Lintnerized Starches. Gel Filtration and Enzymatic Studies of Insoluble Residues from Prolonged Acid Treatment of Potato Starch[1]

J. P. ROBIN, C. MERCIER, R. CHARBONNIERE, and A. GUILBOT, Station de Biochimie et Physico-Chimie des Cereales de l'Institut National de la Recherche Agronomique, 91305 Massy, France

ABSTRACT

Granular potato starch was hydrolyzed with 2.2N hydrochloric acid (HCl) at 35°C. for periods of time up to 40 days. After removal of the acid and soluble carbohydrates, the residues (lintnerized potato starches) were washed with deionized water and dried to 10% moisture. When the percentage of solubilized carbohydrate was plotted vs. time, the curve showed two different steps. The first step, with a high rate, was attributed to hydrolysis of the amorphous part of starch granules, and the second step, with a lower rate, to hydrolysis of the more organized areas. X-ray patterns, apparent amylose content, and iodine absorption spectra of the HCl residues were determined. Sephadex G-50 gel-permeation chromatography of the dissolved residues was performed directly and after successive enzymatic digests with pullulanase and β-amylase. The results show the progressive appearance of two major chain populations: II, $\overline{DP}$ 25, and III, $\overline{DP}$ 15. II was identified enzymatically as being singly branched, and III, primarily as linear chains. III appears as the more acid-resistant and crystalline part of the starch granules, as confirmed by X-ray diffraction. From the molecular size of III, the calculated dimension of the starch crystallites would be about 60 Å in the chain axis direction. The amylose fraction is rapidly degraded, and there is no evidence for amylose participation in the crystalline areas. Amylopectin chains are primarily responsible for the crystallinity of starch, imposing the crystallite dimension. The chains of $\overline{DP}$ 15 which constitute these crystallites could originate from degraded amylose chains, or from the external A-chains or internal chains of amylopectin. These results lead to a new model of amylopectin containing clusters of highly ordered $\overline{DP}$ 15 chains.

During the past 10 years, acid hydrolysis of starch according to Lintner (1) has been used mainly to study, by electron microscopy, the structure of the granule (2–6). There have been few studies on the chemical characteristics of the residues obtained after lintnerization, except the very recent investigations of Kainuma and French (7,8) on Nägeli amylodextrins. The present study was undertaken to investigate the composition and the nature of the residues of potato starch during lintnerization. Results obtained by gel chromatography and enzymatic treatment lead to a new model for amylopectin structure.

MATERIALS

The starch used was commercial potato starch, with a moisture content of 18.9% and apparent amylose content of 20%, determined by the amperometric method (9). Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Pullulanase of *Enterobacter aerogenes* (*Aerobacter aerogenes*), a specific enzyme for α-1,6 linkages, was purified according to Mercier et al. (10). Crystallized sweet potato β-amylase, purified *Aspergillus niger* glucose oxidase (grade II), and horse radish peroxidase (grade I) were obtained from Sigma Chemical Co., St. Louis, Mo. *A. niger* glucoamylase was prepared according to Qureshi (11).

METHODS

Lintnerization of Potato Starch

Ungelatinized potato starch was hydrolyzed at 35°C. at a concentration of 1.67 g. of dry starch in 100 ml. of 2.2N hydrochloric acid. After various times up to 40 days, the insoluble residue was obtained by vacuum filtration on a porous glass filter (5 to 15 μ). The residue was washed with deionized water to remove chloride ions, residual acid, and soluble carbohydrate. Each residue was dried to 10% moisture under vacuum, at 20°C., over phosphorus pentaoxide. The residue was called "lintnerized starch" although the acid treatment was more drastic and more extensive than described by Lintner (1).

Extent of Starch Hydrolysis

The extent of starch hydrolysis with time was followed by measuring the carbohydrate in the filtrate using the anthrone method (12). Results are expressed as percent of the initial starch. For example, "15% lintnerized starch" represents degradation of 15% of the original polysaccharide to soluble form.

Fine Structure of Starch Residues

The fine structure of native and 15, 70, and 85% lintnerized potato starches was studied with a combination of enzymatic action (pullulanase and β-amylase) and chromatography as illustrated in Fig. 1.

Enzymatic Hydrolysis of Native and Lintnerized Starch

Native and lintnerized starches were dispersed in water at 100°C. Debranching with pullulanase and β-amylolysis were carried out as described by Mercier and Whelan (13). Before chromatography and between successive enzymatic actions, the enzyme was inactivated by heating the digest at 100°C. for 20 min. Debranching and β-amylolysis were followed by determining the increase in reducing power (14). Enzymatic action was stopped when the reducing power became constant (24 to 48 hr.).
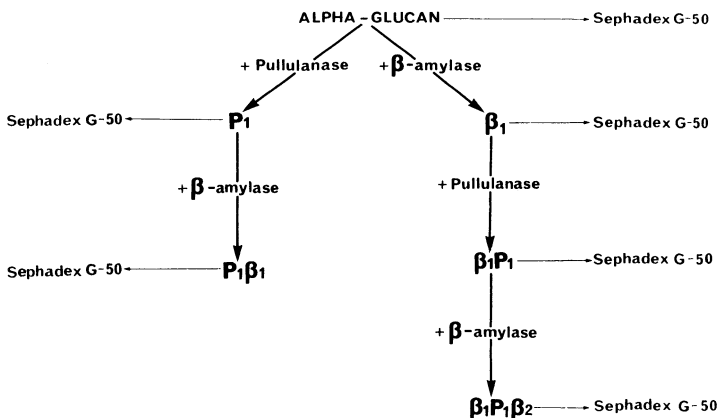

Fig. 1. Enzymatic method for the study of α-glucan structure.

Chromatographic Analysis

Solutions of native and lintnerized starch before and after enzymatic actions were chromatographed on a Sephadex G-50 column (2.5 X 100 cm.) at 20°C. The elution was carried out with an upward flow using a 10mM phosphate buffer, pH 7.0, containing 0.02% sodium azide to prevent bacterial growth. The column was calibrated with debranched waxy maize starch by obtaining the $\overline{DP}$ (ratio of the total carbohydrate to reducing value) of fractions at various elution volumes (13).

For chromatography of solutions of native and lintnerized starch before any enzymatic action was performed, 2-ml. aliquots containing 1 to 2 mg. of polysaccharide were introduced at the lower part of the column with a peristaltic pump. Elution flow was adjusted to 20 ml. per hr. The elution patterns were recorded directly with a Technicon AutoAnalyzer (transmittance at 420 nm., orcinol-sulfuric acid reagent) (15).

Chromatography of enzymatic digests was performed at 25 ml. per hr. with 2-ml. aliquots containing 5 to 15 mg. of polysaccharides. To avoid artifacts in the elution patterns from nonstarch carbohydrates in the enzyme preparations, the starch-type polysaccharide in each 30-min. fraction was determined by hydrolysis with fungal amyloglucosidase as described by Lee and Whelan (16).

Liberated glucose was measured with the glucose oxidase-peroxidase system as by Lloyd and Whelan (17). The elution patterns so obtained represent the weight of polysaccharide expressed as mg. of glucose per 100 mg. of polysaccharide recovered vs. elution volume. Thus these patterns were comparable since each corresponded to 100 mg. of polysaccharide. In all cases, the recovery was 90 to 100%.

Other Determinations

Apparent amylose was determined by an amperometric method (9). The wavelength of the maximum absorbance (λ_{max}) of the lintnerized starch-iodine complex was determined by a modification of the method of Bailey and Whelan (18). The X-ray patterns were recorded with a spectrodiffractometer (Compagnie Générale de Radiologie, France) as by Charbonniere et al. (19).
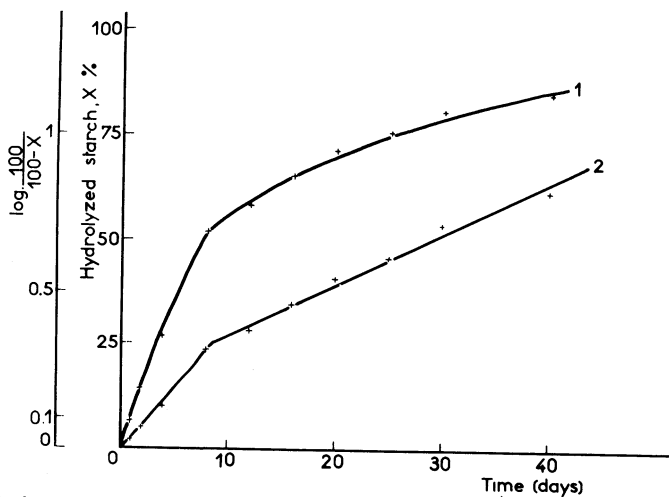

Fig. 2. Hydrolysis of potato starch in 2.2N HCl at 35°C.: curve 1) percentage (X) of hydrolyzed starch vs. time of hydrolysis; curve 2) $\log_{10}$ [100/(100−X)] vs. time of hydrolysis.
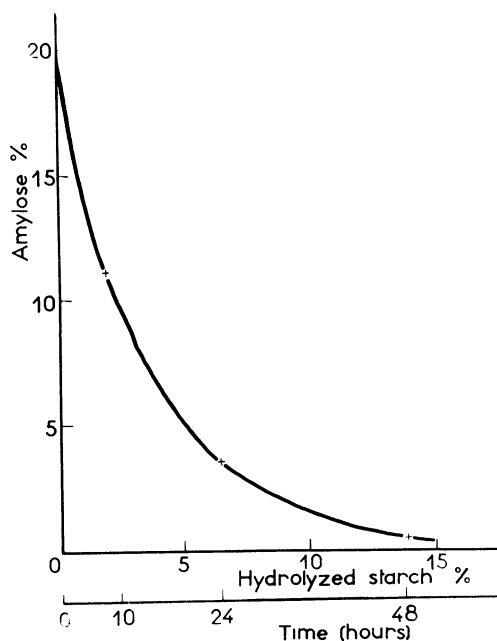
Fig. 3. Decrease in the apparent amylose content of potato starch after various times of lintnerization.

RESULTS

Hydrolysis Kinetics and Some Characteristics of Lintnerized Starch

The extent of hydrolysis (X) vs. time is shown in Fig. 2, curve 1. Curve 2 in this figure shows the change of $\log_{10}$ $[100/(100-X)]$ vs. hydrolysis time.

Figure 3 shows the decrease in apparent amylose content of starch during lintnerization.

The variation of λ_{max} of the iodine complexes of lintnerized starches vs. $\log_{10}$ of hydrolysis time in hours is plotted in Fig. 4, curve 1. The average chain length of the residues, calculated according to Bailey and Whelan (18) and Lee[2], is shown in Fig. 4 , curve 2.

In Fig. 5 are compared the X-ray diffractograms of native potato starch (pattern 1), and 85% lintnerized starch (pattern 2), corresponding to 40 days of treatment.[3]

Gel Filtration of Native and Lintnerized Starch Before Enzymatic Hydrolysis

The aqueous, dispersed native starch is excluded from the Sephadex G-50 (Fig. 6, pattern 1, peak I) since, according to Pharmacia, the maximum molecular weight which will permeate the gel particle is about 10,000 ($\overline{DP}$ about 60). With increasing hydrolysis the elution patterns show the progressive appearance of two peaks II and

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[2]Unpublished results (1967) kindly made available to the author by E. Y. C. Lee.

[3]The complete results of X-ray study of various lintnerized starches, including lintnerized potato starch, will be published elsewhere.
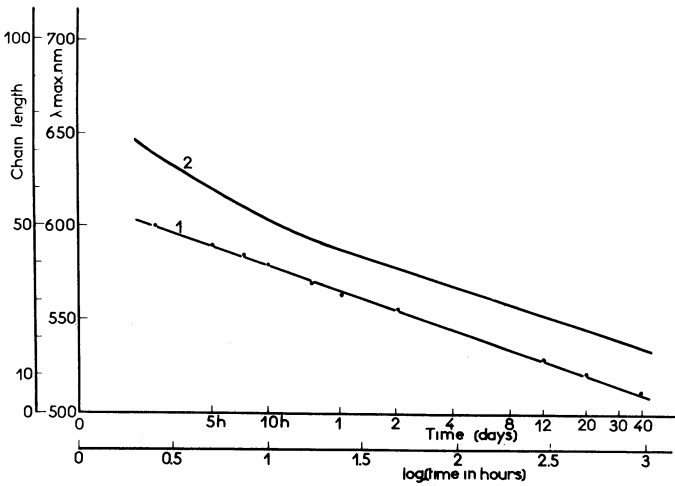
Fig. 4. Lintnerized potato starch iodine complex: curve 1) change of λ_{max} with time (logarithmic scale); curve 2) change of chain length with time as calculated from λ_{max}.
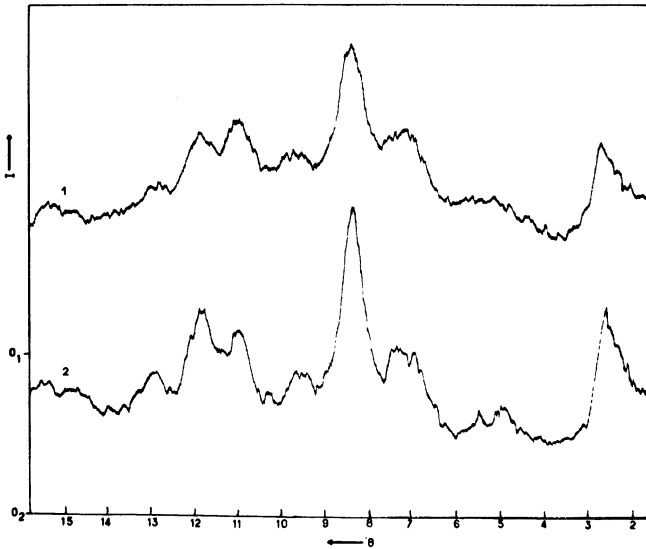

Fig. 5. X-ray diffractograms recorded at 27% H_2O (dry basis): curve 1) native potato starch; curve 2) 85% lintnerized potato starch.

III (Fig. 6) which represent two distributions of chain populations with $\overline{DP}$ independent of the extent of hydrolysis. Calibration of the Sephadex column (13) allows us to assign $\overline{DP}$ 25 to the peak II population and $\overline{DP}$ 15 to peak III. These populations arise from the initial population I and from its "associated fraction" or low-molecular-weight trail located between peaks I and II. Peaks II and III are
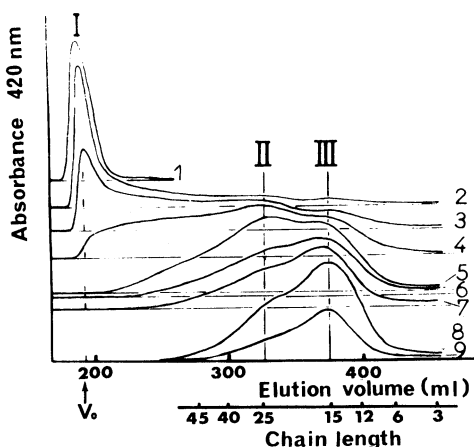
Fig. 6. Elution patterns from a column (2.5 × 100 cm.) of Sephadex G-50 of native and lintnerized potato starch at different extents of hydrolysis. Carbohydrate concentrations in the eluate were recorded with the Technicon AutoAnalyzer. Values are percent hydrolysis and hydrolysis time: 1) native starch; 2) 2.6%, 10 hr.; 3) 15%, 2 days; 4) 27%, 4 days; 5) 52%, 8 days; 6) 58%, 12 days; 7) 70%, 20 days; 8) 80%, 30 days; 9) 85%, 40 days. Population I represents material appearing essentially at the void volume; population II is material with apparent DP 25; population III has DP 15.

already visible at 2.6% hydrolysis, obtained after 10 hr. of treatment (Fig. 6, pattern 2). Then peak I gradually disappears, its "associated fraction" and peaks II and III becoming larger. At 52% hydrolysis, obtained after 8 days of treatment, peak I has completely vanished (Fig. 6, pattern 5). At greater extents of hydrolysis, the components of peak II seem to be more rapidly hydrolyzed than those of peak III. Population III finally dominates the elution pattern.

Gel Filtration of Native and Lintnerized Starch after Pullulanase Debranching

Debranching of native potato starch with pullulanase (P_1) gives three main components (Fig. 7, pattern 2) designated A (45%), B (22%), and C (33%).

The ratio of population A to population B is approximately 2 on a weight basis and 5.4 on a molecular basis. The presence of A and B populations after debranching of starch is in agreement with previous results obtained on waxy maize starch (13,20).

Consecutive action of pullulanase and β-amylase $(P_1\beta_1)$ transforms the chains of all three populations into glucose, maltose, and maltotriose (Fig. 7, pattern 3, peak *b*), these products being identified by TLC. This result proves the linearity of A, B, and C. The A-population, which has the same position as the peak III population in lintnerized starch, consists of linear chains of DP 15, and the intermediate B-population contains linear chains of DP 45. Peak C consists of linear chains of DP > 60 excluded from the gel matrix. The difference between the proportion of C (33%) and the apparent amylose content in native starch (20%) indicates that C contains 13% of long linear chains liberated during the pullulanase debranching of the branched fraction.

The elution pattern of debranched 15% lintnerized starch (2 days of treatment) shows only A and B populations (Fig. 7, pattern 5). Population C, observed in elution pattern of debranched native starch (Fig. 7, pattern 2) has disappeared completely. Compared with pattern 4, obtained before debranching, pattern 5
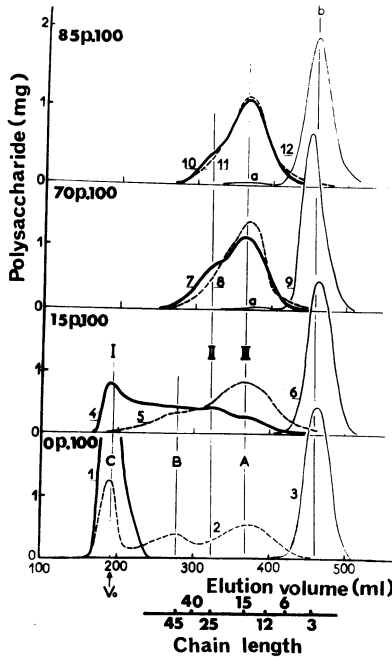
Fig. 7. Elution patterns from a column (2.5 × 100 cm.) of Sephadex G-50 of native and 15, 70, and 85% lintnerized potato starch after treatment with pullulanase (P_1) or consecutive treatment with pullulanase and β-amylase ($P_1\beta_1$). For each pattern, the enzyme treatment and percent hydrolysis are: 1) native; 2) P_1, native; 3) $P_1\beta_1$, native; 4) 15%; 5) P_1, 15%; 6) $P_1\beta_1$, 15%; 7) 70%; 8) P_1, 70%; 9) $P_1\beta_1$, 70%; 10) 85%; 11) P_1, 85%; 12) $P_1\beta_1$, 85%. Populations I, II, and III as in Fig. 6: populations A, B, and C are resistant to pullulanase action; populations a and b are products of pullulanase and β-amylase action.

shows that peak B is located as the "associated fraction" between peaks I and II and that peak A is larger than the initial peak III. This increase results from the contribution of linear chains coming from the debranching of certain chains from population I, the "trail", and population II. The linearity of these chains is verified, as in the case of native starch, by β-amylolysis ($P_1\beta_1$) yielding glucose, maltose, and maltotriose (Fig. 7, pattern 6).

The elution pattern of debranched 70% lintnerized starch (20 days of treatment) shows the complete disappearance of peak B (Fig. 7, pattern 8), only peak A remaining. Comparison with pattern 7 proves that peak A was produced in part by debranching peak II.

The apparent degree of hydrolysis $(T_1)^4$ of the 70% lintnerized starch, after

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[4]The apparent degree of β-amylolysis, T_1, is the ratio of carbohydrate in peak b to the total carbohydrate. This ratio is also used when the last digest is a debranching to estimate the yield, T_3, of oligosaccharides of DP 2 through 10 which are included in peak b along with glucose, maltose, and maltotriose produced by β-amylolysis.

The real β-amylolysis ratio, T_2, is calculated from the reducing power of a β-amylase digest after 20 hr., and expressed as percent maltose. It indicates more exactly the proportion of polysaccharide material transformed into glucose, maltose, and maltotriose.

The β-amylolysis ratios T_1 and T_2, the oligoside yields T_3 or the proportions of polysaccharides contained in peaks a and b, are calculated as percent of the weight ($C_6H_{12}O_6$ basis) of polysaccharide material contained in the digests or recovered after chromatography.

The amounts of starting material and product are reported on a $C_6H_{12}O_6$ basis.

consecutive action of pullulanase and β-amylase $(P_1\beta_1)$, is 98.2%. An enzyme-resistant fraction is revealed on the elution pattern (Fig. 7, pattern 9) as a broad, weak peak a, DP between 30 and 10. Under our experimental conditions peak a is not observed for native starch or for the 15% lintnerized starch after $P_1\beta_1$.

The elution pattern of debranched 85% lintnerized starch (40 days of treatment) shows only peak A (Fig. 7, pattern 11). The complete disappearance of peak II, still faintly present on the elution pattern of the lintnerized starch before debranching (Fig. 7, pattern 10), indicates its branched nature. The ratio T_1 after β-amylolysis $(P_1\beta_1)$ is 98.1%. As with the 70% residue, there is also an enzyme-resistant population a (Fig. 7, pattern 12).

Gel Filtration of Native and Lintnerized Starch after β-Amylolysis

The real limit $T_2{}^4$ of direct β-amylolysis (β_1) of native starch is 57%. The β-limit dextrin is completely excluded from the gel matrix (peak I) and the reaction products, essentially maltose and maltotriose, appear in peak b (Fig.8, pattern 2). After removal of maltose and maltotriose by dialysis, the β-limit dextrin was debranched by pullulanase (β_1P_1) and the elution pattern shows three main peaks (Fig. 8, pattern 3). Linearity after β_1P_1 (pattern 3) was confirmed by further β-amylolysis $(\beta_1P_1\beta_2)$ which gave only peak b material (pattern 4). The first peak
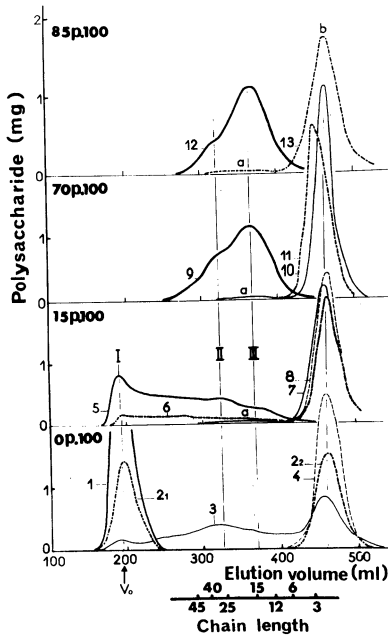

Fig. 8. Elution patterns from a column (2.5 × 100 cm.) of Sephadex G-50 of native and 15, 70, and 85% lintnerized potato starch after β_1, β_1P_1, and $\beta_1P_1\beta_2$ treatments (see text for explanation of enzyme treatments). For each pattern, the enzyme treatment and substrate are: 1) native; 2_1) and 2_2) β_1, native; 3) β_1P_1 of native β-limit dextrins under 2_1; 4) $\beta_1P_1\beta_2$ of native debranched β-limit dextrins; 5) 15%; 6) β_1, 15%; 7) β_1P_1, 15%; 8) $\beta_1P_1\beta_2$, 15%; 9) 70%; 10) β_1, 70%; 11) β_1P_1, 70%; 12) 85%; 13) β_1, 85%.

TABLE I. APPARENT AND REAL β-AMYLOLYSIS LIMITS (T_1 AND T_2) AFTER β_1 AND
$\beta_1 P_1 \beta_2$ TREATMENTS, YIELD (T_3) AFTER $\beta_1 P_1$ TREATMENT,
AND PERCENTAGES OF β-LIMIT DEXTRINS OBTAINED FROM
15% LINTNERIZED STARCH

Enzyme treatment	T_2[a] %	T_1 or T_3[a] %	β-Limit Dextrins	
			DP > 10[b] %	DP 2 to 10[c] %
β_1	63.5	73.5	26.5[d]	10.0
$\beta_1 P_1$	...	$73.5 + 19.5 = 93.0$ —P₁	7.0[e]	...
$\beta_1 P_1 \beta_2$	96.0	$93.0 + 3.0 = 96.0$ β₂	4.0[f]	0

[a] T_2 was obtained by measurement of total reducing value. T_1 and T_3 were obtained by measuring the area under peak *b*. See footnote 4.
[b] Obtained from $100 - T_1$ (or $100 - T_3$).
[c] From $T_1 - T_2$; represents the short β-limit dextrins contained in peak *b*.
[d] Amount of β-limit dextrin with DP > 10, from Fig. 8, pattern 6.
[e] Undebranched β-limit dextrins and debranched chains of DP > 10 (Fig. 8, pattern 7, peak *a*).
[f] Undebranched β-limit dextrins of DP 10 to 30 (Fig. 8, pattern 8, peak *a*).

of pattern 3 (peak I, 6%) consists of long linear chains excluded from the gel matrix. The intermediate second peak (50%) contains essentially linear chains of $\overline{DP}$ 30 through 35. The third peak (44%) contains maltose and maltotriose derived from the external stub branches of the β-limit dextrin and smaller β-amylase-treated internal chains of branched fraction of starch (Fig. 8, pattern 3, peak *b*). The second and third peaks of pattern 3 corroborate the profile obtained by Lee et al. (20) and Akai et al. (21) from the pullulanase-debranched waxy maize starch β-limit dextrins.

The main results obtained from 15% lintnerized starch after β-amylolysis, pullulanase debranching, and a second β-amylolysis are summarized in Table I. The limit T_2 of direct β-amylolysis (β_1) of the 15% lintnerized starch is 63.5%. However, the apparent limit T_1, obtained from the Sephadex fractionation, is 73.5%, indicating that peak *b* contains short β-limit dextrins with $\overline{DP}$ < 10 (Fig. 8, pattern 6). Debranching ($\beta_1 P_1$) of the β-limit dextrin increases to 93% the yield T_3[4] of oligosaccharides of DP 2 through 10, and a resistant fraction appears as peak *a* (Fig. 8, pattern 7) already described (Fig. 7, pattern 9). After further β-amylolysis ($\beta_1 P_1 \beta_2$), both T_1 and T_2 finally rise to 96%. The enzyme-resistant fraction *a* is seen in Fig. 8, pattern 8, as in Fig. 7, patterns 9 and 12, and is equal to 4.0% of the total carbohydrate.

The ratios T_1 and T_2 of direct β-amylolysis (β_1) of 70% lintnerized starch are 98.0 and 86.4%, respectively. But in contrast to 15% lintnerized starch, debranching ($\beta_1 P_1$) after β-amylolysis does not increase the reducing power or the yield T_3 of oligosaccharides of DP 2 through 10. The remaining *a*-fraction is only 2.0% of the total polysaccharide in β_1 or $\beta_1 P_1$ digests (Fig. 8, patterns 10 and 11). A further β-amylolysis ($\beta_1 P_1 \beta_2$) neither modifies the quantity of this resistant fraction nor the real or apparent β-amylolysis ratios, respectively equal to 86.7 and 97.8%.

The ratios T_1 and T_2 of direct β-amylolysis (β_1) of 85% lintnerized starch are 97.8 and 87.5%, respectively. The remaining *a*-fraction is 2.2% (Fig. 8, pattern 13). As with 70% lintnerized starch, a further debranching ($\beta_1 P_1$) and a final β-amylolysis ($\beta_1 P_1 \beta_2$) do not modify the β-amylase limits.

The differences observed between T_1 and T_2 after direct β-amylolysis (β_1) are 10.0, 11.6, and 10.3%, respectively, after 15, 70, and 85% lintnerization. This

difference is explained by the presence of short β-limit dextrins of DP 2 through 10 included in peak b, as shown by paper chromatography. These short β-limit dextrins come from branched chains of population II. For 15% lintnerized starch, these dextrins corresponding to 10% of peak b are further debranched, with final limits T_1 and T_2 near 96% after $\beta_1 P_1 \beta_2$ treatments. In the case of the 70 and 85% lintnerized starch, approximately 10% of peak b consists of pullulanase-resistant dextrins.

The T_1 and T_2 β-amylase limits after β_1, $\beta_1 P_1 \beta_2$, and P_1 β_1 treatments, and the percentages of residual enzyme-resistant fractions of DP 10 through 30 (peak a) and of DP 2 through 10 (peak b) are summarized in Table II.

DISCUSSION

The results show that, from the beginning of acid hydrolysis, potato starch is transformed into a branched chain population, II, of apparent $\overline{\mathrm{DP}}$ 25 and into an essentially linear population, III, of DP 15. These fractions are progressively produced from amylose and amylopectin present in peak I and from the "trailing" chains eluted between peaks I and II. Then, as the extent of hydrolysis increases, the proportion of residual linear chains increases as shown by the β-amylolysis limit (Table II). Simultaneously, the entire chain population is degraded to $\overline{\mathrm{DP}}$ 15, which corresponds to the acid-resistant population III.

Pullulanase-debranching of native and lintnerized starch transforms the branched material of population I, the "trail," and population II into linear chains.
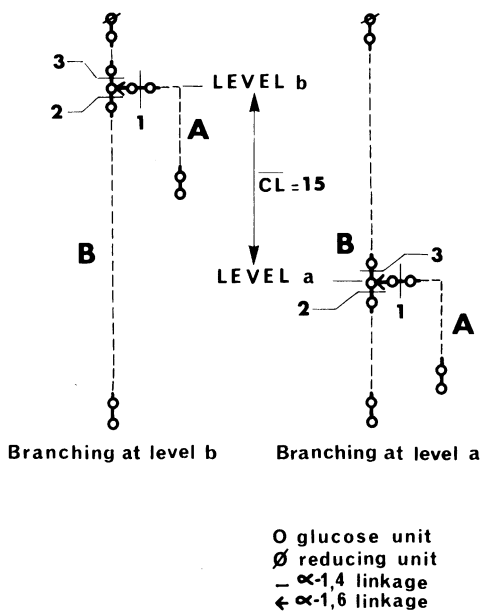

Fig. 9. Three possible modes for acid-catalyzed rupture on an α-1,4 linkage (position 1, 2, or 3) around an α-1,6 branching point located at two different levels (a or b) on the same amylodextrin chain: A) A-chain; B) B- (or C-) chain.

TABLE II. APPARENT AND REAL β-AMYLOLYSIS LIMITS (T_1 and T_2) AND
PERCENTAGES OF ENZYME-RESISTANT β-LIMIT DEXTRINS OBTAINED
FROM 15, 70, and 85% LINTNERIZED STARCHES

| Starch Substrate | β-Amylolysis Limits, % | | | | | | Enzyme-Resistant β-Limit Dextrins, % | | | |
| | after β_1 | | after $\beta_1 P_1 \beta_2$ | | after $P_1 \beta_1$ | | after $\beta_1 P_1 \beta_2$ | | after $P_1 \beta_1$ | |
	T_1[a]	T_2[a]	T_1[a]	T_2[a]	T_1[a]	T_2[a]	DP 10 to 30[b]	DP 2 to 10[c]	DP 10 to 30[b]	DP 2 to 10[c]
Native	...	57.0	100	100	100	100	0	0	0	0
15% Lintnerized	73.5	63.5	96.0	96.0	100	100	4.0	0	0	0
70% Lintnerized	98.0	86.4	97.8	86.7	98.2	95.8	2.2	11.1	1.8	2.4
85% Lintnerized	97.8	87.5	97.8	87.5	98.1	100	2.2	10.3	1.9	0[d]

[a]See footnote 4.
[b]Obtained from 100-T_1; represent the undebranched long β-limit dextrins contained in peak *a*.
[c]Obtained from T_1-T_2; represent the undebranched short β-limit dextrins contained in peak *b*.
[d]Nonsignificant result.

The linear chains obtained with native starch are distributed into two populations: A ($\overline{DP}$ 15) and B ($\overline{DP}$ 45). Remarkably, population A, formed by pullulanase debranching, has the same $\overline{DP}$ as population III, formed by direct acid hydrolysis. The chains of peak A, being the sum of the initial population III plus the linear chains formed by debranching populations I, "trail," and II, are more and more numerous as hydrolysis progresses (Fig. 7, pattern 5). This fact indicates that the material of $\overline{DP}$ 45 (population B) is preferentially hydrolyzed to the linear chains of population A. Furthermore, the debranching of population II (Fig. 7, patterns 7 and 8) into a linear material of $\overline{DP}$ 15 (peak A) suggests the singly branched nature of this population, where both the main chains and branches would be of $\overline{DP}$ 15.

Acid hydrolysis of the singly branched chains of population II seems to occur in preference to hydrolysis of the chains of population III (Fig. 6). If acid rupture of population II occurs near the branching point, it could give two chains of $\overline{DP}$ 15, increasing population III, plus some acid-soluble material.

The undebranched, short β-limit dextrins (USβ-LD) shown in Figs. 7 and 8, peak b (DP 2 through 10) and the undebranched, long β-limit dextrins (ULβ-LD) in peak a (DP 10 through 30), mainly appearing after a further β-amylolysis of the singly branched chains of population II, indicate that the α-1,6 branching points can be located rather near the reducing group for the USβ-LD, and spaced by about 15 glucose units from the reducing group of the carrier chains for the ULβ-LD.

Formation and Nature of Undebranched Limit Dextrins

Resistance to pullulanase action could result from the presence of a pullulanase-stable branching linkage, such as α-1,2 or α-1,3, from a steric obstacle caused by the presence of two adjacent branches linked α-1,6 on the same main chain, or from the presence of a single glucose unit stub (22). Although the presence in starch of α-1,3 linkages was suggested by Wolfrom and Thompson (23), there has been no further evidence for such an origin of pullulanase-resistant limit dextrins. Recently Kainuma and French (24) showed the absence of two adjacent α-1,6 linkages in starch. Therefore the suggestion of single glucose unit stubs seems to be most likely.

Under our experimental conditions, the ULβ-LDs are not observed after debranching of native starch or after $\beta_1 P_1 \beta_2$ treatment of waxy maize starch (13). These results suggest that the two types of limit dextrins originate directly from lintnerization or indirectly after β-amylolysis of the lintnerized residue. In the latter case, the acid treatment could produce a structure in the residual chains which would prevent the debranching after the action of β-amylase.

The enzyme-resistant structure could be explained by considering Figs. 9 and 10. In Fig. 9, we show the three different positions for possible acid cleavage of an α-1,4 link near the α-1,6 branching point, at two different levels (a and b) separated by 15 glucose units. In Fig. 10 we show the three initial structures, resulting from the acid rupture at positions 1, 2, or 3 indicated in Fig. 9, which could lead to the formation of USβ-LD and ULβ-LD.

Whenever cleavage occurs in position 1, the resulting "stubbed" dextrin would be resistant to pullulanase action. Hydrolysis in position 2 results in resistance only with odd-membered A-chains after action of β-amylase (25, 26). When acid hydrolysis occurs at position 3, the β-limit dextrins are eventually debranched by pullulanase giving maltose and maltotriose (27).
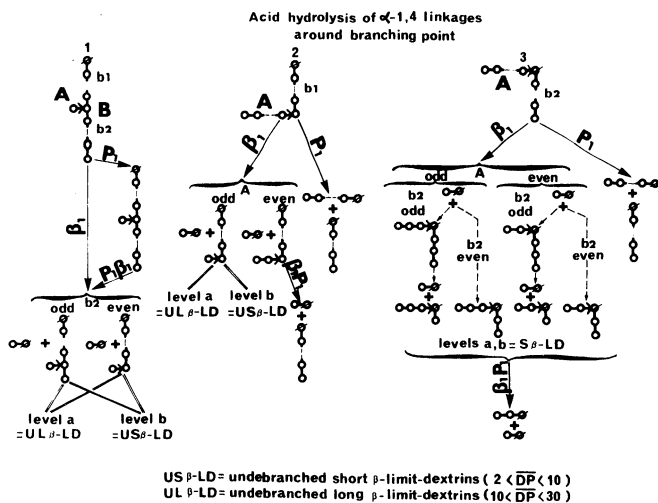
Fig. 10. Three possible structures obtained from acid rupture of an α-1,4 linkage in position 1, 2, or 3 in an amylodextrin chain, leading to formation of ULβ-LD or USβ-LD.

The USβ-LD and ULβ-LD with glucosyl stubs are analogous to stubbed oligosaccharides obtained from starch after successive actions of acid, glucoamylase and pullulanase (24).

The schemes proposed in Figs. 9 and 10 account for the higher percentage of resistant dextrins when the enzymatic treatment begins with β-amylolysis rather than with debranching (Table II). Thus, after $P_1\beta_1$ of the 15% lintnerized starch, the ULβ-LDs are absent, but represent 4% after $\beta_1 P_1 \beta_2$. This also suggests that at this period of acid hydrolysis, α-1,4 cleavages occur more frequently in position 2 than in position 1, position 1 rupture being observed more with 70% lintnerized starch. Also it is suggested that for 15% lintnerized starch, the ruptures in position 2 preferentially occur at level a, since short β-limit dextrins obtained after β_1 treatment of this residue (10% of peak b) are further debranched, whereas the short β-limit dextrins obtained with 70 and 85% lintnerized starches are pullulanase resistant. Indeed if a rupture in position 2 at level b occurs, the USβ-LD should be obtained after β_1 in peak b, which is not the case, since the T_1 and T_2 ratios after $\beta_1 P_1 \beta_2$ are identical (96%).

Origin and Nature of Acid-Resistant Fraction

The X-ray study of lintnerized starch (Fig. 5) clearly shows that the acid hydrolysis preferentially acted on the amorphous part of starch, and progressively produced an increasingly crystalline material represented by populations II and III. This result corroborates the findings of Kainuma and French (7) for sulfuric acid-treated starches (Nägeli amylodextrins). Thus it was evident that the first stage of hydrolysis shown in Fig. 2 (0 to 8 days) corresponds to the hydrolysis of the more amorphous part of the starch granules, while during the second stage (8 to 40 days) the more crystalline material is slowly hydrolyzed. The constant molecular size of populations II and III during the course of lintnerization can be explained by the absence of degradation within the crystallites, suggesting that water or H_3O^+

ion could not penetrate into the crystalline phase. Very slow hydrolysis could proceed by the progressive erosion of external crystallite surfaces.

Our data indicate that the starch crystallites consist of closely packed chains of about 15 glucose units. According to Senti and Witnauer (28) the distance between glucose units in a fully extended amylose helix is about 4 Å. Thus the calculated dimension of a starch crystallite, consisting of a compact association of chains of DP 15, is close to 60 Å along the chain axis. This figure agrees with the one estimated by Meyer and Bernfeld (29) and calculated from electron micrographs by Sterling and Pangborn (30) for the diameter of microfibrils observed in lintnerized
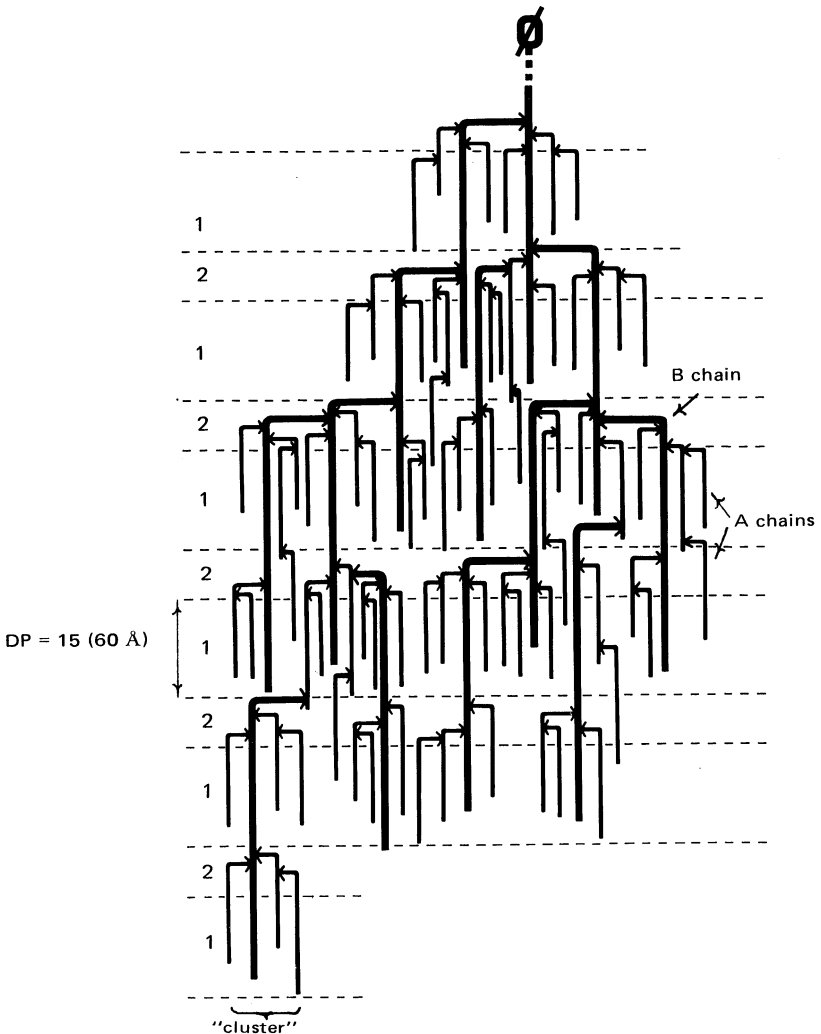

Fig. 11. Proposed structure for potato amylopectin. 1 = compact area; 2 = less compact area, rich in branching points; ϕ = reducing unit.

potato starch, but it is lower than the figure of 146 Å for globoid amylopectin micelles estimated from X-ray diffraction data by Hizukuri and Nikuni (31).

The complete disappearance of peak C (Fig. 7, pattern 5) after P_1 of 15% lintnerized starch indicates that amylose, which constitutes the main part of this peak in native starch (Fig. 7, pattern 2) is strongly acid-hydrolyzed. Thus the peak I in Fig. 7, pattern 4, corresponds to residual amylopectin. Figures 3 and 4 indicate the rapid hydrolysis of amylose, the apparent amylose content becoming almost nil for 15% lintnerized starch (λ_{max} 555 nm. and $\overline{CL}$ 38). The curve in Fig. 4 can be attributed only to the hydrolysis of the amylose fraction of starch since the λ_{max} of the purple amylopectin iodine complex is not greatly affected by hydrolysis[5] or by debranching (32). Figure 4 also corroborates the decrease of $\overline{DP}$ with time as obtained by gel chromatography.

It is difficult to account for the preferential hydrolysis of amylose and its complete elimination in residual polysaccharide after 15% lintnerization. Our results only indicate the rapid splitting of amylose into chains of DP < 60, too short to form a blue iodine complex. It is possible that some of the degraded amylose chains could eventually appear in population III. They might also associate with residual amylopectin chains to constitute the "crystallites."

Irrespective of whether the amylose participates in crystallites, two important facts are evident:

1. The external chains of amylopectin with $\overline{DP}$ 15 (20,33) could be considered as precursors of population III and thus as crystallite constituents.

2. Amylopectin chains are primarily responsible for the crystallinity of starch (29), the crystallite dimension being the length of the amylopectin external chains. The amylose chains, being initially at least in part outside the crystallite regions, are rapidly hydrolyzed and the resistant fragments would have the same length as the crystallite itself.

Hypothesis for the Structure of Potato Amylopectin

The gel filtration and enzymatic studies of lintnerized potato starch confirm one of the structural schemes of amylopectin, specifically the "cluster" model proposed by French (8). Such an organized structure is shown in Fig. 11. This structure explains most of the results obtained in this study. Particularly, it accounts for the following observations:

1. The linear populations A and B which appeared after debranching of native starch. The B chains would form the backbone of the amylopectin molecule and should extend over two or more clusters, each cluster resulting from the association of two or three A-chains (A/B = 5.4). The long chains obtained in the excluded volume (peak C or I) after debranching or after $\beta_1 P_1$ treatment of native starch could come from the slightly branched amylose chains as previously suggested (34, 35, 36).

2. The debranching of the β-limit dextrins of native potato starch. During β-amylolysis, B-chains are reduced by about 10 glucose units up to the first branching point of an A-chain. An analogous result was obtained by Mercier et al. (13,20) and Akai et al. (21) for waxy maize starch.

3. The formation of the USβ-LD and ULβ-LD during lintnerization.

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[5]Unpublished result.

4. The formation of the acid-resistant populations II and III.

In such a crystalline model of amylopectin structure, it is conceivable that associated clusters of A-chains, 60 Å in length, could constitute a crystalline layer 60 Å in thickness in the chain axis direction. Such associated clusters then comprise the acid-resistant fraction in starch granules. The intercrystalline areas between successive clusters or crystalline layers would contain the majority of α-1,6 linkages (A → A, A → B, B → B, or B → A) and would be much more acid susceptible. Effectively, the acid acting in the intercrystalline areas could promote the formation of USβ-LD and ULβ-LD and allow the release of singly branched chain population II and linear chain population III, after aqueous dispersion. The presence of the singly branched chains in lintnerized starch could be explained by the presence of any α-1,6 linkages less acid susceptible and more involved in crystalline areas than the others.

As waxy maize starch consists only of amylopectin, and nevertheless contains an amorphous fraction, amylopectin chains in potato starch also contribute to the amorphous parts. The structure of the amylopectin chains in these areas can be essentially the same as the one previously described but possibly more "opened" and more or less mixed with amylose chains.

The proposed model is different from the classical Meyer model (37) and from the more recent model of Gunja-Smith et al. (38). In our model, the chains A of $\overline{DP}$ 15 and B of $\overline{DP}$ 45 are not entirely comparable with the external "A" chains and with the internal "B" chains of the Meyer model. Our A-chains represent the external "A" chains and any internal "B" chains of DP 15 of the Meyer model. Our B-chains only represent the internal "B" chains of DP 45. This distinction in long and short internal "B" chains of the Meyer model is in agreement with the assumption of Akai et al. (21).

In a starch granule, such amylopectin molecules could be radially oriented as the crystalline fiber shown from microscopic studies by Sterling (6) and by Hess et al. (39). Acid-treated starch granules often show a separation into concentric layers (3,5,40) owing to the hydrolysis of tangentially oriented amorphous layers. With our hypothesis, such hydrolysis would also occur at a submicroscopic scale from the hydrolysis of the layers rich in α-1,6 linkages located between successive, concentric, 60-Å layers of associated clusters in the amylopectin molecule.

Literature Cited

1. LINTNER, C. J. Studien über Diastase. J. Prakt. Chem. 34: 378 (1886).
2. BUTTROSE, M. S. Submicroscopic development and structure of starch granules in cereal endosperms. J. Ultrastruct. Res. 4: 231 (1960).
3. INNAMORATI, M. Transformazioni strutturali e formazione degli Strati nei granuli di amido di Triticum coltivato in condizioni costanti e in ambiante naturali. Caryologia 19: 345 (1966).
4. MUSSULMAN, W. C., and WAGONER, J. A. Electron microscopy of unmodified and acid-modified corn starches. Cereal Chem. 45: 162 (1968).
5. GALLANT, D., and GUILBOT, A. Etude de l'ultrastructure du grain d'amidon au moyen de nouvelles méthodes de préparation en microscopie électronique. Staerke 21: 156 (1969).
6. STERLING, C. Fibrillar structure in lintnerized potato starch by scanning electron microscopy. Staerke 23:193 (1971).

7. KAINUMA, K., and FRENCH, D. Nägeli amylodextrin and its relationship to starch granule structure. I. Preparation and properties of amylodextrins from various starch types. Biopolymers 10: 1673 (1971).

8. FRENCH, D. Fine structure of starch and its relationship to the organization of starch granules. Denpun Kagaku (J. Jap. Soc. Starch Sci.) 19: 8 (1972).

9. BEMILLER, J. N. Iodometric determination of amylose, amperometric titration. In: Methods in carbohydrate chemistry, Vol. IV, No. 165 (1964).

10. MERCIER, C., FRANTZ, B. M., and WHELAN, W. J. An improved purification of cell-bound pullulanase from *Aerobacter aerogenes.* Eur. J. Biochem. 26: 1 (1972).

11. QURESHI, M. Y. Specificities of enzymes of glycogen metabolism. Ph.D. thesis, University of London (1967).

12. TOLLIER, M. T. Contribution à l'étude de l'action du rayonnement gamma sur les caractères physicochimiques de l'amidon et sa sensibilité aux amylases. Thèse d'Ingénieur du C.N.A.M., Paris (1965).

13. MERCIER, C. and WHELAN, W. J. The fine structure of glycogen from type IV glycogen-storage disease. Eur. J. Biochem. 16: 579 (1970).

14. NELSON, N. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375 (1944).

15. KESLER, R. B. Rapid quantitative anion-exchange chromatography of carbohydrates. Anal. Chem. 39: 1416 (1967).

16. LEE, E. Y. C., and WHELAN, W. J. Enzymic methods for the microdetermination of glycogen and amylopectin, and their unit-chain lengths. Arch. Biochem. Biophys. 116: 162 (1966).

17. LLOYD, J. B., and WHELAN, W. J. An improved method for enzymic determination of glucose in the presence of maltose. Anal. Biochem. 30: 467 (1969).

18. BAILEY, J. M., and WHELAN, W. J. Physical properties of starch. I. Relationship between iodine stain and chain length. J. Biol. Chem. 236: 969 (1961).

19. CHARBONNIERE, R., MERCIER, C., TOLLIER, M. T., and GUILBOT, A. Etude diffractométrique des amidons de maïs à teneur variable en amylose. Staerke 20: 75 (1968).

20. LEE, E. Y. C., MERCIER, C., and WHELAN, W. J. A method for the investigation of the fine structure of amylopectin. Arch. Biochem. Biophys. 125: 1028 (1968).

21. AKAI, H., YOKOBAYASHI, K. MISAKI, A., and HARADA, T. Structural analysis of amylopectin using *Pseudomonas* isoamylase. Biochem. Biophys. Acta 252: 427 (1971).

22. ABDULLAH, M., CATLEY, B. J., LEE, E. Y. C., ROBYT, J., WALLENFELS, K., and WHELAN, W. J. The mechanism of carbohydrase action, 11. Pullulanase, and enzyme specific for the hydrolysis of alpha-1,6-bonds in amylaceous oligo- and polysaccharides. Cereal Chem. 43: 111 (1966).

23. WOLFROM, M. L., and THOMPSON, A. Occurrence of the (1→3)-linkage in starches. J. Amer. Chem. Soc. 78: 4116 (1956).

24. KAINUMA, K., and FRENCH, D. Action of pancreatic amylase on starch oligosaccharides containing single glucose side chains. FEBS Letters 5: 257 (1969).

25. SUMMER, R., and FRENCH, D. Action of β-amylase on branched oligosaccharides. J. Biol. Chem. 222: 469 (1956).

26. KAINUMA, K., and FRENCH, D. Action of pancreatic alpha-amylase and sweet potato beta-amylase on 6^2- and 6^3-α-glucosylmalto-oligosaccharides. FEBS Letters 6: 182 (1970).

27. FRENCH, D., and ABDULLAH, M. Specificity of pullulanase. Biochem. J. 100: 6P (1966).

28. SENTI, F. R. and WITNAUER, L. P. X-ray diffraction studies of addition compounds of amylose with inorganic salts. J. Polymer Sci. 9: 115 (1952).

29. MEYER, K. H., and BERNFELD, P. Recherches sur l'amidon VII. Sur la structure fine du grain d'amidon et sur le gonflement. Helv. Chim. Acta 23: 890 (1940).

30. STERLING, C., and PANGBORN, J. Fine structure of potato starch. Amer. J. Bot. 47: 577 (1960).

31. HIZUKURI, S., and NIKUNI, Z. Micelle dimension of potato starch. Nature 180: 436 (1957).

32. GUNJA-SMITH, Z., MARSHALL, J. J., SMITH, E. E., and WHELAN, W. J. A glycogen-debranching enzyme from *Cytophaga.* FEBS Letters 12: 96 (1972).

33. MEYER, K. H., and FULD, M. Recherches sur l'amidon XII. L'arrangement des restes de

glucose dans le glycogène. Helv. Chim. Acta 24: 375 (1941).
34. BANKS, W. and GREENWOOD, C. T. Physicochemical studies on starches, Part XXXII. The incomplete β-amylolysis of amylose. Staerke 19: 197 (1967).
35. OKA, S., SHIGETA, S., and SATO, S. Size distribution of dispersing units in corn amylose. Agr. Biol. Chem. (Tokyo) 35: 1216 (1971).
36. MERCIER, C. The fine structure of corn starches of various amylose percentage: waxy, normal and amylomaize. Staerke 25: 78 (1973).
37. MEYER, K. H., and BERNFELD, P. Recherches sur l'amidon V. L'amylopectine. Helv. Chim. Acta 23: 875 (1940).
38. GUNJA-SMITH, Z., MARSHALL, J. J., MERCIER, C., SMITH E. E., and WHELAN, W. J. A revision of the Meyer-Bernfeld model of glycogen and amylopectin. FEBS Letters 101: 96 (1970).
39. HESS, K., MAHL, H., and GUTTER, E. Elektronenmikroskopische Beobachtungen an Schnittflächen von Weizenkörnern. Mikroscopie 10: 329 (1955).
40. BUTTROSE, M. S. The influence of environment on the shell structure of starch granule. Staerke 15: 213 (1963).