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STRUCTURE OF THE POLYSACCHARIDE FORMED BY INCUBATING GLYCOGEN WITH D-[^{14}C]GLUCOSE IN THE PRESENCE OF THE GLYCOGEN DEBRANCHING ENZYME [AMYLO-(1 → 6)-GLUCOSIDASE-4-α-GLUCANOTRANSFERASE]*

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ABSTRACT

[^{14}C]Glycogen has been synthesized *in vitro* by incubating D-[^{14}C]glucose with rabbit-liver glycogen in the presence of a pure preparation of the glycogen-debranching enzyme [amylo-(1 → 6)-glucosidase-4-α-glucanotransferase]. The course of the reaction has been monitored and ^{14}C-products isolated after 30 min and 5 h. The distribution of D-[^{14}C]glucose groups in the polysaccharides has been determined by debranching the molecules with a crystalline isoamylase from *Pseudomonas*. The quantities of unlabeled and ^{14}C-linear unit chains containing D-[^{14}C]glucose at their reducing ends have been determined by paper chromatography followed by enzymic degradation and analysis. In the 30-min product, between 65 and 85% of the D-[^{14}C]glucose groups were covered by unlabeled groups because of transferase action. In the 5-h product, the extent of covering approached 100%. Extensive redistribution of unlabeled groups also was found to have occurred, even in the early stages of the reaction. It is concluded that the D-[^{14}C]glucose incorporation assay for amylo-(1 → 6)-glucosidase, as ordinarily carried out, is probably not specific just for the hydrolytic action of this enzyme, but that it depends indirectly on its transferase activity as well.

INTRODUCTION

The action of the glycogen-debranching enzyme, amylo-(1 → 6)-glucosidase (E.C.3.2.1.33)-4-α-glucanotransferase (E.C.2.4.1.25), is required to achieve total degradation of glycogen in the phosphorolytic pathway for its catabolism. It has been established that this debranching enzyme can be purified from rabbit skeletal

*Dedicated to Professor Dexter French on the occasion of his 60th birthday, in appreciation of his many contributions to knowledge of the structure of polysaccharides and of his fruitful investigations of the mechanism of action of carbohydrases.

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muscle as a protein containing a single polypeptide chain having a molecular weight of 160,000–170,000 and that it has two different enzymic activities[1-7]. Acting as a 4-α-glucanotransferase it preferentially moves maltotriosyl, and to a lesser extent, maltosyl residues from some donor oligosaccharides and from glycogen to other linear or branched acceptors of suitable structure[1,2,8]. However, the enzyme is unable to move single D-glucose residues from one (1 → 4)-α-D-glucosidically bonded position to another[8]. Acting as a glucosidase, the enzyme has been shown[9] to act on the single (1 → 6)-α-D-glucosidically bonded group that becomes exposed at each outer branch point of glycogen, following degradation of this polysaccharide to a limit dextrin by glycogen phosphorylase (E.C.2.4.1.1) and subsequent transfer of maltotriosyl groups by the debranching enzyme's transferase activity. Purified preparations of the glycogen-debranching enzyme from rabbit liver[4,10] and from the yeast, *S. cerevisiae*[11], have also been shown to have these two types of enzymic activity. Children and adults who have Type III glycogen-storage disease lack the debranching enzyme in affected tissues[12]. The discovery that, under normal circumstances, the single polypeptide chain of this enzyme catalyzes two different reactions, both of which are essential for glycogenolysis, raised the question of whether the congenital lack of this enzyme can involve only one of its two activities or whether deletion of both functions always occurs[13,14]. At least ten different assays for one or the other of these activities, or for the two in combination, have been proposed. In one investigation, by Hers and coworkers[15,16], seven of these methods were applied to human tissues and compared, and it was concluded that there was enough disparity in the results of some of the different assay-methods to support the possibility that in some cases of Type III glycogen-storage disease, the debranching enzyme may have partial residual transferase or glucosidase activity. Recently, limited proteolysis of purified debranching enzyme from rabbit muscle has been used to show the existence of two distinct active sites on the polypeptide chain[17]. This demonstration rests upon the finding that there is a difference in the extent to which the altered protein can degrade a phosphorylase limit-dextrin as compared with its ability to to catalyze the incorporation of D-[^{14}C]glucose into glycogen. The latter assay was described first by Hers[18] and depends ultimately upon the slight reversibility of the hydrolytic action of the debranching enzyme[19]. This method has also been widely used by other workers, especially since the development of a modified procedure[20] that makes it suitable for the analysis of small amounts of tissue. However, the question of whether this assay really depends only upon glucosidase activity or whether the D-[^{14}C]glucosyl groups introduced are covered subsequently and, thus, possibly "locked in" by the action of transferase, has not yet been answered definitively. In 1964, structural evidence was presented from two laboratories that covering action by transferase occurs to a substantial extent[1,14], while the recent publication[17] from the laboratory of Cohen concludes that transferase activity is not required for the net incorporation of D-[^{14}C]glucose residues. Resolution of this problem would assist in better understanding the enzymic basis for the apparent existence of subgroups of Type III glycogen storage disease[16,21].

This paper reports the results of a new study of the structure of the ^{14}C-labeled polysaccharide produced during the D-[^{14}C]glucose incorporation-assay using purified debranching enzyme from rabbit muscle. The [^{14}C]polysaccharide has been degraded to its component unit-chains by the debranching action of crystalline isoamylase from *Pseudomonas* SB15. The usefulness of this enzyme in elucidating the structure of such polysaccharides as glycogen and amylopectin has been established already by the extensive studies of Harada, Yokobayashi, and their coworkers[22–26]. Our results indicate that subsequent covering by transferase action of the D-[^{14}C]glucose residues that had been introduced by the glucosidase is so extensive during the incorporation assay of the purified enzyme that the method probably cannot be regarded as being dependent upon glucosidase activity alone, especially as another aspect of the results presented here is that profound redistribution of unlabeled, outer-chain D-glucose residues has also occurred as the result of transferase action. Because of this, the structure of the acceptor undergoes continuing change during the assay, and even the extent to which the incorporation of the first D-[^{14}C]glucose group depends solely upon glucosidase action is uncertain.

MATERIALS AND METHODS

Materials. — Rabbit liver glycogen was the product of Sigma Chemical Co. D-[U-^{14}C]Glucose (234 mCi/mmol, 99+% radiochemical purity, 90% v/v ethanol) was obtained from New England Nuclear. The radiochemical purity of a solution (70% ethanol v/v) of 16 mM D-glucose containing this D-[U-^{14}C]glucose (final sp. act. 9.06×10^6 d.p.m./μmol) was verified by descending chromatography on Whatman No. 1 paper in 1-butanol–pyridine–water (3:2:1.5 v/v). When 160 nmol of D-glucose (1.45×10^6 d.p.m.) were chromatographed in this way, no radioactivity remained at the origin and none was found in any area of the paper except that containing glucose itself. Amylo-α-(1 → 4)-α-(1 → 6)-glucosidase ("amyloglucosidase") (E.C.3.2.1.3), prepared from *Aspergillus niger*, was obtained from Boehringer–Mannheim.

Amylo-(1 → 6)-glucosidase-4-α-glucanotransferase was prepared from rabbit skeletal muscle and assayed by its action on a phosphorylase limit-dextrin according to described methods[2,4]. The final preparation was free of alpha amylase, as judged from the exact correspondence between the quantity of D-glucose (assayed spectrophotometrically in the presence of added $NADP^+$, Mg^{2+}, ATP, hexokinase (E.C. 2.7.1.1), and D-glucose-6-phosphate dehydrogenase (E.C.1.1.1.49)), and the amount of reducing power (assayed by the method of Nelson[27] using chromatographically purified maltose as a standard) in a solution containing 1% rabbit-liver glycogen, 40 mM sodium chloride, and 40 mM sodium citrate buffer, pH 6.1, which had been incubated with 1 unit per ml of the debranching enzyme for 24 h at 37°. The amount of D-glucose formed during this incubation was 1.7% of the total glycogen present, and corresponded to the removal of about 25% of all of the branch points, originally present in the polysaccharide, by successive action of the transferase and glucosidase

activities of the pure enzyme. That the enzyme has such a debranching action on glycogen itself was shown[28,29] in 1963. As 1 unit of enzyme activity is defined here as the amount of enzyme required to produce 1 μmol of D-glucose per min from a 1% solution of a phosphorylase limit-dextrin in pH 6.1 citrate buffer at 37°, the observed average rate of debranching action on glycogen is less than 0.1% of that on a limit dextrin.

Phosphorylase *b* was obtained as a by-product in the preparation of the debranching enzyme[2]. This phosphorylase was without detectable debranching-enzyme activity and, hence, was suitable for use in preparing a limit dextrin of the [^{14}C]glycogen as described later, as well as for determining the average length of outer chains of various polysaccharide products.

Crystalline isoamylase (E.C.3.2.1.68) prepared from *Pseudomonas* SB15 was a generous gift from Dr. K. Yokobayashi of the Hayashibara Co. Ltd., Okayama City, Japan.

Enzymic determination of polysaccharide structure. — The percent of nonreducing-end groups in the polysaccharide (branch-point content) was determined by a modification of the method of Illingworth *et al.*[30] with phosphorylase *b* and added amylo-(1 → 6)-glucosidase in the presence of inorganic phosphate and 5′-AMP. The modification involved the direct assay of D-glucose in the total enzymic digest by the spectrophotometric determination of NADPH in the presence of $NADP^+$, Mg^{2+}, ATP, hexokinase, and D-glucose 6-phosphate dehydrogenase. D-Glucosyl phosphate was determined by a similar assay, except that phosphoglucomutase (E.C.2.7.5.1) was used instead of hexokinase and ATP. The extent of enzymic degradation was determined by comparing the sum of the quantities of D-glucose and D-glucosyl phosphate formed with the total initial content of polysaccharide, as determined by enzymic assay for D-glucose following complete hydrolysis by 100 μg per ml of "amyloglucosidase" (from *A. niger*) in 20 mM potassium acetate buffer, pH 5.5 (3 h, 37°). The average chain-length of the polysaccharide is the reciprocal of its content of branch points. The average outer chain-length was determined by incubating the polysaccharide in the presence of pure phosphorylase *b*, 5′-AMP, and inorganic phosphate and determining the amount of D-glucosyl phosphate, as already described, at various times until the reaction appeared to be essentially complete. The average outer chain-length was calculated by dividing the percentage of the molecule susceptible to direct phosphorolysis by the percentage of branch points and then by adding 4 to correct for the residual outer-chain D-glucose groups in the limit-dextrin structure; these are known to be resistant to degradation by phosphorylase[31].

Debranching of exhaustively dialyzed [^{14}C]glycogen and of its ^{14}C-phosphorylase limit-dextrin to their constituent unit-chains was achieved by incubating from 30–150 μmol of polysaccharide per ml with 120 μg per ml of *Pseudomonas* isoamylase in 10 mM sodium acetate buffer, pH 4.0, for 24 h at 37°. The total content of linear chains in this digest was estimated from a determination of their reducing power by the copper reduction method of Nelson[27], with chromatographically pure

maltose as a standard. It was found by Robyt and Whelan[32] and confirmed in unpublished work from this laboratory that α-D-(1 → 4)-linked glucose chains up to at least 7 units in length have equal reducing power, relative to maltose, on a molar basis. Accordingly, it was possible to calculate an apparent branch-point content of the polysaccharide in the isoamylase digest and to compare this value with that found by total degradation with phosphorylase plus amylo-(1 → 6)-glucosidase. It is known from the work of Harada, Yokobayashi, and their coworkers[23-26] that outer side-chains (A chains) of two units or less are cleaved only with difficulty by isoamylase. Such chains would not be expected to occur with significant frequency either in the parent rabbit-liver glycogen or in the [^{14}C]glycogen derived from it, even if 4-α-glucanotransferase had acted to cover the newly introduced ^{14}C-branch point groups, in view of the known specificity of this enzymic activity[8]. Accordingly, essentially complete degradation of the [^{14}C]glycogen was expected[24]. Whether D-[^{14}C]glucose branch points not covered by transferase action would appear as D-[^{14}C]glucose in the isoamylase digest was uncertain, from previously published work[23-26].

Separation and analysis of the unit chains of the polysaccharides. — After incubation and determination of reducing-power content, the isoamylase digests were neutralized to pH 6 and boiled. The ^{14}C-content was determined by liquid-scintillation counting. Aliquot portions were chromatographed on Whatman No. 1 paper (descending) in 1-butanol–pyridine–water (3:2:1.5 v/v) for varicus lengths of time, with markers of pure linear and branched oligosaccharides derived by partial acid hydrolysis or treatment of glycogen with alpha amylase. By allowing sufficient time for the chromatography (up to 6 weeks), it was possible to achieve the complete separation of linear chains up to 10 glucose units in length. The analysis of the polysaccharides' structures therefore is confined to unit chains whose length is in this range. After drying the papers, each chain, whose position was revealed by scanning in a Packard Auto Strip scanner and by benzidine–trichloroacetic acid spray of the appropriate markers, was eluted with distilled water by capillary action. Experience with this method has shown that elution is achieved with about 70% efficiency in this chain-length range. However, the data presented below are given without any correction for possible incomplete elution, as such corrections could not be determined with precision. The quantity of each pure chain present in its eluate was found by digesting it totally to D-glucose with "amyloglucosidase" from *A. niger*, as already described, and then determining the amount of D-glucose by the hexokinase–D-glucose 6-phosphate dehydrogenase assay. The total ^{14}C-content of each eluate was found by liquid-scintillation counting of replicate aliquots. From these results, calculation could be made of the fraction of the total chains, on a molar basis, that was present in the isoamylase digest for each chain length, as well as of the fraction of each ^{14}C-labeled chain as compared with the total ^{14}C-chain content of the digest. These calculations were based on the assumption that each labeled chain contained only one D-[^{14}C]glucose unit (at its reducing end), and that the specific activity of such

a chain on a molar basis was equal to that of the [^{14}C]glucose used in the original incorporation assay (9.06 × 10^6 d.p.m./μmole).

Preparation of [^{14}C]glycogen. — Rabbit-liver glycogen (166 μmol/ml) was incubated with 3.2 μmol/ml of D-[U-^{14}C]glucose (9.06 × 10^6 d.p.m./μmole) in 50 mM sodium maleate buffer, pH 6.5 with 0.24 unit/ml of pure amylo-(1 → 6)-glucosidase at 30°. The progress of the ^{14}C-incorporation reaction was monitored by the method of Nelson and Larner[20] by removing 15 μliter aliquots at various times and adding each of them to 45 μliters of 0.2M hydrochloric acid to stop the reaction. Aliquots (50 μliter) of the acidified mixture were spotted on squares of Whatman 31 ET filter paper which then were washed as described and dried. The [^{14}C]glycogen present on them was determined by immersing the papers in a universal counting solvent (3a70, Research Products International). In the first preparation, incubation was continued for 5 h in order to obtain a substantial level of incorporation. In a second preparation, incubation was for 30 min only. In both cases, the reaction was terminated by heating the solution in boiling water and the solution was dialyzed exhaustively at 5° against numerous changes of distilled water until the dialyzate had been entirely free of ^{14}C for at least 24 h. In addition to this demonstration that the ^{14}C-glycogen had been freed thoroughly of dialyzable D-[^{14}C]glucose which might have contaminated it, an amount of the [^{14}C]glycogen which contained 12,960 d.p.m. of ^{14}C was subjected to descending chromatography on Whatman No. 1 paper, as has been already described. Not only did all of this ^{14}C remain at the origin with the glycogen, but an area of the chromatogram that would have contained glucose, had it been present, was eluted and found to have no ^{14}C whatsoever. Furthermore, direct, spectrophotometric enzymic assay of the [^{14}C]glycogen solution for free D-glucose showed that it contained less than 0.005 μmol per ml, which was at the limit of detection. By these criteria, the [^{14}C]glycogen was essentially free of occluded D-[^{14}C]glucose. The solution of [^{14}C]glycogen was analyzed for its total polysaccharide content by "amyloglucosidase" digestion and for ^{14}C.

The sample prepared by incubating with amylo-(1 → 6)-glucosidase for 5 h was recovered quantitatively and it contained 1300 d.p.m./μmol of polymeric glucose. This extent of [^{14}C]glucose labeling corresponded to the introduction of 0.015% of new branch points. The total branch-point content of the sample was found to be 6.79% (100% degradation), which corresponds to an average chain-length of 14.7 glucose units.

The sample prepared by incubating with amylo-(1 → 6)-glucosidase for only 30 min contained 516 d.p.m./μmole and had a total branch-point content of 6.76%.

Both ^{14}C-products were tested for their chromogenicity with 0.02% iodine–0.04% potassium iodide in 0.1M sodium citrate, pH 5.6. The two samples were indistinguishable both as regards the wavelength of the maximum extinction (490–500 nm) and the absorbance of the solution (1-cm path length) at 495 nm (absorbance of 1.0 per μmol of polymeric glucose). The average outer chain-lengths of the two products were also the same (10.3 glucose groups), as calculated from the fact that 42.5% of each polysaccharide was degradable in a single incubation with phosphory-

lase alone. The average inner chain-length of the products was 4.4 glucose residues. These gross structural features are reasonable for rabbit-liver glycogen. The extent of incorporation of D-[^{14}C]glucose branches to make new A chains (if covered by transferase action) was so small that no detectable effect on branch point content or outer chain-length could have been expected.

RESULTS

When rabbit liver glycogen was incubated with D-[^{14}C]glucose at pH 6.5 in the presence of a pure preparation of amylo-(1 → 6)-glucosidase-4-α-glucanotransferase, D-[^{14}C]glucose groups were incorporated into the glycogen. Figure 1 shows the relationship between the extent of incorporation of such D-[^{14}C]glucose groups and the time of incubation of the substrates with the enzyme. It is clear that the reaction proceeds initially more rapidly than at later times. The extent of the reaction seems to be limited by the availability of suitable acceptor-sites on the glycogen molecule. That the net incorporation of D-glucose is a rare event is apparent from the fact that even at 5 h, only one such ^{14}C-branch point has been introduced for every 230 outer branch points initially present. The structural specificity of the acceptor chains required for this incorporation must be quite rigid. Some information that bears on this point could come from a comparison of the distribution of unit chains found in the isoamylase digests of the two products prepared by two greatly different times of incubation. These results are discussed next.

Near limit-dextrins were prepared by a single treatment of each [^{14}C]glycogen sample with pure phosphorylase. In the case of the sample of ^{14}C-glycogen that had been prepared by 5 h of incubation with [^{14}C]glucose and amylo-(1 → 6)-glucosidase, the specific activity of ^{14}C in the "limit dextrin" (39.2% removed by phosphorylase) remaining after exhaustive dialysis was 2013 d.p.m./μmol. The specific activity that

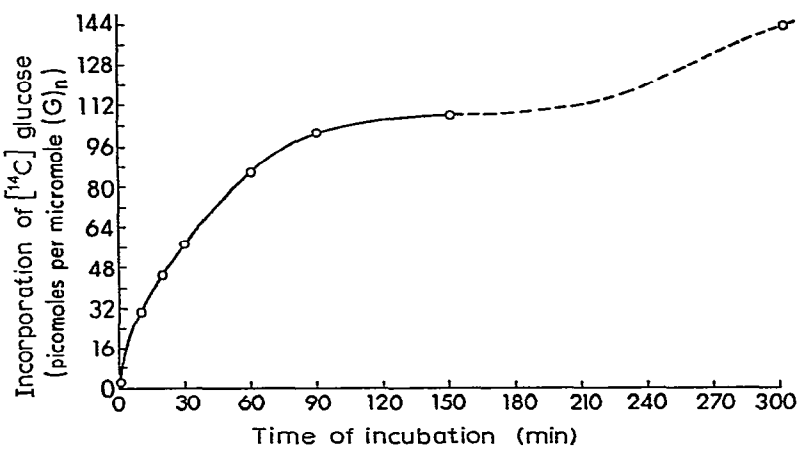

Fig. 1. Rate of incorporation of D-[^{14}C]glucose groups into rabbit liver glycogen. See text for the composition of the reaction mixture.

was expected from the removal of unlabeled exterior D-glucose groups would have been 2138 d.p.m./μmol. Thus, at least 94% of the ^{14}C-glucose incorporated by the action of amylo-(1 → 6)-glucosidase was as glucose residues that were not susceptible to subsequent phosphorolysis. The specific activity of the "limit dextrin" (36.3% degraded by phosphorylase) prepared from the 30-min [^{14}C]glycogen was 822 d.p.m./μmol (101% of expected) which indicated that there were no ^{14}C-labeled D-glucose residues in its exterior α-D-(1 → 4)-linkages.

The data in Table I show that the ^{14}C-glycogen present after 30 min of incubation is very much less susceptible to debranching by isoamylase than the polysaccharide present at 5 h. The latter was virtually completely degraded into its constituent unit-chains as is normal rabbit-liver glycogen[24]. The meaning of the large difference in susceptibility to isoamylase action could be that the ^{14}C-product present at 30 min contained a substantial number of uncovered [^{14}C]glucose groups in α-D-(1 → 6)-linkage on external chains, and that these could not be removed by isoamylase. That they might constitute a significant block to total degradation of the molecule requires that the isoamylase be assumed to have the properties of an enzyme acting from the periphery of the glycogen molecule toward its core and that it also be assumed that the enzyme is virtually unable to bypass any branch point that it cannot remove. Even with these assumptions, however, it does not seem possible to account for the 35% deficit in degradation. Interestingly, however, Harada and coworkers have reported[25] that isoamylase seems to be "like an exo-enzyme when acting on glycogen" and they have concluded that it "does not readily penetrate the glycogen molecule" although it is ultimately capable of bringing about its complete degradation. The formation of a large proportion of [^{14}C]glucose from the limit dextrin prepared from the 30 min [^{14}C]glycogen sample (Table I) was a surprising result in view of the very small proportion of [^{14}C]glucose formed from the parent [^{14}C]glycogen. It is not clear from the data of Harada, Yokobayashi, and coworkers whether significant amounts of D-glucose are to be expected from the action of their crystalline isoamylase. Tests for maltase, isomaltase, and D-enzyme were negative[23], but we have found that when 100 μmol per ml of the parent rabbit-liver glycogen is incubated with 120 μg per ml of isoamylase under the same conditions as those used preparatively to debranch the [^{14}C]polysaccharide products, up to 0.1% of the glycogen is converted into D-glucose in 24 h. The source of this glucose, namely, α-D-(1 → 4)- or α-D-(1 → 6)-linked residues, is unknown. We have considered the possibility that the isoamylase preparation might contain traces of an α-D-(1 → 6)-glucosidase related in substrate specificity to the glycogen-debranching enzyme of yeast and animal origin. Overnight incubation of the enzyme with 1 μmol per ml of the specific 1 → 6-α-D-glucosidase substrate, 6^3-α-glucosylmaltotetraose ("Fast B_5")[33], resulted in the formation of less than 1% of D-glucose. Hence, the presence of a contaminant having the specificity of amylo-(1 → 6)-glucosidase seems highly unlikely. Finally, the possibility has been considered that the specificity of isoamylase is such that it can easily hydrolyze (1 → 6)-bonded-α-D-[^{14}C]glucose residues when they are attached to the fifth or sixth glucose residue from the non-reducing end of an

TABLE I

DEGRADATION OF [^{14}C]POLYSACCHARIDES BY ISOAMYLASE

Sample	*Extent of degradation (% of total branch points)*	*^{14}C-Glucose in digest (% of total ^{14}C-chains)*
Glycogen (5 h)[a]	97.0	0
Limit dextrin (5 h)	83.5	0.23
Glycogen (30 min)	61.7	0.15
Limit dextrin (30 min)	80.3	14.0

[a]The times given in column 1 refer to the incubation time of the incorporation assay. The limit dextrin is the product of phosphorylase action (see text).

outer chain, as they might be in the phosphorylase limit-dextrin prepared from [^{14}C]-glycogen, and that such residues are shielded from hydrolytic action by the long outer chains of the [^{14}C]glycogen itself. This possibility has not been investigated by direct experiment, as suitable substrates of defined structure are not available.

A comparison of the data in Table I relating to the 5-h product with those for the 30-min product indicates that important structural changes have occurred with time in the labeled polysaccharide such that all of the branch points of the glycogen have become more available to isoamylase, and, further, that the exposed [^{14}C]glucose groups appear to have essentially disappeared, as shown by the very small amount of [^{14}C]glucose formed from the 5-h limit dextrin.

In view of the fact that the 5-h product was essentially completely debranched to its constituent unit-chains by isoamylase, the chain-length distribution of the ^{14}C-linear chains in its digest is shown in the lower panel of Fig. 2. As expected from the known specificity of 4-α-glucanotransferase and from that of isoamylase itself, only a very small proportion of [^{14}C]maltose was found. The presence of [^{14}C]maltotriose and [^{14}C]maltotetraose is due to single transfer by 4-α-glucano-transferase of maltosyl and maltotriosyl groups, respectively, to [^{14}C]glucose branch-points. The ratio of the quantity of G_4 to that of G_3 is 1.3, which is an indirect expression of the relative frequency of 3-unit and 2-unit transfers by the enzyme. The quantity of [^{14}C]maltohexaose in the digest exceeds that of [^{14}C]maltopentaose, because the latter can result only from a second maltosyl transfer to G_3, whereas the former can result either from maltosyl transfer to G_4 or maltotriosyl transfer to G_3. Similar considerations apply to the formation of the longer chains up to G_{10}. The ^{14}C-linear chains from G_1 to G_{10} amounted to 76% of all ^{14}C-chains in the isoamylase digest. Consideration of the fact that this result is calculated from the observed data, without correction for loss on elution from the paper chromatograms, leads to the additional conclusion that there are very few ^{14}C-chains longer than G_{10}. Hence, all of the ^{14}C-chains analyzed probably were side chains (A chains) in the [^{14}C]glycogen after 5 h of preparation. Furthermore, there must have been

very few, if any, uncovered [^{14}C]glucose groups remaining in the molecule at that time. The upper panel of Fig. 2 shows the chain-length distribution of ^{14}C-linear chains arising from the action of isoamylase on the phosphorylase near limit-dextrin of the [^{14}C]glycogen shown in the bottom panel. As expected from the fact that the extent of phosphorylase action during its preparation (39.2%) was less than that which could be achieved by more-complete treatment (42.5%), all of the maltopentaose and maltohexaose side-chains (A chains) were not converted into maltotetraose chains. The observed increase in the percentage of maltotriosyl chains from 10.4% in the parent glycogen to 17.4% in the limit dextrin is interesting, as it could mean that some phosphorylase degradation of 4-unit A chains had occurred. However, it must be pointed out that the limit dextrin was only 83.5% debranched, and that the chain-length distribution of its unit chains represents an analysis of a smaller percentage of its total structure, and this may have led to a skewing of the distribution. The persistence of ^{14}C-chains eight to ten D-glucose residues in length in the limit-dextrin structure may reflect the presence of a few second-tier (inner) ^{14}C-branch points formed because of a tendency of amylo-(1 → 6)-glucosidase to add a second [^{14}C]glucosyl group in α-D-(1 → 6)-linkage to an already existing A chain 6 to 9

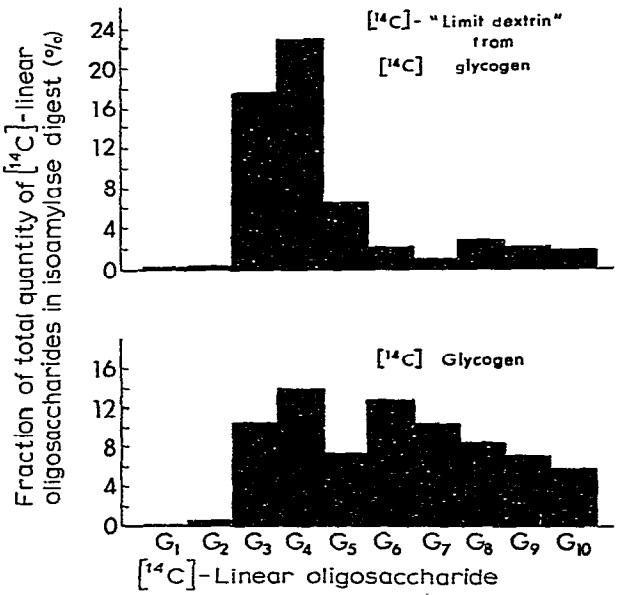

Fig. 2. Distribution of ^{14}C-lateled unit-chains in *Pseudomonas* isoamylase digests of [^{14}C]glycogen prepared by 5-h incubation with amylo-(1 → 6)-glucosidase-4-α-glucanotransferase and of a near phosphorylase limit-dextrin prepared from it. In the case of the [^{14}C]*glycogen (lower panel)*, the isoamylase digest contained 6.56 μmol/ml of total linear chains (calculated from their reducing power as described in the text) and 0.0133 μmol/ml of singly labeled, ^{14}C-linear chains (calculated from the total ^{14}C content of the digest and the original specific activity of the ^{14}C-glucose, 9.06 × 10^6 d.p.m./μmol). Thus, 0.203% of all of the oligosaccharide chains were ^{14}C-labeled. In the case of the [^{14}C]*limit dextrin (upper panel)*, the digest contained 2.94 μmol/ml of total linear chains, of which 0.00684 μmol/ml were singly labeled with ^{14}C (0.233% of the total). Aliquots of these solutions were chromatographed and their oligosaccharide components were analyzed as described in the text.

TABLE II

SIZE DISTRIBUTION OF THE UNIT CHAINS IN [^{14}C]GLYCOGENS AND DERIVED ^{14}C-LIMIT DEXTRINS[a]

Chain	*30-Min product % of total linear chains*		*5 h Product % of total linear chains*	
	Glycogen	*Limit dextrin*	*Glycogen*	*Limit dextrin*
G_1	1.12	10.22	5.73	8.13
G_2	8.54	9.31	1.25	2.18
G_3	1.87	3.74	1.30	4.70
G_4	2.90	22.25	0.74	34.89
G_5	4.57	16.62	5.39	15.32
G_6	6.62	7.65	12.60	5.20
G_7	4.20	1.21	17.28	5.58
G_8	6.93	6.41	14.09	4.78
G_9	6.32	3.07	10.95	4.13
G_{10}	N.D.	N.D.	9.72	3.47
Sum	43.07	80.48	79.05	88.39

[a]The data in this Table refer to the total quantity of each unit-chain (^{14}C-labeled plus unlabeled) which was found (see text for further details).

units in length, which itself had been formed by earlier [^{14}C]glucose incorporation followed by repeated 4-α-glucanotransferase action as already discussed. The lower panel of Fig. 2 shows that chains of this length do exist in the glycogen. However, the present findings do not provide proof for the existence of second-tier ^{14}C-branch points.

The data in Table II show apparent differences in the numbers of unlabeled, relatively short (presumably) A chains that were present in the 30-min and the 5-h [^{14}C]glycogen products. The 5-h polysaccharide seems to have had twice as many side chains that were 6, 8, and 9 residues long and 4 times as many 7-residue side-chains as the 30-min product. There was a large difference in the extent of total degradation of these samples and, hence, it is possible that these results are misleading. However, there is no reason to expect that there would have been a special selection against the debranching of side chains of these lengths in the 30-min product. If the analyses are considered to be representative of the total structures of both polysaccharides, the data indicate that there has been a substantial net transfer by 4-α-glucanotransferase of α-D-(1 → 4)-linked maltosyl and maltotriosyl groups from outer side-chains (A chains) to main chains during the 4.5-h period of continued incubation. Although the extent of debranching of the total number of unlabeled and labeled unit-chains of the 5-h sample (79%) was essentially equal to that of the ^{14}C-labeled unit chains (76%) a greater proportion of the unlabeled chains (42%) were composed of G_7, G_8, and G_9 than of the ^{14}C-chains (26%). This evidence for considerable redistribution of outer glucosyl groups in the acceptor polysaccharide during the [^{14}C]glucose incorporation assay for the debranching enzyme raises the

question of how significant these structural modifications may be in creating favorable sites for addition of [^{14}C]glucose by amylo-(1 → 6)-glucosidase. In view of the very limited amount of such incorporation that occurs, even after considerable time, this question should be considered seriously. Glycogen is known to be a much better acceptor for incorporation of [^{14}C]glucose than is a phosphorylase limit-dextrin[34]. This surprising fact must be related to the differences in structure of these polysaccharides. It is possible that a sufficient number of outer-chain glucose residues must be available in a peripheral position on the same or on neighboring chains to provide a source of maltosyl and maltotriosyl groups to cover the newly introduced [^{14}C]glucose branch-points. Although such covering action clearly is not required for the incorporation step itself, the retention of a significant percentage of such newly introduced [^{14}C]glucose groups may indeed require that they be covered. In the present work, it was found that the ratio of 3-unit transfer (^{14}C-maltotetraose side-chains) to 2-unit transfer (^{14}C-maltotriosyl side-chains) was the same (1.42) in the 30-min product as that (1.32) in the 5-h product.

We conclude that there is no compelling reason to believe that the D-[^{14}C]glucose incorporation assay, as usually carried out with the native enzyme, can be regarded as an assay specific for the amylo-(1 → 6)-glucosidase activity of the glycogen-debranching enzyme. In order that such a specificity could be established beyond question it would be necessary that a [^{14}C]glucose-labeled product be shown to have all of its newly introduced ^{14}C-branch points present as exposed α-D-(1 → 6)-linked glucose stubs. In the present work, the maximum number of such exposed groups seems to have been between 14 and 35% of all of the ^{14}C-branch points in the product after 30 min of incubation. Actually 40% more of the [^{14}C]glucose residues were covered by 2 or 3 unlabeled glucose residues at 30 min than at 5 h. There also is structural evidence that the acceptor glycogen undergoes substantial redistribution of outer-chain units during the incorporation assay, and, therefore, the possibility exists that such changes, catalyzed by the transferase property of the pure enzyme (4-α-glucanotransferase), must occur prior to incorporation of [^{14}C]glucose in order that a favorable structure exists for the reverse action of amylo-(1 → 6)-glucosidase.

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