Transfer of Glucosyl Units to Oligosaccharides and Polysaccharides by the Action of Uridine Diphosphoglucose-α-glucan Transglucosylase*

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ABSTRACT: The question has been studied whether uridine diphosphoglucose-α-glucan transglucosylase makes a preferential glucosyl transfer to one of the two outer chains of singly branched oligosaccharides and of multibranched polysaccharides. The oligosaccharides were isolated from α-amylase digests of glycogen. The polysaccharides were glycogen samples with various outer chain lengths as well as phosphorylase limit dextrins prepared from them. A debranching enzyme has been prepared from the culture medium of a strain of *Aerobacter aerogenes*. This enzyme splits the α-1,6-glucosidic bond by which the side chain is attached to the main chain, provided that the side chain is two or more glucose units long. A single branch-point glucose unit can be cleaved by the action of amylo-1,6-glucosidase but not through the action of the debranching enzyme of *Aerobacter*. Chromatographic separation of the products formed by the latter enzyme from oligosaccharides and polysaccharides before and after enlargement from uridine diphosphoglucose gave the following results. Transfer of glucosyl units to singly branched, low molecular weight oligosaccharide acceptors occurs almost exclusively to the main chains of these molecules.

The transglucosylase acts principally by a multichain reaction mechanism. When polysaccharides are the acceptors, the enzyme extends some outer chains more than others. Evidence has been obtained that the favored site of glucosyl transfer in these cases is also the main chain. These findings are important with respect to the subsequent action of α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase, since the specificity of this branching enzyme and that of uridine diphosphoglucose-α-glucan transglucosylase must together account for the formation of the arboreal structure of the glycogen molecule.

The biosynthesis of the α-1,4-glucosidic linkages in glycogen is known to be due to the action of UDPG-α-glucan transglucosylase (UDPG:α-1,4-glucan-α-4-glucosyltransferase, E.C. 2.4.1.11) by which a glucose unit is transferred from the nucleotide-linked sugar to a suitable oligosaccharide or polysaccharide acceptor. The transglucosylase was first detected in rat liver by Leloir and Cardini (1957). A stimulatory effect of glucose-6-phosphate (Glc-6-P) in the transglucosylase reaction was subsequently described by Leloir *et al.* (1959). Larner and co-workers (Rosell-Perez *et al.*, 1962; Friedman and Larner, 1963; Rosell-Perez and Larner, 1964) have shown that the enzyme exists in two interconvertible forms which differ markedly in their sensitivity to Glc-6-P stimulation. We have reported (Kornfeld and Brown, 1962) the purification from rabbit skeletal muscle of that form of the enzyme which is relatively insensitive to Glc-6-P and have described some of its kinetic properties. With this preparation we have now studied the reaction of branched-chain oligosaccharides and polysaccharides of glucose as acceptors for glucosyl transfer from UDPG. In particular we have studied the question of whether the enzyme makes a preferential transfer to the outer main chains or to the outer side chains of these molecules. The term "side chain" refers here to any outer chain of α-1,4-linked glucose units which is attached by its anomeric carbon atom in α-1,6- linkage to one of the glucosyl residues of another chain. The branch point formed in this way is a structural feature common to glycogen and amylopectin. Knowledge of the site of glucosyl addition by the transglucosylase to an acceptor containing branched chains is needed for an understanding of the mechanism of enlargement of the glycogen and amylopectin molecules, since elongation of the outer chains occurs prior to the introduction of new branch points by the action of the "branching enzyme" (α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase, E.C. 2.4.1.18) (Hobson *et al.*, 1951a; Larner, 1953). Neither the mode of action of the branching

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enzyme nor its specificity for substrate structure is known. These problems may be somewhat clarified by information about the polysaccharide structure which results from the synthetic action of UDPG-α-glucan transglucosylase.

Experimental Methods and Procedures

Preparation of UDPG-α-glucan Transglucosylase. This enzyme was prepared from rabbit skeletal muscle as previously described (Kornfeld and Brown, 1962). The assay system used to determine the activity of the enzyme had the following composition: 1.5×10^{-3} M UDPG, 10 mg/ml glycogen, 1.25×10^{-2} M $MgCl_2$, 2.5×10^{-3} M Glc-6-P (when added), 150 μg/ml bovine plasma albumin (Armour, Lot No. N67110), 6×10^{-2} M Tris (Cl^-) buffer, 1×10^{-3} M EDTA, and 1.5×10^{-2} M 2-mercaptoethanol, *p*H 7.5. From 4 to 7 μg of purified enzyme was incubated in 0.40 ml of this assay solution. After 15 minutes at 30° the solution was heated in boiling water for 1 minute. The UDP content of the solution then was measured spectrophotometrically via the coupled pyruvic kinase–lactic dehydrogenase reaction (Kornberg, 1951). When assayed in this manner, the purified transglucosylase had a specific activity in the presence of Glc-6-P of 2.4 μmoles of glucose transferred to glycogen per minute per mg protein. The ratio of activity assayed in the presence of Glc-6-P to that in its absence was from 1.6 to 1.9 for different fractions in the peak of activity eluted from the DEAE-cellulose column. All preparative enlargements of oligosaccharide and polysaccharide acceptors by the enzyme were carried out in the presence of added Glc-6-P.

Preparation of α-Amylase Limit Dextrins. The low molecular weight, singly branched glucose oligosaccharides used in this work as acceptors for glucosyl transfer from UDPG were prepared by α-amylase degradation of glycogen as described previously (Illingworth and Brown, 1962). Their structures and the designations used for them are shown in Figure 1. The structure assignments are based on our previous studies as well as on information obtained from experiments described here.

Preparation of Bacterial Debranching Enzyme. A "debranching enzyme" which acts to cleave the α-1,6 branch points of dextrins and polysaccharides of the glycogen and amylopectin type was prepared from the culture fluid of *Aerobacter aerogenes.* The fact that an undefined *Aerobacter* species produced an extracellular enzyme of this type when grown on maltose, maltotriaose,[1] or on pullulan was first found by Bender and Wallenfels (1961), who described the enzyme as a "pullulanase" on the basis of its cleaving the polysaccharide, pullulan, quantitatively to maltotriaose (Wallenfels *et al.*, 1963). These investigators also

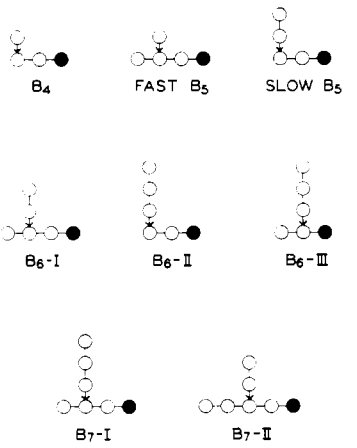


FIGURE 1: Structures of some singly branched oligosaccharides produced by α-amylase action on glycogen. The assignment of structures to B_6-II, B_6-III, and B_7-II is provisional, as discussed in the text. ○, a glucose residue; ●, a reducing-end glucose unit; —, the α,1,4-glucosidic bond; ↓, the α-1,6-glucosidic bond.

recognized the similarity of their enzyme to the plant R-enzyme which was found by Hobson *et al.* (1951b) to debranch amylopectin, and which has been used by French (1960) to study the structure of some α-amylase limit dextrins. In the case of the debranching enzyme used in the present work, its possible action on pullulan was not tested. In preparing the enzyme from *A. aerogenes* (strain ATCC 9621) the procedure of Bender and Wallenfels (1961) was followed, using a culture medium containing 0.5% maltose to induce enzyme formation. The cells were removed by centrifugation in the cold, and 1.5 volumes of ice-cold acetone were added to the supernatant fluid. The enzyme was extracted from the precipitate with cold 0.02 M potassium phosphate buffer, *p*H 6.8. The extract was stored at 5° under toluene vapor and was stable for at least 6 months.

Results

Action of the Debranching Enzyme. The activity of the debranching enzyme prepared in this way was investigated by incubating it with the branched oligosaccharides shown in Figure 1. Glucose formation was measured spectrophotometrically via hexokinase and Glc-6-P dehydrogenase. Reducing power was measured using the Somogyi 60 copper reagent (Shaffer and Somogyi, 1933) and an arsenomolybdate reagent (Nelson, 1944) for development of color. Paper chromatographic separation of the oligosaccharides present in reaction mixtures after the action of the bacterial debranching enzyme was carried out on Whatman No. 1 paper using butanol-pyridine-water (3:2:1.5) as the descending solvent. Reaction mixtures were deionized before chromatography by the use of short columns of the mixed-bed resin, Amberlite MB-3. Care was

[1] In the case of linear oligosaccharides having more than two glucosyl residues in maltosidic (α-1,4) linkage, the number of such units is indicated by the name, as e.g., maltotriaose (3) and maltotetraose (4).

taken to use very little resin and to wash the column with a large volume of water after addition of the sample in order to minimize losses due to nonspecific adsorption of oligosaccharides. Even with these precautions, such losses were sometimes as much as 20% with amounts of polysaccharide of the order of 0.5–3 μmoles. The separated oligosaccharides could be eluted quantitatively from the paper with water and then determined by enzymatic assay for glucose following hydrolysis of the eluate in 1 N HCl for 3 hours at 100°. The total quantity of each substance originally present in an enzyme digest could be calculated from the amount eluted from the final chromatogram by applying a correction for the amount of substance lost in the deionization procedure, on the assumption that the measured loss was unspecific. In order to detect ^{14}C-labeled oligosaccharides, the chromatograms were scanned in a Vanguard low-background autoscanner with an automatic data system. Elution of the ^{14}C-labeled substances and estimation of their quantity permitted a determination of their specific activity. Visualization of standard reference polysaccharides on chromatograms was made possible through the use of a benzidine–trichloroacetic acid spray (Bacon and Edelman, 1951).

By these techniques the purity of the *Aerobacter* debranching enzyme was studied by measuring any possible hydrolytic action on α-1,4-glucosidic bonds using maltose, maltotriaose, and [^{14}C]maltodecaose as substrates. Incubation of the first two substances (4×10^{-3} M) with the enzyme in 0.01 M citrate–0.002 M phosphate buffer, *p*H 6.5, for 3.5 hours at 37° resulted in no glucose formation and no change in reducing power of the solutions. These observations show that the enzyme preparation is free from any detectable maltase or glucamylase activities. Incubation of [^{14}C]-maltodecaose (2070 cpm; 1.6×10^{-3} M) under the same conditions, but for 5.5 hours, led to virtually no formation (less than 1%) of smaller or larger [^{14}C]-oligosaccharides. This shows that the enzyme preparation has no or only insignificant amounts of α-amylase as well as of any transglycosylase acting to redistribute glucosyl residues in α-1,4- linkage. Evidence that the enzyme used in this work is likewise free of any hydrolytic action on single glucose units in α-1,6- linkage was obtained by showing that there was no glucose formation upon incubating it with panose (and no change in reducing power). The latter characteristic of debranching enzymes of this class has been found previously for R enzyme (Hobson *et al.*, 1951b) and for pullulanase (Bender and Wallenfels, 1961). That the debranching enzyme prepared in this work has no amylo-1,6-glucosidase activity was shown by incubating it with a pure preparation of Fast B_5 (Figure 1), which has been shown to be a specific substrate for the assay of this glucosidase (Illingworth and Brown, 1962). No glucose formation occurred, a fact which also confirms the structure of the branched pentasaccharide.

Action of Debranching Enzyme on α-Amylase Limit Dextrins. The action of the *Aerobacter* debranching enzyme on some of the singly branched α-amylase limit dextrins shown in Figure 1 was studied by incubating the substances at 2–4 $\times 10^{-3}$ M with the enzyme fraction in the citrate-phosphate buffer described for 6 hours at 37°. Quantitative measurement of the products formed showed that virtually complete debranching had occurred in the case of every substance in which the side chain is two or more glucose units long (Table I). In the case of the branched hexasaccharide, study of the products of the debranching reaction together with information obtained previously (Illingworth and Brown, 1962; Brown and Illingworth, 1962) shows that the B_6 fraction consists of approximately 60% of a compound with the structure of B_6-I and 40% of a substance with the structure of B_6-II or B_6-III. It should be emphasized that the B_6 fraction cannot be clearly resolved even after a prolonged time (3 months) of descending chromatography on Whatman No. 1 paper using butanol-pyridine-water (3:2:1.5). Studies of this fraction as a possible substrate for phosphorylase have shown that it cannot be degraded further with the production of Glc-1-P. This observation together with the knowledge that Slow B_5 can be completely converted to B_4 (Figure 1) by phosphorylase suggests that B_6-III is more likely than B_6-II to be the structure of the principal second component in the B_6 fraction. On the other hand, the fact that the B_6 fraction does not yield any glucose when incubated with amylo-1,6-glucosidase and oligo-1,4→1,4-glucantransferase (Brown and Illingworth, 1962) is not explained, since the structure of B_6-III has two glucose units covering the branch point and, hence, might react as does B_7-I with this enzyme system. The structure(s) of the component(s) of the B_6 fraction which yields maltotriaose in the *Aerobacter* debranching reaction will remain uncertain until a means is found to resolve the fraction. The flavazole derivative method of Nordin and French (1958) might then be used to establish the structures of the various B_6 compounds.

Of interest are the data which show that the products formed when the debranching enzyme acts on Slow B_5 are those which other studies of its structure predicted (Illingworth and Brown, 1962). Similarly, the B_7 fraction, which has previously been used as the substrate for the demonstration of the action of oligo-1,4→1,4-glucantransferase, yields products upon debranching which show that about 95% of the fraction consists of a compound with the structure, B_7-I, which was previously assigned to it, and that there is a small amount of a second compound which is likely to have the structure of B_7-II.

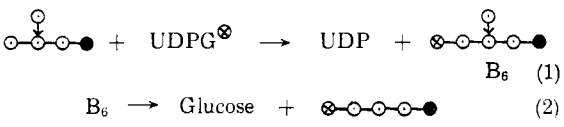
Enlargement of Oligosaccharides by UDPG-α-glucan Transglucosylase. The relative effectiveness of glycogen, of phosphorylase limit dextrins, and of β-amylase limit dextrins as acceptors in the UDPG-α-glucan transferase reaction has been studied in detail. The ability of short linear oligosaccharides of the maltose series to act as acceptors in the transglucosylase reaction was also reported (Kornfeld, 1961). A similar study has been made by Goldemberg (1962), who concluded that the transglucosylase acts by a multichain addition mechanism.

TABLE I: Action of *Aerobacter* Debranching Enzyme on α-Amylase Limit Dextrins.[a]

Oligosaccharide Incubated[b]	Amount Taken (μmoles)	Substance Isolated (μmoles)	Recovery of Branched Oligosaccharide (%)
Fast B_5	1.73	Fast B_5, 1.67	96
Slow B_5	0.92	B_5, 0.027	3
		Maltose, 0.84 Maltotriaose, 0.68	91[c]
B_6-I		Maltose, 0.76 Maltotetraose, 0.75	64
In mixture with	1.18	B_6, 0.015	1
B_6-II or B_6-III		Maltotriaose, 0.89	38
B_7-I		Maltotriaose, 0.47 Maltotetraose, 0.42	95
In mixture with	0.47		
B_7-II		Maltose, 0.05 Maltopentaose, 0.04	9

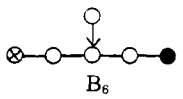
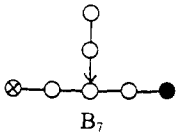
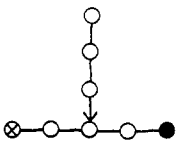
[a] See text for experimental details on incubation conditions and methods of measurement of products formed. [b] See Figure 1 for structures of compounds. In the case of B_6 and of B_7 only the mixture of compounds with each of these degrees of polymerization was incubated in the total amount shown in column 2. [c] Calculated from the recovery of the product maltose on the assumption that the handling losses of maltotriaose were greater.

The availability of the branched α-amylase limit dextrins shown in Figure 1 made it possible to use these as acceptors in the transglucosylase reaction. No demonstrable glucosyl transfer occurred when either B_4 or Slow B_5 (3×10^{-3} M) was incubated for 3 hours with transglucosylase (thirty units/ml) and UDPG (7.5×10^{-3} M) in the presence of all of the usual components of an assay mixture (*vide supra*). Thus branched oligosaccharides in which no α-1,4-linked glucose residue is peripheral to the branch point have apparently very little affinity for the enzyme. The effect on the acceptor function of having one such glucose unit on the main chain beyond the branch point was shown by incubating Fast B_5 (7.4×10^{-3} M) with a 2-fold excess of UDPG (1.8×10^{-2} M) and sixteen units of transglucosylase for 3 hours in 1 ml of reaction mixture. Ten μmoles of UDP was formed. Owing to the presence of amylo-1,6-glucosidase as a contaminant of the transglucosylase preparation, 2.4 μmoles of free glucose was also formed (Illingworth and Brown, 1962). The other product of the action of the glucosidase on Fast B_5 is maltotetraose, which is known to serve as an acceptor for glucosyl units transferred from UDPG. The products of the transglucosylase (plus amylo-1,6-glucosidase) action on Fast B_5 were isolated chromatographically and found to be maltotetraose, maltopentaose, small amounts of other linear chains up to maltodecaose, residual B_5 (3.2 μmoles), and a new singly branched oligosaccharide of the B_6 series (3.3 μmoles). It was of considerable interest to find out whether the new B_6 product had been formed by glucosyl transfer to the main chain or to the side chain, which in this case is the α-1,6-linked branch-point unit of Fast B_5. If the transfer were to occur to the side chain, the product would be B_6-I (see Figure 1) which is not a substrate for amylo-1,6-glucosidase. The alternative possibility in which the main chain is elongated by one unit would lead to a different B_6 which would be expected to be a substrate for the direct action of amylo-1,6-glucosidase. Accordingly, the isolated B_6 product from the transglucosylase reaction was tested at 1.6×10^{-3} M as a substrate for glucosidase. The relative rate of glucose formation was eight times faster than that from the parent compound, Fast B_5, and 1 mole of glucose per mole of B_6 was eventually produced. These facts, together with the finding that maltopentaose could be quantitatively isolated from such a reaction mixture as the only oligosaccharide present, show that the sequence of enzymatic reactions studied is:



in which reaction (1) is catalyzed by the transglucosylase and reaction (2) by amylo-1,6-glucosidase. In view of the fact that no B_6-I was formed by the transglucosylase reaction and that 88% of the Fast B_5 acceptor was accounted for either as unchanged substance or as the B_6 whose structure is shown in equation (1), the pref-

TABLE II: Action of *Aerobacter* Debranching Enzyme on Oligosaccharides Enlarged by Glucosyl Transfer from UDPG⊗.[a]

Compound	Quantity Incubated with Enzyme	Products Isolated
 B_6	153 cpm	Unchanged B_6
 B_7	0.09 μmole	Maltose Maltopentaose
 B_8	(a) 0.28 μmole	Maltotriaose, 0.31 μmole Maltopentaose, 0.28 μmole
	(b) 399 cpm	Maltotriaose, unlabeled Maltopentaose, 396 cpm

[a] Designation for glucose unit transferred from UDPG: ⊗.

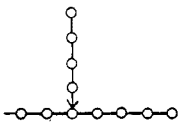
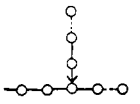
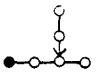
erence of the transglucosylase for main-chain addition is clearly demonstrated.

The reaction of B_6-I (see Figure 1) as an acceptor in the transglucosylase reaction was studied to find out whether only the main chain of this compound, like that of Fast B_5, is available to the enzyme. When B_6-I at 1.0×10^{-3} M (calculated from the data in Table I) was incubated with a 10-fold excess of UDPG in the presence of transglucosylase for 20 hours, the only new compounds demonstrable in the reaction mixture and purified from it were a branched heptasaccharide and a branched octasaccharide. The former substance, resulting from the addition of a single glucose unit, was present in ten times the quantity of the latter. To find whether the heptasaccharide had the structure of B_7-I or of B_7-II (see Figure 1) the compound was incubated with amylo-1,6-glucosidase containing oligo-1,4→1,4-glucantransferase activity. No glucose was produced in even 23 hours of incubation, showing that B_7-I could not have been present (Brown and Illingworth, 1962). The debranching enzyme prepared from *A. aerogenes* then was used to show that the compound formed by glucosyl transfer to B_6-I was, in fact, B_7-II. Table II shows the results of this experiment. The presence of maltose and maltopentaose after debranching proves that the transglucosylase acted by adding a glucosyl unit to the main chain. These data also show that B_6-II and/or B_6-III (present with B_6-I to the extent indicated in Table I) are not acceptors for glucosyl transfer from UDPG.

That the preference of the transglucosylase for main-chain addition extends to its action on B_7-I was shown by isolating the branched octasaccharide from a reaction mixture (1 ml) containing 4.7×10^{-3} M B_7-I and a 2-fold excess of UDPG and incubated with the enzyme for 8 hours. At that time, 5.9 μmoles of UDP had been formed, and isolation of the products by chromatography showed that the octasaccharide product (B_8) was four times more abundant than the nonasaccharide formed by transfer of two units from UDPG. The B_8 product slowly yielded 1 mole of glucose when treated with amylo-1,6-glucosidase and oligo-1,4→1,4-glucantransferase, and the principal linear oligosaccharide formed was maltopentaose. In view of the known mode of action of this combined enzyme system on B_7-I (Brown and Illingworth, 1962), these facts favor the conclusion that the B_8 product of glucosyl transfer has the added unit on its main chain. Direct confirmation of this conclusion was obtained from two experiments in which (a) unlabeled B_8 and (b) ^{14}C-labeled B_8 (specific activity 11,400 cpm/μmole) (formed by transfer from [^{14}C]UDPG, 12,430 cpm/μmole) were incubated separately with the *Aerobacter* debranching enzyme. The results are shown in Table II. The formation of unlabeled maltotriaose and labeled maltopentaose having all of the radioactivity of the B_8 substance, together with evidence for the quantitative formation of only these two compounds from it, shows that transfer of the glucosyl unit had occurred exclusively to the main chain.

Enlargement of Polysaccharides by UDPG-α-glucan Transglucosylase. The transglucosylase has a much greater apparent affinity for polysaccharides than for even those oligosaccharides whose structure is similar to that of the outer branches of the polysaccharides (Table III). In the case of B_6-I as compared to the β-

TABLE III: Comparison of Various Substances as Acceptors in the UDPG-α-glucan Transglucosylase Reaction.

Substance	Typical Structure in Vicinity of Outer Branch Point	Relative V_{max}	K_m
Rabbit liver glycogen	[a]	100	1.7×10^{-6} M[b]
Phosphorylase limit dextrin of glycogen		25	2.1×10^{-6} M[b]
β-Amylase limit dextrin of glycogen[c]		12	4.2×10^{-6} M[b]
B_6-I		24	0.075 M
B_4		*ca.* 0	
Maltooctaose		26	0.02 M

[a] The average outer-chain length of this sample was 9.3. [b] The molar concentration is that of the end groups of the polysaccharide. The K_m value is calculated from rate measurements made at 5–50 μg polysaccharide per ml. [c] The dashed line in the structure signifies that an extra glucose unit may be present or absent depending upon whether the original chain contained an odd or an even number of glucose units.

amylase limit dextrin which has a similar outer structure, the K_m value differs by four orders of magnitude, whereas V_{max} is not markedly different. The fact that maltooctaose has a K_m value similar to that of B_6-I indicates that the presence of a branch point in the acceptor is not in itself sufficient to account for the much greater affinity of the polysaccharides for the enzyme. The results suggest that the molecular weight of the acceptor also plays an important role. On comparing the three polysaccharides in Table III, it is of interest to note that the progressive shortening of the outer chains has a stronger effect on V_{max} than on K_m. Although the β-amylase limit dextrin is about one-half the size of the parent glycogen, it is still a macromolecule when compared with B_6-I.

In view of these large differences in affinity, it could not be predicted whether the transglucosylase would enlarge polysaccharides by the same mechanism as oligosaccharides, i.e., by addition exclusively to the main chains, or whether, perhaps, the enzyme could add glucosyl units to both the side and the main chains of polysaccharide acceptors. To study this question several polysaccharides were enlarged by transglucosylase action using [^{14}C]UDPG as described in the footnote to Table IV. After 4 hours of incubation the [^{14}C]polysaccharides were isolated by dialyzing the boiled reaction mixtures against large volumes of distilled water, followed by alcohol precipitation (1 volume), and then by 20 minutes of digestion in 15% NaOH. The polysaccharides were precipitated from this alkaline solution by alcohol, reprecipitated once from 0.1 N HCl, and then twice from water. After drying with absolute alcohol and ethyl ether, the solid products were recovered in yields of from 90 to 100% of those expected from the weights of parent substances taken and the measured extents of enlargement.

Data on the structures of the parent and of the enlarged preparations are given in Table IV. The extent of enlargement from UDPG was calculated from the known quantity of parent polysaccharide used and the measured amount of UDP produced in the transglucosylase reaction. The experimentally determined decrease in the end-group percentage and increase in the outer-chain length of the derived polysaccharides is in good agreement with the values which can be predicted from the measured extent of enlargement. This fact shows that there was no detectable branching action and no amylolytic degradation by enzymatic contaminants of the purified transglucosylase preparation. The data in Table IV show that the enlarged polysaccharides are quite chromogenic when tested with I_2. Some of the products described (e.g., sample D.L.-A) were not as easily soluble following their isolation in the solid state as was anticipated from their degree of branching and their average outer-chain length. Such relative insolubility was the property especially of a product (not described in Table IV) which had been formed by enlarging a β-amylase limit dextrin of liver glycogen to the extent of 62% from UDPG. Although the molecular weight of this polysaccharide was not much greater than that of the original soluble liver glycogen from which the β-

TABLE IV: Structure and I_2 Staining Characteristics of Glycogen Samples with Modified Outer-Chain Length.

Sample	Substance	Extent of Enlargement from UDPG (%)	I_2 Complex Absorption		End Group (%)		Degradation to Glc-1-P by Phosphorylase (%)
			$\epsilon^{1\,cm}_{max}$/ μmole "Glucose"	λ_{max} (mμ)	Found	Calculated	
36	Human pectoralis muscle glycogen	0	0.14	460	8.9		27.2
36-C	Human pectoralis muscle glycogen	40.6[a]	0.80	500	6.1	6.3	51.7
D.L.	Type III glycogenosis liver glycogen	0	0.11	460	11.0		15.2
D.L.-A	Type III glycogenosis liver glycogen	47.9[a]	1.08	520	6.6	7.4	43.5
B.K.	Incomplete phosphorylase limit dextrin of liver glycogen	0	0.084	410	11.6		7.3
B.K.-B	Incomplete phosphorylase limit dextrin of liver glycogen	52.6[a]	1.30	510	7.5	7.6	

[a] Specific activity of [^{14}C]UDPG used for enlargement, 2750 cpm/μmole. The composition of the reaction mixtures in which the enlarged polysaccharides were prepared was: parent polysaccharide, from 42 to 60 μmoles total polysaccharide glucose per ml; UDPG, 3.03×10^{-2} M; Glc-6-P, 2.8×10^{-3} M; Tris, 5.6×10^{-2} M; EDTA, 9.1×10^{-4} M; 2-mercaptoethanol, 1.7×10^{-3} M; $MgCl_2$, 1.4×10^{-3} M; transglucosylase, 2.1 units per ml (1 unit = 1 μmole glucose transferred to glycogen in 15 minutes); total vol, 2.15 ml; *p*H 7.5; 30°. In the case of each of the three preparative enlargements the initial ratio of the concentration of UDPG to that of the end group of the polysaccharide was from 5.6 to 6.2. For details on the isolation of the enlarged, radioactive polysaccharide products (36-C from 36; D.L.-A from D.L.; B.K.-B from B.K.) see text.

amylase limit dextrin had been prepared, the UDPG-enlarged substance was so insoluble that it precipitated from the reaction mixture during its preparation. When a dilute solution of it was tested with I_2, a deep-blue granular precipitate formed. These solubility and I_2 staining properties suggest that, when glycogen preparations having short outer chains are enlarged from UDPG, the transglucosylase acts in such a way that the outer chains become quite unequal in length. This result could occur if the enzyme tended to make repeated transfers to the same chain.

The possibility of asymmetric addition of glucosyl units near branch points was studied by examining the products formed when the *Aerobacter* debranching enzyme was allowed to act on the ^{14}C-enlarged polysaccharides whose preparation has been described. Table V shows, first of all, that the debranching enzyme can cleave about one-half of all the outer branch points of a phosphorylase limit dextrin of glycogen (sample 1954), and that in addition to maltotetraose, which is the major product expected from the limit dextrin structure proposed by Walker and Whelan (1960), definite amounts of maltotriaose and maltose are also formed. When such a debranched limit dextrin is treated with amylo-1,6-glucosidase having oligo-1,4→1,4-glucan transferase activity, there is an immediate and rapid formation of free glucose (B. Illingworth and D. H. Brown, unpublished experiments; Hers and Verhue, 1964). This observation supports the possibility that many of the branch points not cleaved by the *Aerobacter* enzyme are really single glucose units, and these are known not to be split by the bacterial debranching activity. The presence of such exposed glucose units in a phosphorylase limit dextrin has already been proposed (Cori and Larner, 1951).

When an incomplete phosphorylase limit dextrin (sample B.K.) was enlarged to the extent of 53% from [^{14}C]UDPG, the resulting polysaccharide (sample B.K.-B) was debranched nearly as completely as the unenlarged limit dextrin sample (sample 1954), showing that the two substances served almost equally well as substrates for the *Aerobacter* enzyme. It was striking, however, that in the case of the enlarged polysaccharide only about 2% of the radioactivity present in the outer branches was present in the linear chains from two to nine glucose units long which were produced. Since these products were originally outer side chains of the polysaccharide molecule, the conclusion can be drawn either that 98% of the label had been added exclusively to main chains, or that the length of

TABLE V: Action of *Aerobacter* Debranching Enzyme on Polysaccharides.

Sample	Substance	Outer Branches Removed as Chains[a] (% of total outer branches)	Total ^{14}C in All Linear-Chain Products (% of total ^{14}C in polysaccharide)	Oligosaccharide Formed and Its Specific Activity[b] (μmole per μmole of linear-chain products)					
				Maltose	Maltotriaose	Maltotetraose	Maltopentaose	Maltohexaose	Maltoheptaose
1954	Phosphorylase limit dextrin of liver glycogen	47		0.05	0.035	0.84	0.051	*ca.* 0	0.023
B.K.-B	Incomplete phosphorylase limit dextrin enlarged from [^{14}C]UDPG[c]	40	2.1	+ (0)	<0.20 (*ca.* 1300)	0.38 (1510)	0.12 (*ca.* 1000)	0.14 (*ca.* 3800)	0.058 (*ca.* 4500)
D.L.	Type III ("limit dextrinosis") liver glycogen	*ca.* 20			+	++++		+	
D.L.-A	Type III ("limit dextrinosis") liver glycogen enlarged from [^{14}C]-UDPG[c]	[d]	2.3			++++ (*ca.* 400)	*ca.* 0 (0)	+ (4000)	*ca.* 0 (0)
36	Human pectoralis muscle glycogen	<20		+	+	+	+	+	+
36-C	Human pectoralis muscle glycogen enlarged from [^{14}C]UDPG[c]	<20	Uncertain	Products from G_6 to G_{12} not radioactive				+ (0)	*ca.* 0

[a] Calculated from measured change in end-group percentage after debranching, assuming that one-half of all branch points are outer branch points. [b] The specific activity (cpm/μmole) is given in parentheses below the mole fraction of each radioactive product. [c] Specific activity of [^{14}C]UDPG, 2750 cpm/μmole. [d] Structural analysis of polysaccharide unsatisfactory because of poor solubility.

the extended side chains was so great that they could not be cleaved by the debranching enzyme. This latter possibility is made somewhat unlikely by the observation already mentioned: that the number of branch points split was nearly as great in the case of the enlarged polysaccharide as in that of a phosphorylase limit dextrin. The fact that the maltotriaose and maltotetraose formed upon debranching had specific activities which were about one-half that of the UDPG shows that the parent limit dextrin contained some outer side branches which were two and three glucose units in length and which were able to act as glucosyl acceptors. The specific activities of G_6 and G_7 show that more than one glucosyl unit had been transferred from UDPG. The absence of any ^{14}C in maltose shows that UDPG-α-glucan transglucosylase cannot transfer a glucosyl residue to an exposed branch-point glucose unit in a phosphorylase limit dextrin. Failure of such a unit to act as a glucosyl acceptor has already been shown here in the case of oligosaccharides.

A result similar to that just discussed was also obtained when a human glycogen sample with short outer chains isolated from a case of Type III glycogenosis ("limit dextrinosis") was enlarged by transglucosylase action (sample D.L.-A) and was then subjected to the action of the debranching enzyme (see Table V). As in the case of sample B.K.-B, maltotetraose was isolated as the major product, and only 2.3% of the radioactivity introduced from [^{14}C]UDPG was found in all dialyzable products following debranching. The low specific activity of maltotetraose is due to dilution by the much larger quantity of unlabeled maltotetraose present in the parent "limit dextrinosis" glycogen sample. The reason for the absence of any labeled maltopentaose among the products of the debranching reaction is not apparent.

Table V shows that the extent of debranching of an enlarged glycogen preparation by the *Aerobacter* enzyme was less complete than in the cases of the enlarged limit dextrins. However, even in the glycogen case, no substantial radioactivity was present in the outer side branches up to twelve glucose units in length which were removed. Among the most abundant of such nonradioactive chains was maltohexaose, but some other native, unenlarged human glycogen samples have not shown such a preponderance of this product (B. Illingworth and D. H. Brown, unpublished experiments). In most of these other glycogen samples side chains of two to seven glucose units and longer seem to occur in approximately equal amount (e.g. sample 36, Table V).

Discussion

The experimental data reported above show that transfer of glucosyl units from UDPG to singly branched, low molecular weight oligosaccharide acceptors occurs almost exclusively to the main chains of these molecules. When UDPG is present initially in severalfold molar excess over the amount of acceptor, the principal reaction consists of the transfer of only one, and, to a lesser extent, two units to the main chain of each acceptor molecule without detectable formation of long-chain compounds. Under these conditions, then, the enzyme seemingly acts by a multichain reaction mechanism, as has been found for it when linear oligosaccharides serve as glucosyl acceptors (Goldemberg, 1962). However, the strong preference for main-chain addition has not been reported previously. When high molecular weight polysaccharides act as the acceptors, the favored site of glucosyl transfer also seems to be the main-chain side of outer branch points. Inasmuch as the transfer reaction from UDPG is essentially irreversible, it is not accompanied by any redistribution of glucosyl units. The chromogenic properties of the iodine complexes of the enlarged polysaccharides and their solubility characteristics indicate that the enlargement is asymmetric, especially when they have been formed by glucosyl transfer to glycogen preparations which had initially rather short outer chains, such as partial limit dextrins formed by phosphorylase degradation *in vitro* or *in vivo*. An example of the latter polysaccharide is the glycogen isolated from the tissues of patients with Type III glycogenosis, in which there is a demonstrable absence of amylo-1,6-glucosidase. The evidence that the asymmetrically enlarged polysaccharides actually have most of the added glucosyl units on the main chain side of the outer branch points is less conclusive than is that in the case of the enlarged oligosaccharides, because of seemingly incomplete action of the debranching enzyme on the polysaccharides. For this reason, especially in the case of enlarged glycogen, the location of the added glucosyl units has been determined with less certainty than in the case of the enlarged limit dextrin or partial limit dextrin. It is to be expected that the influence of a branch point in affecting the preferential binding of only the main chain of the glucosyl acceptor at the active site of the transglucosylase will be less marked the more remote such a branch point is from the nonreducing (acceptor) end of the chain. Thus, it might be predicted that if the outer chains of a glycogen sample are rather long, the probability will be equal that side and main chains will both be enlarged by glucosyl transfer from UDPG. However, even in such a case there seems to be a preference for main-chain addition, but more evidence on this point is certainly required.

The finding that the transglucosylase acts preferentially to elongate the main chains of glycogen molecules having short outer branches, such as might be formed *in vivo* during limited glycogenolysis, suggests that many features of the specificity of action of the "branching enzyme," α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase, with respect both to the chain length of segment moved and the site to which it is to be attached in α-1,6- linkage, remain to be discovered before it will be possible to understand fully the enzymatic steps by which the arboreal structure of glycogen is achieved. These problems related to the mode of action of the branching enzyme are under investigation.

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