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ACTIONS OF GLYCOGEN SYNTHASE AND PHOSPHORYLASE OF RABBIT-SKELETAL MUSCLE ON MODIFIED GLYCOGENS*

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ABSTRACT

The high reactivities exhibited by rabbit-muscle synthase and phosphorylase for unmodified glycogen-acceptors decrease progressively, presumably because of a large increase in apparent K_m as the glycogen molecule is converted into its component maltosaccharide chains by the debranching enzyme, isoamylase. Elongation of the outer chains of glycogen acceptor also results in decreased reactivities of the two transglucosylases and this is shown, for phosphorylase acting in the direction of glucan synthesis, to be caused by a decrease in the V_{max} of the reaction. A partial restoration of the degradative reactivity of phosphorylase by a limited alpha-amylolysis of the long outer-chains of modified glycogen suggests a role of cytoplasmic alpha-amylase in mammalian glycogen metabolism.

INTRODUCTION

The superiority of the highly branched glycogen molecule over linear amyloses and maltosaccharides as donor or acceptor in the transglycosylation reactions catalyzed by glycogen phosphorylases and synthases of mammalian origin has been well documented[1–5]. The specificity of rabbit-muscle phosphorylase (EC 2.4.1.1) for the branched structure of glycogen has been attributed to the high concentration of suitably oriented chain-ends at the surface of the polysaccharide molecule[2], or to the ability of the dimeric enzyme to bind at two chain-ends of the branched structure[3]. The specificity of rabbit-muscle glycogen synthase (EC 2.4.1.11) for glycogen acceptor has also been related to its ability to associate with a second glucosidic chain of the glycogen molecule at a binding site remote from the catalytic site[5].

This paper describes additional studies on the action of rabbit-muscle phosphorylase on structurally modified glycogens. Similar studies on glycogen synthase confirm that both transglucosylases act less readily on debranched glycogen and on glycogen possessing elongated outer-chains.

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

MATERIALS AND METHODS

Materials. — Five-times crystallized glycogen phosphorylase *b* was prepared from rabbit-skeletal muscle by the procedure of Fischer and Krebs[6] and was assayed by the method of Hedrick and Fischer[7], slightly modified to allow pre-incubation (10 min) of the enzyme with glycogen. Glycogen synthase, enriched in the D-glucose 6-phosphate-independent form, was isolated from rabbit-skeletal muscle[8] and was assayed by the incorporation of radioactivity from UDP-D-[U-^{14}C]glucose into glycogen[9]. Rat-liver branching enzyme was isolated and assayed as by Krisman[10]. The preparation contained hydrolytic activity that was removed completely by three treatments with washed corn-starch granules. *Cytophaga* isoamylase and salivary alpha-amylase were prepared and assayed as described by Gunja-Smith, Marshall, Smith and Whelan[11], and by Fischer and Stein[12], respectively.

α-D-Glucosyl phosphate, D-glucose 6-phosphate, UDP-D-glucose, adenosine 5-monophosphate (5'-AMP), bovine serum albumin, and oyster glycogen were purchased from Sigma Corporation and UDP-D-[U-^{14}C]glucose was purchased from New England Nuclear. The commercial oyster glycogen was deionized with mixed-bed resin (Bio-Rad AG501-X8 CD) in the carbonate form and was twice precipitated from 70% ethanol solution. Rabbit-liver glycogen was extracted from fresh tissue with 30% potassium hydroxide solution for 1 h at 100° and was purified by three precipitations from 70% ethanol solution. Maltopentaose was separated from a partial acid hydrolyzate of amylose. The different oligosaccharide fractions were eluted sequentially from a charcoal–Celite column with increasing concentrations of ethanol solution[13].

Analytical procedures. — Protein concentrations of phosphorylase solutions were determined from the absorbancy at 278 nm after solutions had been treated with Norit A charcoal to remove 5'-AMP. Other protein concentrations were measured by the Lowry method[14]. Concentrations of polysaccharide and maltopentaose were measured by the phenol–sulfuric acid procedure[15] and reducing sugars were measured by a modified Nelson procedure[16].

The average chain lengths ($\overline{c.l.}$) of glycogen samples were determined by using isoamylase[17] and the increase in $\overline{c.l.}$ of chain-extended polysaccharide was determined from the amount of D-glucose transfer that occurred in the synthetic reactions. D-Glucose transfer in the glycogen-synthase reaction was monitored by the incorporation of the D-[^{14}C]glucose moiety of UDP-D-[U-^{14}C]glucose into products, and D-glucose transfer in the phosphorylase reaction was determined from the amount of inorganic phosphate released from α-D-glucosyl phosphate[18].

Glycogen synthase reactions. — Glycogen synthase was routinely incubated at 25° with acceptor (glycogen or maltosaccharides) in mixtures (150 μl) containing UDP-D-[^{14}C]glucose (1.9×10^4 c.p.m./μ mole), 8mM glucose 6-phosphate, 10mM (ethylenedinitrilo)tetraacetate, 3mM mercaptoethanol, and 100mM Tris–HCl (pH 7.8). There was no detectable reaction in the absence of acceptor under these conditions, and the enzyme remained fully active (96%) during 30 min. However, a trace of

alpha-amylase activity in the synthase preparation became inactivated completely after 30 min and, therefore, all reactions were started by addition of acceptor after preincubation of the synthase preparation for 30 min.

Incorporation of D-[^{14}C]glucose into glycogen was measured in portions (25–50 μl) of the mixtures applied to 12 × 12 mm filter-paper squares. The papers were washed in 70% ethanol solution, dried, and then submerged in toluene (10 ml) containing 0.05% PBBO and 0.8% butyl PBD (Isolab.). The ethanol-insoluble radioactivity on the papers was counted in a Packard Tri-Carb scintillation spectrometer. The incorporation of D-[^{14}C]glucose into debranched glycogen and maltosaccharides was measured in portions (0.1 ml) of mixtures that were applied to a Pasteur pipette containing 0.5 ml of Bio-Rad AG (Dowex) 1-8X anion-exchange resin (200–400 mesh). The resin was washed with water (2 ml), the exact volume of eluate was measured, and the radioactivity in a portion (1 ml) of the eluate measured in scintillation-fluor solution (10 ml) containing 2 ml of Scintisol (Isolab). Control experiments confirmed that only the D-[^{14}C]glucose incorporated into acceptor was recovered in the resin eluate and that recovery was complete.

Phosphorylase synthetic reaction. — Phosphorylase *b* was routinely incubated at 20° with acceptor (glycogen or maltosaccharides) in mixtures containing 75mM α-D-glucosyl phosphate, 2mM 5′-AMP, bovine serum albumin (0.5 mg/ml), 40mM mercaptoethanol, and 40mM sodium glycerophosphate (pH 6.8). The enzyme was preincubated for 5 min before α-D-glucosyl phosphate was added to start the reaction.

The external chains of oyster and rabbit-liver glycogens (1.5 mg/ml) were extended by the action of phosphorylase *b* (0.3 units/ml) under the foregoing conditions, except that bovine serum albumin was omitted from the mixtures. After suitable reaction periods, the phosphorylase was inactivated by heating for 3 min at 100° and the chain extension was determined from the amount of inorganic phosphate released. Extended glycogens and control glycogen that had been incubated under the same conditions with heat-inactivated phosphorylase were twice precipitated with 67% ethanol, and the precipitates were washed with 95% ethanol and anhydrous ether, and dried under diminished pressure.

Phosphorolysis of glycogen and treatment with alpha amylase. — Phosphorylase *b* (1.5 units/ml) was incubated at 20° with equimolar amounts of chain-extended rabbit-liver glycogen ($\overline{\text{c.l.}}$ 17, 355 μg/ml) and unextended glycogen ($\overline{\text{c.l.}}$ 12, 250 μg/ml) in mixtures containing 2mM 5′-AMP and 100mM sodium phosphate (pH 7.0). The extent of phosphorolysis was determined at intervals by measurement of the formation of α-D-glucosyl phosphate with use of a coupled phosphoglucomutase D-glucose 6-phosphate dehydrogenase system[19]. After 30 min the phosphorylase in the mixtures was inactivated by heating for 3 min at 100° and a solution of salivary alpha-amylase (0.1 ml, 0.005 units) was added to 1.4 ml of the mixture containing extended glycogen. To serve as controls, water (0.1 ml) was added to a second 1.4-ml portion and also to 1.4 ml of the mixture containing unextended glycogen. The three solutions were incubated for 10 min at 20°, heated for 3 min at 100° to destroy the alpha-amylase activity, and phosphorylase *b* solution (0.1 ml, 1.5 units) was added to each. The

mixtures were again incubated at 20° and the formation of D-glucosyl phosphate measured as before (see Fig. 6).

Debranching of glycogen. — Rabbit-liver glycogen (c.l. 12, 4.5 mg/ml) was incubated at 37° with *Cytophaga* isoamylase (0.1 units/ml) in a mixture (6 ml) containing 20mM sodium acetate–HCl (pH 5.5). The debranching reaction was monitored by measuring the release of reducing sugars in aliquot portions (0.4 ml) that were removed and heat-inactivated (10 min at 100°). Debranching of glycogen was complete within 21 h. The relative acceptor-activities of the debranched glycogen fractions (0.1 ml, 450 μg) were then tested in the standard glycogen synthase and phosphorylase reactions.

EXPERIMENTAL AND RESULTS

The progressive decrease in reaction rates as the outer chains of glycogen are elongated is shown in Fig. 1 for the glycogen synthase reaction. As previously demonstrated for the reaction catalyzed by mammalian glycogen phosphorylase[2], the decrease in rate of the synthase reaction was more marked in mixtures containing lower concentrations of glycogen acceptor. The addition of fresh glycogen during the reaction increased the rate, as did the addition of a small amount of rabbit-liver branching enzyme (not shown). Initial reaction-rates from these non-linear curves were used to calculate an apparent K_m value for glycogen acceptor of 120 μg/ml (6.2×10^{-5} M chain-ends). Reaction rates from linear time–reaction-progress curves obtained for different concentrations of maltopentaose gave an apparent K_m value of 2.5×10^{-2} M for the single-chain acceptor. However, when glycogen at concentrations in the range of the calculated K_m value was incubated for the same time (10 min) with amounts of glycogen synthase such that the concentration of glycogen acceptor in each mixture was directly proportional to the amount of enzyme present, the extent of synthesis was directly proportional to enzyme activity and, apparently, was not

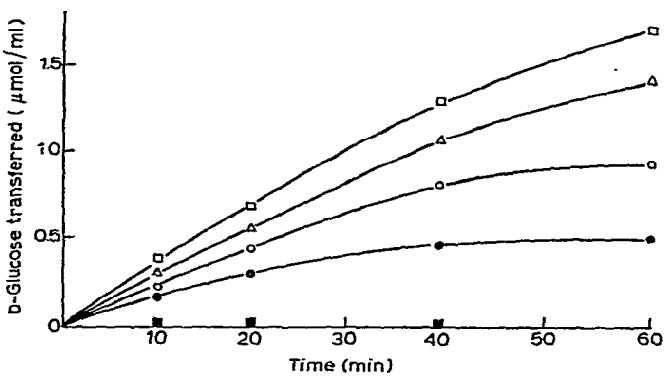

Fig. 1. Rates of reaction of rabbit-muscle glycogen synthase in the presence of different amounts of rabbit-liver glycogen (c.l. 12). Different amounts of glycogen were incubated with glycogen synthase (0.05 units/ml) and 6.7mM UDP-D-[^{14}C]glucose as described in Materials and Methods. Glycogen concentrations (μg/ml): 500, □; 250, △; 125, ○; 62, ●; no glycogen, ■.

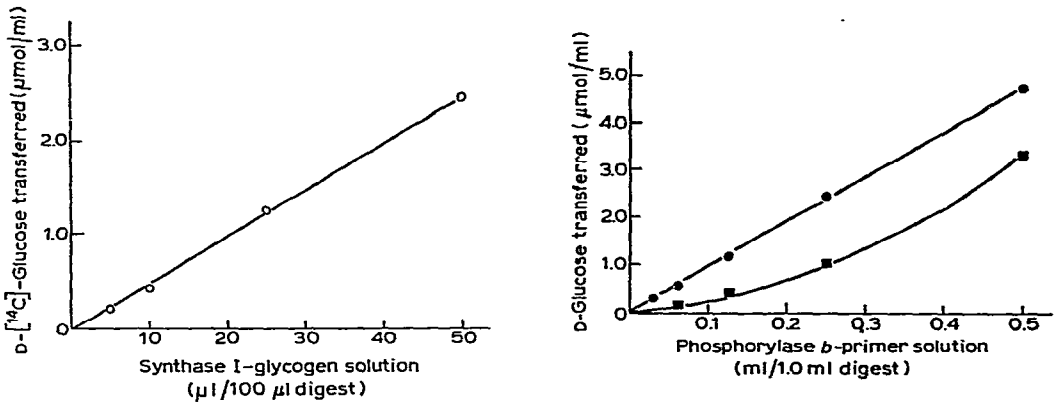

Fig. 2. Effect on total synthesis of equal and concomitant changes in rabbit-muscle synthase activity and glycogen concentration. The indicated volumes of a solution of glycogen synthase (0.4 units/ml) and rabbit-liver glycogen (1.5 mg/ml), were incubated for 10 min with 10mM UDP-D-[^{14}C]glucose in 100-μl reaction mixtures under the conditions described in Materials and Methods.

Fig. 3. Effect on total synthesis of equal and concomitant changes in rabbit-muscle phosphorylase activity and glycogen or maltopentaose concentrations. The indicated volumes of a solution of phosphorylase *b* (5 units/ml) and rabbit-liver glycogen (2.4 mg/ml) were incubated for 3 min with α-D-glucosyl phosphate as described in Materials and Methods. A second phosphorylase *b*-primer solution, in which glycogen had been replaced by maltopentaose (12 mg/ml), was similarly incubated for 30 min. Glycogen primer, ●; maltopentaose primer, ■.

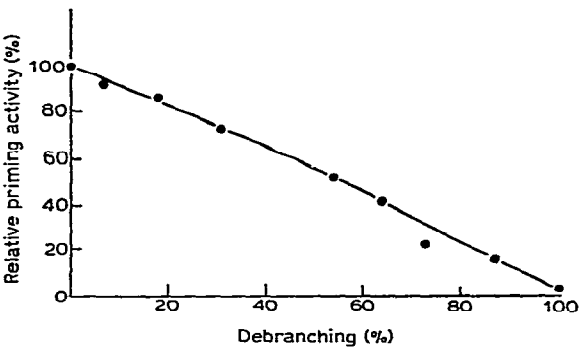

Fig. 4. Effect of debranching of glycogen on its acceptor activity in the rabbit-muscle glycogen-synthase reaction. Rabbit-liver glycogen was debranched to different extents with *Cytophaga* iso-amylase and the products tested as acceptors in the glycogen-synthase reaction. Experimental details are given in Materials and Methods.

affected by differences in the concentration of glycogen acceptor (Fig. 2). Under similar conditions, the rate of the synthetic reaction of phosphorylase *b* was also not affected by glycogen concentrations in about the same range. However, in a control experiment, a rate-limiting effect was caused by the dilution of maltopentaose at a concentration near to the value of the apparent K_m constant (Fig. 3).

There was a dramatic loss of acceptor efficiency in the glycogen synthase reaction (Fig. 4) and the synthetic reaction of phosphorylase (not shown) when

rabbit-liver glycogen was converted into its component maltosidic chains by the debranching action of isoamylase. The loss of acceptor efficiency was progressive and paralleled the extent of debranching in each case.

To establish that the fall in rate of the synthetic reaction of phosphorylase *b* as the outer chains of glycogen are extended is not caused by the formation of an inactive enzyme complex with a water-insoluble product, we tested the phosphorylase *b* activity that remained in the supernatant solution of a mixture in which extensive chain-elongation of the glycogen acceptor decreased the reaction rate to a low value (Table I). Although a significant amount of insoluble product was removed by centrifugation, at least 90% of the original phosphorylase *b* activity remained in the supernatant. When glycogen possessing elongated outer-chains ($\overline{\text{c.l.}}$ increased about three-fold) was included in the mixture before the phosphorylase reaction was started, the initial rate was about 30% lower than that of the reaction with the unmodified glycogen acceptor alone (not shown). The initial reaction was also slower when an equimolar amount of glycogen possessing a 37% increased $\overline{\text{c.l.}}$ was substituted for unmodified oyster glycogen ($\overline{\text{c.l.}}$ 11) as the only acceptor-substrate (Fig. 5). In an experiment, also not shown, the initial rates of the synthetic phosphorylase reaction in the presence of 0.5 and 1.0 mg of elongated glycogen-acceptor ($\overline{\text{c.l.}}$ 17) were identical, and equalled about one half of the reaction rate catalyzed by the same amount of phosphorylase in the presence of 0.5 mg of the unmodified rabbit-liver glycogen ($\overline{\text{c.l.}}$ 12). The elongated glycogen ($\overline{\text{c.l.}}$ 17) also exhibited a lower limit of phosphorolysis than the unmodified glycogen, but a limited hydrolysis of the long outer chains by treatment with alpha amylase increased the susceptibility of the

TABLE I

SOLUBLE PHOSPHORYLASE *b* ACTIVITY DURING THE SYNTHETIC REACTION IN THE PRESENCE OF GLYCOGEN ACCEPTOR AND α-D-GLUCOSYL PHOSPHATE[a]

Reaction time (min)		D-*Glucose transferred (μmol/ml)*	*Increase (%) in average chain-length glycogen*	*Soluble phosphorylase* b *(% of initial activity)*
(at 25°)	(at 4°)[b]			
0	0	0	0	100
0	15	1.1[c]	73	100
3	15	1.8	120	97
10	15	1.9	130[d]	110
20	15	2.2	150	90

[a]Rabbit-liver glycogen (250 μg/ml) and rabbit-muscle phosphorylase *b* (0.3 units/ml) were incubated in a mixture (3.0 ml) as described in Materials and Methods. At the indicated times, samples (0.5 ml) were cooled in ice–water, centrifuged at 12,000*g* for 15 min at 4° to remove insoluble material, and inorganic phosphate and phosphorylase *b* activity in the supernatant solutions were determined. [b]Times given are centrifugation times. [c]Inorganic phosphate released during cooling and centrifugation period. [d]The mixture contained insoluble material in suspension after incubation for 10 min at 25°.

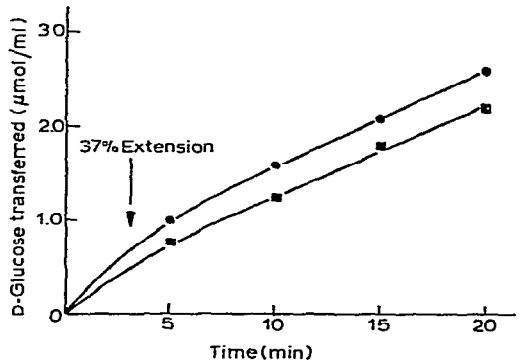

Fig. 5. Rate of the synthetic reaction of rabbit-muscle phosphorylase *b* in the presence of oyster glycogen having extended outer-chains. Equimolar amounts of chain-extended glycogen (415 μg/ml and the unextended glycogen (300 μg/ml) were each incubated with phosphorylase *b* (0.3 units/ml) and α-D-glucosyl phosphate as described in Materials and Methods. Mixtures contained: 37% chain-extended glycogen, ■; unextended glycogen, ●. The arrow indicates the point at which the unmodified glycogen has been extended by 37% under these conditions.

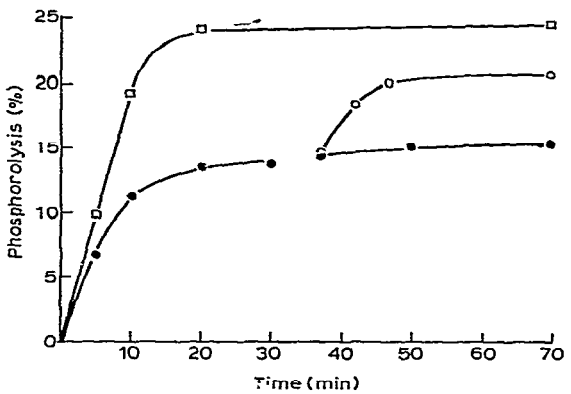

Fig. 6. Increase in the degree of phosphorolysis of chain-extended rabbit-liver glycogen after treatment with salivary alpha amylase. Equimolar amounts of chain-extended glycogen (355 μg, $\overline{\text{c.l.}}$ 17), and unextended glycogen (250 μg, $\overline{\text{c.l.}}$ 12) were each incubated with phosphorylase *b* and sodium phosphate (pH 7.0). Samples (0.1 ml) were removed at intervals, added to water (0.1 ml), and heated for 3 min at 100° to inactivate phosphorylase *b*, and portions (0.1 ml) of the cooled solutions were used to determine α-D-glucosyl phosphate as described in the text. After 30 min, a portion of the mixture containing long-chain glycogen was treated with salivary alpha amylase and again incubated with phosphorylase *b*. Chain-extended glycogen ($\overline{\text{c.l.}}$ 17) before treatment with alpha amylase, ●; after treatment with alpha amylase, ○; unextended rabbit-liver glycogen ($\overline{\text{c.l.}}$ 12) not treated with alpha amylase, □. Experimental details are given in Materials and Methods.

modified glycogen, so that the limit of degradation by phosphorylase was increased (Fig. 6).

The formation of branch linkages concomitant with the lengthening of the outer chains of glycogen acceptor by the combined actions of glycogen-branching enzyme and phosphorylase *b* resulted in a constant rate of synthesis in the presence

of as little as 20 μg of glycogen acceptor (Fig. 7). Under these conditions, the constant reaction-rate was about equal to the initial rate catalyzed by the same amount of phosphorylase in a mixture containing 200 μg of glycogen and no branching enzyme, and was almost as high as the maximum rate observed in the standard phosphorylase assay-mixtures containing an excess of glycogen acceptor. Although the same amount of phosphorylase catalysed a lower rate of glycogen synthesis when the amount of branching enzyme was decreased, the rate remained constant during the reaction, so that linear time–reaction-progress curves were obtained at the two levels of branching enzyme (Fig. 7). The $\overline{\text{c.l.}}$ values of the glycogens produced at the high and low branching-enzyme activities were 12 and 14, respectively. These were determined by complete debranching of the products with isoamylase and measurement of the amount of reducing sugars released after all of the (1 → 6)-branch points had been hydrolyzed. Complete debranching was confirmed by a 100% conversion into maltose when the debranched products were incubated with beta amylase. In contrast, isoamylase did not completely debranch glycogen whose component chains had been extended by as little as 15% by the action of glycogen synthase or glycogen phosphorylase acting in the absence of branching enzyme. For example, rabbit-liver glycogen ($\overline{\text{c.l.}}$ 12) was incubated with glycogen synthase in the presence of UDP-D-[U-^{14}C] glucose until the $\overline{\text{c.l.}}$ had increased to about 13.8 D-glucosyl residues, as calculated from the D-[^{14}C]glucose incorporated. Subsequent treatment of the polysaccharide with isoamylase gave a release of reducing sugar corresponding to $\overline{\text{c.l.}}$ 33, and the product was 64% converted into maltose by beta amylase. No further debranching occurred on addition of more isoamylase, and separation of the products of the debranching reaction revealed that about 80% of the D-[^{14}C]glucose incorporated by glycogen synthase was eluted in the void volume of a column of Biogel P-6 (not shown).

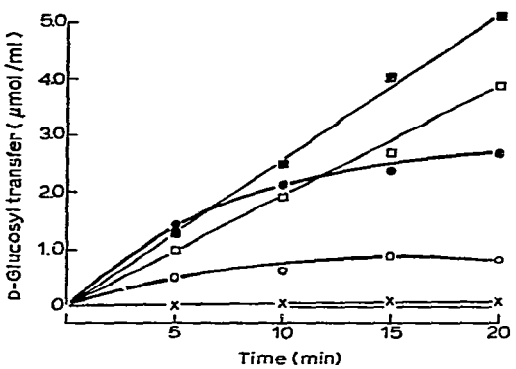

Fig. 7. Rate of the glycogen-primed synthetic reaction of rabbit-muscle phosphorylase *b* in the presence of rat-liver glycogen-branching enzyme. Rabbit-liver glycogen was incubated with phosphorylase *b* (0.3 units/ml) and α-D-glucosyl phosphate in the absence and presence of branching enzyme (0.6 or 0.3 units/ml) under conditions similar to those described in Materials and Methods. Phosphorylase *b* reaction-mixtures contained: glycogen (200 μg/ml), ●; glycogen (20 μg/ml), ○; glycogen (20 μg/ml) plus branching enzyme (0.6 units/ml), ■; glycogen (20 μg/ml) plus branching enzyme (0.3 units/ml), □; branching enzyme (0.6 units/ml) and no glycogen, ×.

DISCUSSION

There is a close similarity of the acceptor requirements of glycogen synthase and glycogen phosphorylase acting in the direction of polysaccharide synthesis. Thus, both enzymes act more rapidly on unmodified glycogen than on glycogen modified by debranching or by elongation of its outer chains. The apparent K_m value derived for maltopentaose (25mM) in the synthase reaction is near to literature values[4,5,20], and this value and that for glycogen (6.2×10^{-5} M chain-ends) are almost identical to the corresponding K_m values derived for the synthetic reaction of rabbit-muscle phosphorylase with maltosaccharide and glycogen acceptors[2,3]. However, when the ratio of enzyme activity and glycogen concentration is held constant, glycogen is not rate-limiting at the concentration corresponding to its K_m value (120 μg/ml, Figs. 2 and 3). The reason for the anomaly is unknown, but it is relevant that in both reactions the molarity of the macromolecular glycogen-acceptor is of the same order as that of enzyme, so that the use of classical Michaelis–Menten kinetics may be impermissible. In any event, it is likely that the real affinity between glycogen and the two enzymes may, as has been suggested by Larner *et al.*[5] for glycogen synthase, be more accurately reflected by affinity constants[12] in the range 10^{-6}–10^{-7} M.

Structural diversity of branched α-D-glucan acceptors is probably responsible for the wide range of their reported kinetic constants in reactions catalyzed by glycogen phosphorylase and glycogen synthase. Hu and Gold[3], using synthetic α-D-glucan acceptors of equal $\overline{\text{c.l.}}$ containing different numbers of chains per molecule, showed that a decrease in the number of chain-ends resulted in an increase in the apparent K_m values, without significantly affecting the V_{max} of the synthetic reaction of rabbit-muscle phosphorylase *a*. The decreasing acceptor-efficiency in the synthase (Fig. 4) and phosphorylase reactions as glycogen is debranched probably reflects, therefore, an increasing K_m value as the branched molecule is converted into maltosaccharide chains, for which the enzymes have low affinities.

Larner *et al.*[5], using beta amylase-degraded amylopectin acceptors of different $\overline{\text{c.l.}}$ containing equal numbers of chains per molecule, showed that there was not a large difference in the affinity ($S_{0.5}$ values) of rabbit-muscle synthase (I-form) for these polysaccharides, but there is a progressive increase in the relative V_{max} values for acceptors possessing average external chain-lengths ($\overline{\text{e.c.l.}}$)increasing from 2–9.9 D-glucosyl residues, followed by a significant decrease in V_{max} for acceptors having $\overline{\text{e.c.l.}}$ greater than 9.9. This apparent requirement of rabbit-muscle synthase for an optimal $\overline{\text{e.c.l.}}$ in branched acceptors is consistent with the greater relative V_{max} for shell-fish glycogen (100%) than for amylopectin (73%)[5], and with the progressive decrease in rate as the outer chains of rabbit-liver glycogen are extended (Fig. 1). By analogy with our observation for the synthetic reaction of phosphorylase (Table I), it is unlikely that this decrease in reaction rate is caused by a loss of synthase activity. A similar requirement by phosphorylase for an optimal $\overline{\text{e.c.l.}}$ is evident from its lower initial reaction-rate with oyster glycogen possessing extended outer-chains (Fig. 5), and the marked reduction (50%) in its V_{max} observed after the $\overline{\text{e.c.l.}}$ of rabbit-

liver glycogen is increased by five D-glucosyl residues ($\overline{c.l.}$ 12–17, in text). This observation again is consistent with the greater relative V_{max} of phosphorylase for glycogen (100%) than for amylopectin (66%)[2].

The $\overline{c.l.}$ of glycogen synthesized by the combined actions of phosphorylase and branching enzyme (Fig. 7) is controlled by the ratio of the two enzymes[22] and, in the presence of an excess of phosphorylase, the rate of glycogen synthesis is dependent on the activity of branching enzyme[23]. The concentration of glycogen is not limiting in these reaction mixtures, as there is no change in rate, despite a change in the glycogen concentration from 20 μg/ml to much higher levels. It follows, therefore, that the different rates of the reactions in the presence of the two levels of branching enzyme represent relative V_{max} values for phosphorylase *b* (and, by analogy, for synthase) that are characteristic of the $\overline{c.l.}$ (and hence $\overline{e.c.l.}$) of the glycogens being synthesized ($\overline{c.l.}$ 12 and 14). The increased phosphorolysis of long-chain glycogen (Fig. 6) after the preferential hydrolysis of its outer branches by alpha amylase[24] illustrates that the $\overline{e.c.l.}$ of the polysaccharide substrate is equally critical for the physiological action of phosphorylase in the degradative direction.

A high reactivity in the phosphorylase reaction of chemically synthesized, multi-chain α-D-glucans that lack α-(1 → 6)-branching linkages[3], and the low reactivity in both the phosphorylase and synthase reactions of the component α-D-glucan chains released from glycogen by isoamylase (Fig. 4), establish that neither the presence of (1 → 6)-branch linkages nor the presence of α-D-glucan chains of an optimal size is the intrinsic factor responsible for the reactivity of unmodified glycogen. It is more likely that the unmodified glycogen samples resemble "native" glycogen in possessing an external structure endowing the molecule with a topography that allows efficient interaction of enzymes with closely packed chain-ends at the polysaccharide surface. Glycogen synthase and, to a lesser extent, glycogen phosphorylase transfer D-glucosyl groups in a repetitive way to a limited number of the outer chains of glycogen[25], so that a small increase in $\overline{e.c.l.}$ (representing the average increase in length of all of the outer chains) in the absence of branching enzyme activity, substantially increases the length of a number of the more-accessible outer chains. As already suggested for the phosphorylase reaction[2], the ability of these initially growing chains to hinder the efficient interaction of glycogen synthase with D-glucosyl chain-ends remaining at the polysaccharide surface would explain the progressive decrease in reaction rate (V_{max}) that occurs as glycogen is enlarged. The same explanation is valid, even if enzyme interaction at the glycogen surface involves two active sites[3] or an additional polysaccharide binding-site[5].

Thus, the increased phosphorolysis of modified glycogen after limited alpha-amylolysis is consistent with the removal of longer outer-chains that occlude the surface of the polysaccharide and hinder further phosphorolysis (Fig. 6). Similarly, occlusion of the polysaccharide surface may explain the marked resistance to enzymic debranching of a glycogen sample whose outer chains have been minimally extended by glycogen synthase in the absence of branching enzyme ($\overline{c.l.}$ increased to 13.8). As the debranching enzyme, isoamylase, has to penetrate the glycogen molecule in

order to hydrolyze the outer tier of branching linkages, its action would be expected to be highly sensitive to inhibition by steric hindrance at the substrate surface.

Although the *in vivo* action of branching enzyme in glycogen synthesis minimizes an excessive elongation of individual chains of the polysaccharide by glycogen synthase, metabolic regulation of the synthase activity probably changes the ratio of enzyme activities sufficiently to alter the structure of the glycogen molecule, particularly in the external regions, so that a structurally heterogeneous population of glycogen molecules is produced. The effect of alpha-amylolysis on the reactivity of phosphorylase with long-chain glycogen lends strong support to our earlier suggestion[26] that cytoplasmic alpha-amylase may supplement branching enzyme action in modifying the outer-chain structure of mammalian glycogen. Thus, by preferentially hydrolyzing longer outer chains, alpha amylase would ensure that newly synthesized glycogen molecules possess the ordered external structure that these studies show is essential for their efficient and rapid degradation by phosphorylase.

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