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On the non-random nature of amylopectin branching

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Abstract

The cluster model of amylopectin (AP) provides a useful conceptual basis for understanding of the structure of the molecule, and this model guides current thinking related to AP biosynthesis and physical behavior. The chain length profile of constituent linear regions of the molecule is commonly determined, and the cluster model guides the interpretation of results with an emphasis on the clustering of the linear regions of these chains. Less attention has been given to the related but distinct question of the clustering of the branch points in AP, perhaps because this work is methodologically more difficult than for determination of the chain length profile and interpretation of the results is less straightforward. However, the unique aspect of AP is that its branching is non-random, and the physical properties of AP may be considered to result from this unique structure. This review addresses the available information on the distribution of branch points in AP. Emphasis on this aspect of the cluster model has ramifications for future research on AP biosynthesis and AP physical behavior.

Keywords: Amylopectin; Cluster model; Chain length profile

1. Introduction

The first depiction of a cluster model for amylopectin (AP) was by Nikuni (1969, 1978), who proposed that periodic clustering into units could account for an AP molecule of indefinite molecular weight by linking of cluster units. French (1972) noted that the evidence strongly indicated that uniformity of inner branch lengths was impossible, and proposed two different models which might account for this evidence: a modified trichitic structure, and a cluster (racemose) hypothesis. French (1972) noted that the two models differed in that the former lacked a "regular rebranching pattern." French pointed out that the cluster model appeared to be more consistent with structures of Nageli amylodextrins, but he nevertheless noted that the cluster theory of AP was speculative and needed much testing. The cluster model was one scheme which could accommodate non-uniform inner branch lengths.

Robin, Mercier, Charbonniere, Guilbot and Duprat (1974, 1975) used the cluster concept to interpret their chromatograms of acid-hydrolyzed waxy starch granules. As did French, they noted that the concept was consistent with observed crystallinity of granules and amylodextrins. The cluster model gained greater credence when Hizukuri (1986) proposed that the observed periodicity in size distribution of constituent 1,4-linked chains might be accounted for by postulating that these linear chains were either located within a single cluster or served to connect two or more clusters. Much recent starch research has generated chain length profiles of debranched starch, and interpretation of this work in terms of the cluster model is now the norm.

The cluster model may be envisioned in two ways: (1) exterior linear regions of AP occur in physical clusters of crystalline double helices; and (2) 1,6 branch points are distributed with periodic variation in branch point density. Relatively little recent work has addressed the question of the nature of the distribution of branch points in AP (Jane, Wong & McPherson, 1997). Compared with synthetic polymers, AP is unusual in that it is an extremely large, highly branched molecule. AP is unique relative to synthetic polymers in that it is non-randomly branched (Galinsky & Burchard, 1995, 1996). Consideration of AP as a non-randomly branched polymer rather than as a polymer with periodicity in length of the linear regions provides a fresh perspective to our understanding of the molecule. For this reason it is useful to consider current information about AP structure in the context of a careful discussion of the ways in which AP branching may be considered to be non-random. This approach may provide insight for research on AP synthesis and the physical behavior of the molecule.

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Hizukuri's (1986) idea relating the polymodal patterns in chromatograms obtained by size-exclusion chromatography (SEC) of debranched starch to the idea of periodicity of linear and branched regions was an important advance, providing fresh insight about the cluster model of AP. This work has led researchers to focus on the chromatography of debranched starch as a means of describing AP structure. However, the polymodal chain length profiles provide little information concerning the random or nonrandom nature of the branching: i.e. these profiles do not allow differentiation between the modified trichitic model and the cluster model. These profiles show only that the distribution of chain lengths is non-random in that it is composed of two major populations of chains: short, with peak degree of polymerization (DP) of about 15, and long, with peak DP of about 45. This chain length profile relates to physical behavior, as Hizukuri (1985) has noted that the X-ray diffraction pattern correlated with the nature of the chain length profiles. Although the presence of the two major populations shows that the AP structure is in some sense non-random, these chain length profiles could occur in AP by means of numerous different branching schemes, including a random attachment of the constituent chains.

The polymodal chain length distribution of debranched AP was recently considered by Erlander (1998a) to be related to a tiered structure of a putative precursor glycogen molecule. The length of an AP chain was considered a function of the number of tiers the chain occupied in the precursor molecule. The tiers in the model do not correspond to the clusters envisioned by Hizukuri (1986), as each of the major peaks in the chromatogram was considered by Erlander (1998a) to result from several chain distributions, each peak including chains from more than a single number of tiers. Erlander assumed a constant "internal degree of branching" for both the precursor glycogen and the AP, meaning a constant average distance between branch points. For this model to be consistent with known A:B chain ratios and β-amyloysis limits (see below), it was necessary to postulate "interior A chains" hidden from the debranching enzymes which putatively converted the precursor glycogen to AP, and also external branches in the precursor glycogen which were not elongated. Erlander's approach to AP structure is based on a "statistical model", a randomly branched structure with no systematic bias toward either the Haworth or Staudinger models (see below). The tiered model led Erlander to posit that only the last tier was accessible to β-amylase, just as only the exterior A chains were believed to be accessible to the debranching enzyme. The interior, inaccessible A chains were postulated to be the basis for observed clusters in AP. Erlander (1998a) concluded that the clusters were randomly branched structures, with random addition of glucose units to each chain.

Matheson and Caldwell (1999) recently considered whether AP could be described by either a regularly branched or a random dendrimeric structure, and they concluded that a mathematical transformation of the random dendrimeric structure was necessary to produce a chain length profile resembling that of AP. A dendrimer has been described as having a "high degree of branching and strictly spherical shape", with each generation (tier) fully reacted (Stutz, 1995). Thus a strict dendrimeric model of AP would be regularly branched at each tier, with all A chains (see below) associated with the outer tier. The random dendrimeric model of Matheson and Caldwell (1999) is one in which the distance between branch points is not fixed at a particular length, but determined by probability; furthermore, A chains may also exist toward the interior of the molecule. The modification of dendrimeric structure by both randomization of inter-branch chain lengths and a two-part transformation (a weighting procedure applied differentially to short and long chains) results in a model that is neither regular nor random.

AP has also been characterized as a non-randomly branched molecule, and the variable concentrations of branch points, rather than the chain length profile, was stressed as a critical feature of AP structure (Burchard & Thurn, 1985; Galinsky & Burchard, 1995, 1996; Hanselmann & Burchard, 1996; Thurn & Burchard, 1985). Others have recently accounted for AP crystallinity by suggesting that double helices may behave as mesogens in a liquid crystal (Waigh, Perry, Riekel, Gidley & Donald, 1998), with double helices forming from pairs of external chain segments joined by a branch point. The mesogens could move relative to each other to pack laterally in crystallite regions. Sufficient length of the flexible linkers connecting the double-helical mesogen to the polymer would be important for mesogen mobility, but a local concentration of branch points might not be necessary for crystallite formation. Since the length of a flexible linker would be related to the distance between the exterior branch point and the next branch point, an understanding of the relationship among branch points would be critical to evaluation of this concept. Predictions about loss or gain of crystallite structure could be made based on differences in this distance. Recent workers have suggested that debranching enzymes may be important in starch synthesis (Ball et al., 1996; Erlander, 1998b; Martin & Smith, 1995). This reduction in the extent of branching would be accomplished in specific, but as yet undocumented ways. Manners (1997) has argued that debranching enzymes are not necessary for AP synthesis. It is appropriate to reconsider the body of literature related to the details of the structural architecture of AP, what many in the literature have termed the AP "fine structure" (Manners, 1989; Manners & Matheson, 1981), as it relates to the non-random nature of the branching in AP.

Branching of polymers in general has been described according to several imprecise and at times overlapping dichotomies: homogeneous/heterogeneous, regular/irregular, periodic/non-periodic, and random/non-random. With respect to AP, homogeneous branching would imply a uniform architecture throughout the molecule, as distinct from a molecule with regions of differing architecture.

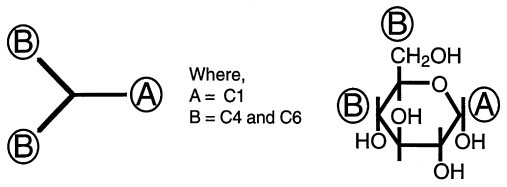

Fig. 1. The glucose unit as an example of a B2-A trifunctional monomer. The reducing end, at carbon 1, corresponds to the A functionality.

Regular branching would imply some pattern to the location of branch points, whereas irregular branching might imply a random relationship among branch points. Periodic branching implies some regular higher-order variation in the branching. And random branching implies no pattern at all. It is the thesis of this review that AP branching is non-random, and that an understanding of the patterns related to branching will lead to a better understanding of important questions related to AP synthesis and to behavior of AP molecules and their derivatives.

2. Branched structures from condensation of trifunctional monomers

Flory (1953) suggested that starch branching may be accounted for by the random condensation of trifunctional groups of the type B2-A, as each glucose monomer can potentially condense through the OH group at carbon 1 (functionality A) to carbon 4 or carbon 6 of another glucose monomer, and also can potentially condense through its OH groups at carbons 4 and 6 (functionalities B2) to carbon 1 of two other glucose units (Fig. 1). Condensation at all three sites would result in the unit becoming a branch point. If the condensation were to proceed in sequence toward the non-reducing terminus (NRT) without always condensing at both B sites, and there were no preferences for condensing the new A functionality (carbon 1) to carbons 4 or 6, then the branching pattern would be random. The two oligosaccharides shown in Fig. 2 illustrate possible outcomes. Structure "a" is consistent with known structures within an AP molecule, but structure "b" is not (see below). Flory also stated that if bifunctional B-A units were interspersed into a branching scheme, there would be no effect on "the essential character of the molecular plan." This idea is illustrated in Fig. 3 (assuming that the B functionality is always through carbon 4), using the structure of Fig. 2a as the basis for two additional structures with the same molecular plan as in Fig. 2a. These two structures of Fig. 3 are also consistent with structures within an AP molecule.

3. Constraints on random condensation structures

Rather than think in terms of bifunctional spacer units, one might equally well consider AP the result of condensation of *potentially* trifunctional glucose monomers, but with a few constraints on randomness. The following constraints are necessary for consistency with what is known about AP:

1. Condensation through carbon 6 is allowed only when condensation through carbon 4 also occurs. In other words, no linear 1,6-linked regions occur.
2. The probability of condensation through carbon 4 is far greater than through carbon 6 (~25 times so).
3. A glucose monomer condensed through its carbon 1 to carbon 6 of another glucose monomer cannot itself have another glucose monomer condensed to it through carbon 6 (see Fig. 4a).
4. A glucose monomer with another glucose monomer linked to it through carbon 6 may not be condensed through carbon 4 of another glucose monomer which has a glucose monomer condensed through its carbon 6 (see Fig. 4b).

The first two constraints describe one aspect of the non-random nature of the branching of the potentially trifunctional monomers. Constraints (3) and (4) simply mean that two branch points may not be adjacent (see below) (Umeki & Yamamoto, 1975). Each of the latter two constraints limits the random nature of the branching by introducing a bias favoring one type of branch; together, they represent a symmetrical pair of constraints that limit randomness in branching, but with no net bias toward allowing a subsequent branch on one chain or the other. Since these four structural constraints are consistent with reported evidence, then at least these above-mentioned aspects of the non-random nature of AP follow. In addition to these points, another indication of the possible random nature of the branching would be whether a particular branch is further branched or not; i.e. whether there is a bias toward or against further branching of those chains that emanated from carbon 4 as opposed to those that emanated from carbon 6 of the previous branch point.

4. Chain length and chain length profiles

Considerable evidence related to starch fine structure may be brought to bear on the problem of the non-random branching of AP. For some time it has been known that, on average, about 4% of the glucose units in APs from normal starches represent branch points in the molecule (Meyer & Bernfeld, 1940). From this information (and the high MW of the polymer) one may calculate what has been termed the average chain length (CL) to be about DP 25. The average CL is the average length of the linear chains, i.e. regions with anhydroglucose units (AGUs) connected only by 1,4 linkages. The same value may be obtained after enzymatic debranching with isoamylase, by dividing the total glucose units by the reducing glucose units of the chains. The average CL is a number-average value. It is also possible to chromatograph the debranched AP and process the data to calculate the number-average DP (DP_n) (the

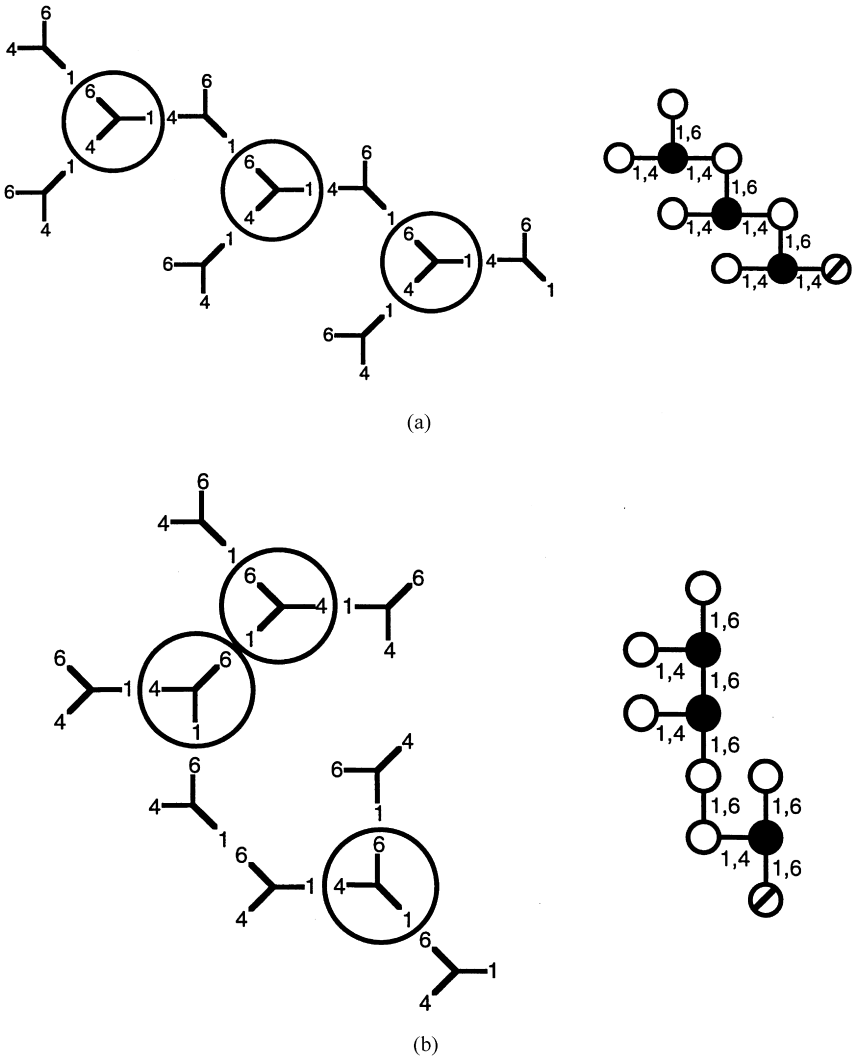

Fig. 2. Two possible outcomes of the random condensation of B2-A trifunctional monomers. Structure *a* might be observed in starch, whereas structure *b* would not be.

value should be identical to the average CL calculated by these other methods), and the weight-average DP (DP_w).

The constituent linear chains have been categorized according to their relationship to the rest of the molecule (Peat, Whelan & Thomas, 1952): *A chains* are linked only through their reducing terminus to carbon 6 of a glucose unit of another chain; *B chains* are linked in the same way, but in addition, a B chain has at least one other chain attached at carbon 6 of one of its glucose units (this other chain could be either A or B type). The *C chain* is the only chain having a free reducing end in the intact molecule. (In an AP sample the C chains will not be distinguishable in practice by most analytical approaches, and will be observed with the B chains.)

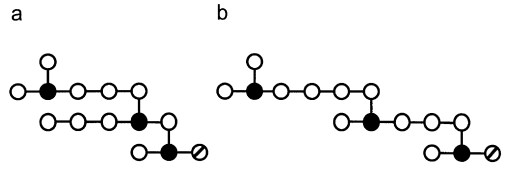

Fig. 3. Structure *a* of Fig. 2 is used as the basis for insertion of bifunctional B-A units (based on the OH groups at carbon 4 and carbon 1, respectively) in two different ways, producing the two structures (*a* and *b*) which thus have in common the same molecular plan for branching.

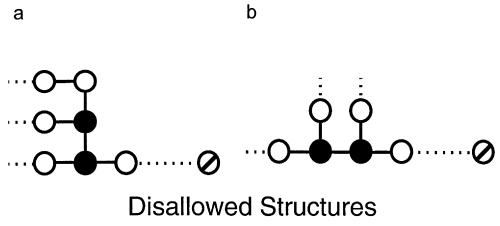

Fig. 4. Two structures thought to be disallowed in starch. Filled circles correspond to branch points. In each case, the branch point closest to the reducing terminus has another branch point directly attached.

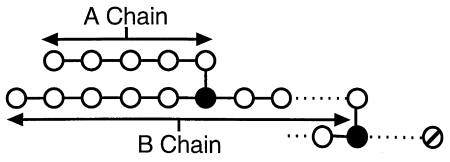

Fig. 5. A branch point in relation to an A chain and the B chain attached to it.

5. A:B chain ratios

The labeling of constituent chains as either A, B or C chains implicitly focuses attention on the chain length profile and detracts from a focus on the arrangement of branch points. However, the molar ratio of A:B chains provides insight into one aspect of the randomness of branching: whether there is a bias concerning further branching of chains emanating from carbon 4 versus chains emanating from carbon 6. We may consider a branch point to be a glucose unit internal to a 1,4-linked chain and also having a chain linked at the carbon 6 position, as indicated in Fig. 5. The ratio of those linear 1,4 chains linked only through carbon 6 to those linear 1,4 chains containing one or more branch points is the A:B chain ratio. Several readily distinguishable models of starch branching were initially proposed, and the A:B chain ratio determined by experiment was a useful way of distinguishing among them. The Haworth model predicted that each branched chain (except the single most external one) would have a branch attached, i.e. that all but one chain attached through carbon 6 would itself contain a branch point (Haworth, Hirst & Isherwood, 1937). The Staudinger model predicted that all branch points would be part of the same 1,4-linked backbone chain, i.e. that no chain attached at a branch point would itself contain a branch point (Staudinger & Huseman, 1937). These two models describe the extremes of non-random branching with respect to a bias toward further extending a chain emanating either from carbon 4 or carbon 6 of a branch point.

Degradation by purified β-amylase to produce the β-limit dextrin and maltose has been employed as a means of distinguishing A and B chains. β-Amylase is an exo-enzyme hydrolyzing maltose from the NRTs until it encounters a branch point. The A chains are hydrolyzed to within 2 or 3 glucose units (depending upon whether the original chain had an even or odd number of glucose units) of the branch point. The exterior portions of B chains are hydrolyzed to within 1 or 2 glucose units of the branch point (depending upon whether there originally were an odd or even number of glucose units beyond the branch point) (Manners, 1989). When the β-limit dextrin is subsequently completely debranched, the maltose and maltotriose indicate the previous presence of A chains, and the longer chains represent the residual portions of the B chains. In this way the A and B chains can be distinguished (there is also some maltotriose released from very short residual B chains, based on the first structure in Fig. 10). By this and other means, determination of the A:B chain ratio to be approximately 1 has ruled out the Staudinger and the Haworth models. This ratio implies no net bias, after the branch point, in whether one chain or the other would contain another branch point. A ratio of 1 is also not inconsistent with random branching of chains. Several branching schemes other than the original Meyer model (Meyer & Bernfeld, 1940) are also consistent with an A:B ratio of unity (Manners, 1989). Lee, Mercier and Whelan (1968) showed that although the Meyer model could be drawn in the most ordered form possible, a regularly rebranched structure (similar to the regular dendritic structure described by Matheson & Caldwell, 1999), this form of the model was inconsistent with the chromatography evidence, particularly of the debranched β-limit dextrins (see below). Later modifications to the Meyer model suggested that not all B chains carried A chains, and some B chains ended prior to the final tier (Gunja-Smith, Marshall, Mercier, Smith & Whelan, 1970).

6. β-Limit dextrins, and interior and exterior chain length

β-Limit dextrins contain all the branch points of the intact AP molecules. Based on knowledge of the average CL of the intact molecules, and the extent to which the intact molecules are hydrolyzed by β-amylase (the "β-limit%"), one can calculate the portion of the original molecules that is from the NRTs to the first branch points encountered by the enzyme. This portion is in a sense *exterior* to the branching structure (even though it may be located in the interior portion of the volume of the three dimensional molecule) and therefore the chains are in theory accessible to an exo-acting enzyme acting at the NRTs (this assumption of accessibility of all external chains has been questioned (Erlander, 1998; Manners, 1989). Assuming that every constituent AP chain is accessible, and based on the idea that every chain is partially hydrolyzed by β-amylase, one may calculate the average length of the portion of the chains that is external to the branch points, as the average "exterior chain length," ECL:

$$\mathrm{ECL} = [\mathrm{CL}(\beta\text{-limit}\%/100)] + 2.$$

The *2* carries the implicit assumption that there are equal numbers of A and B chains, as the average stubs from A and B chains are 2.5 (from the stubs of the linear A chains) and 1.5 (from the B chain branch stubs), respectively. Of course, it is possible to use a number other than 2 to correct for an A:B ratio other than one. Manners (1989) has pointed out that this correction is generally not of practical importance.

Having calculated the ECL, it is a simple matter to calculate a corresponding average "interior chain length", ICL:

$$\mathrm{ICL} = \mathrm{CL} - \mathrm{ECL} - 1.$$

The *1* appears as a means of the considering the branch point

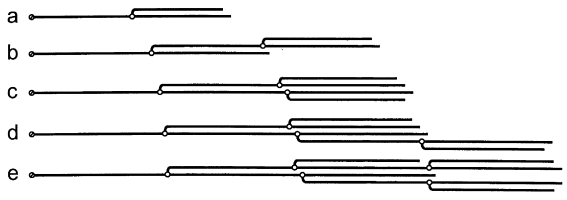

Fig. 6. A series of branched molecules as described in Table 1. The open circle represents a branch point. The length of a line between or outside the circles represents the length of a linear segment. Molecule *a* depicts total DP of 25, with one branch point. Molecule *b* has a DP of 50, with two branch points, molecule *c* has a DP of 75 with three branch points, and so on.

glucose unit as neither exterior nor interior. While the ICL is simple to calculate, its meaning is not immediately evident, as an A chain has no interior length, and a B chain contains at least one, but often more than one, interior segment, depending upon whether there is only one or more than one branch point in the 1,4 sequence. The meaning of the ICL is fundamental to establish before discussing the relationship among branch points.

The ECL and ICL values obtained from the β-limit dextrin data for APs are important in that they show another way that branching is non-random. Although this point will not be rigorously evident until the meaning of the ICL value is clear (see below), the commonly determined limits of β-amylase digestion of AP (about 55%, see for example Manners, 1989; Yun & Matheson, 1993) lead to the conclusion that there is a bias toward location of the branch points toward the interior (away from the NRTs) of the molecule.

In order to illustrate this bias, consider a series of AP molecules constructed by a simple algorithm, with the branches regularly distributed as far from each other as possible. In this illustration, the structures are constrained to having an A:B ratio of about one, and it is assumed that there is one branch point per 25 AGUs. Fig. 6 shows a series of molecules with increasing MW, and thus increasing numbers of branch points. As part of this exercise, the linear *segments* of chains, defined as either *between or external to the branch points*, are calculated. This segment concept is distinct from the A or B chain concept, and is evident in Fig. 6. The total number of linear segments, the length of these linear segments, the number of interior segments (including the unique segment from the RT to the first branch), and the number of exterior segments are also calculated. Fig. 6 illustrates the exercise visually, and Table 1 shows the outcome of the calculations. It is evident that as the MW increases, the value for the average CL approaches 25, and the length of a linear segment approaches 12. It is obvious that the ECL will be equal to the length of a linear segment. When the CL is 25, the ICL may be readily calculated to be 12 as well. By inspection of Table 1 it is clear that in this exercise the ICL represents the average length of what we have defined to be the interior linear segments, and not the length of the interior portion of a B chain.

The hypothetical molecules of CL 25 may be digested with virtual β-amylase. In this way one answers the question, "what is the limit of β-amylase digestion that is consistent with an AP model with regular, homogeneous branching?" Since the ECL is 12 and the CL is 25, we may solve

$$12 = [25(X\%/100)] + 2 \qquad \text{and } X = 40\%.$$

This value is at variance with the typical limit of β-amylase digestion of AP of about 55% (Manners, 1989). For the typical AP sample with the typical β-limit value and an average CL of 25, we can calculate the ECL to be 15.8, and the ICL to be 8.2. For this typical model, the difference between the ECL and the ICL values provides a sense of the extent to which the branch points are not located regularly and homogeneously throughout an AP molecule, but tend to be more concentrated toward the interior of the molecule. Fig. 7 illustrates this difference for a portion of the high-MW hypothetical completely regular, homogeneously branched AP (Fig. 7a) as compared to a portion of a similar AP, but with a structure that is also constrained by the data for the limit of β-amylase digestion (Fig. 7b).

In the above argument the hypothetical molecules in Fig. 7a or b have been drawn to have homogeneous structures, with each having identical exterior segments and identical interior segments. For an actual AP sample, the ECL and ICL values represent only average lengths, and provide no information about the distribution of segment lengths of each type.

Baba and Arai (1984) showed that the APs of *wx* starch and of *ae* starch have similar β-amylolysis limits; consequently even though both the ECL and the ICL for *ae*-type AP are greater than these values for *wx* AP, these values are in about the same proportion: 29.4 and 20 for *ae* AP, vs. 20.3 and 14.3 for *wx* AP. Yun and Matheson (1993) also observed similar β-amylolysis limits for *ae* AP and *wx* AP, as well as for the AP of *ae wx* starch. They also showed that the ECL and ICL of *ae* AP and *ae wx* AP were greater than for *wx* AP in about the same proportions. Thus, both ECL and ICL increase for *ae*-type APs, but the overall bias toward branches being located away from the NRTs is maintained.

7. Clustering of branch points

No insight is provided by ECL and ICL concerning the extent to which the branches may be unevenly concentrated within local regions of greater or lesser branching density. Bertoft (1989a,b) has addressed the question of branching concentration by employing a limited initial α-amylolysis, in an effort to isolate clusters by the preferential hydrolysis of the long B chains which link clusters. A cluster in this work is operationally defined as an arrangement of branch points sufficiently close to hinder α-amylase digestion of the connecting segments. Additional work has been done to isolate and characterize the individual clusters so that

Table 1
Linear segments in a series of hypothetical branched polymers with A/B of 1, one branch point for every 25 glucose units, and branch points uniformly distributed

Total DP	Branch points	Linear chains	A chains	B chains	Linear segments	Interior linear segments	Exterior linear segments	Mean DP of linear segments	Average CL (DP)
25	1	2	1	1	3	1	2	8.0	12.5
50	2	3	1	2	5	2	3	9.6	16.7
75	3	4	2	2	7	3	4	10.3	18.8
100	4	5	2	3	9	4	5	10.7	20.0
125	5	6	3	3	11	5	6	10.9	20.8
150	6	7	3	4	13	6	7	11.1	21.4
200	8	9	4	5	17	8	9	11.3	22.2
400	16	17	8	9	33	16	17	11.6	23.5
800	32	33	16	17	65	32	33	11.8	24.2
1600	64	65	32	33	129	64	65	11.9	24.6
160,000	6400	6401	3200	3201	12,801	6400	6401	12.0	25.0
960,000	38,400	38,401	19,200	19,201	76,801	38,400	38,401	12.0	25.0

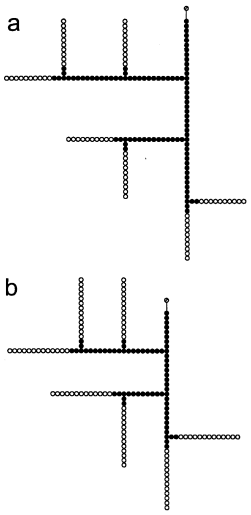

Fig. 7. Molecule *a* is a hypothetical molecule with six branch points regularly distributed, at a maximum distance from each other and from the chain ends. Molecule *b* is the hypothetical molecule with branch points regularly distributed an equal distance from each other, but with external chains of a length that would result in a β-amylolysis limit of 55%. Glucose units that would be removed by β-amylase are indicated by open circles.

cluster branching patterns can be better understood (Bertoft, Zhu, Andtfolk & Junger, 1999; Zhu & Bertoft, 1996; Bertoft & Koch, 1999). In their study of Naegeli dextrins, Jane et al. (1997) showed that APs with A-type X-ray diffraction patterns tended to produce more singly-branched molecules than starches with B-type diffraction patterns. They concluded that the branch points were more scattered in the A-type starches, and they suggested that their work was consistent with Bertoft's studies of clusters in waxy maize (Bertoft, 1991a) and potato (Zhu & Bertoft, 1996) starches.

Others have evaluated cluster branching parameters based on light scattering by intact molecules, and observed a lower calculated branching density than found by chemical means, an inconsistency which they attributed to a non-random branching pattern (Galinsky & Burchard, 1995; Hanselmann & Burchard, 1996). Although Burchard has described clusters of branch points with 4–5 branch points per cluster (Burchard & Thurn, 1985; Thurn & Burchard, 1985), the calculation appears to be based on an incorrect premise (they use the % of AP digestion by β-amylase as if it were the % of the AP remaining after β-amylase treatment) that when corrected leads to an estimate of nearly double their number of branches per cluster. Their calculation also assumes that a cluster has exceptionally high branching density (see below), with every second glucose unit in a cluster carrying a branch point.

8. The cluster model of Robin et al.

Robin et al. (1975) drew their cluster model with nine short chains (which they termed "A chains" by their unique

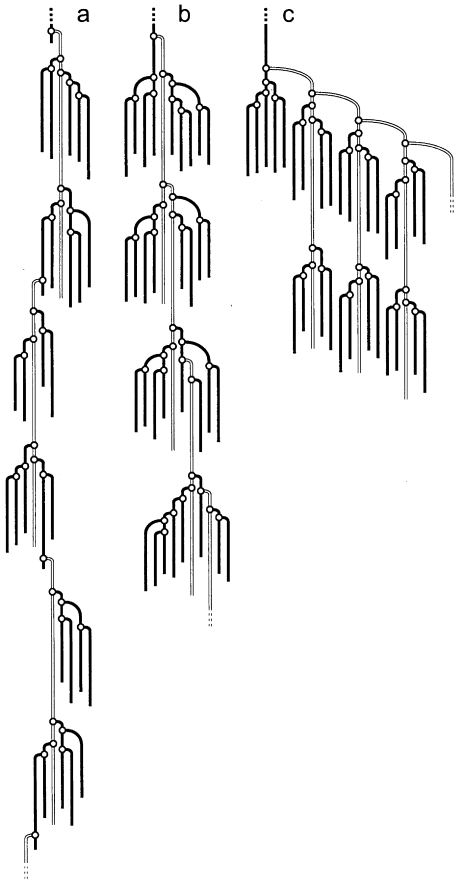

Fig. 8. Three structures with identical ratios of long B chains to short chains, but differing in the way the long B chains are connected to each other. For simplicity, all long B chains are of identical length. The number of branch points per cluster varies for the three attachment schemes. The cluster model depicted by Robin et al. (1975) contained a mixture of the three types of connections (see Fig. 9). These three schemes cannot be distinguished by the chain length profile.

nomenclature) per long chain (which they termed a "B chain"). While they clearly envisioned their long chains to interact with short chains in two clusters, it is not clear from their model how many branch points would be in each cluster, as there are three ways in which their long chains can link clusters (see Fig. 8). The average number of branch points per cluster would be a *minimum* of 4.5 (resulting from the linkages illustrated in Fig. 8a), with the actual average depending upon the relative proportion of the three ways of linking clusters. One way of linking clusters (see Fig. 8a) is to have the cluster-connecting chain linked near the NRT of a chain of the last cluster in which that previous long chain participates. A second way (see Fig. 8b) is to have the long chain linked between the two clusters of the previous long chain. A third way (see Fig. 8c) is to link the long chain at the RT end of the first cluster by which the previous long chain is attached. These three possibilities are each represented in the original Robin model, as shown in Fig. 9. Based on a chain length profile, there is no way to distinguish among these three possible ways of linking

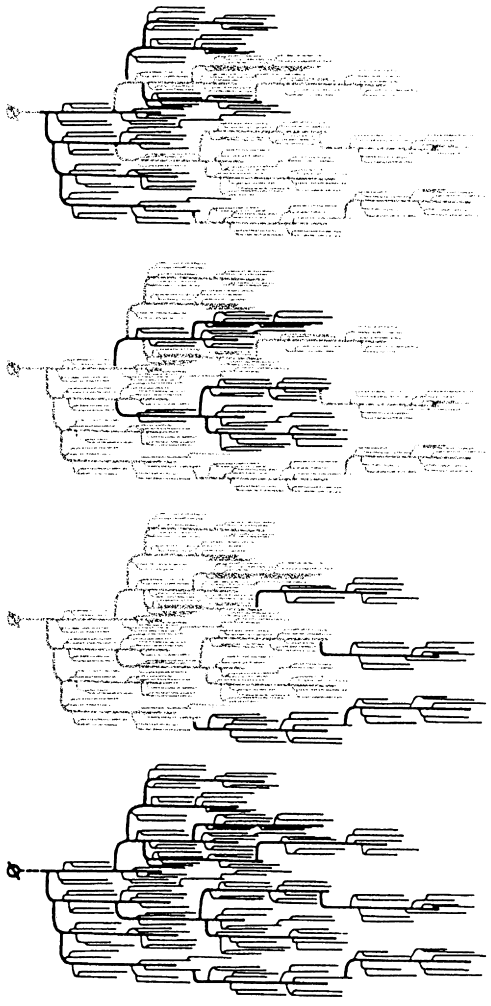

Fig. 9. The cluster model of Robin et al. (1975) is illustrated in the bottom depiction. Portions connected by the three different schemes of Fig. 8 are emphasized in the other three depictions.

clusters. Perhaps a combination of the three is the most appropriate model, as Robin et al. (1975) originally drew the model. A combination of the three schemes would allow for flexibility in filling available volume as granule synthesis proceeded in a radial direction. It is worth noting that if the third scheme (Fig. 8c) predominated, periodicity in chain lengths and generally radial orientation of chains could both result, even though biosynthesis would proceed with individual chains added tangentially. The result could

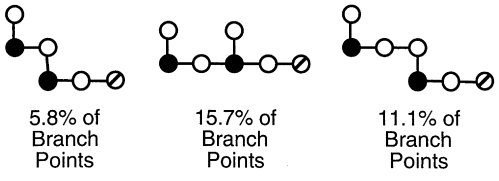

Fig. 10. Three multiply branched α-limit dextrins observed by Umeki and Yamamoto, with the percentage of the total branch points accounted for by each.

theoretically be a cluster with the number of branch points and linear chains per cluster approaching infinity. On the other hand, even by this third branching scheme, if some interior segments were somewhat longer than others, the structure might be conceived of as a series of smaller clusters. This analysis highlights the idea that a precise definition of a cluster based on the nature of the concentration of branch points is not available. Bertoft (1989a,b) has pointed out that the size of a unit cluster is a matter of definition.

9. The ICL profile

If clusters obtained by α-amylolysis were subjected to exhaustive β-amylolysis, ICL-type values could be obtained ($ICL_{cluster}$), and we would gain insight into the extent of further branching concentration, as these $ICL_{cluster}$ values could be compared to ICL values for the intact molecule (although as illustrated in Table 1, the same ratio of branch points to DP will produce shorter segments as DP decreases, leading to a somewhat smaller ICL). Regions of higher branching density were implied in the models presented to explain the *periodicity* of CLs in Hizukuri's (1986) original illustrations of his revised cluster model, but direct evidence for periodicity in branching density was not gathered in that work.

Banks and Greenwood (1975) describe formation of macrodextrins, multiply branched structures resistant to porcine pancreatic α-amylase, from glycogen and AP. They pointed out that the existence of these structures implied heterogeneity of branching, a concept further supported by the lower ICL values for these molecules than for the starting material. Kainuma (1988) summarized other earlier work to characterize the residual branched structures in the α-limit dextrin of maize AP (Kainuma, 1988; Kainuma & French, 1970) (see also French, 1972). They found that 65% of the branch points were observed in singly branched oligosaccharides, with the remaining 35% in more highly branched oligosaccharides which could not be further digested because the branch points were too close to each other. Umeki and Yamamoto (1972) quantitated the various doubly and triply branched α-limit dextrins of rice AP (in this work accounting for 44% of the total branch points), and found that nearly 33% of the total branches were in three forms of multiply branched α-limit dextrins (Fig. 10). These structures provide evidence that some interior linear segments are far shorter than the average ICL values for the AP. However, from this work it is not clear that the presence of these highly branched materials necessarily indicates non-random distribution of branch points, since random, heterogeneous location of branch points would be expected to generate some short interior linear segments as well as some that were longer than the average. In order to distinguish a broad unimodal distribution of interior segments from a polymodal distribution of interior segments, it

would be best to know the actual distribution of interior segment lengths, but information required to determine the ICL profile is not available by current analytical methods. Nevertheless, with one-third or more of the branches contributing to interior segments of DP 1 or 2, the argument appears strong for a non-unimodal distribution of branches within the β-limit dextrin. Bertoft and Koch (1999) have recently provided further evidence of the relative importance of very short internal chain lengths.

Based on computer simulation of molecular models of AP, O'Sullivan and Perez (1999) showed that only some internal chain lengths allow low-energy and parallel orientation of two adjacent double helices. They showed that certain combinations of the two internal chain lengths needed to connect two double helices are favored: 1 and 3, 4 and 6, 6 and 4, and 7 and 7. Perhaps equally important, they showed that several other combinations do not produce parallel double helices when the internal chains are in stable configurations. If the organization of the AP chains in a granule were dictated by energetics, this work would argue for a non-random distribution of internal chain lengths. Although the synthesis of AP may not produce the appropriate internal chain lengths for granule organization to be based on the lowest-energy configuration, the work of O'Sullivan and Perez suggests that differences in the ICL profile among starches could well influence the ability of disordered AP molecules to develop crystallinity during retrogradation.

Although the above research does not exclude a non-random distribution of branch points based on periodicity in branching density, no regular *periodic* differences in branching density may be directly inferred from these data. It is possible that the most highly branched regions are all in one region of the molecule. As Bertoft has recently suggested, the branching may also vary among AP molecules in a sample or even in different parts of the same granule (Bertoft et al., 1999; Bertoft & Koch, 1999).

10. Core chain profiles

The average CL for the β-limit dextrin includes both the residual A chains and the residual B chains. Yun and Matheson (1993) calculated this average CL (CL_{β}) as well as the average CL of the residual B chains ($CL_{\beta B}$). Considering only the residual B chains, they then calculated the average "core chain length", CCL, as the average value for the linear region between the first branch point and the reducing terminus (RT) of a chain, as follows:

$$CCL = CL_{\beta B} - 2.5.$$

The *2.5* includes 1.5 for the average external stub length and 1 for the branch point itself. (Bertoft (1991b) had earlier described the internal portion of a B chain as including the branch point.) To obtain insight into the branching pattern, it would be helpful to know the *distribution* of the core chain lengths. It would also be informative to know whether the number of branches on a B chain is a linear function of the core chain length, and whether these branch points are distributed randomly along the chain.

Just as debranched AP may be subjected to chromatography to show the chain length profile of the constituent chains, so may debranched β-limit dextrin be chromatographed and its chain length profile observed. And just as the chain length profile of debranched normal or waxy-type maize AP shows two major populations of linear chains, so does the chain length profile of residual B chains in debranched β-limit dextrin from these APs (Boyer, Garwood & Shannon, 1976; Inouchi, Glover & Fuwa, 1987; Manners, 1989; Shi & Seib, 1995; Yuan, Thompson & Boyer, 1993). The distribution of the residual B chains is closely related to the distribution of the core chain length (it represents the core chain length distribution shifted by 2.5 DP). For a given residual B chain there is no way to know how much of the original chain might have been exterior to the last branch point, and there is no way to know from this evidence whether the branch points on the chains are randomly distributed. There is also no way to know how many chains may have been attached to it. Nevertheless, one may obtain some insight into the distribution of branch points by focusing on the shorter of the two residual B chain populations. In this region the likelihood is greatest that the original B chains contained only one branch point (a likelihood that increases as DP decreases); to the extent that only one branch point had existed for these short residual B chains, the core chain length distribution in this region would represent a unique portion of the population of the interior segments between adjacent branch points. That there is a population of short residual B chains and a separate population of longer residual B chains (Bertoft & Koch, 1999) is itself strongly suggestive of a non-random branching pattern. Lee et al. (1968) have suggested that the "anomalous peak" at higher DP for SEC of β-limit dextrins means that the B chains are not distributed in regular fashion. If the branches were randomly distributed along B chains of random length, a unimodal distribution of CCL would result.

11. Span length: attachment of A to B chains

Hizukuri (Hizukuri, 1996; Hizukuri & Maehara, 1990) has described an analysis based on sequential treatment with β-amylase, isoamylase, and β-amylase to evaluate patterns of attachment of A chains to B chains. He differentiates between those B chains with only B chains attached (B_b) and those B chains with A chains attached (B_a), and he estimates the number of A chains per B_a chain (Hizukuri & Maehara, 1990). In a subsequent discussion of this work (Hizukuri, 1996), he calculates the average "span length" (SL) (defined as the average number of

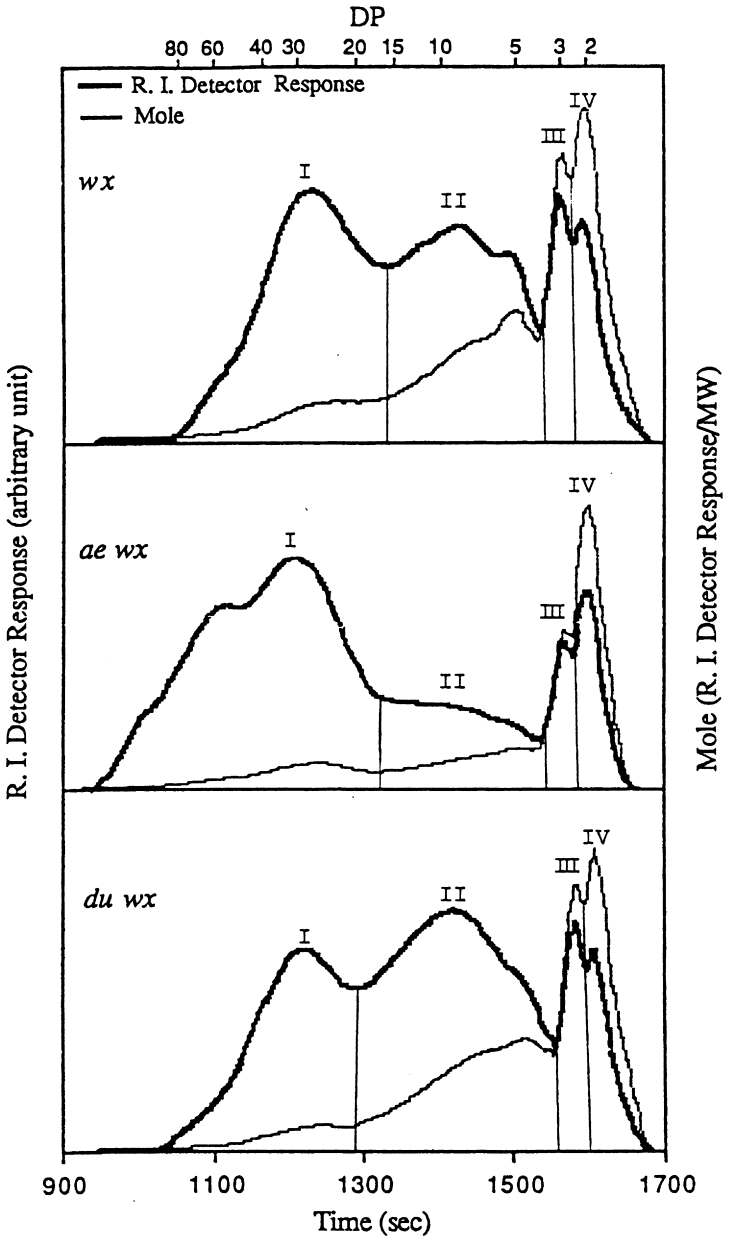

Fig. 11. Size-exclusion HPLC chromatograms of the debranched β-limit dextrins from three *wx*-containing genotypes from the W64A maize inbred line (from Yuan, 1991). The fraction corresponding to the short residual B chains is labeled II.

glucose units separating A-type branch linkages on a B chain) for the B_a chains. He shows that span length decreases disproportionately as the chain length of the residual B_a chain decreases, varying from 10.9 for 43.1 DP_{Ba} to 2.7 for 7.9 DP_{Ba}; the average SL was 6.9. While it must be kept in mind that the span length applies only to B_a-type branching, this information shows systematic changes in branching density, with greater density of branching for shorter residual B_a chains. The number of A chains attached to a B chain is therefore not a simple function of residual B_a chain length; instead the concentration of this type of branch point is biased toward shorter residual B chains. Thus the A chains are not randomly distributed along the B chains.

12. Short residual B chains

Based on the work described above with α-limit dextrins, in which 44% of the branches were as doubly or triply branched dextrins, a large molar proportion of relatively short internal segments would be expected. These segments might or might not be observed as short residual B chains. To appreciate the molar distribution of residual B chains

after β-amylolysis, one can view the chain length profile for the β-limit dextrin on a molar basis, as Yuan et al. (1993) have done. From that work (Fig. 11), it is clear that there is a large molar proportion of short residual B chains for the β-limit dextrin of the AP of *wx* starch, as about 70% of the total moles of residual B chains occur as DP $3 < X < 16$ (Yuan et al., 1993), with the modal value about DP 5. A residual B chain of DP 5 would correspond to an internal segment length of 2 or 3 when only one branch was attached. Thus, in addition to the evidence gathered for the branched molecules recovered in α-limit dextrins, the distribution pattern of residual B chains also strongly suggests that there is a strong bias toward interior segment lengths considerably shorter than the calculated ICL of about DP 8.2. At the same time, some interior segment lengths will necessarily be much longer than 8.2 to obtain the ICL average, a point made in general much earlier by French (1972). Given the large number of interior segments of DP 2 or 3 observed by Yuan et al. (1993) for *wx* starch, at least some of the chains in the longer of the two populations of residual B chains have unusually long interior segments, as Bertoft has shown (1989a,b; Bertoft et al., 1999; Bertoft & Koch, 1999). Burchard and Thurn have suggested that longer interior segment lengths may link clusters of branch points, while the shorter segments represent intra-cluster linkages (Burchard & Thurn, 1985; Thurn & Burchard, 1985).

These chain length distributions of residual B chains are of further interest as they vary among those starches (see Fig. 11) for which there is strong evidence of variation in other aspects of molecular architecture, as well as in physical behavior. Among maize starches the *ae wx* genotype has a much lower proportion of short residual B chains (about 50% of the chains) compared to the *wx* genotype (about 70% of the chains), and the *du wx* genotype has a relatively higher proportion of short residual B chains (about 86% of the chains) (Yuan et al., 1993). Potato AP also shows a relatively low proportion of the short residual B chain population in its β-limit dextrin (Zhu & Bertoft, 1996). While it is known that the chain length profiles differ for potato and normal cereal starches (Hanashiro, Abe & Hizukuri, 1996), only by looking at the chain length profiles for the β-limit dextrins do we gain insight into differences in the distribution of branch points.

13. Periodic concentration of branch points

The values for ECL and ICL are average values that can contribute insight into the overall bias toward branching being located toward the interior. The further evidence from α-limit dextrins and from the chain length profiles of the β-limit dextrins gives some insight into the distribution of the branch points within the core of the molecule, but no direct evidence for *periodicity* in concentration of the branch points. Periodicity in branching concentration is inferred from other types of evidence in combination with the fine structure data. Periodicity of branching concentration cannot be excluded on the basis of observed chain length distributions, and appears to be consistent with other data. If an ICL profile could be determined, the question would be addressed directly. The constant fractal dimension observed by Hanselmann and Burchard (1996) for degraded maize APs of decreasing MW suggests similar branching at various structural levels; consequently the distribution of branch points might help understand the higher-order structure of AP as well. Because evidence for periodicity in branching concentration is lacking, the nature of any periodicity in branching concentration may be different than what is illustrated in the various depictions of the cluster model.

14. The cluster model and physical behavior

The idea that the double helices can act as mesogens in a liquid-crystal type arrangement (Waigh et al., 1998) depends both on exterior chains of sufficient length to form double-helical mesogens and on the existence of sufficiently flexible linkers. It seems likely that an A chain and the B chain to which it is attached could readily form a double helix, as the branch point does not adversely affect energetics of the double helix, and the branch may actually serve to nucleate the structure (Imberty, Chanzy, Pérez, Buléon & Tran, 1987; O'Sullivan & Perez, 1999). The distance between the branch point terminating the double helix and the next branch point would be the length of the flexible linker joining the mesogen to the rest of the molecule (Waigh et al., 1998). Mesogens could have the mobility to align into crystalline array without the need for periodic clustering of branch points. The chain length profile of debranched AP could provide some insight into the length of the flexible linker, if one assumes that the main peak and the high-MW shoulder represent the modal lengths of A and short B chains (B1, by the terminology of Hizukuri, 1986); Hizukuri (1986) made this assumption in the analysis of the chromatograms in his seminal paper on the periodicity of the cluster model. However, the reasoning that the length of flexible linker segments can be inferred from the difference in DP between the modal lengths of A and B1 chains is indirect and tenuous. The distribution of residual B chain lengths in a β-limit dextrin bears more directly on the question of the length of possible linker segments, but the logic is still tenuous since the interior segment length is not identical with the core chain length. Potato starch (Zhu & Bertoft, 1996) and *ae wx* maize starch (Boyer et al., 1976; Inouchi et al., 1987; Shi & Seib, 1995; Yuan et al., 1993) have relatively less of the short residual B chains, as compared to *wx* starch. On this basis one might suggest that the length of flexible linker segments would tend to be longer for these starches since some long B chains might be involved. The β-limit dextrin of *du wx* starch

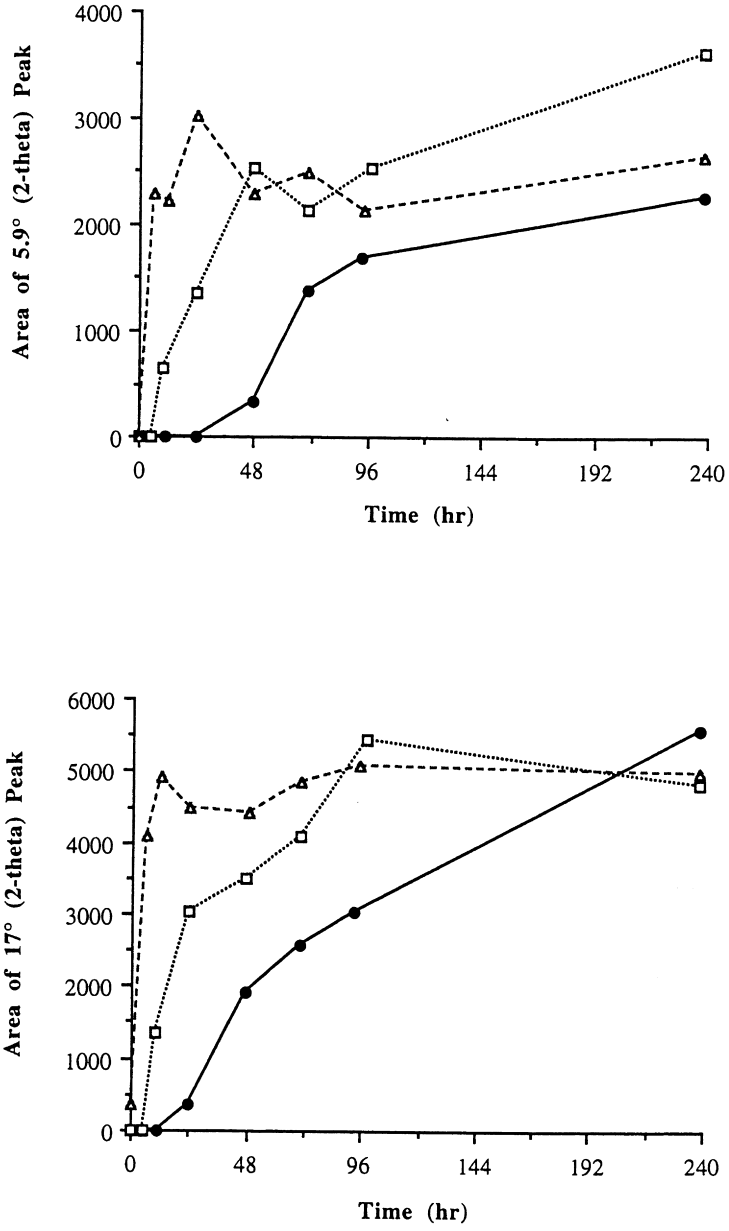

Fig. 12. Retrogradation of three *wx*-containing starches as observed by X-ray diffraction, from Thompson and Blanshard (1995). Starches at 27% solids were gelatinized and stored for various times at 4°C. Triangles, squares, and circles correspond to *ae wx*, *du wx*, and *wx* starches, respectively.

has an even larger proportion of short residual B chains than does *wx* starch. In addition, the minimum separating the chromatogram peaks for the short and long residual B chains is at a higher DP (~20) for *du wx* starch than for *wx* starch (Fig. 11). Shi and Seib (1995) also observed a greater proportion of short residual B chains of DP > 10 for the β-limit dextrin of *du wx* starch as compared to *wx* starch. Thus one might argue that the difference between A chain length and B1 chain length was greater, leading to longer flexible linker segments, but this argument does not account for possible multiple branching to B1 chains.

Yuan proposed that the relatively longer length of the interior chains of *ae wx* starch (Yuan, 1991) might allow it the flexibility to retrograde (as observed by DSC) in a manner not as strongly influenced by concentration as for *wx* starch (Yuan et al., 1993). This argument would be consistent with the longer ICL for *ae wx* starch than for *wx* starch (however, one must bear in mind that the ICL is an average value). Fisher and Thompson (1997) have shown that for *ae wx* starch some enthalpy is recovered so quickly after gelatinization that it is evident on an immediate DSC rescan. Liu and Thompson (1998a) have recently studied the different effects of concentration on retrogradation kinetics by DSC in greater detail for these starches. Retrogradation of these starches was earlier studied by wide-angle X-ray diffraction (Thompson & Blanshard, 1995), and the *ae wx*

starch was observed to develop crystallinity far more rapidly that the *wx* or *du wx* starches (Fig. 12). This observation would be consistent with Yuan's earlier suggestion that the longer interior chains could provide important flexibility for retrogradation, allowing double helices to associate readily in crystalline register. This observation would also be consistent with the liquid-crystal model proposed recently (Waigh et al., 1998), based on the unusually long flexible linker region for *ae wx* starch. However, because *du wx* starch also retrogrades faster than *wx* starch as observed by DSC (Liu & Thompson, 1998b; Shi & Seib, 1992; Thompson & Blanshard, 1995; Yuan et al., 1993) and X-ray diffraction (Thompson & Blanshard, 1995), application of this liquid-crystal model to retrogradation of *du wx* starch is more complex. One might suggest that the higher proportion of the DP $>$ 8 chains in the population of short residual B chains might be related to longer flexible linker chains for *du wx* than for *wx* starch. By this reasoning the less rapid retrogradation of *wx* starch as compared to *du wx* and to *ae wx* starch might be understood based on relatively high proportion of the very short internal linear segments in *wx* starch, functioning as less flexible linkages for association of double-helical mesogens.

Alternatively, linkers of particular lengths might serve to stimulate association of mesogens by virtue of the most energetically stable orientation of the linker, as described by O'Sullivan and Perez (1999). Other specific lengths would tend to hinder association. The different retrogradation behavior of these *wx*-type starches might partly reflect the proportion of linker lengths stimulating vs. hindering association of double helices. The extent to which the organization of a native starch granule could be improved through annealing might also be related to the precise distribution of the lengths of linker regions.

15. The cluster model and biosynthesis

While a better understanding of starch fine structure may help refine the cluster model or guide evaluation of modifications to it, improved understanding of the relationship between the fine structure and the organization of AP in the native granule is also important to better understand the nature of starch synthesis. The chain length profiles that appear to be related to altered branching enzyme activities (Martin & Smith, 1995) may actually be an indirect effect on an altered distribution of branch points. Application of Occam's razor would suggest that if branching enzyme activities are altered in starch synthesis, then one would look first for a change in the distribution of branch points, rather than a change in the chain length profile that would be secondary to an altered relationship among branch points. The recent hypothesis by Ball et al. (1996) that debranching enzyme is critical to development of normal AP structure, as well as the glycogen precursor hypothesis by Erlander (1998b), would both be amenable to evaluation based on the nature of the distribution of branch points.

The liquid-crystal modification of the cluster model is intriguing in its implications for synthesis. Just as with the proposed superhelical structures for AP in potato starch granules (Oostergetel & vanBruggen, 1993), the superhelical structure proposed to account for crystalline and amorphous regions in the liquid crystal model (Waigh et al., 1998) would allow simultaneous activity of synthases and branching enzymes at an exposed radial surface of an AP molecule. By such an architecture it would not be necessary to postulate discrete cycles of branching, debranching, and synthesis for each additional lamellae, as has been proposed (Ball et al., 1996). Instead, once double helices formed, they could maneuver into crystalline position oriented radially as the crystallites spiralled obliquely outward. This mechanism would be consistent with the third scheme of connecting clusters according to Robin et al. (1975).

Descriptive statistical models of the structure of AP are useful to gain insight into the nature of the molecule, but they are not necessarily consistent with a mechanism of synthesis. For example, the Flory model is based on a condensation reaction of polyfunctional monomers, as in elongation of synthetic polymers, and does not take into account constraints imposed by enzymes with specific action patterns. Banks and Greenwood (1975) pointed out that "remarkably little attention appears to have been given to the kinetic schemes necessary to produce these branched polymers." While a mechanism for starch synthase activity is analogous to a bifunctional condensation reaction in amylose synthesis, the mechanism for branching enzyme activity in the synthesis of AP is not analogous to a monomer condensation reaction. Any proposed model for AP synthesis must take into account the mechanism of action of starch branching enzyme (Jespersen, MacGregor, Henrissat, Sierks & Svensson, 1993; Manners, 1997). This transferase cleaves a glucosidic bond in a linear 1,4-linked chain and transfers the new reducing end to the 6 position on a previously existing chain (perhaps the same one that was cleaved) (Jespersen et al., 1993). Since a branch point is formed by moving a chain, while attached to a branching enzyme, toward the RT and thus other branch points, it is difficult to explain how additional branches might be located in very close proximity to existing branches; and yet the evidence suggests a bias toward just this sort of arrangement. Hypothesizing an initially highly branched molecule (or portion of one) which is then partially debranched (Ball et al., 1996; Erlander, 1998b) leads to even more difficult questions concerning how the excess of branching would originate. The following mechanism is a different way to account for the observed bias toward closely spaced branch points. The assumptions are that one or more synthases act to elongate accessible chains at the NRTs, and that one or more branching enzymes cleave a chain only when the chain has reached a sufficient distance from the previous branch point. The distance required for

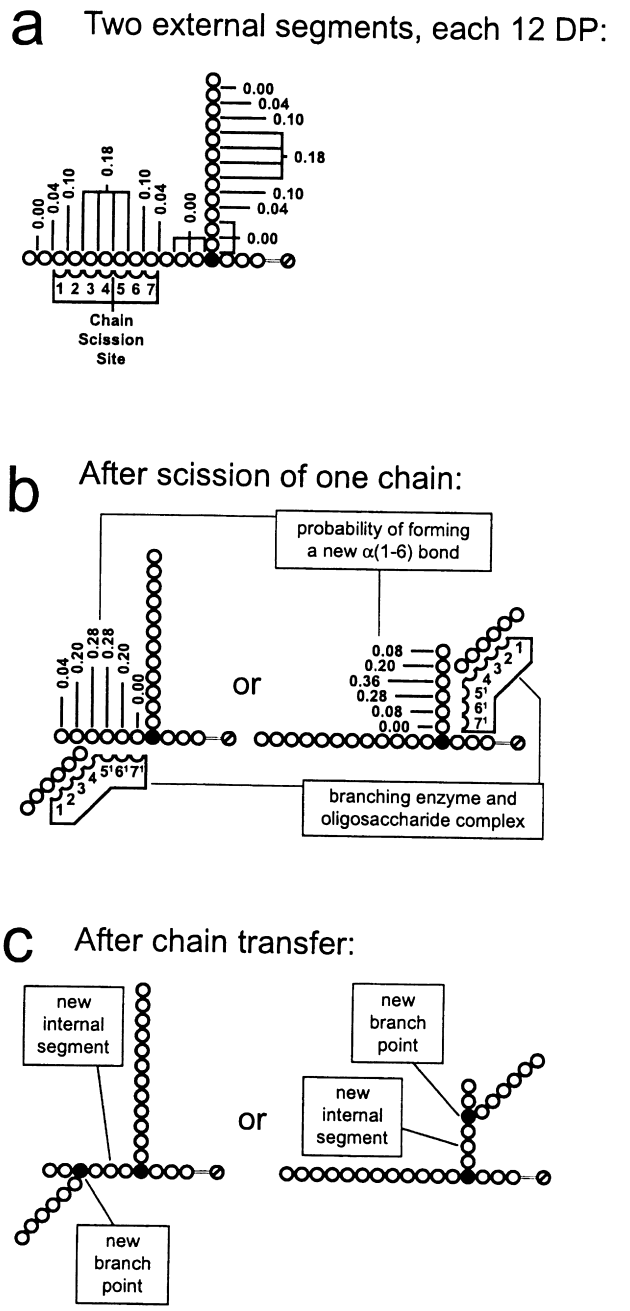

Fig. 13. (a) An exterior portion of a portion of an AP molecule. 1,4 linkages of the B chain are shown as horizontal. The branch point is the filled circle. The A chain extends vertically from the branch point. A branching enzyme with seven subsites is shown with the cleavage site between subsites 4 and 5. The enzyme is positioned with the cleavage site in a region of high probability for binding to the external portion of the B chain, as indicated by the probability values for location of the cleavage site. Although not indicated, the enzyme would be equally likely to act on the A chain, according to the probabilities shown for the location of the cleavage site. (b) After cleavage, the branching enzyme is shown with the short chain still attached at subsites 1–4, and binding to the now-shortened external chain (or some other external chain) is suggested to occur according to the indicated probabilities for creation of the new 1,6 linkage. The probabilities are no longer identical for the shortened B and A chains. (c) The new branch point is indicated, as is the new interior segment. All exterior segments would be extended by a starch synthase, and perhaps cleaved again by a branching enzyme once a sufficient exterior segment length is achieved.

cleavage, and the proximity of the new branch point to the previous branch point, would describe the action pattern of the particular branching enzyme. Jespersen et al. (1993) suggested that differences in the nature of two specific loops in the $(\alpha/\beta)_8$ domain of a branching enzyme could account for the different specificities of the glycogen and AP branching enzymes. Different action patterns for different branching enzyme isoforms might account for the more subtly different structures observed when the relative activities of different branching enzymes are altered for AP synthesis, as with the *ae* mutation. The mechanism envisioned is illustrated in Fig. 13. Two chains external to an exterior branch point are illustrated. Because there is no evidence otherwise, the variation in probabilities of cleavage as a function of distance from the branch point is assumed to be the same for each chain (part a, Fig. 13). However, after cleavage, the variation in probability of fixing a new RT to create a new branch point is assumed to differ according to which chain is involved (part b, Fig. 13), because the distance between branch points is known to differ for the two chains in the immediate proximity to the branch point (see Fig. 10). The key feature of this mechanism is that the interior segment length would be a direct function of the branching enzyme action pattern.

This type of mechanism might also explain why exterior segments of AP chains are longer on average than interior segments, i.e. why ECL > ICL. A requirement for a minimum length of linear chain for the cleavage activity of branching enzyme would mean that any chain shorter than the minimum (12 in the hypothetical example above) would not be subject to cleavage activity of branching enzymes, whereas the lengths of short interior segments would be fixed by the branching activity action pattern of the branching enzyme. In the example above, two new exterior chains would form from the original exterior chain of DP12: a new branch chain of 6 glucose units, and a residual chain extending 2 glucose units beyond the new branch point. The new interior segment would be 3 glucose units. The two exterior chains could be further extended by a synthase. The lengths of the various chains in Fig. 13 are important only as illustrations of a type of mechanism capable of introducing the systematic bias that would result in numerous short interior segments and in ECL > ICL.

16. Conclusion

The non-random nature of the AP molecule must be related to the specific action patterns of enzymes which elongate or branch the molecule. The nature of the branching pattern varies among starches from different sources, and may be equally as important as the chain length distribution in determining the physical behavior of the molecules. While the ECL and ICL provide some insight into the nature of the linear segments, knowledge of the

distribution of the interior segments will be important for an understanding of the branching pattern.

Focusing on the nature of the distribution of branch points provides a useful perspective from which to evaluate the various cluster models, and also stimulates a fresh approach to the fundamental question of how a cluster might be defined. After gelatinization and loss of all residual non-covalent order, a cluster might be considered as a local concentration of branch points. A quantitative expression of what constitutes a sufficient concentration of branch points would be necessary to define a cluster. It seems likely that only a general tendency toward two populations of interior segments will be observed, and that the presence of interior segments of intermediate length will make the definition of a cluster somewhat arbitrary.

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