which is liberated gradually during the hydrolysis.

The α-D-galactosidase which is present in the brewers' yeast invertase preparations has been used in a comparative study of the hydrolysis of melibiose, α-methyl-D-galactoside, α-phenyl-D-galactoside and the configurationally related β-methyl-L-arabinoside.

The galactoside linkages in stachyose and manninotriose were hydrolyzed by the preparations from brewers' yeast which contain α-D-galactosidase but not β-D-galactosidase. Stachyose, based upon the methylation data of Onuki, can therefore be formulated completely as 6-α-D-galactopyranosido-4-α-D-galactopyranosido-2-α-D-glucopyranosido-β-D-fructofuranoside and manninotriose as 6-α-D-galactopyranosido-4-α-D-galactopyranosido-D-glucose (see formulas I and II of the text).

The purest invertase preparation, from brewers' yeast, contained a small amount of β-D-glucosidase which was capable of hydrolyzing amygdalin gentiobiose and β-phenyl-D-glucoside, but not cellobiose, or lactose.

Invertase preparations from both brewers' and bakers' yeasts contained small amounts of a new enzyme, a β-D-mannosidase which hydrolyzes β-phenyl-D-mannoside.

No evidence was obtained to indicate the hydrolysis of an α-D-fructofuranoside (isosucrose), or of any β-D-galactoside, α-D-glucoside (including the α- and β-dextrins), or α-D-mannoside by these highly purified invertase preparations. Melezitose and α-methyl-D-manno-D-gala-heptoside also were not hydrolyzed.

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[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, NATIONAL INSTITUTE OF HEALTH, U. S. PUBLIC HEALTH SERVICE, AND FROM THE RESEARCH LABORATORY OF CORN PRODUCTS REFINING COMPANY]

The Action of *macerans* Amylase on the Fractions from Starch

BY E. JUSTIN WILSON, JR., THOMAS JOHN SCHOCH AND C. S. HUDSON

A starch fractionation recently has been reported[1] which utilizes the selective precipitation of one component of the starch by normal butyl alcohol. The fractions so obtained are characterized by marked differences in alkali lability, solubility, gelation and retrogradation tendencies. On the basis of X-ray evidence, iodine adsorption and flow polarization, Rundle and Baldwin[2] suggest that the fraction so isolated by butanol precipitation represents linear chains of glucose units, and that the material not precipitated by butanol comprises molecules of highly branched character.

Tilden and Hudson[3] have investigated the conversion of starch by the enzyme derived from *B. macerans*, and have suggested that the resulting crystalline Schardinger dextrins are derived from some basic configuration pre-existing in the starch molecule. The present study was undertaken to ascertain whether the seeming differences in the chemical nature of the butanol-separated fractions would be reflected in their behavior toward *macerans* amylase.

Isolation of Starch Fractions.—Since the butanol method for starch fractionation requires use of a continuous supercentrifuge, a procedure was developed for more general application which does not necessitate such specialized equipment. By treating a hot autoclaved starch sol with a mixture of isoamyl and normal butyl alcohols, a fraction of the starch precipitates on cooling which is readily removed by low speed centrifuging; a non-precipitated fraction is recovered from the supernate by the addition of excess methanol. While this procedure is somewhat tedious and does not lend itself to the processing of large quantities of starch, it gives substantially the same fractions as are obtained by supercentrifugal separation, and it was accordingly employed for the preparation of the starch fractions used in this study.

The yields of crude precipitated fraction from four runs of potato starch averaged 24.4 ± 0.9%; the average yield from defatted corn starch (five runs) was 24.9 ± 1.4%.[4] Waxy maize gave no precipitated fraction. Where supercentrifugal equipment is available, the use of mixed butanol and isoamyl alcohol affords somewhat improved mechanical separation of the precipitated fraction; the yields are identical with those obtained by the method herein described,

(1) Schoch, THIS JOURNAL, **64**, 2957 (1942).
(2) Rundle and Baldwin, *ibid.*, in press.
(3) Tilden and Hudson, *ibid.*, **61**, 2900 (1939).
(4) All weights and percentages in this article are corrected to the dry weight of starch substance.

averaging 24.9 ± 1.0% on four 400-g. batches of defatted corn starch. While these yields are slightly higher than those obtained from corn starch by use of butanol alone (*viz.*, 22.8 ± 1.2%), no significant difference has been observed between the respective fractions as obtained by the two methods. The relative purity of the crude corn starch fractions has been evaluated by a modification of the iodometric method of Bates, French and Rundle[5] (Table I).

TABLE I

PERCENTAGE IODINE IN COMPLEX FORMATION

Precipitant	Precipitated fraction[a]	Non-precipitated fraction
Butanol	16.3	1.74
Butanol-isoamyl alcohol	16.5	1.80

[a] One recrystallization of the precipitated fraction raised its affinity for iodine to 18.2%, a second recrystallization to 18.7%.

Conversion of the Starch Fractions.—Seven starch substances were converted with *macerans* amylase, and the ultimate yield of crude Schardinger dextrins was determined by duplicate runs in each case: defatted corn starch, the precipitated and non-precipitated fractions from corn starch, potato starch, the precipitated and non-precipitated fractions from potato starch, and waxy maize starch. Thirty-gram samples of each substance were treated with excess enzyme and the dextrins recovered as described in the experimental section. The residual limit dextrin was also isolated from each conversion and its alkali number determined by the usual procedure.[6]

From the results summarized in Table II, it is apparent that the precipitated fractions from both corn and potato starches give very high yields of Schardinger dextrins and comparatively little limit dextrin. The non-precipitated fractions, and likewise waxy maize starch, give less Schardinger dextrins and more limit dextrin than do whole corn and potato starches. These results are in accord with the viewpoint[2,7] that the precipitated fractions are relatively simple and uniform in molecular configuration, while the non-precipitated fractions are much more complex in character, possibly representing linear glucopyranose chains and highly branched molecules, respectively.

TABLE II

DEXTRINS FROM STARCH FRACTIONS

Substrate	Alkali no.	% Yield of crude trichloroethylene precipitate[a]	% Yield of limit dextrin	Alkali no. of limit dextrin
Defatted corn starch	11.0	54.4	37.3	7.2
		55.9	34.1	6.4
Potato starch	7.0	54.1	38.8	7.5
		53.4	42.0	7.2
Defatted waxy maize starch	4.3	43.4	51.6	3.6
		43.8	50.4	3.7
Non-precipitated fraction from corn starch	5.9	49.0	39.7	4.0
		50.5	41.5	4.6
Non-precipitated fraction from potato starch	5.5	47.0	43.0	5.7
		49.4	44.0	5.0
Precipitated fraction from corn starch	20.2	70.1	11.5	16.0
		72.4	12.1	23.2
Precipitated fraction from potato starch	9.3	71.7	9.7	21.3
		69.2	10.8	20.4

[a] This consists of a mixture of Schardinger *alpha* and *beta* crystalline dextrins.

γ-Amylose.—No significant amount of insoluble material was formed during any of the above conversions. Neither potato starch, nor the individual fractions from potato starch, nor the non-precipitated fraction from corn starch gave any trace of insoluble floc with *macerans* conversion. Autoclaved sols of defatted corn starch gave less than 0.1% of insoluble material, while the precipitated fraction from corn starch gave 0.7% of insolubles (or less than 0.2% calculated on the original starch basis). Kerr has reported recently a third starch component (γ-amylose), isolated as the insoluble floc resulting from conversion of corn starch with β-amylase[8] or with *macerans* amylase.[9] Similar products have been described in the early starch literature under such designations as amylocellulose or amylohemicellulose, representing the insoluble coagulum deposited during enzymolysis of starch pastes. Using the method outlined by Kerr[9] for isolation of γ-amylose (by gelatinization in

(5) Bates, French and Rundle, THIS JOURNAL, **65**, 142 (1943). This method involves solution of the starch sample in potassium hydroxide, followed by neutralization with hydriodic acid. Since the latter reagent requires frequent redistillation over red phosphorus, the following simplification was employed for convenience. The starch sample was dissolved in 10 ml. of 0.5 *N* potassium hydroxide, then neutralized to methyl orange with 0.5 *N* hydrochloric acid; 10 ml. of 0.5 *N* potassium iodide was added and the solution diluted to 100 ml. This was titrated electrometrically with an iodine reagent 0.001 *N* with respect to iodine, 0.05 *N* to potassium iodide and 0.05 *N* to potassium chloride. This procedure differs from the published method only by the presence of 0.05 *N* potassium chloride in the starch solution and in the iodine reagent. Consequently, calibration of free iodine against potential must be run in the presence of 0.05 *N* potassium chloride. Results on various starch materials check with values by the Rundle method. Satisfactory titration curves were obtained with a Coleman potentiometer, with a precision of ± 1 mv.

(6) Schoch and Jensen, *Ind. Eng. Chem., Anal. Ed.*, **12**, 531 (1940).

(7) Meyer, "Advances in Colloid Science," Interscience Publishers, Inc., New York, N. Y., pp. 143–182.

(8) Kerr and Trubell, *Cereal Chem.*, **18**, 530 (1941).

(9) Kerr, THIS JOURNAL, **65**, 188 (1943); Kerr and Severson, *ibid.*, **65**, 193 (1943).

0.67 *N* sodium hydroxide solution, followed by neutralization and *macerans* conversion), defatted corn starch gave 1.4 to 1.7% of insoluble material. Variations in Kerr's procedure (as by longer time of alkali peptization, use of stronger alkali, mechanical stirring, increased amounts of enzyme, or gelatinization in boiling water) gave yields of insoluble material from defatted corn starch ranging from 0.1 to 4.8%. By Kerr's procedure, raw corn starch (not defatted) gave 9–10% of insolubles. While potato starch gave no insoluble floc, potato starch impregnated with 1% of oleic acid gave 18% of insoluble residue. From these results, it appears that this insoluble material does not imply the existence of a third amylose, but is related primarily to the presence of fatty material in the substrate and secondarily to the degree of dispersion of the starch substance. It is suggested that the action of other amylases may be similarly influenced.

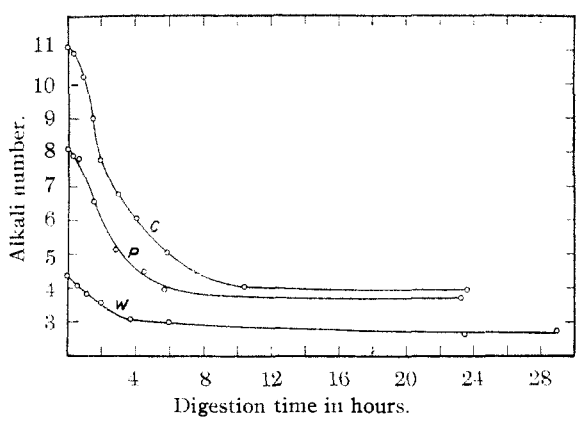

Fig. 1.—Change in alkali lability of various starches with *macerans* enzymolysis: C, corn starch; P, potato starch; W, waxy maize.

Alkali Lability Studies of the *macerans* Enzymolysis.—It has been shown[10] that the action of *macerans* amylase does not increase the copper reducing value of whole starch to any appreciable extent. Alkali lability measurements during *macerans* enzymolysis afford further confirmation of this point. Exactly 1.00% sols of autoclaved potato starch and of defatted corn and waxy maize starches were converted at 40° with the enzyme; at intervals, 50-ml. portions of the digest (representing 500 mg. of the original starch) were withdrawn and analyzed directly for alkali number value.[6] Corrections were made for consumption of alkali by the enzyme, and the resulting alkali number is plotted against time of digestion (Fig. 1). It is apparent that the reducing properties of the digest actually *decrease* as conversion progresses.

Purified samples of the Schardinger alpha and beta dextrins show negligible degradation in hot dilute alkali over extended periods of time, as estimated by alkali number evaluation (Table III).

TABLE III

RESISTANCE OF SCHARDINGER DEXTRINS TO ALKALI

Digestion time in hot alkali, hours	Ml. of 0.1 *N* alkali consumed per g. of dextrin α-Dextrin	β-Dextrin
1	0.93 ± 0.05	0.48 ± 0.03
2	1.19 ± 0.04	0.58 ± 0.09
5	1.28 ± 0.06	0.97 ± 0.02

These values are comparable with those similarly obtained with β-methylmaltoside and calcium maltobionate.[11] The non-crystalline limit dextrins, however, show a relatively high alkali lability (Table II). The above data are in accord with the generally accepted view that *macerans* amylase attacks the non-aldehydic terminus of the starch molecule, progressively producing crystalline Schardinger dextrins from some basic configuration in the starch, and stopping in this action only when some irregularity is encountered in the molecular structure. In this connection, it is suggested that the limit dextrin from this enzymolysis may afford a suitable starting material for isolation of new starch fractions and the identification of "irregularities" in configuration.

Experimental Part

Details of Fractionation.—An 80-g. sample of potato starch or of thoroughly defatted corn starch[12] is gelatinized in a mixture of 4 liters of boiling water and 400 ml. of butanol, then autoclaved for two hours at 18–20 lb. pressure. An additional 200 ml. of butanol and 200 ml. of commercial isoamyl alcohol are added, and the mixture allowed to cool slowly to room temperature, the container being wrapped with cloth to retard cooling. The precipitated fraction separates as a gelatinous floc of microscopic spherocrystals or hair-like needles. The mixture is stirred at room temperature for several hours to break up the crystalline mass, which is then centrifuged in 250 ml. bottles at 2000 r. p. m. in a laboratory centrifuge. The supernate is decanted carefully, and the precipitated material resuspended in cold water previously saturated with butanol and then centrifuged. This washing procedure is repeated until the supernatant liquid is substantially free of solids, as indicated by the absence of turbidity when treated with excess methanol. The crude pre-

(10) McClenahan, Tilden and Hudson, THIS JOURNAL, **64**, 2139 (1942).

(11) Schoch, Wilson and Hudson, *ibid.*, **64**, 2871 (1942).

(12) Schoch, *ibid.*, **64**, 2954 (1942).

cipitated fraction may be further purified by "recrystallization" from boiling water in the presence of excess butanol.[1] The recrystallized product is readily separated by low speed centrifuging. The non-precipitated fraction is flocculated from the original supernate by the addition of a large volume of methanol.

Conversion with *macerans* Amylase.—Accurate 30-g. samples of potato starch and of defatted corn and waxy maize starches were gelatinized in 3 liters of boiling water, autoclaved for one hour at 20 lb. pressure, cooled to 40° and each treated with 6.3 ml. of *macerans* enzyme having a Tilden value of 20 units per ml.[13] Thirty-gram samples of the non-precipitated fractions from potato and corn starches were dissolved in 3 liters of boiling water with vigorous stirring, then cooled to 40° and treated with the same amount of enzyme. The precipitated fractions from potato and corn starches were similarly processed, using the wet precipitated product to avoid retrogradation, in amount equivalent to 30 g. on a dry substance basis. The amount of enzyme added in each case corresponds to 10 times the calculated amount for forty hours conversion at 40°[13]; all samples were converted at this temperature for forty hours. The digests were then concentrated *in vacuo* to 600 ml., heated to boiling and subsequently refrigerated with 25 ml. of trichloroethylene. After several days, the initial crop of crude Schardinger dextrins was removed by filtration and washed with a small amount of cold water. The filtrate and washings were combined, evaporated to 200 ml., and again refrigerated with trichloroethylene. After filtering and washing the second crop of crude dextrins, the filtrate and wash waters were evaporated to 100 ml. and treated for a third time with trichloroethylene, to ensure maximum recovery of the dextrins. These operations extended over a period of several weeks. The combined trichloroethylene precipitates were dried *in vacuo* at 50° and weighed. No attempt was made to separate the individual α- and β-dextrins from these mixtures, but the presence of both substances was confirmed in every case by means of the iodine test.[13]

The accuracy of the dextrin yields is demonstrated by the following computation. The precipitated fractions from corn and potato starches averaged 25.3% of the whole starch, and gave an average yield of 70.9% of trichloroethylene precipitate. The non-precipitated fractions, comprising 74.7% of the original starch, gave an average yield of 49.0%. Hence their respective contributions to the yield from whole starch are calculated to be 0.253×70.9 and 0.747×49.0, or a total of 54.5% dextrins. This coincides with the observed average yield of 54.4% from whole corn and potato starches.

Isolation of Limit Dextrins.—Each of the final filtrates from the above conversions was stirred with 1.5 liters of anhydrous methanol, a few drops of saturated sodium chloride solution being added to assist flocculation. After settling and decantation, the damp sticky precipitates of limit dextrin were dehydrated with fresh methanol to give white amorphous powders which were dried *in vacuo* and weighed.

(13) Tilden and Hudson, *J. Bact.*, **43**, 527 (1942). The enzyme used had been concentrated and purified as previously described by Tilden, Adams and Hudson, THIS JOURNAL, **64**, 1432 (1942).

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Summary

1. Selective precipitation of starch sols with mixed *n*-butyl and isoamyl alcohols permits separation of the fractions by low speed centrifuging.

2. With *macerans* amylase, the precipitated fractions from corn and potato starches give high yields of Schardinger dextrins and minor amounts of non-crystalline limit dextrins. In contrast, the non-precipitated fractions give less Schardinger dextrins and more limit dextrins than do the parent whole starches. This suggests a relatively uniform molecular configuration for the precipitated fraction, and a more complex structure for the non-precipitated fraction. The behavior of waxy maize starch is similar to that of the non-precipitated fractions from corn and potato starches.

3. The alkali lability of a starch digest decreases during *macerans* enzymolysis. The Schardinger alpha and beta dextrins are stable toward alkali, while the limit dextrins are alkali labile.

4. Any insoluble precipitate (γ-amylose) resulting from *macerans* conversion of corn starch is attributed to the presence of fatty material and to incomplete dispersion of the starch. Under proper conditions, the amount of such insoluble material is negligible.

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