch. farmacol. sper. 56, 383 (1933); Chem. Abst. 28, 1721 (1934)
4. Teller, J. Biol. Chem. 114, 425 (1936)
5. Newton and Naylor, Cereal Chem. 16, 71 (1939)
6. Artom and Orestano, Bull. soc. chim. bio. 13, 516 (1931)
7. Schultz and Landis, J. Am. Chem. Soc. 54, 211 (1932)
8. Martin and Newton, Cereal Chem. 15, 456 (1938)
9. Newton, Farley and Naylor, Cereal Chem. 17, 342 (1940)
10. Martin, Doctoral Dissertation, Ia. State Coll. Library, Ames, Iowa (1938)
11. Denny, J. Assoc. Official Agr. Chem. 6, 175 (1922)
12. Martin, Naylor and Hixon, Cereal Chem. 16, 565 (1939)
13. Caldwell, Doctoral Dissertation, Ia. State Coll. Library, Ames, Iowa (1940)
14. Beckmann and Landis, J. Am. Chem. Soc. 61, 1504 (1939)
15. Schoene, Fulmer and Underkofler, Ind. Eng. Chem. 32, 544 (1940)
16. Payen and Persoz, Ann. Chem. Phys. 53, 73 (1833); cited by Bayliss, Principles of General Physiology, 1915, p. 307
17. Dubrunfaut, Compt. rendus 66, 274 (1868)
18. Gorup-Besanez, Ber. 7, 1478 (1874)
19. Osborne, J. Am. Chem. Soc. 17, 587 (1895)
20. Loew, Ber. 20, 58 (1887)

21. Michaelis and Ehrenreich, Biochem. Z. 10, 283 (1908)

22. Peters, J. Biol. Chem. 5, 367 (1908)

23. Holmbergh, Biochem. Z. 258, 134 (1933); 266, 203 (1933)

24. Caldwell and Doebbeling, J. Biol. Chem. 98, 553 (1932)

25. Sherman, Caldwell and Adams, J. Biol. Chem. 88, 295 (1930)

26. Kitano, J. Soc. Chem. Ind., Japan 39, Suppl. binding, 163, 164, 165 (1936)

27. Hemmi and Inami, J. Agr. Chem. Soc. Japan 5, 660 (1929); Chem. Abst. 24, 1655 (1930)

28. Waldschmidt-Leitz and Mayer, Z. physiol. Chem. 236, 168 (1935)

29. Hemmi and Inami, J. Agr. Chem. Soc. Japan 7, 133 (1931); Chem. Abst. 7, 133 (1931)

30. Holmbergh, Arkiv Kemi, Mineral Geol. 11A, No. 20 (1935)

31. Waldschmidt-Leitz and Zeigler, U. S. Patent 2,121,549, June 21 (1938)

32. Young and Hartman, Proc. Indiana Acad. Sci. 48, 79 (1939)

33. Myrbäck, Enzymologia 1, 280 (1936)

34. Myrbäck and Myrbäck, Biochem. Z. 258, 158 (1933)

35. Sallans and Anderson, Can. J. Research 16C, 405 (1938)

36. Sallans and Anderson, Can. J. Research 17C, 361 (1939)

37. Bray and Naylor, Unpublished work. This laboratory (1935)

38. Naylor and Dawson, Ia. State Coll. J. Sci. 10, 267 (1936)

39. Iwasa, J. Agr. Chem. Soc. Japan 13, 225 (1937); Chem. Abst. 31, 5607 (1937)

40. Blish, Sandstedt and Mecham, Cereal Chem. 14, 328 (1937)

41. Hixon and Martin, Ind. Eng. Chem., Anal. Ed. 11, 395 (1939)

42. Sherman and Neun, J. Am. Chem. Soc. 41, 1855 (1919)

43. Klinkenberg, Z. physiol. Chem. 212, 173 (1932)

44. Hanes, Can. J. Research 13B, 185 (1935)

45. Freeman and Hopkins, Biochem. J. 30, 446 (1936)

46. Liebermann, Ber. 11, 1619 (1878)

47. Tollens-Elsner, Kurzes Handbuch der Kohlenhydrate, Johann Barth, Leipzig, 1935.

48. Hudson and Johnson, J. Am. Chem. Soc. 37, 1276 (1915)

49. Brown and Morris, J. Chem. Soc. 67, 709 (1895)

50. Kuhn, Ann. 443, 1 (1925)

51. Ohlsson, Z. physiol. Chem. 189, 17 (1930)

52. Klinkenberg, Proc. Acad. Sci. Amsterdam 34, 893 (1931)

53. Wijsman, Rec. trav. chim. 9, 1 (1890)

54. Giri, J. Indian Inst. Sci. 17A, 127 (1934); Chem. Abst. 29, 5139 (1935)

55. Samec, Kolloidchemie der Stärke, Steinkopff, Dresden, 1927, p. 314

56. Wohlgemuth, Biochem. Z. 9, 1 (1908)

57. Creighton and Naylor, Ia. State Coll. J. Sci. 7, 253 (1933)

58. Sherman and Schlesinger, J. Am. Chem. Soc. 35, 1784 (1913)

59. Oparin and Manskaja, Biochem. Z. 260, 170 (1933)

60. Oparin, Manskaja and Magaram, Biochem. Z. 265, 21 (1933)

61. Ohlsson, Compt. rend. trav. lab. Carlsberg 16, 1 (1926)

62. Sherman and Tanberg, J. Am. Chem. Soc. 38, 1638 (1916)

63. Sherman and Schlesinger, J. Am. Chem. Soc. 34, 1104 (1912)

64. Sherman and Schlesinger, J. Am. Chem. Soc. 37, 1305 (1915)

65. Ono, J. Agr. Chem. Soc. Japan 12, 191 (1936); Chem. Abst. 30, 6771 (1936)

66. Yamagishi, J. Agr. Chem. Soc. Japan 13, 1275 (1937); Chem. Abst. 32, 4614 (1938)

67. Patwardhan, *J. Indian Inst. Sci.* *A12*, 165 (1929); *Chem. Abst.* *24*, 113 (1930)

68. Bulbrook, Thesis, Ia. State Coll. Library, Ames, Iowa (1928)

69. Kneen and Sandstedt, *Cereal Chem.* *18*, 237 (1941)

70. Wijsman, *Rec. trav. chim.* *9*, 5 (1890)

71. Sabalitschka and Weidlich, *Biochem. Z.* *210*, 414 (1929)

72. Waldschmidt-Leitz, *Enzyme Actions and Properties* (translation by Walton), John Wiley and Sons, New York, 1929, p. 204.

73. Baker, *J. Chem. Soc.* *81*, 1177 (1902)

74. Syniewski, *Ann.* *441*, 285 (1925)

75. Blom, Bak and Braae, *Z. physiol. Chem.* *241*, 273 (1936)

76. Baker and Day, *Analyst* *33*, 393 (1911)

77. Harding, *Sugar* *25*, 350 (1923)

78. Brown and Millar, *J. Chem. Soc.* *75*, 286 (1899)

79. Myrbäck, *Biochem. Z.* *285*, 290 (1936)

80. Örtenblad and Myrbäck, *Svensk. Kem. Tid.* *50*, 72, 168 (1938)

81. Somogyi, *J. Biol. Chem.* *134*, 301 (1940)

82. Somogyi, *J. Biol. Chem.* *119*, 741 (1937)

83. Sherman and Walker, *J. Am. Chem. Soc.* *39*, 1476 (1917)

84. Pringsheim and Schapiro, *Ber.* *59*, 996 (1926)

85. Gottschalk, *Z. physiol. Chem.* *152*, 132 (1926)

86. Lohmann, *Biochem. Z.* *178*, 444 (1926)

87. Blitz, *Ber.* *46*, 1532 (1919)

88. Fernbach and Wolff, *Compt. rend.* *139*, 1217 (1904)

89. Sallinger, *Kolloid Z.* *25*, 79 (1919)

90. Ling and Nanji, J. Chem. Soc. 127, 636 (1925)

91. Chrzaszcz, Z. Nahr. Genussem. 48, 306 (1924)

92. Sherman and Punnet, J. Am. Chem. Soc. 38, 1877 (1916)

93. Clayson and Schruyver, Biochem. J. 17, 493 (1923)

94. Schruyver and Thomas, Biochem. J. 17, 447 (1923)

95. Malloch, Can. J. Research 1, 111 (1929)

96. Hermano and Rask, Cereal Chem. 3, 361 (1926)

97. Taylor and Walton, J. Am. Chem. Soc. 51, 3431 (1929)

98. Taylor and Sherman, J. Am. Chem. Soc. 55, 258 (1933)

99. Taylor and Kereeztesy, Ind. Eng. Chem. 28, 502 (1936)

100. Taylor and Nelson, J. Am. Chem. Soc. 42, 1726 (1920)

101. Lehrman and Kabat, J. Am. Chem. Soc. 55, 850 (1933)

102. Ling and Nanji, J. Chem. Soc. 123, 2666 (1923)

103. Samec, Z. physiol. Chem. 236, 103 (1935)

104. Haworth, Hirst and Waine, J. Chem. Soc. 1935, 1299 (1935)

105. Haworth, Hirst, Kitchen and Peat, J. Chem. Soc. 1937, 791 (1937)

106. Hopkins, Cope and Green, J. Inst. Brew. 39, No. 8, 487 (1933)

107. Hanes, New Phytologist 36, 101, 189 (1937)

108. Samec and Blinc, Kolloid-Beihefte 49, 75 (1939)

109. Freeman and Hopkins, Biochem. J. 30, 442 (1936)

110. Stamberg and Bailey, J. Biol. Chem. 126, 479 (1938)

111. Blom, Braae and Bak, Z. physiol. Chem. 252, 261 (1938)

112. Myrbäck, Skand. Arch. Physiol. 77, 58 (1937)

113. Myrbäck, Biochem. Z. 304, 147 (1940)

114. Myrbäck, Örtenblad and Ahlborg, Enzymologia 3, 210 (1937)

115. Dammann, Ber. 71B, 1865 (1938)

116. Vonk and Braak, Proc. Acad. Sci. Amsterdam 27, 188 (1934)

117. Klinkenberg, Z. physiol. Chem. 209, 253 (1932)

118. Ahlborg and Myrback, Biochem. Z. 297, 172 (1938)

119. Myrbäck, Svenska Bryggarefören. Månadsbl. 53, 93 (1938); Chem. Abst. 34, 3542 (1940)

120. Ortenblad and Myrback, Biochem. Z. 303, 335 (1940)

121. Waldschmidt-Leitz and Reichel, Z. physiol. Chem. 223, 76 (1934)

122. Beckmann, Z. physik. Chem. 2, 683 (1888)

123. Kline and Acree, Ind. Eng. Chem., Anal. Ed. 2, 413 (1930)

124. Bergmann and Machemer, Ber. 63, 316 (1930)