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CRYSTALLISATION OF MALTO-OLIGOSACCHARIDES AS MODELS OF THE CRYSTALLINE FORMS OF STARCH: MINIMUM CHAIN-LENGTH REQUIREMENT FOR THE FORMATION OF DOUBLE HELICES

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ABSTRACT

The multigram preparation of malto-oligosaccharides of average d.p. ~11, by the debranching of glycogen using *Cytophaga* isoamylase is described. Debranched glycogen and fractions derived therefrom readily crystallise from hot, concentrated aqueous solution to give 40–70% of crystalline materials having sharp X-ray diffraction patterns characteristic of A-, B-, and C-type (intermediate) starch polymorphs. The polymorphic form obtained is dependent on chain length, concentration, and temperature, the A-type being favoured by shorter chain-length, higher concentration, and higher crystallisation temperature. For pure oligomers, the minimum chain-length required for crystallisation (formation of double helices) is 10. In the presence of longer chains, oligomers as short as maltohexaose can co-crystallise. These results explain the known differences in aggregation properties of glycogens and amylopectins.

INTRODUCTION

A common feature of native starches is the presence of regions of long-range ordering (crystallinity) within granules which gives rise to characteristic X-ray diffraction patterns. Two major crystalline forms, A and B, have been observed for cereal starches and tuber, fruit, and stem starches, respectively[1]. X-Ray diffraction analysis of amylose fibres[2,3] shows that both polymorphs contain ordered arrays of double helices. Individual double helices in the two crystalline forms are similar, if not identical, but helix-packing arrangements show substantial differences[2,3]. The A-type structure adopts a close-packed arrangement[3], whereas the B-type has a more open structure containing significantly more water between helices[2]. A third polymorph (C-type) is occasionally observed in native starches and is thought to be a mixture of A- and B-type structures[4]. There is strong evidence to suggest that it is the amylopectin component which is responsible for the crystallinity of starch granules[1].

Studies of low-molecular-weight model compounds have provided valuable information on the factors affecting starch crystallisation[5–15]. Model compounds are

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usually derived from the insoluble residue remaining after extensive acid hydrolysis and are called Nageli[5–9] (or Nageli-type[10]) amylodextrins or lintnerised starch[11,12], depending on the conditions of hydrolysis used. These materials contain[7–9,11,12] three types of structure. Thus, fraction I is multiply branched and of high molecular weight, fraction II (d.p. 25–30) has a single α-(1→6) branch near the reducing end, and fraction III is largely linear with a d.p. of 12–16.

These fractions can be conveniently prepared from acid-degraded starches by step-wise precipitation from pyridine–methanol–water mixtures[9]. Crystallisation from hot aqueous solution of such low-molecular-weight model compounds can lead to either A-, B-, or C-type polymorphic forms depending on the conditions employed. All other factors being constant, the A-type crystalline form is favoured by (*a*) shorter chain-length[13], (*b*) increasing concentration[14], (*c*) higher temperature[14], (*d*) the presence of salts of high lyotropic number[15], and (*e*) the presence of water-soluble alcohols and organic acids[10]. The chain length (amylopectin branch length) is thought to be the major polymorphic determinant in native starches[13].

We now report on the the crystallisation behaviour of malto-oligosaccharides [(1→4)-α-D-glucan oligomers] as model compounds for the crystalline forms of starch. We also describe a novel method for the large-scale preparation of such oligomers (with d.p. 6–14) based on the debranching of mussel glycogen with *Cytophaga* isoamylase.

EXPERIMENTAL

Materials. — The cell-free *Cytophaga* preparation was a gift from Dr. I. D. Fleming (Glaxo Research, Stoke Poges), mussel *(Mytilus edulis)* glycogen (Type VII) was obtained from Sigma, and amyloglucosidase (EC 3.2.1.3, lyophilised), mixed hexokinase (EC 2.7.1.1)/glucose 6-phosphate dehydrogenase (EC 1.1.1.49), maltohexaose, and maltoheptaose from Boehringer.

Preparation of isoamylase and debranching of glycogen. — A solution of isoamylase (EC 3.2.1.68) was obtained by a modification[16] of a published method[17]. To a solution of cell-free *Cytophaga* preparation (200 mg) in 50mM sodium acetate buffer (pH 5.0, 9 mL) was added 10mM phenylmethylsulphonyl fluoride in propanol (1 mL), the mixture was incubated at 4° overnight, and 0.1M calcium acetate in 50mM sodium acetate buffer (pH 5.0, 10 mL) was added. Isoamylase activity was then assayed[17] and gave a value of 0.55 U/mL.

For the debranching of glycogen, isoamylase solution (20 mL) was desalted by passage through a column (2.5 × 40 cm) of Sephadex G-50 equilibrated with deionised water. Material eluted in the void volume (~30 mL) was added immediately to a solution of mussel glycogen (20 g) in water, the pH was adjusted to 5.5, and the volume was adjusted to 2 L. The solution was incubated at 37° and the reaction was monitored on the basis of increase in reducing power[18]. When the reaction was complete (~24 h), as shown by constant reducing power, the enzyme

was inactivated by heating to 100° for 20 min, the solution was filtered through 1.2-, 0.45-, and 0.22-μm Millipore filters in succession and then freeze-dried (yield, 18.9 g).

A solution of a portion (10 g) of this material in deionised water (150 mL) was dialysed against deionised water (1.5 L) at 4° for 16 h, and then lyophilised to yield dialysate 1 (3.3 g). Further dialysis of the retained material against deionised water (1.5 L) at 4° for 60 h gave a dialysate which was lyophilised to give dialysate 2 (1.2 g).

Characterisation of the malto-oligosaccharides. — Debranched glycogen (15 mg) was dissolved in 20mM ammonium hydrogencarbonate (5 mL) with gentle heating, the solution was applied to either two columns (1.5 × 95 cm) of Sephadex G-50 in series at 25° or to a column (2.6 × 100 cm) of Bio-Gel P4 (−400 mesh) at 60° equilibrated with 20mM ammonium hydrogencarbonate, and fractions (5 mL) were collected by elution at 20 mL/h with the same buffer. Samples (0.5 mL containing up to 80 μg) of fractions were incubated with amyloglucosidase (0.5 mL, 0.33 mg/mL) in 0.2M sodium acetate buffer (pH 4.8) for 2 h at 37°. The glucose produced was assayed[19] after adding 0.5M Tris-HCl buffer (pH 7.6, 2 mL) containing NADP (1 mg), ATP (5 mg), sodium hydrogencarbonate (5 mg), magnesium chloride (8 mg), and a hexokinase/glucose 6-phosphate dehydrogenase mixture (20 μL), and incubating at room temperature for 30 min.

For ^{1}H-n.m.r. analysis, solutions of samples in D_2O were lyophilised, and the residues were re-dissolved in D_2O to give ~2% solutions. Spectra were recorded at 90° with a Bruker WP 200 instrument operating at 200.13 MHz with a pulse repetition time of 15 s. Chemical shifts are referenced to the signal for sodium 4,4-dimethyl-4-silapentane-5-sulphonate (0 p.p.m.).

Crystallisation experiments. — Mixtures of malto-oligosaccharides (200–400 mg) and water to give the desired concentration were sealed in tubes, and left for at least 16 h to ensure complete hydration[14]. The tubes were then heated at 90° until clear solutions were obtained (2–10 min) and incubated at the desired temperature for 10–14 days during which time solid material deposited. The crystalline solid was collected on a 5-μm Millipore filter, washed with ice-cold water (1–2 mL), and air-dried (20–30 min). As the crystalline materials contained various amounts of water, yields were estimated by difference after lyophilisation of the mother liquor and washings.

X-Ray diffraction patterns were obtained by using a Phillips powder diffractometer (PCW 1050/1390) mounted on a PW 1730/10 sealed-tube X-ray generator operating at the Cu-K_α wavelength (1.542 Å).

RESULTS

The elution profile from Sephadex G-50 of material produced by the action of *Cytophaga* isoamylase on mussel glycogen (Fig. 1) is similar to that obtained for rabbit-liver glycogen after analogous treatment[20]. As untreated mussel glycogen is

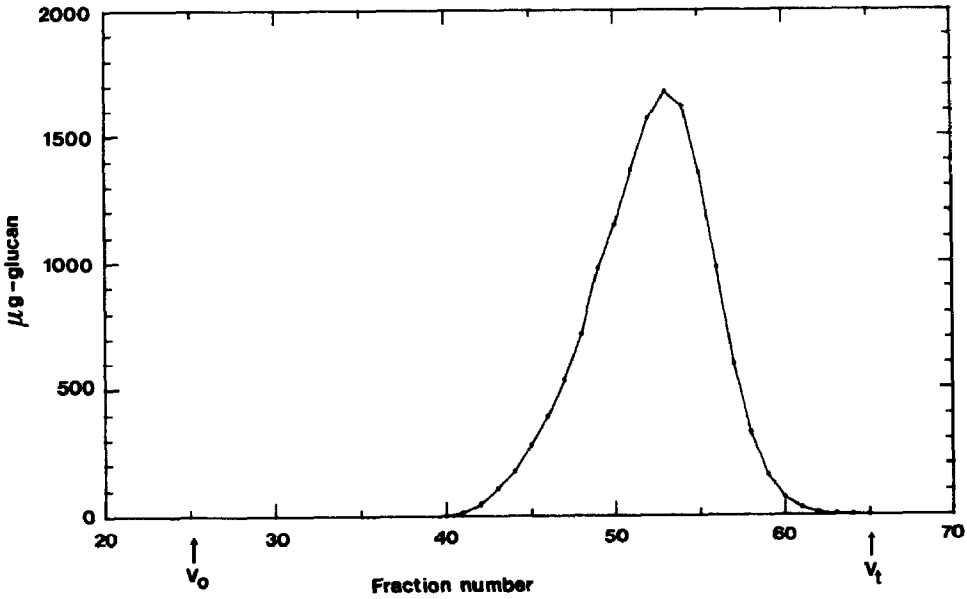

Fig. 1. Fractionation on Sephadex G-50 of isoamylase-treated mussel glycogen.

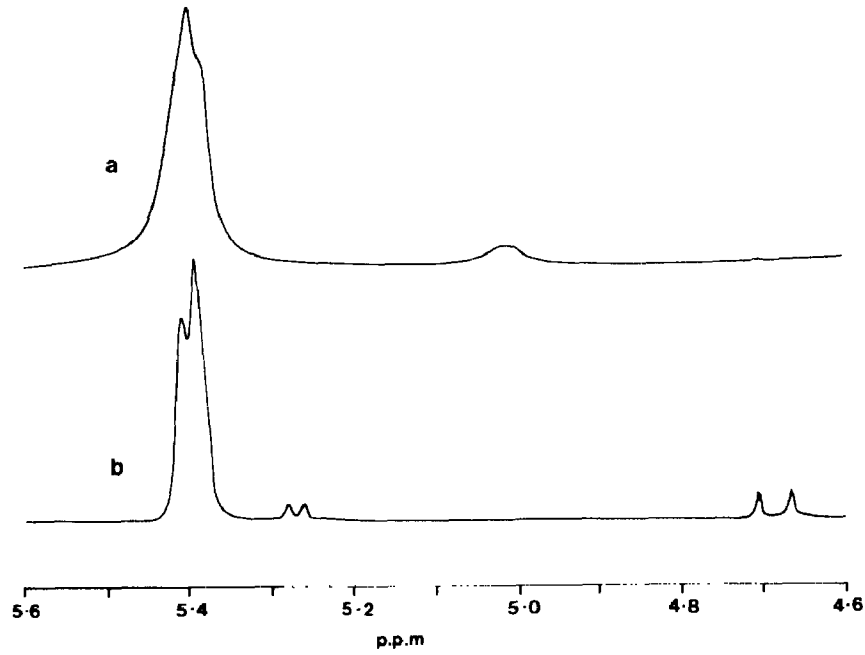

Fig. 2. Partial 200-MHz ^{1}H-n.m.r. spectra of (a) mussel glycogen and (b) isoamylase-treated mussel glycogen.

eluted in the void volume (data not shown), all of the glycogen sample had been degraded. A comparison of the low-field ^{1}H-n.m.r. spectrum of mussel glycogen before and after treatment with isoamylase is shown in Fig. 2. For untreated glycogen (Fig. 2a), the peaks at 5.4 and 5.0 p.p.m. are due to H-1 adjacent to α-(1→4) and α-(1→6) linkages, respectively[21]. After degradation with isoamylase (Fig. 2b), there was no signal at 5.0 p.p.m. which showed the absence of branching α-(1→6) linkages, but there were two additional doublets (5.27 and 4.68 p.p.m.) due to H-1 of the α and β forms of reducing terminal glucose residues, respectively[21]. Integration of this spectrum (Fig. 2b) showed the average chain-length of debranched mussel glycogen to be 11.2 (±0.5), in close agreement with values determined for a range of glycogens[22].

Chromatography of debranched glycogen on Bio-Gel P-4 (Fig. 3), in comparison with that on Sephadex G-50 (Fig. 1), effected superior resolution. Thus, peaks due to individual oligosaccharides of d.p. 5–18 were resolved (chain lengths being determined by ^{1}H-n.m.r. analysis[21] of isolated fractions) and there was a significant amount of material of d.p. >20. ^{1}H-N.m.r. analysis[21] showed the material in the void volume to have an average d.p. of ~40 in line with the observation[20] of a maximum unit chain-length for glycogen of ~50.

Crystallisation of debranched glycogen from hot, concentrated aqueous solution gave good yields of solids which gave sharp X-ray diffraction patterns of either A-, B-, or C-type polymorphic forms depending on the conditions of crystallisation

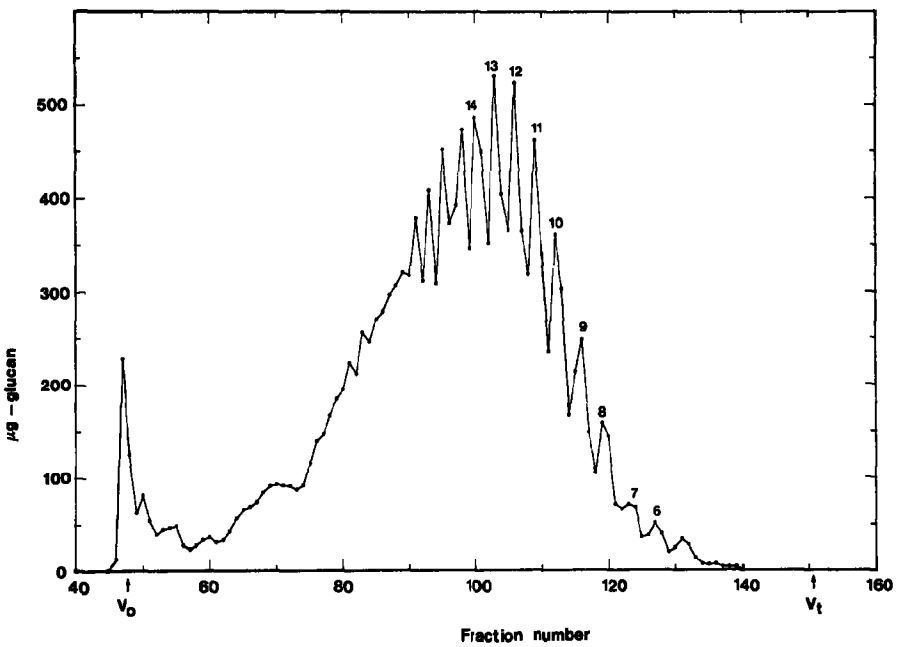

Fig. 3. Chromatography on Bio-Gel P-4 of debranched mussel glycogen. The chain lengths of resolved oligomers (as found by ^{1}H-n.m.r. analysis) are given above the relevant peaks.

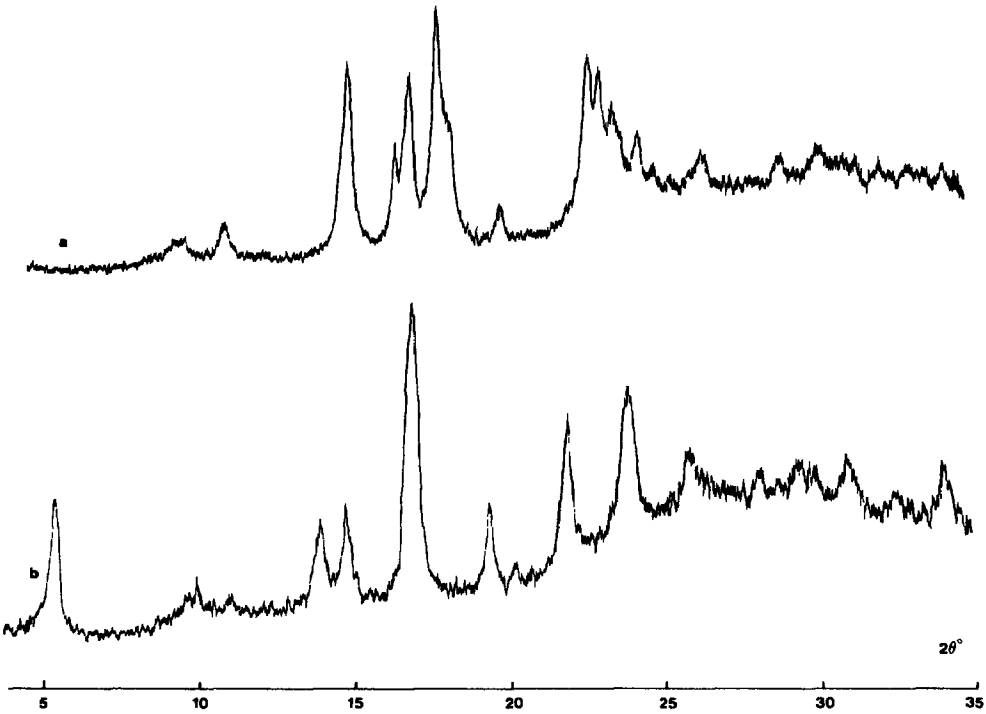

Fig. 4. Typical A- and B-type X-ray diffraction patterns obtained from (a) crystallisation of an aqueous 50% solution of dialysate 2 at 30° (A-type) and (b) crystallisation of an aqueous 40% solution of debranched mussel glycogen at 15° (B-type).

employed. Typical A- and B-type X-ray diffraction patterns are shown in Fig. 4. Table I shows the effect of concentration and incubation temperature on the yield and nature of polymorphic form of crystalline debranched glycogen. The A-type polymorph was favoured over the B-type by increases in concentration and temperature. These effects are discussed in the following paper[23].

TABLE I

THE EFFECT OF INITIAL CONCENTRATION AND TEMPERATURE OF INCUBATION ON THE POLYMORPHIC FORM AND YIELD (IN PARENTHESES) OF DEBRANCHED GLYCOGEN CRYSTALLISED FROM HOT AQUEOUS SOLUTION[a]

Concentration (%)	*Temperature*	
	15°	*30°*
30	B (70)	Cb (55)
40	B (67)	Ca (58)
50	Cb (67)	A (52)

[a]X-Ray diffraction patterns intermediate between A- and B-types (Fig. 4) are designated[14] Ca, Cc, or Cb based on the relative intensity of diffraction peaks at ~17 and ~18 2θ°. The latter peak is absent in B-type patterns (Fig. 4b), whereas both peaks are present in A-type patterns (Fig. 4a). If the intensity ratio of peaks at 18 and 17 2θ° is <1:3, the pattern is Cb; Cc patterns have ratios between 1:3 and 2:3, and Ca patterns have[14] a ratio >2:3.

TABLE II

EFFECT OF (1→4)-α-D-GLUCAN CHAIN-LENGTH ON CRYSTALLISATION[a] BEHAVIOUR

	Average chain-length[b]	*Temperature (degrees)*	*Yield (%)*	*Type*	*Average chain-length of crystalline material*[b]	*Average chain-length of material in mother liquor*[b]
Debranched glycogen	11.2	15	67	Cb	13.2	7.2
Dialysate 1	7.0	15	41	A	9.0	5.8
Dialysate 2	8.0	15	62	A	9.0	6.0
Dialysate 2	8.0	30	52	A	9.9	6.3

[a]From hot 50% aqueous solution. [b]Estimated[21] errors $<\pm 5\%$.

In order to examine the effect of chain length on crystallisation behaviour, two lower-molecular-weight fractions (dialysates 1 and 2) of debranched mussel glycogen were prepared, the average chain-lengths of which were found to be 7.0 and 8.0, respectively, by ^{1}H-n.m.r. analysis[21] (Table II). These fractions crystallised from hot, concentrated aqueous solution, giving good yields of materials having very sharp A-type X-ray diffraction patterns (Fig. 4a). Average chain-lengths of material which crystallised and which remained in the mother liquor were deter-

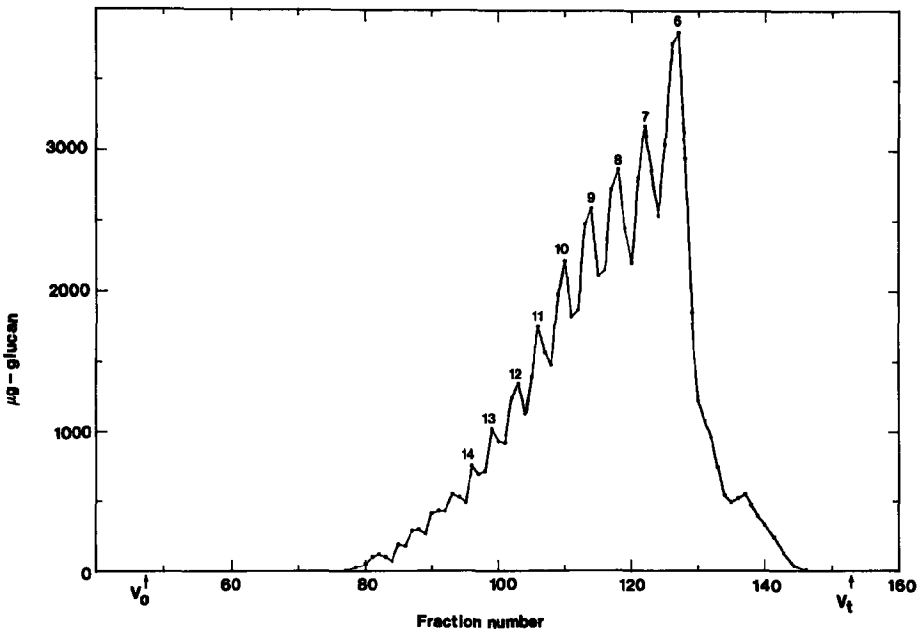

Fig. 5. Fractionation on Bio-Gel P-4 of material crystallised from dialysate 1 (130 mg) (see Table II). The chain lengths shown for resolved oligomers were determined by ^{1}H-n.m.r. analysis of isolated fractions.

mined by ^{1}H-n.m.r. analysis for both debranched glycogen and dialysates 1 and 2. The results of these experiments are summarised in Table II.

The range of oligosaccharides present in the crystallised material was assessed by chromatography on Bio-Gel P-4. Fig. 5 shows the profile of material crystallised from dialysate 1 (average d.p. 9.0). Oligosaccharides of d.p. 5–20 were present, with most material having d.p. 6–12. As this preparation was obtained from crystallised material which gave a sharp X-ray diffraction pattern (essentially identical to that in Fig. 4a), the majority of the material must have been involved in the formation of double helices. Thus, malto-oligosaccharides of $\geq$6 units can participate in the formation of double helices in the presence of longer chains. Chromatography of crystallised material from dialysate 2 and unfractionated debranched glycogen on Bio-Gel P4 also showed the hexaose to be the lowest oligosaccharide present in substantial quantities.

In order to determine the lowest chain-length capable of forming double helices in the absence of higher oligomers, pure oligomers of d.p. 6–14 were prepared by chromatography on Bio-Gel P4. Up to 350 mg of a mixture of oligosaccharides could be fractionated on a single column (2.6 × 100 cm) without significant loss of resolution. By combining fractions from several fractionations, 50–200-mg samples of pure oligosaccharides were obtained[24]. Chain lengths were verified by ^{1}H-n.m.r. analysis and purity was confirmed by re-chromatography. Crystallisation was attempted from aqueous 35% and 50% solutions at 4°. Oligomers of d.p. >10 readily crystallised (1–2 days), whereas those of d.p. $\leq$9 were stable in 35% and 50% solutions for at least 2 months at 4°. Crystallisation from aqueous 50% solution gave the A-type polymorph for each oligosaccharide (d.p. 10–14). From the 35% solutions, the A-type polymorph was obtained for the oligosaccharides with d.p. 10–12, but X-ray diffraction showed partial B-type character for the compounds of d.p. 13 and 14. These mixed diffraction patterns were of the Ca (d.p. 13) and Cc (d.p. 14) types as defined by Hizukuri[14] (see Table I). These results, as well as those in Table II, show the expected effect[13] of chain length on polymorphic form (*i.e.*, A-type favoured by shorter chains) and are discussed in the following paper[23].

DISCUSSION

Crystallised debranched glycogen as a model of crystalline forms of starch. — The debranching of glycogen using *Cytophaga* isoamylase is a convenient preparative approach to multigram quantities of malto-oligosaccharides of d.p. 5–50. Fractionation on Bio-Gel P-4 of this material yielded pure oligosaccharides on the hundred milligram scale. Debranched glycogen and fractions derived therefrom crystallised readily from hot aqueous solution to give, in good yield (40–70%), highly crystalline materials of the same polymorphic forms as those observed in native starches. Therefore, these materials may be used as model compounds for the crystalline (double helical) regions within starch granules. For example, solid-

state ^{13}C-n.m.r. (c.p.–m.a.s.) studies of crystalline debranched glycogen[25] have proved valuable in the analysis of spectra of native starches and have provided insights into the structural differences between A- and B-type polymorphs[25].

Previous model compounds for the crystalline forms of starch have been produced by the degradation of starches by acid (Nageli dextrins[5–10,13] and lintnerised starches[11,12]) or enzymes[14,15]. These may be compared with debranched glycogen in terms of crystallisation efficiency (yield) and the sharpness of features and presence of fine structure in the X-ray diffraction patterns which reflect the degree of crystalline perfection.

The only reported yields on crystallisation are those of Hizukuri[14] for malto-oligosaccharides of average d.p. 12.6 obtained by enzymic degradation of sweet-potato starch. Yields of 3–40% were obtained[14] (*cf.* 40–70% obtained in our work). Published X-ray diffraction patterns of B-type crystalline model compounds are slightly superior in one case[10] and inferior in others[13–15] to that shown in Fig. 4b, but the A-type pattern shown in Fig. 4a has sharper features and contains more fine structure detail than for any model A-type material previously described[10,13–15]. This finding suggests that studies of crystals of purified malto-oligosaccharides may provide structural information to complement the data available from X-ray fibre-diffraction analysis[2–4].

Minimum chain-length requirement for the formation of double helices. — The results presented above for pure malto-oligosaccharides show that a chain length of at least 10 is required for crystallisation and, by inference, for the formation of double helices. This minimum chain-length corresponds to <2 turns of the 6-fold helix characterised by X-ray fibre diffraction[2–4]. However, in the presence of longer chains (*i.e.*, >10 units), malto-oligosaccharides containing as few as 6 units (*i.e.*, a single turn of the double helix) can co-crystallise.

These observations may explain the differences in aggregation behaviour shown by glycogens and amylopectins. Solid preparations of glycogen show no long-range ordering (X-ray diffraction), and aqueous solutions have long-term stability. Amylopectins, on the other hand, are involved in long-range ordering (of double helices) within granules[1] and in aged starch gels[27,28], and aqueous solutions have a tendency to undergo phase separation (retrogradation) on prolonged storage. Glycogens have[22] a symmetrical distribution of unit chains of average length 10–14, whereas amylopectins typically show a bimodal distribution of chain lengths centred at 15–20 and 40–50 units[20,26]. As studies with molecular models have shown[8] that, adjacent to branch points, at least two residues per chain cannot participate in the formation of a double helix due to steric constraints, there will be very few potential interchain contacts of the required minimum length for double-helix formation in the multiply branched[29] glycogen structure. By contrast, the presence of longer chains and the clustering of branch points in amylopectin would lead to a large number of chains having the potential to form double helices. Extensive formation of interchain double helices in amylopectins, as observed in granules[1] and aged starch gels[28], could also occur in solution, eventually resulting in phase separation.

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