# Studies of Glycogen Branching Enzyme. Preparation and Properties of α-1,4-Glucan–α-1,4-Glucan 6-Glycosyltransferase and Its Action on the Characteristic Polysaccharide of the Liver of Children with Type IV Glycogen Storage Disease*

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ABSTRACT: The glycogen "branching enzyme" (α-1,4-glucan–α-1,4-glucan 6-glycosyltransferase, EC 2.4.1.18) has been purified over 3000-fold from rabbit skeletal muscle. The soluble enzyme is about 90% pure as judged from the results of polyacrylamide disc gel electrophoresis. The apparent molecular weight of the branching enzyme was found by sucrose gradient centrifugation to be from 92,000 to 103,000 depending upon the choice of reference protein. The activity of the enzyme was inhibited to the extent of 50% by 0.4 M urea and 100% by 2 M urea. The inhibition by 2 M urea could be reversed by dilution. Sucrose gradient centrifugation showed that the protein was not dissociated in 2 M urea. The assay for the enzyme at all stages of purification depended upon its ability to increase the measured rate of polysaccharide synthesis from glucose 1-phosphate by phosphorylase, under conditions where the activity of the latter enzyme was made highly dependent upon small changes in the number of polysaccharide chains which serve as acceptors for glucose units. The purified branching enzyme was also assayed directly by using as substrate an amylopectin-like polysaccharide which had been isolated from the liver of a patient with Type IV glycogen storage disease. This congenital disease is known to be due to the absence of branching enzyme, and, in consequence, the characteristic polysaccharide present in these human tissues has long, unbranched outer chains. It has now been found that these outer chains can be branched *in vitro* by the purified rabbit muscle enzyme, and this action was assayed by measuring the rate of decrease in absorbance at 520 nm of the iodine complex of the abnormal human liver polysaccharide. Approximately a twofold stimulation of activity was obtained in 0.15 M sodium citrate or 0.05 M potassium α-D-glucose 1-phosphate at pH 7. The enzyme has a broad pH optimum (pH 6.8–7.8) in citrate buffer but a somewhat sharper one (pH 7.2–7.6) in glucose 1-phosphate. It is maximally active in citrate buffer at 30–35°. The $K_m$ of the Type IV "amylopectin" was $5.6 \times 10^{-6}$ M when calculated as the concentration of its total outer chain ends. Linear oligosaccharides of the maltose series which have from 6 to 10 glucose units were found to inhibit the enzyme. Maltoheptaose was the most inhibitory of these substances (40% inhibition at 15 mM). Type IV "amylopectin," before and after incubation with branching enzyme, was subjected to structural analysis by stepwise degradation by glycogen phosphorylase and oligo-α-1,4→1,4-glucantransferase–amylo-1,6-glucosidase. The results of these studies indicated that the branching enzyme had branched both outer side chains and outer main chains and to nearly the same extent. Furthermore, the structural data were consistent with results which could be predicted if the branching enzyme were assumed to have placed each new outer branch point at an average position which was 4 glucose units away from the nearest preexisting outer branch point of the parent polysaccharide.

The biosynthesis of the branch points in mammalian glycogen is due to the action of an α-1,4-glucan–α-1,4-glucan 6-glycosyltransferase (EC 2.4.1.18) which is often referred to as the "branching enzyme." Larner (1953) found that when this enzyme acted on outer chains of glycogen which had an average chain length of from 11 to 21 glucose units, new α-1,-6-glucosidic linkages were formed as outer branch points. By using a partially purified enzyme prepared from rat liver according to the procedure of Krisman (1962), Verhue and Hers (1966) were able to obtain evidence that the reaction catalyzed was not the transfer of a single glucose unit, but that it appeared to involve the scission of a chain of 6 or more α-1,4-linked glucose units from the terminal portion of the outer chain of a polysaccharide substrate and the reattachment of this oligoglucan fragment in α-1,6-glucosidic linkage as a new outer side chain. Brown and Brown (1966c) showed that a partially purified branching enzyme from rabbit skeletal muscle preferentially transferred chain segments which were 7 glucose units long, when it acted on polysaccharides previously enlarged *in vitro* from UDPG[1] by the action of rabbit muscle UDPglucose–glycogen glucosyltransferase (EC 2.4.-1.11). Whether the branching enzyme has any specificity with respect to its interaction with the outer main chains as distinct from the outer side chains of polysaccharides, either as donors or acceptors in the branching reaction, is unknown, and there is no information about the interbranch point distance which exists after the enzyme transfers an oligoglucan segment to form a new branch point in the polysaccharide substrate. In

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[1] Abbreviations used are: UDPG, uridine diphosphate glucose; G-1-P, α-D-glucose 1-phosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; 5′-AMP, 5′-adenylic acid.

order to study these questions, the branching enzyme has been prepared in a nearly homogeneous state from rabbit skeletal muscle and its molecular weight and some kinetic properties have been determined. New information about the specificity of its action has been obtained by using as a substrate the isolated, amylopectin-like polysaccharide which is present, instead of glycogen, in the tissues of children who have Type IV glycogen storage disease due to the absence of branching enzyme (Brown and Brown, 1966a, 1968).

## Materials and Methods

*Materials.* α-D-Glucose 1-phosphate (Lot No. 55B-2000) was obtained from Sigma Chemical Co. It was tested for the possible presence of contaminating polysaccharide by incubating it (0.08 M, pH 6.6) with glycogen phosphorylase *a* (6 units/ml) in the presence of 2 mM 5′-AMP but without added polysaccharide. No $P_i$ was formed during 90 min of incubation at 30°. Rabbit liver glycogen was obtained from Mann Research Laboratories. Deboned, frozen rabbit muscle was purchased from Pel-Freez Biologicals, Inc. Bio-Gel P-200 (50-100 mesh) was obtained from Calbiochem and Cellex XF-1 (formalated cellulose) from Bio-Rad Laboratories. DEAE-cellulose (Brown Co.) was treated by the method of Peterson and Sober (1962) before use.

Phosphoglucomutase, glycerol-3-phosphate dehydrogenase, yeast hexokinase, glucose-6-phosphate dehydrogenase, and rabbit muscle lactate dehydrogenase were obtained from the Boehringer Corp. Phosphorylase *a* was prepared from rabbit skeletal muscle and recrystallized repeatedly according to the procedure of Illingworth and Cori (1953), except that 5 mM 2-mercaptoethanol–1 mM EDTA was substituted for the 12 mM cysteine used in the original procedure in the recrystallization steps. Assay of phosphorylase was by incubation at 30° with 1% glycogen, 1 mM 5′-AMP, and 0.1 M G-1-P (pH 6.6). One unit of activity is the amount of enzyme which forms 1 μmole of $P_i$/min. $P_i$ was determined by the method of Fiske and Subbarow (1925). Oligo-α-1,4→1,4-glucantransferase–amylo-1,6-glucosidase was prepared by the method of Brown and Brown (1966d).

*Enzymatic Determination of Polysaccharide Structure.* The per cent of nonreducing end groups in polysaccharides (branch point content) was determined by a modification of the method of Illingworth *et al.* (1952) using a pure preparation of phosphorylase *a* to which was added oligo-α-1,4→1,4-glucantransferase–amylo-1,6-glucosidase ("debranching enzyme system"). The modification involved the direct assay of glucose in the total enzymatic digest by the spectrophotometric determination of NADPH in the presence of added $NADP^+$, $Mg^{2+}$, ATP, hexokinase, and glucose-6-phosphate dehydrogenase. Glucose 1-phosphate was determined by an assay which was similar except that phosphoglucomutase was used instead of hexokinase and ATP. The extent of enzymatic degradation was determined by comparing the sum of the quantities of glucose and glucose 1-phosphate formed with the total initial polysaccharide content, as determined by enzymatic assay of glucose following total hydrolysis of the polysaccharide for 3 hr in 1 M HCl at 100°. In order to determine the extent to which phosphorylase alone was able to degrade the polysaccharide in the presence of $P_i$, it was necessary to remove traces of the "debranching enzyme system" by column chromatography of recrystallized phosphorylase *a* on DEAE-cellulose in 5 mM Tris–1 mM EDTA–5 mM 2-mercaptoethanol (pH 7.1) using a salt gradient between 0 and 0.5 M NaCl (Illingworth *et al.*, 1961).

*Preparation of Linear Oligosaccharides.* Soluble starch was hydrolyzed in 0.05 M $H_2SO_4$ for 1 hr at 100°. After neutralization to pH 6.5 with $Ba(OH)_2$, $BaSO_4$ was removed by centrifugation. The supernatant fluid was deionized with Amberlite MB-3 and chromatographed on Whatman No. 1 paper using 1-butanol–pyridine–water (3:2:1.5, v/v) as the developing solvent in a descending system. Each separated linear oligosaccharide was eluted with water and its amount determined by hydrolysis and subsequent assay for glucose. The purity of each product from maltopentaose to maltodecaose was determined by measuring the extent to which it could be degraded to maltotetraose and G-1-P by the prolonged action of rabbit muscle phosphorylase *a* in the presence of 5′-AMP and $P_i$ (Brown and Cori, 1961; Illingworth *et al.*, 1964). By this criterion each isolated oligosaccharide was at least 95% pure.

*Type IV Amylopectin.* The presence of an amylopectin-like polysaccharide is characteristic of tissues from patients with Type IV glycogen storage disease, "amylopectinosis" (Illingworth and Cori, 1952; Brown and Brown, 1966a, 1968). The polysaccharide of this kind which was used in the present work as a substrate for the purified branching enzyme was the same substance as that which had been studied by Illingworth and Cori (1952) after it had been isolated from the liver of a child (P. B.) who had died with Type IV disease.

*Polyacrylamide Disc Gel Electrophoresis.* The enzyme solution (up to 50 μl) was mixed with an equal volume of 40% sucrose and subjected to electrophoresis in a 5.5% gel, using 5 mM Tris–38 mM glycine buffer, pH 8.3, according to the method of Dietz and Lubrano (1967). Electrophoresis was usually for 1 hr at 4° with a current of 2.5 mA/tube. The gels were stained with 0.5% aniline black in 7.5% acetic acid and then destained electrophoretically. Visual comparison was made between the stained protein bands and the location of enzyme activity as revealed by extraction of small segments of unstained gel columns run simultaneously.

*Sucrose Density Gradient Centrifugation.* The branching enzyme was mixed with phosphorylase and samples of commercial preparations of three other reference enzymes, and the mixture was dialyzed overnight at 4° in a buffer containing 5 mM Tris–1 mM EDTA–25 mM KCl–5 mM 2-mercaptoethanol, pH 6.2. The mixture of enzymes was centrifuged in a linear sucrose gradient (7–17%). For experiments carried out in the presence of urea, the sucrose solutions used to form the gradient, as well as the buffer used for dialysis, contained added urea of the appropriate concentration. Centrifugation was in the SW65 rotor of the Spinco L2-65. Fractions from the gradient (0.18 ml) were assayed for the branching enzyme by method 1 described below, for phosphorylase as described above, for glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) by the method of Beisenherz *et al.* (1955), for phosphoglucomutase (EC 2.7.5.1) by the method of Klenow and Emberland (1955), and for lactate dehydrogenase (EC 1.1.1.27) by the method of Kornberg (1955).

*Assay of Branching Enzyme (Method 1).* In this method, the principle of which was described by Illingworth *et al.* (1961), phosphorylase was used as an indicating enzyme, and the rate of its action in synthesizing maltosidic bonds from glucose 1-phosphate was measured. It was shown previously that the action of branching enzyme results in the production of an increased number of chain termini and, thus, is equivalent to increasing the effective concentration of polysaccharide acceptor available for the phosphorylase reaction. The standard assay mixture for branching enzyme contained 80 mM G-1-P (pH 6.4), 2 mM 5′-AMP, and 6 units/ml of phosphoryl-

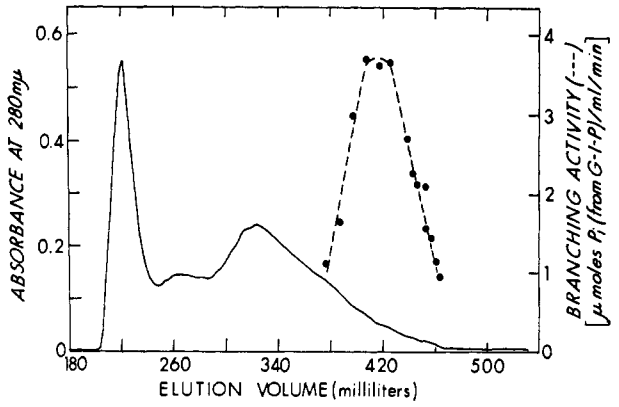


FIGURE 1: Elution profile of Bio-Gel P-200 chromatography of rabbit muscle branching enzyme. Conditions are described in the text.

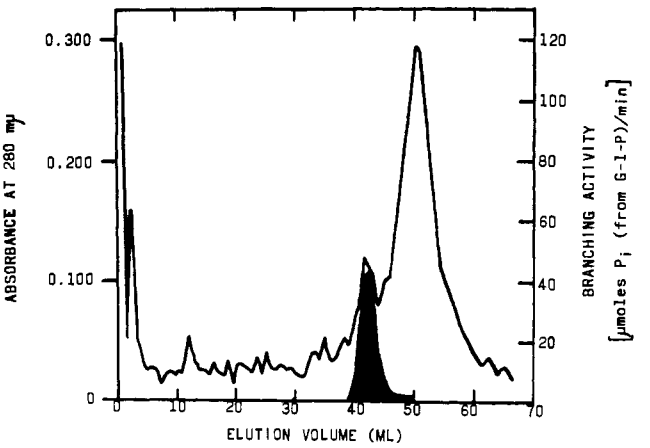


FIGURE 2: Elution profile of a preparative electrophoresis column after 10 hr at 750 V and 3–5 mA. Other conditions are described in the text. The solid line shows the absorbance at 280 nm, and the darkened area shows the location of the branching enzyme. The right-hand ordinate refers to the total enzyme activity in each fraction of 0.82 ml.

ase *a* activity. Incubation with various quantities of branching enzyme was at 30°; 50-μl aliquots were removed at 15-min intervals and analyzed for $P_i$. In the absence of any added branching enzyme, no detectable $P_i$ was formed during at least 90 min of incubation. In the presence of the branching enzyme, the rate of $P_i$ formation from G-1-P by phosphorylase was determined, after an initial lag period, from the linear slope of the straight line obtained when $P_i$ formation was plotted as a function of time. One unit of branching enzyme activity was defined as the amount of enzyme whose presence in the assay mixture described above resulted in the production by phosphorylase of 1 μmole of $P_i$/min. The specific activity of the enzyme was expressed as units/mg of protein as determined by the method of Lowry *et al.* (1951).

*Assay Method 2.* In this method the branching enzyme was allowed to act directly on Type IV amylopectin (P. B.). By virtue of the enzyme's ability to form new α-1,6-glucosidically linked branch points, the chromogenicity of the polymer toward $I_2$–KI was decreased, and the absorption maximum of the $I_2$ complex ($\lambda_{max}$ 530 nm) was shifted toward shorter wavelengths. The rate of such a change, with plant amylopectin as the substrate, was described by Larner (1955) as an assay for the branching enzyme from rat liver. The assay mixture contained 2 mg/ml of Type IV amylopectin (P. B.) in 0.25 M sodium citrate, pH 7.1, 30°. At time intervals up to 30 min after the addition of various amounts of branching enzyme, 75-μl aliquots were sampled directly into 3 ml of 1.3 mM $I_2$–21 mM KI–40 mM sodium citrate, pH 5.6. The rate of decrease in absorbance at 520 nm of the $I_2$ complex was taken as a measure of branching enzyme action. Using several different enzyme preparations at various stages of purity, it was found that 1 unit, assayed as in method 1 above, produced a decrease in the 520-nm absorbance of the $I_2$ complex of the Type IV amylopectin (P. B.) of 0.029 per min when assayed as described here.

## Results

*Purification of Branching Enzyme.* The first steps of the preparation were essentially those described by Brown and Brown (1966b) in the procedure in which the mother liquor from the first crystals of phosphorylase *a*, isolated from rabbit skeletal muscle, served as the source of the branching enzyme. Immediately after treatment with corn starch (1 mg/2 mg of total protein as determined according to Lowry *et al.*, 1951), 3-ml aliquot portions of the clear supernatant fluid containing the enzyme were chromatographed on a 3.2 cm × 100 cm column of Bio-Gel P-200 in 1% sodium glycerophosphate–1 mM EDTA–5 mM 2-mercaptoethanol–5 mM NaF, pH 6.6, at a flow rate of 6 ml/hr. The elution profile of the branching enzyme from the column is shown in Figure 1. Fractions in which the branching enzyme had a specific activity of greater than 20 units/mg were pooled and dialyzed overnight against 5 mM Tris–1 mM EDTA–5 mM 2-mercaptoethanol, pH 7.1, and then poured through a 1 × 2 cm DEAE-cellulose column which had been equilibrated with buffer of the same composition. At a flow rate of 10 ml/hr the effluent was collected as a single fraction which was dialyzed against 0.5% sodium glycerophosphate–1 mM EDTA–5 mM 2-mercaptoethanol, pH 7.4. Disc gel electrophoresis of the resulting solution showed that the short column of DEAE-cellulose had removed a number of different contaminating proteins which were present in small quantities without causing the loss of more than 10% of the branching enzyme. The dialyzed enzyme solutions from three DEAE-cellulose columns, and, hence, from three Bio-Gel P-200 columns, were combined and concentrated to 3 ml by ultrafiltration in a Diaflo cell using an XM-50 membrane (Amicon Corp.).

*Preparative Electrophoresis.* The enzyme solution (3.4 mg/ml) was dialyzed against 16 mM Tricine–0.8 mM EDTA–4 mM 2-mercaptoethanol, pH 7.8 (conductance, 300 μmhos). A 1 cm × 25 cm electrophoresis column, constructed according to Porath (1956), was packed with Cellex-XF-1 formalated cellulose and equilibrated with 20 mM Tricine–1 mM EDTA–5 mM 2-mercaptoethanol, pH 7.8 (conductance, 390 μmhos). The enzyme solution was added to the top of the column and washed into it with 3–5 ml of the more concentrated Tricine buffer. Each electrode vessel contained 3 l. of buffer, and the jacketed column was maintained at 2° during electrophoresis for 10 hr at a constant voltage of 750 V (3–5 mA), during which time the branching enzyme migrated anodically. After discontinuing the run, the top of the column was connected to a buffer reservoir and fractions of 0.8 ml each were collected. Figure 2 shows the protein profile and the location of branching enzyme activity from such a column. If electrophoresis were carried out for a longer time (24 hr), there was a somewhat more complete separation of the branching en-

TABLE I: Purification of Branching Enzyme from Rabbit Muscle.[a]

| Step | Fraction | Vol (ml) | Act. (units/ml) | Total units | Recov (%) | Protein (mg/ml) | Sp act. (units/mg) | Purification factor |
|---|---|---|---|---|---|---|---|---|
| 1 | Aqueous muscle extract | 1500 | 7.93 | 11,900 | 100 | 26.8 | 0.25 | 1.0 |
| 2 | pH 6.0 supernatant | 1490 | 9.60 | 14,300 | 120 | 17.4 | 0.45 | 1.8 |
| 3 | Phosphorylase *a* crystal supernatant | 42.7 | 118.9 | 5,080 | 42.7 | 26.5 | 4.49 | 17.9 |
| 4 | Starch treated, centrifuged | 39.0 | 115.6 | 4,510 | 37.9 | 18.4 | 6.29 | 25.1 |
| 5 | Bio-Gel P-200 pool | 66.3[b] | 4.3 | 3,677[c] | 37.9[c] | 0.088 | 48.7 | 195 |
| 6 | DEAE-cellulose pool | 71.7[b] | 3.6 | 3,350[c] | 28.1[c] | 0.048 | 75.3 | 301 |
| 7 | Electrophoresis | | | | | | | |
| | Tube No. 49 | 0.82 | 9.5 | | | 0.015 | 395 | 1580 |
| | 50 | 0.82 | 25.9 | | | 0.020 | 616 | 2470 |
| | 51 | 0.82 | 50.5 | | | 0.040 | 871 | 3490 |
| | 52 | 0.82 | 53.6 | | | 0.075 | 767 | 3070 |
| | 53 | 0.82 | 35.1 | | | 0.048 | 568 | 2270 |
| | 54 | 0.82 | 18.9 | | | 0.024 | 306 | 1224 |

[a] Based on 1 kg of muscle. [b] Volume after fractionating 3 ml of centrifuged solution (step 4). [c] Recovery calculated for the total volume obtained at step 4.

zyme from other proteins but the loss of total activity was often as great as 45% and the specific activity of the peak tubes was not substantially greater than was found in the experiment shown in Figure 2.

When enzyme eluted from the preparative electrophoresis column was stored at 4°, it was stable for at least 1 month. Replacement of the Tricine-containing buffer by dialysis against 0.5% sodium glycerophosphate–1 mM EDTA–5 mM 2-mercaptoethanol, pH 7.0, stabilized the enzyme for an additional period of many months. However, the branching enzyme activity was destroyed if such solutions were frozen. A summary of the results of the purification procedure is shown in Table I.

*Purity and Properties of the Enzyme.* Polyacrylamide disc gel electrophoresis showed that when 22 μg of protein from the peak tube of the electrophoresis column was added to a gel column, only one protein band could be stained after electrophoresis under the conditions described in Methods. In other tubes from the enzyme peak where the specific activity of the protein was slightly lower, the major stainable band constituted an estimated 90% of the total protein, and only one other minor band was visible. As determined by elution of other disc gel columns, the major protein band was the only one with enzyme activity. Up to 80% of the total units of activity added to the column could be recovered from it by elution of this band, but the alkalinity of the disc gel buffer made the enzyme unstable and the recovery from polyacrylamide of activity in various experiments was variable. An absorption spectrum of the most highly purified branching enzyme preparation (specific activity, 950 units/mg), determined on a solution containing 84 μg/ml, revealed no unusual features except that the ratio of absorbance at 260 nm to that at 280 nm was 0.76. The reason for this rather high value has not been found. Petrova (1964) has claimed that purified preparations of branching enzyme contain 6–12% of RNA which cannot be separated by electrophoresis. More recently, Petrova and Filippova (1971) have described a preparation of the muscle enzyme which seems to have had about 5% of the specific activity of the enzyme which we have prepared. This very much less active preparation was shown to consist of two centrifugally separable protein fractions, one of which contained no bound RNA and was active, and the second of which appeared to be a nucleoprotein of higher molecular weight having more activity. The activity of the less active fraction could be increased by the addition of 3S RNA which had been isolated previously from the whole enzyme preparation. In view of the extremely low absolute activity of both fractions, and the lack of evidence that other enzymes which could affect the iodine color of soluble starch were absent, the significance of the results of Petrova and Filippova is difficult to assess. In the present work there was no direct demonstration of RNA in the purified enzyme.

In view of the methods used to assay the branching enzyme, it was of importance to find whether any hydrolytic enzyme which could act on glycogen remained in the final preparation as a contaminant. Accordingly, as much as 300 times more branching enzyme than that required for assay was incubated for 3.5 hr with 10 μmoles (as total polymeric glucose) of [$^{14}$C]-glycogen having a specific activity of 2660 cpm/μmole in the outer 27% of the molecule susceptible to degradation by phosphorylase, and 2870 cpm/μmole in the residual limit dextrin (Jeffrey *et al.*, 1970). No $^{14}$C-labeled maltose, maltotriaose, maltotetraose, or maltopentaose could be found by paper chromatography of the incubation mixture. Hence, the branching enzyme preparation appeared to be free of α-amylase.

The relationship between the apparent activity of purified branching enzyme and the concentration of phosphorylase used in the coupled assay is shown in Figure 3. The proportionality of branching activity to enzyme concentration is better at low phosphorylase concentration. However, if the indicating enzyme is present in too low a quantity, the assay has an inconveniently long lag period (not shown). In the presence of 6 units/ml of phosphorylase, the rate of $P_i$ formation was proportional to branching enzyme concentration over a two- to threefold range, if the amount of branching enzyme was such that the linear rate of $P_i$ formation attained

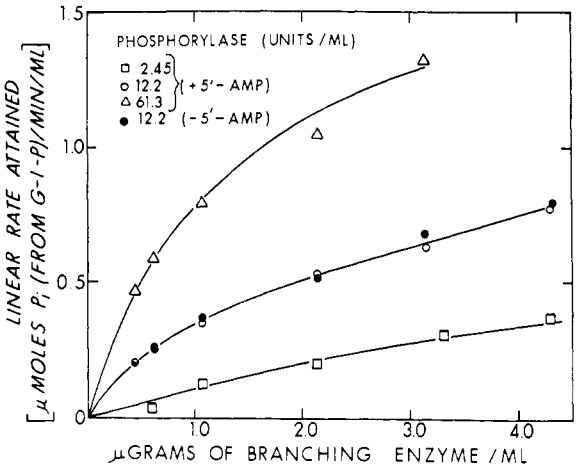


FIGURE 3: Rate of polysaccharide synthesis by phosphorylase in the presence of branching enzyme. The reaction mixtures which contained 80 mM G-1-P (pH 6.4) and various amounts of phosphorylase *a* and branching enzyme as shown, were incubated at 30° with or without 5'-AMP. The ordinate refers to the linear rate of $P_i$ formation attained after an initial lag period. See the text for details of this assay procedure.

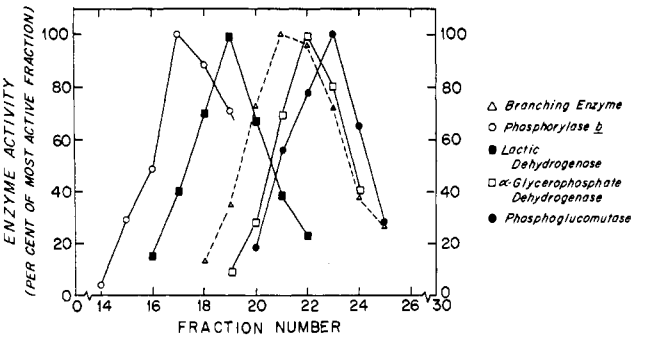


FIGURE 4: Centrifugation of branching enzyme in a 7–17% sucrose gradient with marker enzymes as shown.

after the lag period was between 0.02 and 0.05 μmole per min per ml of reaction mixture.

One series of experiments shown in Figure 3 was carried out in the absence of 5'-AMP, and no difference was found when the results were compared to those of a similar series done with this nucleotide added. Thus, there appears to be no direct effect of 5'-AMP on the activity of the branching enzyme under these assay conditions.

*Molecular Weight.* The sucrose density gradient technique of Martin and Ames (1961) was used to obtain an estimate of the molecular weight of the purified branching enzyme as described in Methods above. The marker enzymes which were mixed with branching enzyme and centrifuged with it in the gradient for 7 hr at 258,000$g_{av}$ were rabbit muscle phosphorylase *b* [mol wt, 185,000 (Seery *et al.*, 1967, 1970)], rabbit muscle lactate dehydrogenase [assumed mol wt 144,000 (Castellino and Barker, 1968; Millar *et al.*, 1969)], rabbit muscle glycerol-3-phosphate dehydrogenase [mol wt, 78,000 (Pfleiderer and Auricchio, 1964)], and rabbit muscle phosphoglucomutase whose molecular weight was taken at 67,000. The molecular weight of this latter enzyme is somewhat uncertain. Values from 62,000 (Filmer and Koshland, 1963) to 74,000 (Taylor *et al.*, 1956) have been reported. A recent determination of the value by Harshman and Six (1969) gave 64,900. The calculated molecular weight based on the amino acid composition reported by Harshman and Six as well as that given by Sloane *et al.* (1964) is 66,900. In spite of the uncertainty of the exact molecular weight of this enzyme, it was used in this work since it has no subunit structure (Harshman and Six, 1969) and could serve as a reference protein in sucrose gradients containing urea in which it was found that no significant irreversible loss of activity occurred (see below). The results are shown in Figure 4. The calculated molecular weight of branching enzyme is 103,100 with reference to the position in the gradient of phosphorylase *b* activity, 99,170 with reference to lactate dehydrogenase, 92,030 with reference to glycerol-3-phosphate dehydrogenase, and 91,160 with reference to phosphoglucomutase.

*Inhibition by Urea.* The branching enzyme's activity, as determined either in the combined assay with phosphorylase, or in the assay involving the direct action of the enzyme on Type IV amylopectin (P. B.), was found to be inhibited by urea. Figure 5 shows the results of an experiment in which the latter assay was used, as described in Methods above, except that 1 mM Tris, pH 7.1, replaced 0.25 M citrate, pH 7.1. It is evident that even quite low concentrations of urea inhibit the enzyme, and that in the presence of 2 M urea it has no activity. In other experiments (not shown) it was found that high citrate ion concentration somewhat protects against urea inhibition. For example, in 0.25 M citrate, pH 7.1, containing 2 M urea, the enzyme retained 13% of its initial activity. The immediate inhibition of activity shown in Figure 5 was found to be largely reversible if the reaction mixtures were diluted fivefold with the Tris buffer to which 2 mg/ml of the Type IV amylopectin had been added. At urea concentrations higher than 2 M the enzyme is also inhibited, but irreversible denaturation of the protein then occurs even at 0°. Inasmuch as the molecular weight of the enzyme was found to be about

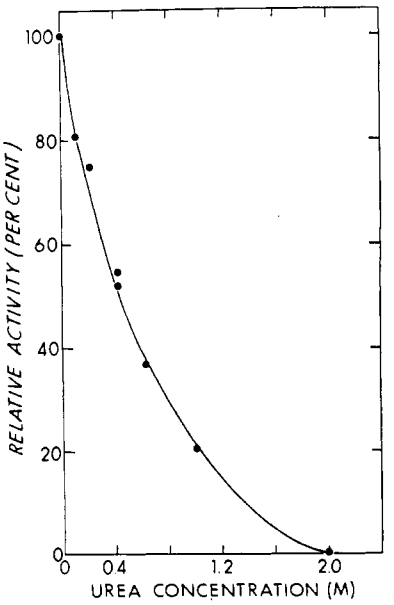


FIGURE 5: Inhibition by urea of branching enzyme activity. Reaction mixtures contained 2.2 mg of Type IV amylopectin (P. B.)/ml and 1.9 μg of enzyme/ml in 1 mM Tris–0.25 mM EDTA–1.3 mM 2-mercaptoethanol, pH 7.1. Urea was present as indicated. Incubation was at 30° for 30 min. Enzyme activity was determined by the decrease in absorbance at 520 nm of the iodine complex of the polysaccharide [165 μg/3 ml of $I_2$–KI–citrate (see text)]. The activity in the absence of urea is plotted as 100%.

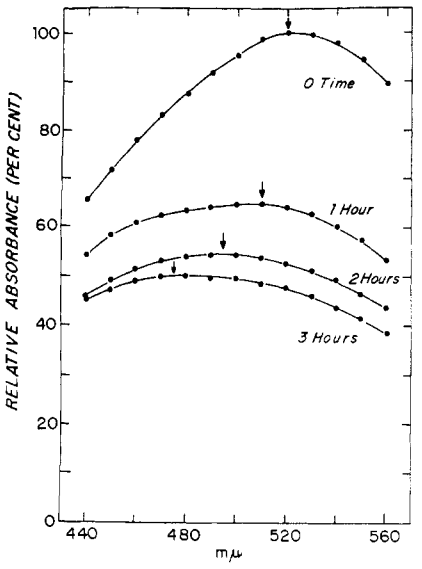


FIGURE 6: Changes in the absorption spectrum of the iodine complex of the amylopectin-like polysaccharide isolated from the liver of a child with Type IV glycogen storage disease as a function of time of incubation with branching enzyme. The reaction mixture (0.6 ml) contained 10 μmoles of polysaccharide (as total polymeric glucose) and 1.5 units of enzyme in 0.25 M sodium citrate, pH 7.1, 30°. Aliquots (30 μl) were added to the $I_2$–KI–sodium citrate reagent whose composition is described in assay Method 2 (see text). Arrows indicate the wavelength of maximum absorbance at each time.

98,000, it was of interest to find whether reversible inhibition of activity by 2 M urea might have been due to the dissociation of the protein into 2 or more subunits. To test this possibility, a mixture of purified branching enzyme and phosphoglucomutase was prepared in 1 mM Tris, pH 7.1, containing 2 M urea. The mixture was centrifuged in duplicate in a linear sucrose gradient which contained a uniform concentration of 2 M urea. Centrifugation was for 11 hr at 274,000$g_{av}$. Harshman and Six (1969) found that reduced and carboxymethylated

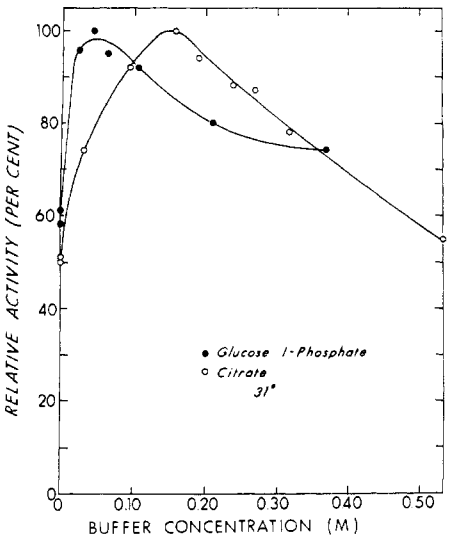


FIGURE 7: Effect of buffer concentration on branching enzyme activity at pH 7.1. The basal activity for both curves was that measured in 1.3 mM Tris–0.26 mM EDTA–1.3 mM 2-mercaptoethanol in the presence of 1.9 μg/ml of enzyme.

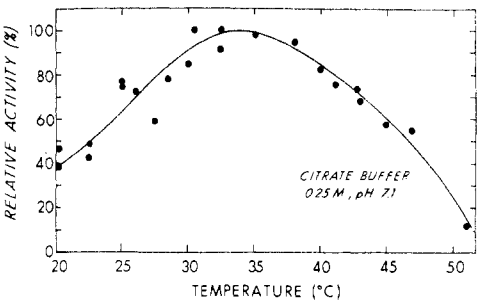


FIGURE 8: Temperature dependence of branching enzyme activity. The activity at the apparent temperature optimum is plotted as 100%; enzyme concentration, 2.1 μg/ml.

phosphoglucomutase has the same molecular weight in 5 M guanidine hydrochloride as the native enzyme has in 50 mM phosphate, pH 6.8, when determined by the method of sedimentation equilibrium. Their finding indicates that phosphoglucomutase probably has no subunit structure. Accordingly, in the present work, it was assumed that mutase would not undergo any dissociation in 2 M urea in the sucrose gradient. It was shown by assay that the position of the enzyme in the gradient could be determined after suitable dilution, which indicated that 2 M urea produced no irreversible inhibition of the activity of mutase. Likewise, the branching enzyme was active when the fractions from the gradient were diluted sufficiently for assay by method 1, although they had no activity in the presence of urea. Calculation of the molecular weight in 2 M urea of the branching enzyme relative to that of phosphoglucomutase gave a value of 90,940 in one gradient and 91,400 in the second. Since the average of these values is approximately the same as that obtained by the same method in the absence of urea, there is evidence that branching enzyme is not dissociated by urea under conditions in which its activity is totally and reversibly inhibited.

*Branching Action on "Type IV Amylopectin."* The action of the purified enzyme in changing the absorption spectrum of the $I_2$–KI complex of Type IV amylopectin (P. B.) is shown in Figure 6. The decrease in absorbance at 520 nm was shown to be proportional to enzyme concentration and to time. In view of the earlier finding (Brown and Brown, 1966b) that partially purified preparations of the branching enzyme from rabbit muscle appeared to be stimulated by anions, and the fact the Krisman (1962) had reported the stimulation of the corresponding rat liver enzyme by borate, acetate, and citrate ions, the effect of G-1-P, present at high concentration in assay method 1, and that of citrate ion on the activity of the purified enzyme toward Type IV amylopectin were investigated. The results are shown in Figure 7, where it is seen that a twofold stimulation occurs in the presence of either 0.15 M citrate or 50 mM G-1-P. A kinetic analysis of the activating effect of citrate and G 1-P has not yet been carried out because of the limited quantity of the human polysaccharide substrate which is available. Because of the considerable basal level of enzyme activity in the absence of either citrate or G-1-P and the high concentration of these substances required to produce even a moderate degree of activation, it does not seem likely that stimulation of branching enzyme by either citrate or G-1-P should be regarded as having possible physiological significance.

*Temperature and pH Optima.* The temperature dependence of branching enzyme activity in citrate buffer is shown in Figure 8 where it is evident that there is a broad optimum.

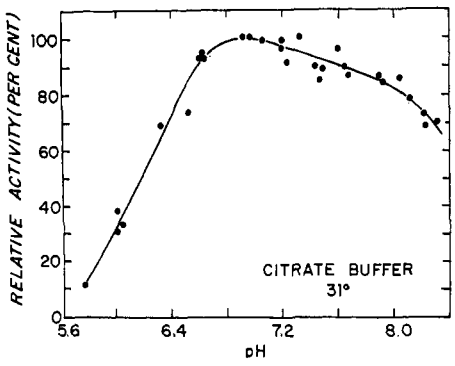


FIGURE 9: Activity of branching enzyme in sodium citrate buffer as a function of pH. The maximal activity is plotted as 100%. Reaction mixtures contained 0.25 M citrate buffer and 1.1 μg/ml of branching enzyme.

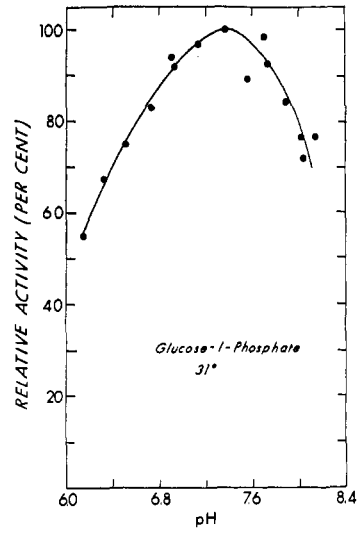


FIGURE 10: Activity of branching enzyme in potassium glucose 1-phosphate–phosphoric acid buffer as a function of pH. The maximal activity is plotted as 100%. Reaction mixtures contained 80 mM buffer and 1.4 μg/ml of branching enzyme.

The pH optimum for activity depends upon the buffer system used for its determination. Figure 9 shows the curve as determined in citrate buffer and at the apparent temperature optimum. Activity falls off rather sharply below pH 6.5 but is well maintained up to a pH of 8. When G-1-P which had been neutralized with phosphoric acid was used as the buffer, maximum activity was at pH 7.4 and the pH–activity curve was sharper than in the case of citrate (Figure 10).

*Apparent Affinity for Type IV Amylopectin.* The $K_m$ of Type IV amylopectin (P. B.) as a substrate for the branching enzyme was determined by the iodine assay method, using two different preparations of enzyme under conditions where the assay had been shown to be linear with time for each concentration of polysaccharide used. The results are shown in Figure 11 as a plot of substrate concentration/initial velocity *vs.* substrate concentration expressed as moles per liter of total polysaccharide outer chain ends. The $K_m$ was found to be $5.6 \times 10^{-6}$ M. Since the number of chains which are inaccessible to the enzyme for steric reasons, as well as the number of chains whose lengths are not in the effective range for substrate activity are both unknown, the actual $K_m$ value for the remaining chains which are truly substrates cannot be calculated accurately, but is probably much lower than that found here. As will be discussed below, there is also some evidence that linear chains of certain lengths may inhibit the action of branching enzyme, although they are not themselves susceptible to branching. Thus, it is possible that in the macromolecular substrate some of the outer chains inhibit the branching of others, and the observed $V_{max}$ may not be at all similar to that pertaining to the physiologically meaningful situation where the branching enzyme acts with UDPglucose–glycogen glucosyltransferase to synthesize glycogen of normal structure. The latter situation requires further investigation.

*Inhibition by Oligosaccharides.* The effect of adding various oligosaccharide chains of maltosidically linked glucose units to reaction mixtures containing the branching enzyme and Type IV amylopectin (P. B.) on the rate of action of the enzyme was investigated by the iodine assay method. The results are shown in Figure 12. Maltoheptaose was the most inhibitory of any substance tested. Chains from one to five glucose units in length had no effect, while longer chains up to maltodecaose were inhibitory, but not as strongly so on a molar basis as maltoheptaose. In other experiments (not shown) chains from one to five glucose units were tested at concentrations up to 20 mM and no inhibition was observed. It is possible that the results in Figure 12 are indirectly a reflection of the specificity of the branching enzyme with respect to the range of lengths of chain segments which can be moved. However, kinetic results such as these cannot be used to prove the mechanism of action of the enzyme, since it may be just as likely that an inhibitory chain combines with the acceptor binding site on the enzyme as with the donor binding site. This question cannot be easily investigated, since the donor and the acceptor chains are present in the same macromolecule and may even be two adjacent regions of the same outer chain.

*Chain Placement by Branching Enzyme.* The question of whether a chain segment which is moved by the branching enzyme is transferred preferentially to a "main chain" (that is, to one which already has a preexisting side chain attached to it), or to a previously unbranched outer "side chain" was

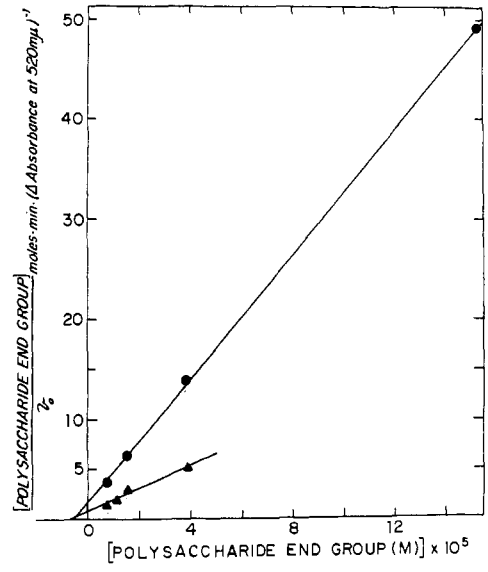


FIGURE 11: Dependence of branching enzyme activity on concentration of Type IV amylopectin (P. B.). Reaction mixtures contained 0.25 M sodium citrate, pH 7.1, 30°: ●—●, 0.30 unit of enzyme/ml; ▲—▲, 0.66 unit of enzyme/ml.

TABLE II: Stepwise Enzymatic Degradation of Type IV "Amylopectin" (P. B.) before and after Incubation with Branching Enzyme.[a]

| Substance | Original | | Limit dextrin after degradation by phosphorylase | | |
|---|---|---|---|---|---|
| | End group (%) | Outer chain units removed by phosphorylase (%)[b] | End group (%) | Glucose formed by debranching enzyme (%)[c] | Digestion by phosphorylase after debranching |
| Type IV amylopectin (P. B.) | 5.4 | 58.4 | 12.6 | 5.0 | 36.6 |
| After incubation with branching enzyme | 7.4 | 41.0 | 12.5 | 6.0 | 39.7 |

[a] The polysaccharide (17 μmoles of total polymeric glucose/ml) was incubated with 2.6 units/ml of branching enzyme for 3.5 hr at 30° in 0.3 M sodium citrate, pH 7.1. The native polysaccharide was treated similarly except that no branching enzyme was added. Both solutions were exhaustively dialyzed against distilled water before determination of polysaccharide structure. [b] Calcd from the total quantity of G-1-P formed after 2 sequential degradations. [c] The phosphorylase limit dextrin (2 μmoles of total polymeric glucose) was incubated with 9 munits of transferase–glucosidase in 1 ml of 22 mM sodium citrate, pH 6.0, 30°.

investigated by using the Type IV amylopectin (P. B.) as substrate. Stepwise enzymatic degradation of the parent polysaccharide and of that derived from it by the action of branching enzyme was done using, first, phosphorylase free of debranching enzyme activity to prepare a limit dextrin, and, then, pure oligo-α-1,4→1,4-glucantransferase–amylo-1,6-glucosidase to remove the outer tier of branch points. The rate and extent of glucose formation by the latter enzyme were measured in order to distinguish between its rapid action on the predominantly four unit long outer side chains which remain after exhaustive phosphorylase degradation, and its slower action on the second, inner tier of branch points (Brown *et al.*, 1966). Thus, an accurate measurement of the number of outer branch points in the phosphorylase limit dextrin was possible.

If the branching enzyme could transfer segments of main chains only to preexisting outer side chains to form new branches, the *number of outer branch points in the polysaccharide would remain unchanged* as compared with that in the parent polysaccharide, on the assumption that each acceptor chain can receive only one new side chain. However, due to an increase in total branch points, a smaller percentage of the polysaccharide would now be susceptible to phosphorylase degradation. Hence, in the case of the polysaccharide after such branching, *the amount of glucose which would result from complete debranching of its outer branch points would be a smaller percentage of the mass of the phosphorylase limit dextrin* than in the case of the parent polysaccharide.

On the other hand, if the branching enzyme could transfer main chain segments only to preexisting main chains, the *number of outer branch points would be greater* than that in the parent polysaccharide. Since, as before, a smaller percentage of the resulting polysaccharide would be susceptible to phosphorylase degradation as compared to the parent, *the amount of glucose which would result from complete debranching of its outer branch points would be a greater percentage of the mass of the phosphorylase limit dextrin* than in the case of the parent polysaccharide, assuming that branching occurred in a manner that maintained the preexisting average inner chain length.

If, as a third possibility, the branching enzyme were not to have either of the extreme specificity characteristics described above, the transfer and placement of chain segments might depend instead upon steric factors largely unrelated to whether the acceptor chains were or were not already branched, and, hence, intermediate results in glucose yield from the phosphorylase limit dextrin would then be expected. In fact, if chain transfer and placement were random with respect to the type of outer chain which could act as acceptor, the amount of glucose formed from the outer branch points of the phosphorylase limit dextrin derived from the parent and the branched polysaccharides would be equal when calculated as a percentage of each.

Table II shows the results of stepwise enzymatic degradations, as described above, of the Type IV amylopectin (P. B.), and of the more branched polysaccharide derived from it by incubation with branching enzyme under the conditions described in the table. From the observed branch point content of the parent polysaccharide (5.4% of which one-half is assumed to be in the outer tier) and the 2.0% increase observed due to branching, and the fact that 41.0% of the

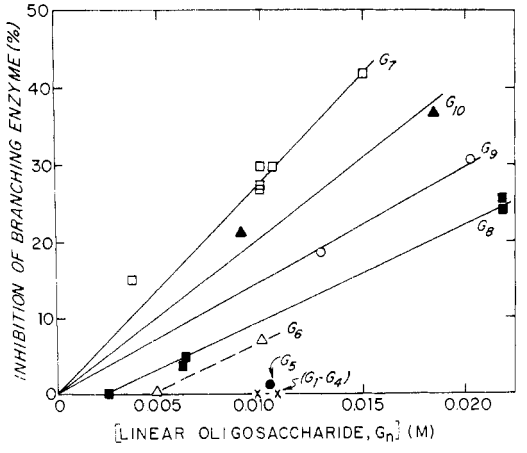


FIGURE 12: Inhibition of branching enzyme activity by linear oligosaccharides of the maltose series. Reaction mixtures contained 2.2 mg of Type IV amylopectin/ml and 2 μg of enzyme/ml in 0.25 M sodium citrate, pH 7.1. Various oligosaccharides were added as indicated. Incubation was at 30° for 15 min. Enzyme activity was determined by the decrease in absorbance at 520 nm of the iodine complex of the polysaccharide.

branched polysaccharide was susceptible to phosphorylase degradation, it can be calculated that the glucose yield from debranching only the outer branch points of the phosphorylase limit dextrin would have been 4.6% if only outer side chains had been branched, and 8.0% if only main chains had been branched. The observed value of 6.0% is intermediate between these two calculated values, and this fact indicates that the branching process was nearly random with respect to chain placement. If the values predicted for the two cases of extreme specificity are corrected for the apparent accessibility to the debranching enzyme of only 40% of the total branch points in the phosphorylase limit dextrin of the parent Type IV amylopectin (40% × 12.6% = 5.0%, see Table II), instead of the value of 50% which is characteristic of glycogen and plant amylopectin, these predicted values become 3.7% for only side-chain branching and 7.1% for only main-chain branching. The observed value of 6.0% is still close to the average of these values, and, so, on this basis also the data in Table I support the conclusion that the rabbit muscle branching enzyme shows no marked preference for either side chains or main chains as acceptors for the newly formed outer branch points.

The data in Table II allow a calculation to be made of the average number of glucose units between the outer branch points which preexisted in the Type IV amylopectin and the adjacent ones which were added peripherally as a result of incubation with the branching enzyme. The data required for this calculation are (1) the branch point content of each polysaccharide, and (2) the extent of phosphorylase degradation of each. In view of the repeated treatment with phosphorylase, the assumption may be made that the limit dextrin finally prepared had only 4 glucose residues remaining on each chain peripheral to their outermost branch point glucose units. On the basis of this assumption and the analytical data of Table II, the predicted extent of degradation of the branched polysaccharide by phosphorylase would be 43.4% if the average interchain branch point distance were 3.5 glucose units, and 41.4% if it were 4.5 glucose units. The value found (41.0%) is in agreement with the latter assumption. However, the accuracy of the analytical data is not great enough to permit considering this estimate to be more than approximate. Likewise, no data have been obtained from which a calculation of the range of new interbranch point distances can be made.

## Discussion

The maintenance of the structure of mammalian glycogen during its synthesis evidently requires that the branching enzyme act in a somewhat regular way on the external linear chains of the polysaccharide after these are elongated by the synthetic activity of UDPglucose–glycogen glucosyltransferase (glycogen synthetase). Presumably the specificity of each enzyme is to some extent adjusted to that of the other, so that the polysaccharide product made by one has a structure which is favorable for action on it by the other. Hence, it is of interest to consider the relationship of the present findings on the action of branching enzyme to the information which is available on the activity of glycogen synthetase toward polysaccharide substrates.

The specificity of glycogen synthetase was investigated by Brown *et al.* (1965) who used the rabbit muscle enzyme and uridine diphosphate [$^{14}$C]glucose to enlarge an incomplete limit dextrin which had been prepared by phosphorylase degradation of rabbit liver glycogen. When the enlarged polysaccharide which contained [$^{14}$C]glucose units only in its outer chains was treated with the bacterial debranching enzyme, pullulanase, 40% of the outer side chains of the molecule was removed and these chains were found to contain only 2% of the total $^{14}$C which was present in the enlarged polysaccharide. This result together with the chromogenic properties of the latter substance when tested with iodine and its solubility characteristics, indicated that enlargement of the limit dextrin by synthetase had occurred asymmetrically, that is, that many more [$^{14}$C]glucose units had been added to some of the external chains than to others. These data were interpreted as meaning that glycogen synthetase from muscle has a marked tendency to add glucose units repetitively to the main chains rather than to the side chains of those polysaccharides whose outer chains are initially short. In the case of glycogen itself as an acceptor, where the outer chains were already much longer, Brown *et al.* (1965) could not obtain conclusive evidence as to the site of [$^{14}$C]glucose addition, since debranching of the product by pullulanase was very incomplete. On the assumption that these results could be extrapolated to conditions *in vivo*, it appeared that the branching enzyme of rabbit muscle might be expected to have specificity such that main chains would be the principal oligoglucan donors and side chains the principal oligoglucan acceptors in the branching reaction which occurs after the outer chains of a phosphorylase limit dextrin of glycogen are lengthened by synthetase action. The data reported in this paper are not helpful in deciding whether the oligoglucan fragment is taken preferentially by branching enzyme from an outer main chain or an outer side chain. They do show, however, that such a chain segment can be transferred either to main chains or to side chains and with approximately equal frequency to each. Transfer has also been shown to occur in such a way as to maintain the average inner chain length of the polysaccharide substrate (3.7 glucose units before branching, and 4.0 glucose units after branching, calculated from the data of Table II). Thus, these new data suggest that the branching enzyme's specificity in interacting with polysaccharides—especially those with long outer chains such as the Type IV amylopectin used here—would enable it to function well in concert with glycogen synthetase if the latter enzyme were to extend both the outer main chains and outer side chains of glycogen.

Recently, Parodi *et al.* (1970) have published the results of an extensive study on the action pattern of glycogen synthetase. By using a chemical method of analysis to find the number of outer chains which can accept glucose units from UDPG, they have been able to show that only about 40% of the total number of outer chains of liver glycogen are available to liver glycogen synthetase. They have also found that the mechanism of action of muscle glycogen synthetase is significantly different from that of the liver enzyme, in that the former enzyme makes repeated transfers to the same chain and to a much greater extent than is the case with the liver enzyme. These results with the muscle enzyme are in agreement with those of Brown *et al.* (1965, 1966) as discussed above. Parodi *et al.* (1970) have now shown also that liver glycogen synthetase probably adds glucose units with about equal frequency to both the outer side chains and outer main chains of glycogen. In view of their demonstration of the differences in action of the liver and the muscle enzymes, it is not possible to extrapolate this latter result with certainty to the synthesis of muscle glycogen. However, the properties of the muscle branching enzyme which are reported here, would allow it to function effectively in the synthesis of

glycogen either with a glycogen synthetase whose action pattern is of the liver type or with one having the somewhat different action pattern of the muscle enzyme.

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