Note

Stereoregularity of chemically synthesized (1→6)-α-D-mannopyranan as revealed by enzymic degradation with *Arthrobacter* α-D-mannanase

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Until recently, it was not possible to synthesize, chemically, carbohydrate polymers comparable, in terms of configurational and structural regularity, to naturally occurring polysaccharides. However, in one of our laboratories (that of C. S.), the synthesis of (1→6)-α-D-glucopyranan[1,2], (1→6)-α-D-mannopyranan[3], and (1→6)-α-D-galactopyranan[4] has now been achieved through the phosphorus pentafluoride-catalyzed polymerization of the appropriate 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-aldohexopyranose derivatives and subsequent debenzylation of the products. On the basis of periodate-oxidation data, optical rotation values, and infrared and n.m.r. spectra, these synthetic polysaccharides of high molecular weight appear to be remarkably stereoregular. It has been estimated, for example, that as many as 98 or 99% of the residues in the D-glucopyranan are α-D-(1→6)-linked[1,2]. The data in the case of the D-mannopyranan indicate that not more than 3% of structural or configurational flaws could be present[3].

Carbohydrases have been used extensively for the elucidation of structural features of natural polysaccharides and of the carbohydrate moieties of glycoproteins. Such enzymes also furnish a convenient and extremely valuable means for analyzing the glycosidic linkages in synthetic polysaccharides. Reese and Parrish[5] investigated the degradation of the aforementioned (1→6)-α-D-glucopyranan by a variety of enzymes, including dextranase (from *Penicillium funiculosum*), β-D-glucosidase (almond), glucamylase, and α-D-glucosidase (sources unspecified), and concluded that linkages other than α-D-(1→6) occurred therein at a frequency of about two per hundred, but that no β-D-linkages were present, thus confirming the earlier stereoregularity estimates that had been based on chemical and physical evidence. The report by Robinson and Goldstein[6] that the (1→6)-α-D-mannopyranan does not

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react with concanavalin A, a lectin from jack beans, provided supporting evidence that the polymer is devoid of branch points, but, until now, no study of the enzymic degradation of this synthetic polysaccharide has been made.

Jones and Ballou[7–9] have isolated a soil micro-organism capable of utilizing the mannan of bakers' yeast as the sole source of carbon. The bacterium, originally reported to be a member of the genus *Arthrobacter* (see ADDENDUM), produces an extracellular α-D-mannanase that acts as an exoglycosidase, releasing single D-mannose units from the nonreducing termini of D-mannan molecules. When degrading *Saccharomyces cerevisiae* mannan, the enzyme preferentially liberates the α-D-(1→2)- and α-D-(1→3)-linked residues that constitute the side chains, leaving the α-D-(1→6)-linked backbone essentially intact. Di- to hepta-D-mannosides that are α-D-(1→6)-linked and that are derived from the backbone are, however, hydrolyzed by the enzyme. The inability of the D-mannanase to degrade *S. cerevisiae* mannan totally is, presumably, due to the occurrence of an obstructing group (probably phosphate) within the D-mannan, rather than to an inherent inability of the enzyme to bring about the hydrolysis of α-D-(1→6) bonds occurring in polymers of high molecular weight, as Gorin *et al.*[10] have found that the D-mannanase attacks the α-D-(1→6)-linked D-mannose backbone of *Schizosaccharomyces octosporus* galactomannan if the D-galactose side-chains have first been chemically removed.

We now describe the degradation of (1→6)-α-D-mannopyranan by *Arthrobacter* α-D-mannanase. The results presented permit estimation of the extent to which structural or configurational flaws occur in this synthetic polysaccharide.

Two preparations of (1→6)-α-D-mannopyranan were employed. The prepara-

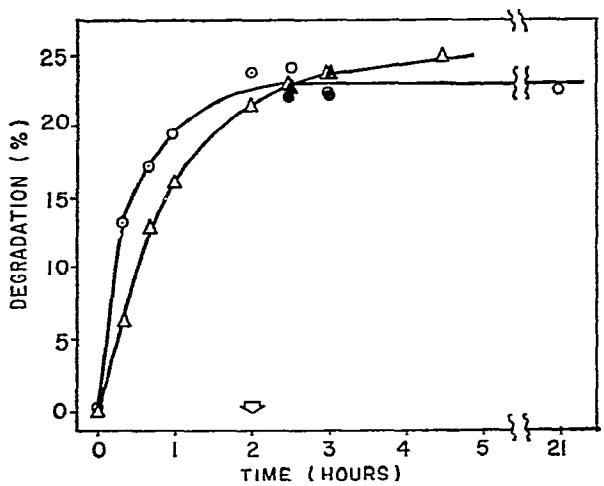

Fig. 1. Hydrolysis of (1→6)-α-D-mannopyranan (batch I) by *Arthrobacter* α-D-mannanase. (Circles–reaction in 0.01M potassium phosphate buffer, pH 6.8; triangles–reaction in 0.1M potassium phosphate buffer, pH 6.8. The arrow shows the time of addition of fresh enzyme, and the filled circles and triangles indicate the degree of degradation that was subsequently attained in the reaction mixtures which received additional enzyme.)

tions had similar specific rotations (+121°, batch I; +122.8°, batch II), but different intrinsic viscosities (0.41 dl/g, batch I; 0.31 dl/g, batch II), indicating that they were approximately equivalent in terms of stereoregularity, but dissimilar with respect to degree of polymerization (d.p.).

The time course for the enzymolysis of batch I in both 0.01 and 0.1M phosphate buffer is presented in Fig. 1. It is evident that the initial rate of reaction is affected by the ionic strength, the release of reducing sugar being more rapid in 0.01M than in 0.1M buffer; the final degree of degradation, on the other hand, is independent of the concentration of the buffer. Between 22 and 25% of the potential reducing groups in this preparation are liberated by the enzyme. When fresh α-D-mannanase is added to the reaction mixture at 2 h (indicated by the arrow), no increase in the rate of hydrolysis is subsequently observed, suggesting that inactivation of the enzyme added at the start of the reaction is not the cause of the observed cessation in release of reducing sugar.

In Fig. 2, the time course of the enzymic degradation of batch II in 0.1M phosphate buffer is shown. In this instance, between 42 and 43% of the reducing groups are susceptible to attack. Again, no stimulation is observed when fresh enzyme is added at 2 h.

Before the implications of these findings are considered, it should be mentioned that the initial rates of release of reducing sugar seen in Figs. 1 and 2 are between a

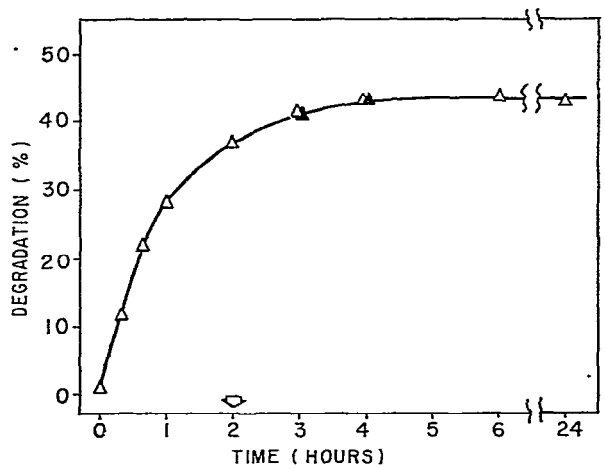

Fig. 2. Enzymolysis of (1→6)-α-D-mannopyranan (batch II). (For explanation of symbols, see legend to Fig. 1.)

sixth and a fourteenth of the rate that would be observed with *S. cerevisiae* mannan as the substrate under identical reaction-conditions. Jones and Ballou[9] found that α-D-(1→2)- and α-D-(1→3)-linked oligomannosides are hydrolyzed by the α-D-mannanase more readily than are their α-D-(1→6)-linked counterparts. In view of this finding, it seems likely that the differences in the initial rates of hydrolysis of *S. cerevisiae* mannan and the synthetic polymer can be accounted for by two structural

features of the *S. cerevisiae* mannan molecule: first, α-D-(1→2)- and α-D-(1→3)-linked D-mannose residues occur at, and immediately adjoining, the numerous nonreducing termini, and second, residues linked in this way constitute 65% of the total residues present[7].

Sarid *et al.*[11] have derived a mathematical expression relating the extent to which synthetic polymers may be enzymically degraded with the frequency of irregularities or flaws in the polymers. For this derivation, it was assumed that (*a*) all polymer molecules are linear and initially uniform with respect to d.p., (*b*) irregularities are randomly distributed within the polymer molecules, and (*c*) the enzyme degrades the polymer by an *exo* type of attack proceeding from only one end of the substrate molecule. Recent data[12] suggest that the preparations of (1→6)-α-D-mannopyranan used in these experiments have ratios of weight-average to number-average molecular weight of ~2, indicating that the distributions of chain length within the polymer populations are relatively narrow. In light of this conclusion and the fact that the *Arthrobacter* α-D-mannanase degrades a D-mannan chain by an *exo* attack from the nonreducing end[7], the equation of Sarid *et al.*[11] seems applicable to the analysis of the data obtained in the present experiments. Accordingly, we have calculated the maximal percent of degradation to be expected upon the enzymic hydrolysis of polymers having d.p. of 100, 200, 300, 500, and 1,000, and irregularities of 0 to 5 mole percent. The results are presented in Fig. 3.

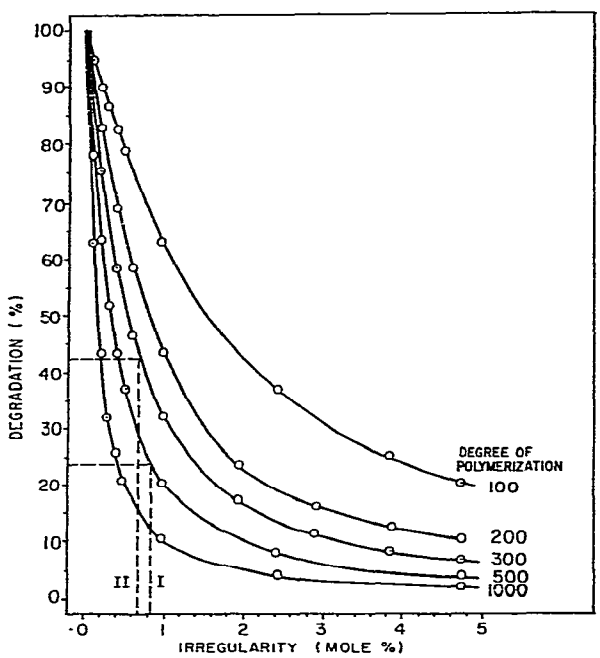

Fig. 3. Theoretical relationship between the maximal percent of enzymic degradation and the mole percent irregularity for polymers having d.p. of 100, 200, 300, 500, and 1,000. (Calculated by the equation of Sarid *et al.*[11] The dashed lines illustrate the calculations for batches I and II.)

Wales *et al.*[13] have proposed a relationship between instrinsic viscosity and molecular weight for linear dextran molecules, and the average chain-lengths of the (1→6)-α-D-mannopyranan preparations can be estimated by using in this relationship the known intrinsic viscosities of the preparations. These estimates should be reasonably accurate, even though the relationship proposed by Wales *et al.*[13] pertains to dextran molecules in aqueous solution, and the viscosity data available were obtained in methyl sulfoxide–water[3], because Glaudemans and Timell[14] found that the intrinsic viscosity–molecular weight relationships for natural xylans in methyl sulfoxide and in aqueous potassium hydroxide are quite similar. Thus, batch I (intrinsic viscosity, 0.41 dl/g) has a viscosity-average molecular weight of 81,200, which corresponds to an average d.p. of ~500, whereas batch II (intrinsic viscosity, 0.31 dl/g) has a viscosity-average molecular weight of 53,700, corresponding to an average chain-length of 330.

On the basis of the theoretical considerations of Sarid *et al.*[11] (see Fig. 3), the observed maximal percentages of degradation, together with these estimates of d.p., suggest that structural or configurational flaws in the (1→6)-α-D-mannopyranan occur at a frequency of 0.8–0.9 per 100 residues in batch I (see Fig. 3), and 0.65–0.7 per 100 residues in batch II (see Fig. 3). The lower occurrence of irregularities in batch II that is indicated by this analysis is consistent with the fact that the specific rotation of batch II is slightly more positive than that of batch I.

These values of percent irregularity are well below the upper limit of 3 per 100 suggested by Frechet and Schuerch[3] on the basis of chemical and physical data. It is noteworthy, in this regard, that, even had number-average molecular weights been used for computing the average chain-lengths, a calculation which would have given values of the d.p. only ~60% of the values obtained from the viscosity-average molecular weights, the frequencies of irregularities calculated by the equation of Sarid *et al.*[11] would still have been <2 per 100. A second point that should be mentioned concerns the specificity of the α-D-mannanase. As the enzyme can release D-mannose residues that are attached by α-D-(1→2)- and α-D-(1→3)-, as well as by α-D-(1→6)-, bonds, it is evident that, although the presence of the two former types of linkage would constitute flaws in the (1→6)-α-D-mannopyranan, they would go undetected in these experiments, with the result that the estimates of stereoregularity obtained by this analysis would be deceptively high. It is, however, unlikely that the synthetic polymer contains α-D-(1→2)-linked D-mannose residues, because the 1,2-anhydro-D-mannopyranose derivatives from which such residues would have arisen could not have survived the conditions of preparation of the monomer, but would have been destroyed either during pyrolysis or benzylation. (A description of the technique for preparation of the monomer was given in Ref. 3.) Similarly, the possibility that α-D-(1→3) bonds occur within the polymer is remote, because the 1,3-anhydro-D-mannopyranose derivatives required for their formation are unknown. On the other hand, results obtained with analogous systems suggest that 1,4-anhydro-D-mannopyranose could have been formed in trace amounts during the pyrolysis step in the preparation of the monomer. As such derivatives would probably not react

stereospecifically under the conditions of polymerization employed[15], they may be responsible for the occurrence of irregularities in the (1→6)-α-D-mannopyranan.

The statistical considerations developed by Sarid *et al.*[11] serve to emphasize the magnitude of the effect that can be exerted, by relatively few flaws, upon the degradation of a polymer by an enzyme exhibiting an *exo*-depolymerization mechanism. Indeed, it is a testimony to the structural and configurational regularity of naturally occurring polysaccharides that methods utilizing *exo*-glycosidases for the estimation of average chain-lengths in the polymers[16,17] yield results in such good agreement with the values obtained by chemical and physical techniques. The deliberate introduction of irregularities into a biopolymer is useful for determining whether a degradative enzyme acts by an *endo* or an *exo* mechanism (that is, by essentially random cleavages of the inter-monomer bonds, or by stepwise cleavages proceeding from one end of the polymer); this was elegantly demonstrated by Drummond *et al.*[18] with pullulan-degrading enzymes. By periodate oxidation, flaws were created, at a frequency of 5 per 100 residues, in a pullulan preparation that had an average d.p. of 1450. Because the bonds on each side of an irregularity are susceptible to attack by *endo*-hydrolases, introduction of this low level of flaws had only a slight influence on the enzymolysis brought about by pullulanase, an *endo*-glycosidase. In contrast, flaws obstruct the action of *exo*-hydrolases, and the oxidized pullulan was, therefore, not appreciably degraded by amyloglucosidase, an *exo*-glycosidase capable of degrading native pullulan. The latter finding is in accord with the information given in Fig. 3; namely, that, in polymers having d.p. >1000 and irregularities of 5 mole percent, less than 2% of the residues are susceptible to attack by *exo*-hydrolases.

In summary, we have found that the α-D-mannanase produced by *Arthrobacter* GJM-1 is capable of degrading chemically synthesized (1→6)-α-D-mannopyranan. The results suggest that flaws occur in this polymer at a frequency of less than 1 per 100 residues.

EXPERIMENTAL

Chemical determinations. — Total carbohydrate was measured by the phenol–sulfuric acid method of Dubois *et al.*[19], and reducing sugar by the copper-reduction procedure of Nelson[20]. D-Mannose was employed as the standard for both determinations.

Arthrobacter α-D-*mannanase.* — The enzyme was prepared according to the procedure of Jones and Ballou[7], the ammonium sulfate fraction being used in the experiments reported here. The α-D-mannanase was assayed as described by the same authors[7], with the exception that the reducing sugar was determined by the Nelson method. One unit is defined as the amount of enzyme that releases 1 μmole of reducing sugar from bakers' yeast mannan in 1 min at 30° (50 mM potassium phosphate buffer, pH 6.8, containing 0.1 μmole of calcium chloride and 50 μg of bovine serum albumin per ml)[7,10].

Enzymolysis of the (1→6)-α-D-mannopyranan. — The reaction mixtures con-

tained 150 to 200 μg of the synthetic polysaccharide, 0.0524 unit of α-D-mannanase, 0.1 μmole of calcium chloride, and 50 μg of bovine serum albumin per ml of either 0.1 or 0.01M potassium phosphate buffer, pH 6.8. The mixtures were incubated at 30°, and, at appropriate intervals (up to 24 h), samples were withdrawn and analyzed for total carbohydrate or reducing sugar, or both. Two hours after the initial addition of the enzyme, a portion of each mixture was supplemented with fresh α-D-mannanase (0.0524 unit per ml of the mixture), incubated for an additional period (up to 2 h) and then analyzed as just described. The percent degradation was computed by dividing the amount of reducing sugar in a sample by the amount of total carbohydrate in that sample, and multiplying by 100.

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ADDENDUM

The culture of *Arthrobacter* GJM-1 employed as a source of α-D-mannanase in the experiments reported here has been examined by Mrs. Mary P. Lechevalier of the Institute of Microbiology, Rutgers University. It is her opinion that the organism should not be considered to be an *Arthrobacter* species, as it is hyphal when growing on agar. The true identity of strain GJM-1 is, however, unknown. With the exception of being non-motile, it exhibits traits characteristic[21] of the genus Oerskovia.

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