[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE CORN PRODUCTS REFINING COMPANY]

On the Significance of the Degradation of Starch by Macerans Amylase[1]

BY RALPH W. KERR

General Discussion

In 1905 Schardinger[1a] made known a new type of starch degradation by the growth of *Bacillus Macerans* upon starch pastes, which yielded products called by him "crystallized dextrins." Since then considerable speculation has arisen from time to time as to what these products are, the mechanism of their formation and the significance of the reaction in respect to the chemistry of the starches.

From the many studies on this subject during the next two decades, particularly the work of Pringsheim,[2] the general conclusion appears to have been drawn that the structure of these dextrins does not occur in the starch molecule, since they are not attacked by the more common starch splitting enzymes.[3]

Recently, interest has again been aroused in this study by the work of Tilden and Hudson,[4] who were the first to show that the so-called Schardinger dextrins could be produced directly from starch by an enzyme preparation whereas previous workers had obtained these dextrins only from starch solutions on which the bacilli Macerans were grown. Interest has also been revived by the work of Freudenberg on the constitution of the dextrins and his speculations on the existence of these configurations in the parent starch.[5]

One of the purposes of the present work is to throw some light on the questions referred to above. The main purpose is to point out the usefulness of the reaction in which starch is degraded in the presence of the Macerans enzyme, in showing certain differences in the composition and possibly constitution of the starches. This work is to further clarify our viewpoint[6,7,8] that the starches are composed of many diverse units.

The Separation and Significance of an Insoluble Fraction from Corn Starch.—In this study, the interest was primarily in corn starch and a conversion procedure was developed accordingly, so that substantial yields of the dextrins are obtained from this source.

If corn starch is alkali gelatinized by thoroughly dispergating the dioxane-extracted starch in dilute alkali at room temperature, neutralized, the paste converted with Macerans amylase, then with suitable conditions of dilution, *p*H, temperature and time, as given in the Experimental Section, it was observed that the large mass of insolubles resulting through the conversion of corn starch agglutinate sufficiently to be centrifuged out of the solution.

After reconverting with a fresh supply of enzyme, these insolubles are again centrifuged, purified by washing, dissolved in alkali, reprecipitated by acid, and finally dried. 9.89% of the weight of corn starch, or about 10%, was isolated as an insoluble amylose by these procedures. It has recently been called to our attention, however,[9] that the quantity of this precipitable material may vary with the amount of fat present in the starch. In respect to superficial properties, at least, this product is the same as that which we have designated as gamma-amylose.[6] If future work on gamma-amylose should show that the two products are identical, then the use of Macerans enzyme would be a more efficient method of isolating the product, than the use of barley diastase; the latter is known to solubilize some of the product in the course of the preparation procedures.

The behavior of potato starch was simultaneously determined by the same conversion procedures used for dioxane-extracted corn starch. After the conversion, however, it was noted that the potato starch liquors were practically clear, indeed as free of suspended material as they were immediately after gelatinization and neutralization, while the corn starch was clear only in alkaline solution. In addition, it was noted that the more highly viscous potato starch lost

(1) Presented at the 102nd meeting of the American Chemical Society, Division of Sugar Chemistry, September 9, 1941.

(1a) F. Schardinger, *Zentr. Bakt. Parasitenk.*, **14**, 772 (1905).

(2) H. Pringsheim, in Walton, "Survey of Starch Chemistry," Chem. Cat. Co., New York, N. Y., 1928.

(3) H. Pringsheim and F. Kissler, *Ber.*, **46**, 2959 (1913).

(4) E. B. Tilden and C. S. Hudson, THIS JOURNAL, **61**, 2900 (1939).

(5) K. Freudenberg, *Ann. Rev. Biochem.*, **8**, 81 (1939).

(6) R. W. Kerr and O. R. Trubell, *Cereal Chem.*, **18**, 530 (1941).

(7) R. W. Kerr, O. R. Trubell and C. M. Severson, *ibid.*, **19**, 64 (1942).

(8) R. W. Kerr and N. F. Schink, *Ind. Eng. Chem.*, **33**, 1418 (1941).

(9) T. J. Schoch, E. J. Wilson, Jr., and C. S. Hudson, private communication.

viscosity at a much more rapid rate after adding the enzyme than did the corn starch; this indicates in the latter instance the presence of some component, at least, which was not converting.

The small amount of insolubles in the potato conversion were filtered off and washed. After drying, a yield of 0.434 g. was obtained from 100 g. of potato starch, dry basis, which amounts to only 0.43%.

By this independent method, therefore, our conclusion[6] is confirmed that there exists a component amylose in corn starch which is lacking in potato starch. If this less soluble material is an enzyme-synthesized product and if starches are composed of the same basic types of molecules, then it seems reasonable to conclude that potato starch, under the same conditions of test, should yield the same less-soluble product. If it is a product of retrogradation, then an answer should be forthcoming to the question of why the corn starch molecule is more disposed to retrograde than the potato starch molecule, especially using conditions where retrogradation effects are near a minimum, as evidenced by the lack of formation of insolubles in the case of potato starch. However, no answer occurs to us which deserves serious thought, except that corn starch differs from potato starch in chemical constitution, in respect to one component at least.

The Separation and Significance of Yields of the Crystalline Dextrins.—After the initial forty-eight hours conversion referred to above, the clarified liquors from both the corn and the potato starches, to which have been added fresh supplies of enzyme, gradually begin to precipitate small quantities of additional insolubles. These, however, are non-gelatinous, being more granular in appearance. After twenty-four hours additional conversion, a yield of about 0.3% insolubles was obtained in both conversions. This material is possibly the "Schlamm" referred to by Schardinger. The amounts, however, were too small to encourage further work with it.

The reconverted liquors, clarified by filtration, were concentrated and the crystalline dextrins separated by well-known procedures detailed in the Experimental Section, using trichloroethylene as the precipitant.

The yield of the mixed dextrins and the distribution between the alpha variety and the beta variety for the two starches are given in Table I.

TABLE I

YIELD OF DEXTRINS

	Mixed dextrins		Ratio × 100 Beta/Alpha
Corn starch	25.3	25.2	26
Potato starch	30.6	30.6	28

The above yields are quite likely not the maximum obtainable inasmuch as substantially higher yields have been reported for potato starch.[4] However, our procedures consistently have given yields of 25% from corn starch and these procedures give sufficiently higher yields from potato starch to permit us to conclude that a difference in composition exists between the two starches in addition to that already observed in respect to gamma amylose content. The distribution obtained between the two types of dextrins may indicate a similarity in that fraction from which the dextrins originate.

To shed further light on these questions the hereinafter described experiments were run.

Origin of the Crystalline Dextrins.—One interpretation of our results on distribution might be that they indicate a constant ratio of the amylose–amylopectin components for the two starches. This is in accordance with Pringsheim, who at one time proposed[2] that amylose gives rise to what he termed the alpha series of polyamyloses (dextrins) and amylopectin, the beta series.

We are reluctant to accept this conclusion, at least as stated in this form, because, first, it is our belief that amylose and amylopectin are only hypothetical substances, the concept of Maquenne being probably an over-simplification of the composition of the starches[7]; second, if they do exist as ideal entities, our results have shown that the ratio of this amylopectin to amylose is higher in both starches than the ratio of beta to alpha dextrin, now obtained; and, third, that even the highest yields reported for these products[4] are too low to justify the conclusion that amylose contains essentially configurations which convert into alpha dextrins, or that amylopectin is made up essentially of beta dextrin nuclei.

Moreover, amylose (as contrasted to amylopectin)[9a] is converted completely to maltose with diastase, whereas the experiment next described shows that both Schardinger dextrins are quite resistant to barley diastase. A sample of mixed Schardinger dextrins, separated in a run on corn starch, was treated with barley diastase in the conversion procedures used by us[7] for estimating

(9a) L. Maquenne and E. Roux, *Compt. rend.*, **140**, 1308 (1905).

the limit of conversion of the starches and starch fractions. Using the increase in reduction of alkaline ferricyanide as a quantitative index of conversion, no conversion took place. Hence, neither dextrin is a true unit of hypothetical amylose.

However, the possibility exists that the dextrins represent the residues of one or more resistant starch components, of which the Schardinger alpha dextrin may be the resistant residue of one, and the beta dextrin of another, or they might represent parts of the same fraction. Thus, should a relationship be shown between the constitution of the Schardinger dextrins and the limit dextrins, then the argument that the former were not true units of starch, because they are not converted to maltose by the diastases, would carry very much less weight. The above possibility appears even more attractive when it is recalled that Pringsheim[2] reported that the Schardinger dextrins are split directly to dextrose by certain fungus amylases, and also since the observation has been made[8] that the limit dextrins are so split with the same type of enzyme.

Accordingly, corn limit dextrin, prepared by procedures given in the Experimental Section, by a barley diastase conversion, was redissolved in water and converted by the method for converting corn and potato starches with the *B. macerans* enzyme preparation. However, no Schardinger dextrins of either type were obtained.

To clarify the discussion which follows it should be pointed out that beta-amylase limit dextrin shows a higher order of molecular magnitude, than alpha-amylase limit dextrins or the average magnitude of limit dextrins resulting from the use of more complex enzyme preparations[10,11] and certainly higher than the molecular magnitude which has been estimated for the Schardinger dextrins.[2,5]

One would expect, then, to obtain the product of lesser magnitude from that of larger magnitude by further degradation of the latter, unless the limit dextrins are synthetic products, not true units of starch. This possibility has not been seriously advocated but, nevertheless, cannot be overlooked. Otherwise, it would seem that the resistant Schardinger dextrins are synthesized from other components of starch, once nonresistant to the action of diastase.

(10) K. Myrbäck, *Biochem. Z.*, **297**, 160 (1938).
(11) C. S. Hanes, *New Phytologist*, **36**, 101, 189 (1937).

To show that the latter explanation is the more probable one, the following starch products were treated with Macerans enzyme as previously described. Three stages in the acid hydrolysis of starch were selected: first, what is commercially known as a 40 fluidity, thin boiling corn starch, which represents the initial stages of acid degradation. (The hydrolysis of primary glucosidic bonds resulting in this product is questionable); second, what is known commercially as a 90 fluidity corn starch, which is essentially a wet dextrinization. The viscosity of the starch has all but disappeared at this point and some reducing substances are in evidence in the 30% fraction of this product which is now cold water soluble; and lastly, an extensive acid conversion of starch where the reducing substances by Fehling test, estimated as dextrose, amount to approximately 40% of the original starch.

These products were treated and converted with the Macerans enzyme in the same manner as the parent starch, except the sirup sample, which was not alkali dispersed, but merely dissolved in water.

Incidentally, it was noted that from the first sample, substantially the same order of yield of insolubles (which we have termed gamma-amylose) was obtained as from parent starch, in this experiment 9.74%. The intermediate sample gave a somewhat reduced yield of 6.41%, and in the most extensively acid converted sample, the gamma-amylose, as such, had all but disappeared. A small quantity, together with extraneous impurities, was removed before adding the enzyme.

The yield of crystalline dextrins, on the other hand, decreased sharply, even with the first sample which gave a yield of only 8.1%. From the 90 fluidity starch only traces were obtained and from the most extensive acid conversion none whatsoever were obtained by our procedures.

It should be recalled at this point that we have shown[8] that in an extensive acid conversion of corn starch, represented by our third sample, the quantity of residues resistant to diastase is still the same as it is in parent starch; that is, the resistant configurations of the limit dextrins are disrupted in the last stages of acid hydrolysis.

Therefore, the conclusion is that the Schardinger dextrins are formed by the enzyme from components of the starches which are most readily modified by acid treatment (and which are, also,

originally convertible by the diastases). It may be that the original configurations present in these components are so closely related to those of the Schardinger dextrins that one may be transformed into the other with little chemical change. This view has been suggested by Tilden and Hudson,[4] who state, "we infer . . . that the crystalline dextrins are components of the starch structure, or are closely related to such components."

While the above conclusion is as yet difficult to prove directly, further thought is being given to it in this Laboratory in the isolation of starch components, and the use of the Macerans enzyme reaction to characterize these components, when isolated.

Some starch fractions present certain difficulties in utilizing the reactions, as for example, gamma-amylose, which is relatively insoluble at the *p*H and temperature at which the enzyme is active; in this state it is apparently even more resistant to the Macerans enzyme than to beta amylase. One fraction, however, which seems to be very well suited to the proposed method of inspection is our preparation of that part of gelatinized corn starch which is more soluble in alcohol–water mixtures.[7] This fraction seems to be distinguished from the remainder of corn starch, and closely allied to the bulk of non-cereal starch, by its high colloidal dispersibility and its low alkali lability. This fraction amounts to 55% of the weight of corn starch. It dissolves in hot water, and hence in preparing this fraction for enzyme conversion, an alkali dispersion was not made. The conversion with Macerans enzyme was performed and the dextrins separated in the manner mentioned above. Only the faintest traces of insolubles resulted from the initial phase of the conversion showing, incidentally, that this fraction is substantially free from gamma-amylose.

A surprisingly large yield of 43.6% crystalline dextrins was obtained, however, sufficient to indicate that the dextrins originate from the simpler configurations present in this fraction. For if we multiply the percentage yield of crystalline dextrins found (43.8%) by 55.6% (which is the amount of this amylose fraction we find in corn starch) we obtain 24.3%. This is approximately the total percentage yield of Schardinger dextrins obtained directly from whole corn starch by the procedures outlined by us. These results should not necessarily be interpreted to mean that it is impossible to produce Schardinger dextrins from other fractions or that the total yield from whole corn could not be increased above 25% by some special procedures. The results show, however, that by using the method outlined in the Experimental Section, the yield of dextrins from whole starch is obtained almost entirely from the more colloidally stable fraction mentioned.

Two diverse inferences may be drawn from the above result: first, that the more alcohol-soluble fraction of corn starch may be a mixture of two or more component amyloses. This explanation would account for the fact that while the Schardinger dextrins appear to originate principally from the fraction separated by us, it is known that a considerable share of the limit dextrins of corn starch also originate from this fraction as well.[7]

An alternative explanation would be that a definite component had been separated by us in our 55% fraction, a part of the chemical configuration of which may be changed into the Schardinger dextrins and the residue of which becomes a limit dextrin during a diastatic conversion.

Further attempts to fraction this component, using the Macerans enzyme reaction as a guide, should aid in solving the question involved.

Experimental Section

Preparation of Macerans Amylase.—The procedures are essentially those of Hudson and his co-workers.

To a kilo of peeled and sliced potatoes, 100 g. of calcium carbonate in a slurry of 10 liters of water is added and the mass cooked for an hour at 20 lb. steam pressure. After cooling, the mash is inoculated, under sterile conditions, with a culture of *B. macerans* and incubated for about three weeks at 100 °F.

The digestion is cooled, the supernatant liquor siphoned off, clarified by filtration and precipitated by adding acetone to a concentration of 33% by volume. The mixture is refrigerated for several hours, then centrifuged.

The precipitate is dissolved in a liter of water and stored in the refrigerator, adding thymol as a preservative.

The converting power is determined by noting the time in minutes required for 1 cc. of the enzyme solution to convert 1 cc. of a 3% potato starch paste at 40°. The end-point is where the addition of one drop of 0.1 *N* iodine to three drops of the conversion liquors on a microscope slide shows a fringe of anisotropic dextrin-iodide complex crystals completely around the edge of the smear as it evaporates. The result is divided by 30.

The converting power of the preparation used in these experiments was 0.3.

The Conversion of Starch with Macerans Amylase.—100 grams of starch, on a dry basis, is moistened with 333 cc. of water, and with constant stirring at 25°, 667 cc. of *N*

sodium hydroxide is stirred in. After thirty minutes the paste becomes nearly clear, only the faintest turbidity remaining. 500 cc. of dilute hydrochloric acid is then slowly stirred in. The acid is of sufficient strength to bring the *p*H of the paste to approximately *p*H 6.0.

200 cc. of a Macerans enzyme preparation of unity converting power is now added, or for example, 80 cc. if the converting power is 0.40, 60 cc. if it is 0.30, etc. The *p*H is adjusted to 6.0. The mixture is then converted at 45° for forty-eight hours, adding a small amount of thymol as a preservative.

After forty-eight hours the *p*H is adjusted to 3.8 to 4.0 with hydrochloric acid, allowed to cool for about an hour, and the insolubles removed by centrifuging.

With corn starch, the insolubles, which form an appreciable fraction, are resuspended in about 800 cc. of water, adjusted to *p*H 6.0, and 16.7 cc. more of enzyme of unity converting power is added. A like quantity of fresh enzyme is added to the centrifugate after adjusting to *p*H 6.0, and both fractions are converted twenty-four hours more at 45°. With potato starch in the second part of the conversion, 33.3 cc. of enzyme of unity converting power was added to the liquors.

The insolubles are adjusted to *p*H 5.0 and, after cooling for about an hour, are centrifuged and washed several times in the centrifugate. The centrifugate and washings are added to the main conversion liquors which are clarified by filtration and evaporated to about 400 cc. at *p*H 6.0 by vacuum distillation.

Separation of Crystalline Mixed Dextrins.—The conversion liquors above are transferred quantitatively to a precipitating vessel, adjusting the volume to 500 cc., and warmed to 45°. 500 cc. of trichloroethylene is now added and the mixture stirred for an hour, after which it is allowed to cool to room temperature. With occasional stirring it is allowed to stand for forty-eight hours, after which it is refrigerated at 5° for forty-eight hours more.

While still cold the precipitated dextrins are filtered, suspended in sufficient ice-water to give a thick cream, and again filtered. Both filtrations are made with suction on a Büchner filter. The precipitate is finally washed on the filter with methanol and air dried.

Isolation of the Individual Dextrins.—For each gram of mixed dextrins, 2 cc. of water is added and the mixture brought to a boil to effect solution. A small quantity of boneblack is stirred in while the solution is still hot, filtered through a heated filter, allowed to cool, and seeded with a few crystals of beta-dextrin. After twenty-four hours at room temperature, it is refrigerated for forty-eight hours. The precipitate is filtered, washed with several small portions of ice-water and air dried.

The filtrate and washings are evaporated to a sirup (solid content of about 50% by weight). The alpha-dextrin is recovered by the addition of methanol, slowly with constant stirring as long as any immediate precipitate forms. The solution is allowed to stand at room temperature for several days, adding alcohol to compensate for that which is evaporated during the crystallization, keeping the alcohol content around 80%. This mixture is finally refrigerated at 5° for forty-eight hours with intermittent stirring. The product is then filtered, washed with 60% cold methanol, then absolute methanol, and finally air dried. For estimating comparative yields, the above procedures are deemed sufficient. For a critical inspection of the products, recrystallization of both is advisable.

Preparation of Limit Dextrin.—Two kilos of corn starch, dry basis, are made up to 32 liters of suspension and the *p*H adjusted to 6.5 with sodium hydroxide. The suspension is then brought to 90° over a period of thirty minutes and without cooling passed through a two-stage homogenizer at 4500 lb. pressure.

It is cooled quickly to room temperature, in the meantime dropping the *p*H to 4.7–4.8. As the temperature passes through the range of about 40°, 1000 cc. of barley diastase is added, then 800 cc. of toluene and the mass converted at 20–25° for four days, stirring as long as an appreciable paste viscosity remains.

The liquors are filtered on gravity filters the first time; about twenty-four hours is required for filtration. As the filtrate collects, the *p*H is adjusted to 5.5 and evaporation *in vacuo* is started. The insolubles are washed with water in the centrifuge and the washings after *p*H adjustments are added to the main conversion liquors.

When the sirup reaches about a 33% solid concentration, the dextrins are precipitated by adding alcohol to a concentration of 80% by volume. The gum is taken up in water and the volume adjusted to 1500 cc. An equal volume of absolute ethanol is slowly stirred in and, after standing for an hour, the gum is again collected and dehydrated in successive portions of absolute ethanol; 591 grams of dextrins, dry basis, was obtained, or a yield of 29.6%.

The preparation of our barley diastase has been previously described.

Preparation of Acid Converted Products from Corn Starch.—All of the products used are standard articles of commerce. The 40 fluidity starch is made by suspending raw starch in approximately 0.1 *N* sulfuric acid at 52° for eight to ten hours, neutralizing with soda ash to approximately *p*H 5.0, filtering and drying. The 90 fluidity starch is suspended in acid at approximately *p*H 2.0, filtered and the cake slowly dried and heated in the drying kiln until 20 to 40% of the starch becomes cold-water-soluble. It is then neutralized with ammonia. The sirup is the C. S. U. of commerce. The starch is acidified to approximately *p*H 2.0 with hydrochloric acid and pressure-cooked at about 30 lb. steam pressure, until the conversion liquors show a dextrose equivalent of approximately 40% using the Fehling test. It is then neutralized with soda ash to *p*H 4.5 to 5.0, clarified and decolorized with carbon and finally vacuum evaporated to a thick sirup.

Summary

The significance of the reaction involving the degradation of the starches in the presence of Macerans amylase is discussed and its usefulness pointed out.

Evidence is given to show that the formation of the diastase resistant configurations present in the Schardinger dextrins is probably a synthetic one, forming by rearrangement of the simpler configurations of starch, in contrast to the limit

dextrins which originate probably as residues from the more complex configurations.

An insoluble amylose fraction is obtained from corn starch by the Macerans enzyme conversion. Potato starch yields negligible quantities of this amylose.

The fraction of gelatinized corn starch more soluble in alcohol–water mixtures has been characterized by the high yield of Schardinger dextrins which can be obtained from it.

ARGO, ILLINOIS RECEIVED DECEMBER 18, 1941

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE CORN PRODUCTS REFINING COMPANY]

On the Multiple Amylose Concept on Starch. III. The Isolation of an Amylose in Crystalline Form

BY RALPH W. KERR AND G. M. SEVERSON

In a preceding paper[1] a method was described for fractionating a corn starch paste into two components by the addition of butanol plus methanol. The less soluble portion of the paste, some 45% of the total, was shown to differ from the more soluble portion, principally in its higher conversion limit[1] with beta-amylase and its higher alkali labile number as determined by the method of Schoch and Jensen.[2] These figures are given again for reference in Table II. From this study and from a study of the characteristics of a component amylose, provisionally called gamma-amylose,[3] it was concluded that corn starch is composed of at least three major constituent amyloses, two of them precipitating in the less soluble fraction. The purpose of the present work is to present two experiments which further confirm this conclusion.

It was shown that the relatively insoluble gamma-amylose collected with the alcohol precipitate as might be anticipated, but it was indicated that the balance of this latter fraction, probably originally soluble, would be found to have a conversion limit well above 70% and an alkali labile number slightly higher than the parent corn starch.

Direct fractionation of the less alcohol-soluble part to decide the point in question has presented certain obstacles. Leaching the product with a limited amount of water gives an unmanageable, gelatinous mass. The use of larger volumes of water, higher temperatures, higher *p*H's, etc., shows that little of this product can be redispersed unless conditions are used which lead to a solution of appreciable quantities of gamma-amylose as well. From this solution it has been impossible as yet to precipitate, fractionally, the originally more insoluble gamma-amylose. Therefore, indirect methods were investigated to answer the question.

(1) R. W. Kerr, O. R. Trubell and G. M. Severson, *Cereal Chem.*, **19**, 64 (1942).

(2) T. J. Schoch and C. Jensen, *Ind. Eng. Chem., Anal. Ed.*, **12**, 531 (1940).

(3) R. W. Kerr and O. R. Trubell, *Cereal Chem.*, **18**, 530 (1941).

Alcohol Fractionation of Potato Starch Pastes

It occurred to us, first, to investigate potato starch, wherein no gamma-amylose is found, so that if a component exists which is comparable to the third corn constituent predicted, the difficulties involved in separating gamma-amylose from this constituent would be avoided.

Accordingly, potato starch pastes were fractioned by methods employed previously on corn starch[1] with the following results

TABLE I

FRACTIONATION OF POTATO STARCH PASTES WITH ALCOHOLS

	Yield, %	Alkali no.	beta-Amylase conversion limit, %	Phosphorus, %
Main fraction sols.	73.5	5.2	56.2	0.088
First wash of insolubles	2.6			
From recrystn. of less soluble	1.8			
Less sol. fraction	22.1	12.9	89.0	.014
Parent starch		6.0	63.6	.081

As might be anticipated, the precipitated fraction is much more readily soluble than the corresponding precipitate from corn starch. Indeed, at boiling temperature, even with double the ratio of water to solids, which was used to redissolve completely the precipitated potato fraction, the corn fraction did not redissolve completely. Furthermore, the potato fraction, when redissolved, remained clear and limpid when