AN ANOMALOUS LOW-MOLECULAR-WEIGHT BRANCHED COMPONENT IN DENT CORN STARCH[1]

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ABSTRACT

Ultracentrifugal schlieren patterns at both acidic and alkaline pH values with or without ethanol show that the peak corresponding to amylose comprises 33 or 34% of the total pattern for normal dent corn starch. The slower peak is absent in waxy corn starch samples, and only a single skew peak is present in samples of high-amylose (70%) corn starch. The percentage of amylose obtained by iodine-iodide absorption spectra was 26 or 27% for normal dent corn starch. The discrepancy between absorption and ultracentrifugal analyses reveals that the greater percentage of amylose obtained from schlieren patterns is due to an anomalous component that has 4.0% branching, a beta-amylolysis limit of 56%, and a weight-average molecular weight of 1.4 million. Other studies such as hydrolysis of amylopectin with proteolytic enzymes or with acid suggest that the anomalous component of normal dent corn starch is amylopectin not aggregated or complexed with protein. Data presented also suggest that the apparent absence of aggregates in high-amylose amylopectin and the absence of low-molecular-weight components in waxy starch are due to an affinity of enzymes or proteins for short-branch lengths. Data also show that the ultracentrifuge cannot be used to obtain the percentage of amylose in starch.

Perlin (1) isolated a component from wheat starch which he called amylopectin-C. He dispersed his wheat starch sample by autoclaving it for 3 hr. at about 120°C. according to the procedure of Schoch (2). After autoclaving, the amylopectin was centrifuged off in a preparatory-type ultracentrifuge. The amylose was then precipitated with n-amyl alcohol and the material remaining, called amylopectin-C or the C-component, with ethanol. Its yield ranged from 1 to 15% with an average of about 5%. It had a beta-amylase limit less than that of amylopectin, i.e., 48 to 53% compared to 55 to 60% for the amylopectin. McConnell *et al.* (3) further studied this C-component in wheat starch. About the same time, but independently, Edna M. Montgomery, in unpublished work (1957), obtained a component similar to that of the C-component from dent corn starch. This component was also obtained by centrifuging the amylopectin to the bottom of the centrifuge tube and precipitating the amylose with amyl alcohol. In addition, Erlander and French (4) observed a low-molecular-weight component in starch from immature, but not from

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mature, waxy corn. These findings suggest that a branched fraction of low molecular weight may exist in certain varieties of starch.

During an investigation of the composition of corn starch by ultracentrifugal analysis, we observed an incongruity between the amount of amylose shown by ultracentrifuge patterns and that by the colorimetric iodine method. Calculations from ultracentrifuge patterns gave 33 to 34% amylose, whereas colorimetric measurements on the same samples recorded 26 to 27%. After we observed this discrepancy a similar inconsistency was reported by Taki (5); that is, the chromatographic method he developed for determining the percentage of amylose gives a value of 35.5% instead of the accepted value of 26 or 27%. We know now that the discrepancies observed by ultracentrifugation are due to the presence of a low-molecular-weight polysaccharide that is branched.

Experimental

Seed-corn kernels of ordinary hybrid dent, waxy hybrid, and *ae* high-amylose (70% apparent amylose) Bear hybrid and their isolated endosperms were used as sources of starch. About 5 g. of kernels or endosperm were mixed with about 25 cc. of water in a Virtis homogenizer cell and stirred for 15 min. The cut-up kernels were centrifuged and washed twice with water to remove water-solubles. A solvent consisting of 1.5*M* lithium thiocyanate plus 6*M* guanidinium chloride was added to the centrifuged pad. The approximately 2% starch solution was stirred for 2 hr. in this solvent system at about 94°C. in a water bath. After dispersion, the solution was filtered to remove insolubles. The same result was obtained whether the pH of the solvent system was maintained at 4.2 or 6.7–7.0. Starch in the lithium thiocyanate-guanidinium chloride solvent mixture was precipitated by adding to 95% ethanol to give 80% v/v ethanol and then centrifuging. The precipitate was then dissolved in water. Since a small amount of material, which appeared to be protein, did not redissolve, it was removed by centrifugation. The supernatant was then placed in a dialysis bag and dialyzed overnight against either alkaline or acid aqueous medium.

A Spinco Model E ultracentrifuge equipped with a Wolter phase plate and RTIC temperature control system was used to characterize the dialyzed solutions. At first, the amylose-sedimenting peak was isolated in a partition cell in this analytical ultracentrifuge. However, to isolate larger quantities, the time and force necessary to sediment the amylopectin peak in the analytical ultracentrifuge were observed. The necessary calculations were made to change from the Model E ultracentrifuge partition cell to a swinging bucket rotor in a Model

L ultracentrifuge. In general, a 1% solution had to be centrifuged approximately 100 min. at 40,000 r.p.m. in the Model L in order to separate amylose-sedimenting material from amylopectin-sedimenting material. A small glass-wool plug in the bottom of the swinging bucket test tube prevented the amylopectin from being stirred after centrifugation.

Amylose was also separated from this starch solution before centrifugation as follows: The aqueous starch solution (about 200 ml., pH 7) was heated to 50° to 60°C., excess thymol was added, and the solution was allowed to cool with rapid magnetic stirring to about 30°C. (about 2 hr.). It was then placed in a refrigerator at 4°C. for another 2 hr. to complete precipitation of the amylose. The aqueous amylopectin and the anomalous component were then added to 95% ethanol (a final value of 80% v/v) to precipitate both components and to remove the thymol; this was centrifuged, redispersed in water by shaking, and heated to remove the ethanol. The amylopectin was then removed by centrifugation.

The anomalous component isolated by separation from it the amylopectin (by centrifugation) and the amylose (by precipitation with thymol) was oxidized by sodium metaperiodate to determine the degree of branching. The method of Manners and Wright (6) was used to determine the correct degree of branching; the apparent chain length was plotted *vs.* time of oxidation and the curve extrapolated to zero time.

The molecular weight of the isolated polysaccharide was obtained in 4*M* guanidinium chloride with the Model E analytical ultracentrifuge at 3,189 r.p.m. and a temperature of 25°C. The weight and Z-average molecular weights were obtained by the methods of Van Holde and Baldwin (7) and Erlander (8). A double-sector cell was used for both. The synthetic-boundary cell was constructed from a double-sector cell by cutting fine grooves close to the top but at the same distances from the bottom, one groove being on one side of the cell and the other on the opposite side. These grooves enable air to pass from one sector to the other. Two other grooves were cut about one-fourth the distance down from the top of the cell, on opposite sides of the cell as before. At about 7,000 or 8,000 r.p.m. the solution from the solvent part of the cell passes through these lower grooves to the other compartment in the double-sector cell, establishing the synthetic boundary.

Iodine absorption curves were obtained in a 5-cm. cell of a Cary spectrophotometer. The iodine-iodide color was developed by using 0.0005% amylopectin or anomalous component in a solution contain-

ing 0.0002*N* KIO_3 plus 0.0008*N* KI, which gives about 3.3 mg. I_2 plus 11.0 mg. of KI per 100 ml. solution. The solutions were made from stock of 0.005128*N* KIO_3 and 0.016337*N* KI.

The concentrations of starch in solution were obtained by the anthrone method and by optical rotation using a specific rotation for the starch of 200 for the sodium D-line. The concentrations were also obtained from the ultracentrifuge patterns as given by the method of Erlander and Foster (9). All areas were corrected for radial dilution with the Trautman Z-scale.

Results and Discussion

Typical ultracentrifuge patterns obtained for dent corn starch are given in Fig. 1. The two peaks correspond to amylose and amylopectin,

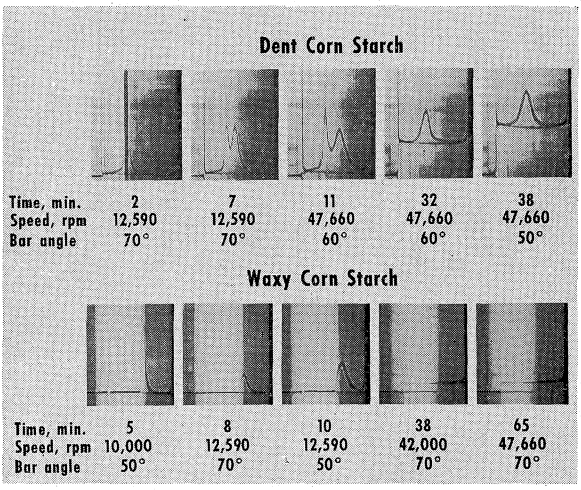

Fig. 1. Ultracentrifugal schlieren patterns of dispersed dent and waxy corn starches in water. Centrifugal force is from left to right in Figs. 1 through 3.

the faster-sedimenting peak being the amylopectin. Percentages of amylose calculated from these types of pattern are given in Table I for solutions of starch in 0.05*M* sodium acetate-acetic acid buffer, pH 4.2, and for solutions in 0.5*M* potassium hydroxide. An average of 33 or 34% apparent amylose was obtained from the ultracentrifugal patterns, compared with the value of about 26 or 27% from iodine sorption. This high percentage is also obtained in alkaline solutions; i.e., a value of 33% is obtained in 0.5*M* KOH just as in pH 4.2 solutions. In addition, if the sample is treated with alkali and then the alkali is removed by dialysis, the same result is obtained, 33.5% ap-

TABLE I

APPARENT PERCENTAGE OF AMYLOSE IN VARIOUS CORN VARIETIES FROM ULTRACENTRIFUGAL PATTERNS[a]

Apparent percent amylose	33.0	32.1	35.5	33.3	33.4	33.5
pH	>11 (0.5*M* KOH)	4.2	4.2	4.2	4.2	0.5*M* KOH→ pH 4.2[b]
Sample source	K	K	K	K	E	E

[a] The "percent amylose" was calculated as discussed in "Experimental." The "pH" refers to that of the solution analyzed in the ultracentrifuge synthetic boundary cell. "K" and "E" refer to kernels and endosperm as the source of corn starch.
[b] Solution originally in 0.5*M* KOH was dialyzed to pH 4.2.

parent amylose. Two starch solutions, both at pH 4.2 and containing 1% ethanol, were prepared from mature dent corn kernels. The apparent percentage of amylose, as obtained from ultracentrifugal schlieren patterns, was 32.6 and 34.7%, in agreement with data in Table I. These results show that no additional low-molecular-weight material can be separated from the amylopectin by using either alkali or ethanol. Therefore, the anomalous component sedimenting with the amylose constitutes approximately 20% of the total amylose pattern or 7 to 8% of the total starch pattern.

The dispersed amylose was separated from amylopectin with thymol as the reagent (see "Experimental"). It was observed that if the thymol was left in the sample for longer periods than 4 or 5 hr., such as overnight, then a large part of the amylopectin was precipitated with the amylose. The amylopectin remaining after thymol precipitation was virtually free of amylose as determined by iodine-absorption methods, i.e., less than 0.1%. However, the sedimentation patterns, as shown in Fig. 2, reveal that both with thymol and butanol precipitation a significant amount of low-molecular-weight component remains in solution. In Table II the calculated percentage of low-

TABLE II

PERCENTAGE OF LOW-MOLECULAR-WEIGHT FRACTION IN CORN AFTER THYMOL PRECIPITATION

Apparent percent amylose[a]	8.5	9.8	14.4
Precipitating agent at pH 7	Thymol	Thymol	Butanol

[a] "Apparent percent amylose" refers to the low-molecular-weight component which does not complex with the precipitating agent.

molecular-weight fraction after thymol and butanol treatment is given. The higher figure obtained by butanol fractionation is due to the presence of amylose impurities. Therefore, about 9% of the amylopectin consists of a low-molecular-weight component that is not precipitated by either thymol or butanol. Since the amylopectin fraction

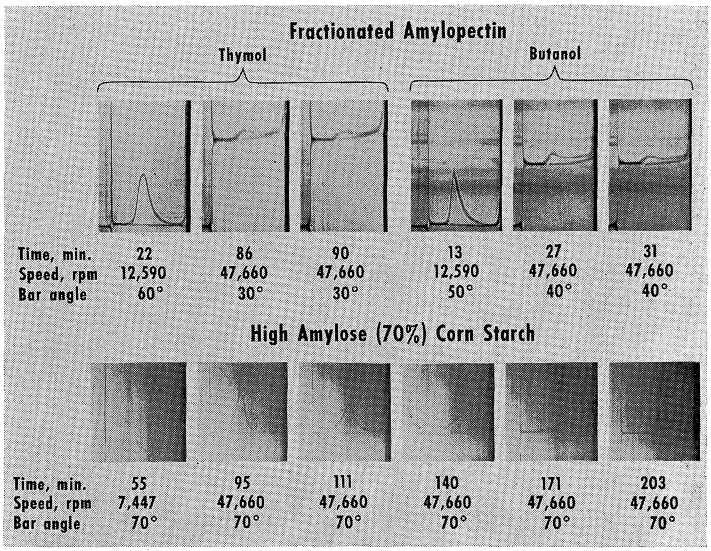

Fig. 2. Ultracentrifugal schlieren patterns of dispersed dent corn amylopectin and high-amylose (70% apparent) corn starch in water. The amylose was separated from the dent corn amylopectin by precipitation with an excess of either thymol or butanol.

comprises about 74% of the total starch, then the percentage of this low-molecular-weight component is approximately 7% for the total starch sample. This value agrees quite well with that obtained from sedimentation patterns of the unfractionated starch, i.e., 33% minus 26% equals 7%.

Waxy starch and high-amylose starch having an apparent percent amylose of 70% were also examined in the ultracentrifuge after dispersion in the solvent system of lithium thiocyanate plus guanidinium chloride. The waxy starch does not possess the low-molecular-weight fraction, as shown in Fig. 1. Even before the speed of the ultracentrifuge rotor can be raised from 12,590 r.p.m. to 46,000 r.p.m., the waxy amylopectin has sedimented to the bottom of the cell leaving no polysaccharide in solution. Therefore, in the case of ordinary dent corn starch, the anomalous component is not due to slight hydrolysis of amylopectin. This conclusion is supported by the observation that the low-molecular-weight component was obtained from dent corn starch by dispersion at pH 7.0 as well as pH 4.2.

The 70% high-amylose starch does not separate into two peaks and, therefore, no analyses could be made. It was observed previously (10) that the molecular weight of amylopectin from 70% high-amylose corn starch is quite low, being around 5 million as compared to about

1 billion for ordinary dent corn amylopectin. Thus, the single skewed peak in Fig. 2 for high-amylose corn starch is accounted for because amylopectin has a molecular weight too low for separation from the amylose.

To obtain large quantities of the low-molecular-weight anomalous polysaccharide, a dispersed ordinary dent corn starch sample was treated with thymol to remove amylose. The supernatant was sedimented in a swinging bucket rotor, as described under "Experimental," to separate the amylopectin from the low-molecular-weight polysaccharide. Sedimentation patterns of the resulting low-molecular-weight polysaccharides are given in Fig. 3 for both aqueous and 4*M*

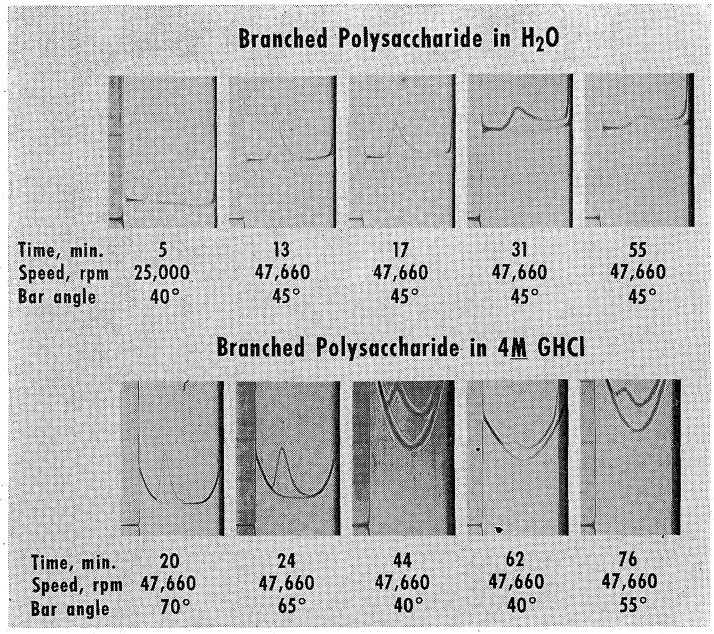

Fig. 3. Ultracentrifugal schlieren patterns of the anomalous dent corn starch component in water (pH 7) and 4*M* guanidinium chloride.

guanidinium chloride media. The patterns are skew, indicating heterogeneity. The degree of branching was 4.05%, which agrees with that obtained for dent corn amylopectin (11). In addition, the beta-amylolysis limits of the anomalous dent corn starch component and of dent corn amylopectin were both 56%. This value is higher than the 48 to 53% value obtained by Perlin (1) for his C-component. Our data indicate that the anomalous component has about the same chemical characteristics as that of its corresponding dent corn amylopectin.

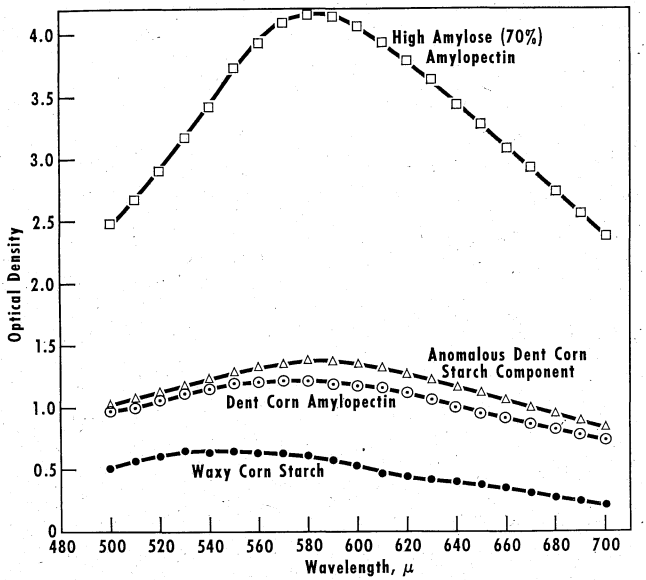

Fig. 4. Optical densities of iodine-iodide-amylopectin complexes obtained from high-amylose (70%) (□), dent (⊙), and waxy (●) corn starches *vs.* wave length compared to that for the anomalous dent corn starch component (Δ).

In Fig. 4 the absorbances (optical densities) of the iodine-iodide solutions of high-amylose, dent, and waxy corn amylopectins are compared with that of the anomalous component. The same concentration (0.0005%) of amylopectins and the anomalous component was used in all four cases. The extinction coefficients ($E^{1\%}_{1\ cm.}$) obtained at the peaks of the curves for the waxy, dent, and high-amylose (70%) corn amylopectins were 30, 51, and 164 as compared to 55 for the anomalous component. The differences between the absorbances of the various samples are due to a variation in exterior chain lengths, as indicated, for example, by the beta-amylolysis limits of approximately 52 and 71% (12,13) for waxy and high-amylose (70%) corn amylopectins, respectively. Hence, the interaction with iodine and the beta-amylolysis limit are in agreement.

A branched heterogeneous structure for the anomalous component was indicated further by data from molecular-weight determinations by equilibrium ultracentrifugation. The weight-average molecular weight of this anomalous component in 4*M* guanidinium hydrochloride was 1.37 million; the Z-average was 4.0 million. Thus, the ratio of the Z-average to the weight-average molecular weight is $\overline{M}_z/\overline{M}_w = 2.9$. For a linear component, the ratio of the Z-average to the

weight-average molecular weight should not exceed 1.5, whereas for a branched component it should not exceed 3.0 (14). Thus, from the ultracentrifugal molecular-weight studies the anomalous component is likely a randomly branched A-R-B_{f-1} type of condensation polymer rather than a linear A-B type.

The ratio of the Z-average to the weight-average molecular weight was also substantiated from the slopes of the concentration dependence of the plot $1/\overline{M}_w$ or $1/\overline{M}_z$ *vs.* the average concentration (Ca + Cb)/2. According to the equations of Erlander (8,15), the ratio of the concentration coefficient from the Z-average to that from the weight-average molecular weight is $B_Z/B_W = 2/(M_Z/M_W)$. The ratio of the Z-average to the weight-average molecular weight obtained from these concentration coefficient terms[2] is 3.9. Although higher than the extrapolated and theoretical values, this value again suggests that the component is appreciably branched, has a broad size distribution, and is not homogeneous.

It should be noted that the anomalous amylopectin in starch isolated by Banks and Greenwood (16) is not the same as this branched component of low molecular weight. Their component is precipitated by thymol, whereas ours is not precipitated by either thymol or amyl alcohols. Likewise, other components isolated by Schoch's precipitation method from various starches as intermediate fractions are not the same as our anomalous component. Their intermediate fractions were obtained by first precipitating the amylose with such agents as water saturated with thymol, amyl alcohol, or 2-nitropropane, and then by reprecipitating with such milder agents as water saturated with butanol or 1-nitropropane. Such intermediate fractions have been examined by Whistler and Doane (17) for various genetic corn starches.

It is known (11,14) from its molecular size distribution that amylopectin is an extremely heterogeneous polymer and behaves as an A-R-B_2- or A-R-B_3-type branched condensation polymer. Because of this broad size distribution, one would expect that components such as those isolated by Whistler and Doane (17) are only part of the normal distribution for amylopectin. The size distribution in exterior chain lengths could even be broadened further if debranching enzymes, such as the R-enzyme (18,19), attack the amylopectin before it becomes a part of the starch granule, since certain molecules would be more susceptible than others to a debranching enzyme. The components of Banks and Greenwood (16) can also be considered as an intermediate fraction even though the degree of branching is apparently higher.

[2] S. R. Erlander and J. P. McGuire, unpublished report, 1963.

The higher degree of branching may be due to some kind of coprecipitation. Further studies are required to characterize their intermediate fractions more fully.

Our anomalous component differs in chemical structure from sweet, dent, waxy, and high-amylose (70%) corn glycogens (12), which also are not precipitated in water with amyl alcohol. The percentage of branching (6 to 8%) and the beta-limit (35 to 46%) of these glycogens (12) differs significantly from that of the anomalous component (4% branching; 56% beta-limit).

Perlin's (1) amylopectin-C was isolated from wheat endosperm starch under conditions similar to those used in obtaining the anomalous component of dent corn starch. That is, the amylopectin was removed by centrifugation and the amylose by fractional precipitation using n-amyl alcohol. The beta-amylolysis limit of Perlin's amylopectin-C is slightly lower than ours. However, this difference may be an artifact or a difference in species. It may, therefore, be concluded that the two components are the same.

The presence of three components in ordinary dent corn amylopectin was demonstrated by Lewis and Smith (20) by glass-paper electrophoresis. The structural relationships of these components have not been investigated, although Whistler and Doane (17) inferred that at least one of the components may be the intermediate fraction which they isolated. Our low-molecular-weight amylopectin might also have contributed to the existence of three components in the amylopectin fraction.

The low-molecular-weight amylopectin may be responsible for the anomalously high value for the amylose content of corn starch reported by Taki (5) on the basis of paper chromatography. His value of 35.5% is remarkably close to the range of 33 to 35% apparent amylose we obtained from the ultracentrifugal schlieren patterns (see Table I and text). Amylose, amylopectin, and their degraded products have been separated from each other by chromatographic methods. Results suggest that chromatography of starch depends on both structure and molecular size (Winkler, 21; Ulmann, 22; Richter, 23; Richter and Stroh, 24; Ulmann and Richter, 25). The high concentration of perchloric acid (40%) used by Taki (5) most likely dissolves the starch in the same manner as lithium bromide or similar salts used in this study because of the high activity coefficient of perchloric acid ($\gamma = 500$ for 16 molal $HClO_4$ and $\gamma = 198$ for 16 molal LiBr (26)). Failure to resolve amylose from the low-molecular-weight amylopectin would then account for Taki's high results for amylose content.

Erlander *et al.* (27) observed that amylopectin can be partially hydro-

lyzed by the proteolytic enzyme Pronase (*Streptomyces griseus* protease). Alpha-amylase appears to be completely absent. The limiting weight-average molecular weights of both dent and waxy corn amylopectin after Pronase action were about 4 million. Moreover, acid hydrolysis studies (11) on waxy and immature sweet corn amylopectins and glycogen and enzymatic hydrolysis (28) of waxy corn starch also give extrapolated molecular weights in the range of 4 million even though the initial molecular weights may have been in the billions. These results strongly suggest that the high molecular weights obtained for various amylopectins are due to the complexing of protein with carbohydrate. Once this complex is destroyed, the molecular weight of an undegraded amylopectin becomes equal to about 4 million or possibly less since the limiting molecular weight has not yet been determined.

In the case of mature waxy amylopectin, the low-molecular-weight component is absent (Fig. 1). In the case of high-amylose starch, the entire amylopectin behaves as the anomalous component (Fig. 2). Consequently, there appears to be a variation in the degree that an amylopectin may complex with protein. Table III shows the correlation of exterior branch lengths of various amylopectins and the approximate percentage of amylose that has complexed with protein as judged by the schlieren ultracentrifuge patterns. This variation is in accord with the length of the exterior chains on the amylopectin, the shorter chains (waxy amylopectin) complexing strongly and the longer chains (high-amylose (70%) amylopectin) complexing only slightly if at all. Studies on the complexing nature of concanavalin-A by Manners and Wright (29) suggest that there is an inverse linear relationship between the average chain length of an amylopectin or glycogen and its ability to complex with this protein. This same sort of relationship appears to hold in our studies with regard to the amount of anomalous component in various amylopectins.

An obvious conclusion that must be drawn from our studies is that the ultracentrifuge cannot be used to determine the percentage of amylose in starch samples.

TABLE III
PROPERTIES OF CORN AMYLOPECTIN[a]

VARIETY	BETA-LIMIT	BRANCHING	APPARENT AMOUNT OF PROTEIN COMPLEX IN AMYLOPECTIN
		%	%
Waxy	52	4.7	100
Dent	56	4.0	91
70% High-amylose	75	2.0	0

[a] Values for beta-limit and percentage of branching obtained from data of Erlander *et al.* (12).

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