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Kinetics of Glycogen Phosphorylase *a* with a Series of Semisynthetic, Branched Saccharides. A Model for Binding of Polysaccharide Substrates†

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ABSTRACT: The requirement of muscle phosphorylase for branched polysaccharide substrates was investigated by kinetic studies on semisynthetic branched saccharides. One series of saccharides was prepared from maltoheptaose by oxidizing the reducing group to a carboxyl group and coupling this with an amino group of ethylenediamine. The resulting aminooligosaccharide was coupled with *p*-nitrophenyl esters of mono-, di-, tetra-, and polycarboxylic acids to produce saccharides containing one, two, four, and approximately 52 maltodextrin chains per molecule. A similar series of saccharides was prepared from a heterogeneous maltodextrin of average chain length 11.7. Kinetic constants were determined for the reaction with phoshorylase *a* in the direction of chain elongation. Michaelis constants are equilibrium constants for dissociation of saccharide from the enzyme-AMP-glucose-1-P-saccharide complex. The Michaelis constants, expressed in terms of the concentration of nonreducing end groups, are independent of maltodextrin chain length but decrease considerably as the number of chains per molecule increases. Maximum velocities do not differ greatly from that for glycogen. Among the synthetic saccharides, only the polymer behaves similarly to glycogen in exhibiting a decreasing reaction rate as the chains are elongated. The kinetic constants are quantitatively consistent with a model in which two chain termini from the same saccharide molecule bind to the phosphorylase molecule simultaneously. Differences in binding between saccharides having different numbers of equally accessible chains are caused solely by statistical factors in the equilibrium. Highly branched substrates bind better because of their greater multiplicity of two end-group pairs.

Glycogen phosphorylase (EC 2.4.1.1, α-1,4-glucan:orthophosphate glucosyltransferase) from rabbit skeletal muscle catalyzes the reversible transfer of an α-glucosyl group, with retention of configuration, between orthophosphate and the 4 position of the nonreducing terminus of an α-1,4-glucan. The enzyme shows considerable specificity for highly branched α-1,4-glucans such as glycogen and amylopectin, which contain α-1,6 branch points, compared to linear glucans like simple maltodextrins and amylose (Goldemberg, 1962; Smith, 1971). Specificity is manifested largely in the Michaelis constants for the glucans; the branched polysaccharides have far smaller K_m values than do maltodextrins, such as maltotetraose or maltopentaose. Maximum velocities are comparable for the two types of saccharide. The kinetic mechanism of phosphorylases *a* and *b* is known to be rapid equilibrium random bi-bi (Gold et al., 1970; Engers et al., 1969, 1970a,b) and the Michaelis constant is the equilibrium constant for dissociation of the saccharide from a central complex.

In contrast, potato phosphorylase, which has physical and kinetic properties very similar to those of muscle phosphorylase (Gold et al., 1971), shows little or no discrimination between branched and linear saccharides. When the natural substrate, amylopectin, is debranched enzymatically the resulting mixture of maltodextrins is as good a substrate as the original amylopectin (Smith, 1971).

In the present work we have attempted to answer the question of why muscle phosphorylase shows such great preference for binding branched α-1,4-glucans by studying the kinetics of phosphorylase *a* with a series of semisynthet-

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ic branched saccharides. These saccharides were prepared from either pure maltoheptaose or a heterogeneous maltodextrin averaging 12 glucose residues per chain, coupling the reducing end, in several steps, to groups in multifunctional "core" molecules. The resulting products contained definite numbers of maltodextrin chains of known length. All the synthetic saccharides served as substrates for phosphorylase *a*, and their kinetic constants were determined for reaction in the direction of polysaccharide synthesis.

The results are consistent with a hypothesis that requires phosphorylase *a* to bind two nonreducing end groups of the same saccharide molecule. This model gives good quantitative agreement if one assumes that the probability of any pair of end groups in the saccharide combining with the two binding sites of the enzyme is the same as that of any other pair. Our conclusion is that there is no intrinsic difference in the binding of simple and complex saccharides to phosphorylase. The reason for the stronger binding of highly branched polysaccharides lies in statistical factors: there is a high multiplicity of two end-group pairs capable of binding to the enzyme.

Materials and Methods

Cycloheptaamylose, maltose, trinitrobenzenesulfonic acid, and TRI-SIL (silylation reagent) were products of Pierce Chemical Company. Poly(glutamic acid) ($\overline{DP}$ 120) was obtained from Miles Laboratories, Inc. 2,2,6,6-Tetrakis(2-carboxyethyl)cyclohexanone and 1,2,3-propanetricarboxylic acid were from K and K Laboratories, Inc. Bio-Gels and ion-exchange materials were purchased from Bio-Rad Laboratories, while Sephadexes were from Pharmacia Fine Chemicals, Inc. Dithiothreitol, *p*-nitrophenyl acetate, AMP, dipotassium glucose 1-phosphate, and glycogen (shellfish, type II) were products of Sigma Chemical Company. Glycogen was deionized by passage through AG 501-X8 mixed-bed resin.

Maltoheptaose (Figure 1, I, a = 7). A modification of the method of French et al. (1949) was used. Cycloheptaamylose (25 g) was dissolved in 100 ml of 5 m*M* HCl and heated in a boiling water bath with stirring for 3.5 hr. In this time ca. 8% of the cyclodextrin had hydrolyzed to linear dextrin as estimated by the reducing power of the solution. The subsequent procedure was the same as described, except that the combined concentrates were desalted on a column of AG 501-X8 mixed-bed resin and lyophilized.

The average chain length of this preparation was found by determining the total hexose content of a stock solution by the phenol–sulfuric acid method of Dubois et al. (1956) and the reducing power by the copper–neocuproine method of Dygert et al. (1965) using maltose as a standard in both analyses. The product gave a $\overline{CL}$[1] of 7.04 glucose units *per* reducing group. Paper chromatography by the method of French et al. (1950) showed essentially no reducing saccharides smaller than maltoheptaose.

Maltodextrin of $\overline{CL}$ 11.7 (I, a = 11.7). A solution of 100 ml containing 1 mmol of maltoheptaose, 10 mmol of glucose-1-P, 0.2 mmol of AMP, and 3.4 mg of phosphorylase *a* (pH 6.8) was incubated at 30° for 4.5 hr. The reaction mixture was heated at 90° to denature the enzyme and, after centrifugation, was concentrated in vacuum and applied to a column (2.5 × 66 cm) of Sephadex G-25 (20–80 μ) which had been equilibrated with distilled water. The column was eluted with distilled water and the fractions containing dextrin of $\overline{CL}$ near 12 were combined and lyophilized. The product had $\overline{CL}$ 11.7 and the yield was 53%, based on maltoheptaose.

Oxidation of Maltodextrins. A modification of the hypoiodite method of Schaffer and Isbell (1963) was used. Maltodextrin (400 μmol) was dissolved in 0.5 ml of distilled water and treated alternately with 0.20 ml of 0.20 *M* I_2 in 1.0 *M* KI solution and 0.16 ml of 1.0 *M* NaOH, until a total of 2.1 ml of the iodine solution and 1.68 ml of the NaOH had been added. After 20 min at room temperature for G_7, or 40° for G_{12}, the reaction mixture was applied to a column (1.3 × 60 cm) of Bio-Gel P-2 (200–400 mesh) that had been equilibrated with distilled water. The column was eluted with distilled water and the void volume peak containing the sodium salt of the oxidized saccharide (II) was lyophilized. This preparation had no detectable reducing power: yield 90%.

Oligosaccharide Amine (III). The sodium salt of the oxidized saccharide (150 μmol) was dissolved in 5 ml of water and treated with a solution of 0.75 mmol of ethylenediamine, 1.35 mmol of constant boiling HCl, and 0.225 mmol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in 1.0 ml of water. After 18 hr at room temperature the reaction mixture was passed through a column (1.3 × 63 cm) of Bio-Gel P-2 in distilled water to remove small molecules. The fractions containing saccharides were combined and applied to a column (1.5 × 30 cm) of carboxymethylcellulose (Cellex-CM) which had previously been treated with excess 0.5 *M* NH_3 and washed with distilled water. The column was eluted with water to remove acidic and neutral substances and then with 0.1 *M* NH_3 to elute amines. The 0.1 *M* NH_3 fractions containing saccharides were combined and again passed through the Bio-Gel P-2 column; the void volume fractions containing saccharides were lyophilized. Yields were ca. 50%.

Preparation of p-Nitrophenyl Esters. a. BIS(*p*-NITROPHENYL) ADIPATE. Sodium *p*-nitrophenoxide (50 mmol) was heated on a hot plate in an evacuated flask to drive off most of the water of crystallization. After cooling, the salt was dissolved in 50 ml of dimethylformamide and cooled in ice, and 25 mmol of adipyl chloride was added in small portions. After 15 min the solution was poured into 500 ml of ice–water mixture and stirred until the precipitate congealed to crystalline form. The precipitate was collected, washed with water, and dried in vacuum over H_2SO_4. This crude product was recrystallized twice from acetonitrile; mp 121°. The equivalent weight was determined by dissolving a sample in aqueous ethanolic NaOH, diluting in 0.01 *N* NaOH, and measuring the absorbance at 400 nm. A sample of purified *p*-nitrophenol gave ϵ_{400} 18,300 under these conditions. The equivalent weight was calculated to be 192; the theoretical value is 194.

b. *p*-NITROPHENYL ESTER OF 2,2,6,6-TETRAKIS(2-CARBOXYETHYL)CYCLOHEXANONE. The tetracarboxylic acid (3 mmol) was dissolved in 20 ml of dimethylformamide and cooled to −5°. Isobutyl chloroformate (18 mmol) and triethylamine (18 mmol) were added in that order and the mixture was held at −5° for 30 min with occasional shaking. Solid *p*-nitrophenol (14.4 mmol) was added and the mixture was left at room temperature overnight. The reaction mixture was diluted with 50 ml of cold water and the gummy product was collected, dried, and recrystallized

[1] Abbreviations used are: $\overline{CL}$, average chain length in terms of glucosyl units; glucose-1-P, α-D-glucopyranose 1-phosphate; G_7, maltoheptaose; G_{12}, heterogeneous maltodextrin of $\overline{CL}$ 11.7.

three times from acetonitrile. The product had mp 109–110°; yield 23%.

The equivalent weight was determined to be 218, as described above; the theoretical value is 217.5.

c. *p*-NITROPHENYL ESTER OF POLY(GLUTAMIC ACID). Poly(glutamic acid) (300 μmol, in terms of glutamic acid residues) of $\overline{DP}$ 120 and 360 μmol of *p*-nitrophenol were dissolved in 0.60 ml of dimethylformamide and treated with 360 μmol of dicyclohexylcarbodiimide in 0.20 ml of dimethylformamide. After 17 hr at room temperature the mixture was filtered and the filtrate was diluted with 2 ml of 0.2 *N* HCl. The gummy product was washed with more HCl and with water, and dried in vacuo over NaOH. The product was redissolved in a small volume of dimethylformamide, and centrifuged, and the supernatant was diluted with 5 ml of 0.2 *N* HCl. The precipitate was washed again with HCl and water, and dried.

A portion of the product was analyzed for glutamic acid residues by hydrolysis and amino acid analysis by the method of Spakman et al. (1958). Another portion was analyzed for esterfied *p*-nitrophenol as described previously. The product contained 0.46 residue of *p*-nitrophenol *per* glutamic acid residue.

Coupling of Oligosaccharide Amines (III) with p-Nitrophenyl Esters. a. WITH ADIPIC ACID. Amino saccharide (III) (40 μmol) in 0.30 ml of dimethyl sulfoxide was treated with ca. 50% of the theoretical amount of *p*-nitrophenyl adipate (0.25 *M* in dimethylformamide) at room temperature. The reaction was followed by determining free amino groups in small aliquots by the trinitrobenzenesulfonate method (Fields, 1972). When the reaction was complete, more *p*-nitrophenyl ester was added to bring the total to 90% of the amount necessary for complete reaction of amino groups. After completion of this reaction, the solution was diluted to 1 ml with cold water and passed through a column (1.2 × 31 cm) of Bio-Gel P-2 equilibrated with distilled water. Fractions containing saccharides were combined and passed through a column (0.9 × 6 cm) of DEAE-cellulose (Whatman DE-52) which had been treated with excess 1 *M* acetic acid and washed thoroughly with water. This column was eluted with water to remove neutral and basic components. Fractions containing saccharides showed no *p*-nitrophenyl ester detectable by absorbance at 278 nm. The combined fractions were passed through a column (0.9 × 8.5 cm) of CM-cellulose (Cellex-CM) in the NH_4^+ form as described above. Elution with water removed neutral substances and the fractions containing saccharides were combined and lyophilized. The yield was ca. 80%; this material (Figure 2, structure V) is referred to as "dimer".

The glucose content of this substance was determined by the phenol–sulfuric acid method of Dubois et al. (1956). Analysis for adipic acid was carried out by hydrolysis with constant-boiling HCl at 110° for 22 hr in vacuo. After evaporation of the HCl, an accurately known amount of suberic acid was added as an internal standard and the mixture was extracted with ether and silylated with TRI-SIL. This was then subjected to vapor-phase chromatography using a Hewlett-Packard Model 5700A gas chromatograph equipped with a Model 18710A flame ionization detector, and a column packed with 3% SE-30 (methylsiloxane polymer) on 100–200 mesh Gas Chrom Q.

b. WITH 2,2,6,6-TETRAKIS(2-CARBOXYETHYL)CYCLOHEXANONE. This condensation was carried out similarly to that described above using the *p*-nitrophenyl ester of the tetracarboxylic acid. Analysis of the product was also carried out similarly, with the exceptions that 1,2,3-propanetricarboxylic acid was used as internal standard, the hydrolysate was extracted with acetone, and the acids were converted to their methyl esters prior to chromatography. This product (VI) was formed in yields of ca. 60% and is termed "tetramer".

c. WITH ACETIC ACID. The procedure was similar to that described above, except that the oligosaccharide amine (III) was treated with a 15% excess of *p*-nitrophenyl acetate in one portion and the passage through DEAE-cellulose was omitted. The yields were ca. 65% and the product (IV) is designated "monomer".

d. WITH POLY(GLUTAMIC ACID). The procedure was similar to that described for adipic acid. After 90% of the free amino groups had reacted, the solution was diluted with 0.5 ml of 2 *N* NH_3 and passed through a column of Bio-Gel P-2. The fractions containing saccharide were combined and passed through the DEAE-cellulose column as before; however, this time the unadsorbed materials eluted with water were discarded and the column was eluted with 1 *N* acetic acid. The fractions containing saccharides were combined, neutralized with 2 *N* NH_3, and chromatographed on a column (1.2 × 26 cm) of Sephadex G-50 which had been equilibrated with distilled water. Saccharides in the void volume were lyophilized. The yield was *ca.* 25% and the compound (VII) will be referred to as "polymer".

Analyses for glucose and glutamic acid residues were carried out as described previously.

Phosphorylase a. Phosphorylase *b* was prepared from frozen rabbit muscle (Pel-Freez, type I) by the method of Fischer and Krebs (1962). Phosphorylase *a* was prepared from three times crystallized phosphorylase *b* using a crude preparation of phosphorylase kinase (Krebs and Fischer, 1962) and recrystallized at least three times.

Kinetics. Concentrations of phosphorylase *a* were determined spectrophotometrically at 279 nm using $A_{1cm(1\%)}$ 13.0. Concentrations of all saccharides are expressed in terms of the concentration of nonreducing chain termini. All experiments were carried out at pH 6.8 and 30°.

Rates were determined in the direction of saccharide synthesis. Reaction mixtures were 50 μl containing 0.025 *M* potassium maleate, 0.10 m*M* EDTA, 3.5 m*M* dithiothreitol, 1.0 m*M* AMP, 75 m*M* potassium glucose-1-P, 0.5–1.0 μg of phosphorylase *a*, and saccharide, unless otherwise indicated. The substrate and enzyme–buffer mixtures were preincubated separately at 30° for 5–10 min, and reaction was initiated by adding enzyme to substrate. After 3–6 min the reactions were stopped by addition of 10 μl of 7 *M* NH_3.

Inorganic phosphate was determined by a modification of the method of Baginski et al. (1967a,b). Reagent A contained 2.4% ascorbic acid, 0.1% EDTA, and 12% trichloroacetic acid, and was stable for several weeks when refrigerated. Reagent B contained 6% ammonium molybdate tetrahydrate, and reagent C contained 3% sodium citrate dihydrate, 3% anyhydrous sodium arsenite, and 3% acetic acid. The sample solution (60 μl) was diluted with 0.50 ml of water and reagents A (0.50 ml), B (50 μl), and C (0.40 ml) were added at 9-sec intervals with thorough mixing after each addition. The reaction mixture was incubated at 30° for 15 min and absorbance at 840 nm was determined. The color was stable for 24 hr at room temperature and the standard curve was linear, at least up to 0.07 μmol of total inorganic phosphate.

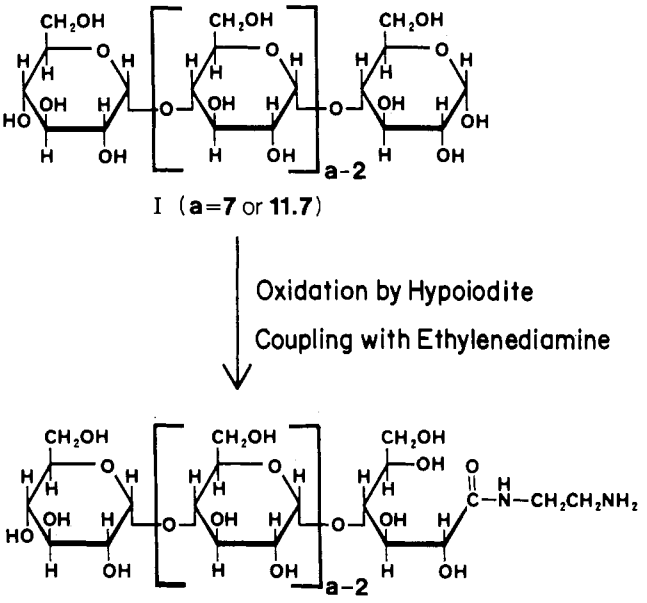

FIGURE 1: Synthesis of aminooligosaccharides. The details of the synthesis are in the Materials and Methods section.

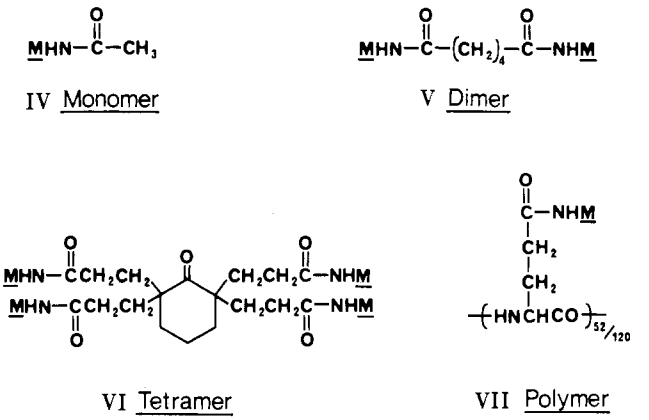

FIGURE 2: Structures of synthetic saccharides. Details of the syntheses are in the Materials and Methods section. The group M is defined in Figure 1.

Table I: Compositions of Semisynthetic Saccharides.[a]

Saccharide	Core	Maltodextrin Chains/Molecule of Polycarboxyl Compound: G_7 Series	Maltodextrin Chains/Molecule of Polycarboxyl Compound: G_{12} Series
Dimer (V)	Adipic acid	2.2	2.4
Tetramer (VI)	2,2,6,6-Tetrakis-(2-carboxyethyl)-cyclohexanone	4.8	4.2
Polymer (VII)	Poly(glutamic acid) $\overline{DP}$ 120	50.7	52.4

[a] Analytical methods are described under Materials and Methods.

Points on double reciprocal plots are averages of duplicate determinations; these usually did not differ by more than 5%. Lines on reciprocal plots were calculated by use of the HYPERBOLA program described by Cleland (1963). This program provides standard errors for the kinetic constants.

Results

The central compound in the synthetic scheme is the aminooligosaccharide III, shown in Figure 1. This was prepared in two forms: one form (III, a = 7) was homogeneous in structure, having a maltohexaose chain linked α-1,4 to N (2-aminoethyl)gluconamide, while the other form (III, a = 11.7) was similar but contained a heterogeneous maltodextrin chain averaging 10.7 glucose residues. These aminooligosaccharides were coupled with p-nitrophenyl esters of several carboxyl compounds to produce a series of saccharides having well-defined numbers of maltodextrin chains with free nonreducing end groups. A neutral saccharide (Figure 2, IV) with only one chain was prepared by condensation with the ester of acetic acid, while condensation with esters of adipic acid and 2,2,6,6-tetrakis(2-carboxyethyl)-cyclohexanone gave saccharides with two (V) and four (VI) chains, respectively. Condensation with partially p-nitrophenylated poly(glutamic acid) of $\overline{DP}$ 120 resulted in derivatives (VII) with an average of 52 chains per molecule.

Some of the substances were characterized in terms of the number of maltodextrin chains per molecule of polycarboxyl "core" (Table I). In the case of the dimer (V) and tetramer (VI), the number of chains appears to be too high by as much as 20%. This is probably caused by low recovery of the acids after hydrolysis; the fractionation methods employed in synthesis should have removed all acidic and basic impurities, and the products contained no detectable p-nitrophenyl ester. Furthermore, the carboxyl "cores" are a small fraction of the mass of the saccharides. We conclude that these substances are nearly pure, and the errors lie with the analytical methods employed.

In order to include glycogen among the saccharides studied, it was necessary to determine its molecular weight. The sedimentation rate was determined at 9.7°, 31,410 rpm, using a 1% solution of glycogen in water. A plot of log x vs. t was linear for movement of the boundary across 75% of the cell. The resulting $s_{20,w}$ of 46 S corresponds to a molecular weight of 1.6×10^6, according to the relationship given by Brammer et al. (1972). These authors report a molecular weight of 3.4×10^6 for a similar preparation.

Kinetic constants were determined for the saccharides with phosphorylase a for reaction in the direction of chain elongation. The concentrations of glucose-1-P and AMP were maintained at saturating levels; this was occasionally checked by repeating particular rate determinations at higher or lower concentrations of these substances. Kinetic constants determined in this way should be true maximum velocities and Michaelis constants. The rate data for all saccharides gave linear double reciprocal plots. Michaelis constants are compiled in Table II and maximum velocities are given in Table III. Glycogen is included under the G_{12} series because the average length of α-1,4 chains is 12 residues, but it could as well have been included under the G_7 series since the average external chain length is ca. 7 residues. In order to compare the constants for the different saccharides, it was convenient to express concentrations in terms of the concentration of nonreducing end groups. On this basis, it is clear that increasing the number of chains per molecule has a significant effect on the Michaelis constant; however, increasing the number of glucose residues per chain has little or no effect. Modification of the reducing terminus of the maltodextrin chains does not affect the

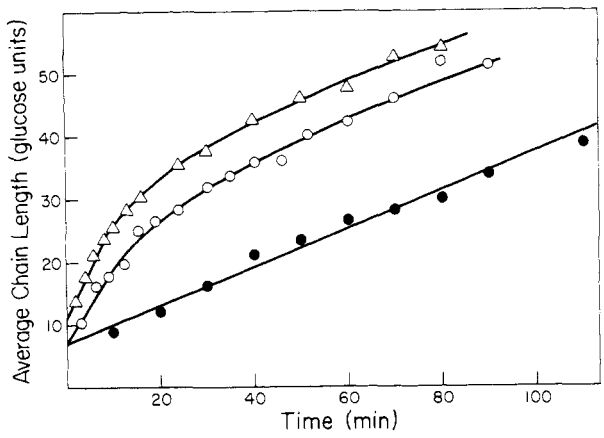

FIGURE 3: Time course of elongation of saccharide chains of tetramer and polymer in the G_7 series and glycogen. Conditions are the same as described for the concentration-dependence studies; however, the total reaction volume was 1 ml and 10-50-μl aliquots were removed at time intervals and quenched with aqueous NH_3. (●) Tetramer in G_7 series, 0.028 mM in chains, enzyme 10.2 μg/ml; (○) polymer in G_7 series, 0.044 mM in chains, enzyme 5.1 μg/ml; (△) glycogen, 0.167 mM in chains, enzyme 5.0 μg/ml.

Table II: Michaelis Constants of Phosphorylase *a* for Saccharides.[a]

Saccharide	n[b]	Michaelis Constant[c] (mM) G_7 Series	Michaelis Constant[c] (mM) G_{12} Series
Maltodextrin (I)	1	33 ± 3	26 ± 3
Monomer (IV)	1	32 ± 8	
Dimer (V)	2	7.7 ± 1.5	12 ± 3
Tetramer (VI)	4	3.6 ± 0.6	3.0 ± 0.3
Polymer (VII)	52	0.34 ± 0.04	0.31 ± 0.03
Glycogen	820[d]		0.069 ± 0.006

[a] The constants are for reaction in the direction of chain elongation. Conditions are given in the text. [b] The number of nonreducing chain termini per saccharide molecule. [c] Michaelis constants are expressed in terms of concentration of nonreducing chain termini. Limits shown are standard error. [d] Calculated using a molecular weight of 1.6×10^6 and assuming that one of every 12 glucose residues is an end group.

kinetic properties of the molecule, since K_m and V_{max} of maltoheptaose and monomer in the G_7 series are similar. Most of the saccharides have V_{max} close to that of glycogen; the two that are significantly smaller seem to be reproducible, but none are less than 50% that of glycogen.

One of the characteristics of glycogen as a substrate for phosphorylase is that the reaction rate decreases as the chains are elongated. The saccharides were tested under conditions where their concentrations are small compared to their respective Michaelis constants and the chains are elongated extensively. Changes in either V_{max} or K_m would be manifested as changes in slope of the plots of average chain length vs. time. Maltoheptaose, dimer, and tetramer in the G_7 series give linear plots up to $\overline{CL}$ ca. 40, while the polymer in the G_7 series and glycogen begin to curve down after the $\overline{CL}$ reaches 20. Curves for the latter three saccharides are shown in Figure 3. The observation that the dimer and tetramer in the G_7 series have significantly lower V_{max} than those in the G_{12} series should be reflected in an upward curvature in the plots for these substances; this may indeed be observed in the early points for tetramer in Figure 3. Over the long run, plots for both of these substances may be approximated as straight lines and show no evidence for downward curvature at long times.

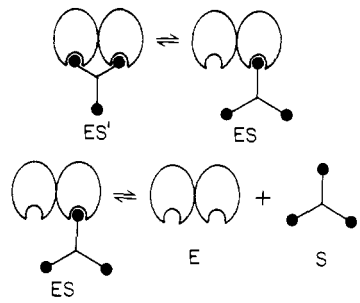

FIGURE 4: Model for binding of branched saccharides to phosphorylase *a*. Symbol E represents phosphorylase *a* dimer saturated with AMP and glucose-1-P. S is branched saccharide; only three nonreducing chain ends are represented in the figure, but it is understood that the saccharide contains n chain ends. ES and ES′ are the two 1:1 complexes of E and S in which one and two of the binding sites, respectively, are saturated.

Table III: Maximum Velocities of Phosphorylase *a* for Saccharides.[a]

Saccharide	n	Maximum Velocity[b] (μmol per min per mg) G_7 Series	Maximum Velocity[b] (μmol per min per mg) G_{12} Series
Maltodextrin (I)	1	26.5 ± 1.3	23.4 ± 1.9
Monomer (IV)	1	25.4 ± 3.6	
Dimer (V)	2	14.1 ± 1.4	24.2 ± 3.1
Tetramer (VI)	4	15.8 ± 1.2	21.6 ± 0.9
Polymer (VII)	52	25.0 ± 0.9	25.4 ± 1.0
Glycogen	820		27.1 ± 0.8

[a] Calculated from the same experiments that provided the data in Table II. [b] Limits shown are standard error.

Discussion

Attempts to define the concentration of branched polysaccharides in experiments with phosphorylase have never been entirely satisfactory. As long as a particular polysaccharide preparation is used, it makes little difference whether the concentration is expressed in terms of grams *per* liter, molar concentration of glucose residues, or molar concentration of nonreducing chain termini. When preparations of different branching or molecular weight are to be compared, the choice of units is significant. In this case, the molar concentration of chain ends is most appropriate; if one polysaccharide molecule combines with each active site independently of other sites, the kinetic constants for the polysaccharides should not depend on the molecular weight or degree of branching so long as all end groups are accessible. The results reported above clearly indicate that this is not the case for phosphorylase *a* in reactions with the semisynthetic saccharides. Saccharides containing more chains per molecule have significantly smaller K_m than less branched saccharides.

A model to rationalize these results is presented in Figure 4; it was suggested by the following observations. (1) Phosphorylase *a* and *b* exist as dimers under the conditions of our rate measurements (Wang and Graves, 1964). (2) The active and allosteric sites of phosphorylase *b* are relatively close to one another and to the subunit interface (Bennick et al., 1971). (3) Homotropic cooperativity has never been observed with glycogen and native phosphorylase, although it has been observed with the other substrates and AMP (Fischer et al., 1970). We assume that the two binding sites for chain termini in phosphorylase dimer are close enough together so that they can simultaneously bind end groups

Table IV: Intrinsic Dissociation Constants of Saccharides Calculated from Their Michaelis Constants.

Saccharide	n	K_1K_2[a] (mM)	
		G_7 Series	G_{12} Series
Dimer (V)	2	7.7	12
Tetramer (VI)	4	11	9
Polymer (VII)	52	17	16

[a] The constants are calculated from eq 5 using the Michaelis constants in Table II.

from the same polysaccharide molecule. In addition, we assume that they only bind chain termini from the same polysaccharide molecule, and that the probability of binding any pair of chain ends is the same as that of binding any other pair.

This model, illustrated in Figure 4, details the two steps of dissociation of saccharide from the enzyme-AMP-glucose-1-P-saccharide complex. In the equilibrium expressions (eq 1 and 2) (S) represents the molar concentration of saccharide, n the number of nonreducing chain termini *per* saccharide molecule, (E) the molar concentration of enzyme dimer saturated with AMP and glucose-1-P, and (ES) and (ES′) the molar concentrations of the two 1:1 complexes of E and S. The equilibrium constants are composed of intrinsic dissociation constants K_1 and K_2 and statistical factors. In the first reaction there are two ways in which one of the bound end groups can dissociate, and there are $n - 1$ ways in which one of the free end groups can combine with the free binding site; therefore, the statistical factor is $2/(n - 1)$. Similarly, the factor for the second step of dissociation is $1/2n$. Bearing in mind that the kinetic mechanism is rapid equilibrium random bi-bi, this gives rise to the rate eq 3 where k is the specific rate constant, (E_T) is the total enzyme concentration, and (C) is the concentration of nonreducing chain termini, according to eq 4. The model supposes that ES′ predominates greatly over ES and, therefore, $K_1 \ll n - 1$. This gives the relationships of eq 5 and 6.

$$\frac{(ES)}{(ES')} = \frac{2}{n-1} K_1 \quad (1)$$

$$\frac{(E)(S)}{(ES)} = \frac{1}{2n} K_2 \quad (2)$$

$$v = \frac{2k(E_T)(K_1 + n - 1)/(2K_1 + n - 1)}{\frac{K_1K_2}{(C)(2K_1 + n - 1)} + 1} \quad (3)$$

$$(C) = n(S) \quad (4)$$

$$K_m = K_1K_2/(n - 1) \quad (5)$$

$$V_{max} = 2k(E_T) \quad (6)$$

In the series of semisynthetic saccharides, we should expect all to have the same V_{max} and to differ in K_m according to eq 5. Calculated values for K_1K_2 are summarized in Table IV. Maltodextrins and glycogen are not directly comparable to the others; the former cannot bind two sites simultaneously, while the structure of the latter is relatively complex and may violate our assumptions. It is clear that although K_m values for the semisynthetic saccharides vary over a wide range, the calculated values of K_1K_2 are remarkably similar. This agreement is strong support for the proposed model.

Glycogen does not fit the model, giving a calculated value of 57 mM for K_1K_2, which is considerably greater than the other saccharides. This is not surprising in view of the structure of this polysaccharide. Brammer et al. (1972) have shown that glycogen is organized into densely branched "macrodextrin" regions which are joined together by regions of relatively low branching. It is reasonable that only a fraction of the chains are available to react with phosphorylase and that even these cannot combine in all possible pairs. If we take 12 mM as the best value of K_1K_2, we can calculate an effective value of n to be 170: this is 20% of the actual number of nonreducing termini.

One discrepancy in the results that is difficult to rationalize in terms of the model is the fact that dimer and tetramer in the G_7 series have V_{max} approximately 50% that of glycogen, while all other saccharides studied have V_{max} similar to glycogen. This may reflect the inability of these compounds to completely span the distance between the two binding sites. If the binding sites consist of series of subsites which are capable of binding several glucose residues at the end of the saccharide chain, it is possible that one chain end binds correctly to one binding site, but the other chain binds incompletely to its site, perhaps one or two subsites short of the catalytic center. This would result in 50% maximum velocity, but might not reduce the apparent affinity of the saccharide molecule for the enzyme by an amount that is significant in the context of this work. Increasing the distance between end groups by using saccharides of $\overline{CL}$ 12 results in full V_{max}.

An addition to the model is possible, and might appear reasonable, in which a second molecule of saccharide can bind to the ES complex to give ES_2. This would result in a rate equation containing a C^2 term and double reciprocal plots should be nonlinear. In fact, all double reciprocal plots were linear, and we must conclude that the ES_2 complex is not present in significant amounts over the concentration ranges that were investigated.

The experiments reported here help to explain why the reaction rate falls with time when low concentrations of glycogen or amylopectin (Smith, 1971) are incubated with phosphorylase and saturating glucose-1-P. Since the rate is zero-order in glucose-1-P and the concentration of nonreducing end groups is constant, one would expect the rate of chain elongation to be constant. We and others (Smith, 1971) have shown with several simple substrates that the length of the saccharide chains does not in itself affect the kinetic constants. The only synthetic saccharide that shows an effect similar to glycogen and amylopectin is the polymer. These substrates have in common a high degree of crowding of the saccharide chains. In the course of chain elongation some chains will grow more rapidly than others and tend to inhibit further growth of the shorter chains by steric hindrance. This will result in yet greater growth of the longer chains and have the effect of reducing the number of chains available to participate in the reaction. In terms of the model, it will reduce the effective value of n. A similar explanation has been proposed by Smith (1971).

Acknowledgments

We thank Miss E. Legrand for technical assistance in developing the synthetic methods.

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Kinetic and Mechanistic Studies on the Reaction of Melilotate Hydroxylase with Deuterated Melilotate†

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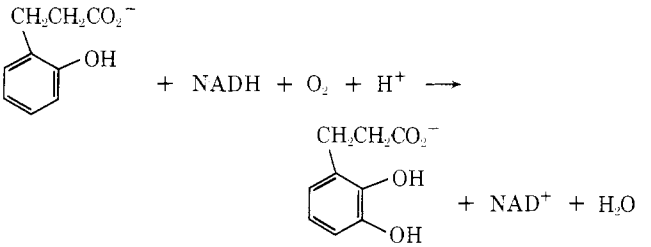
ABSTRACT: [3,5-^{2}H]Melilotate has been synthesized from melilotate by iodination followed by reductive deiodination in the presence of deuterated hydrazine. The deuterated melilotate has been employed in investigations of the reaction mechanism of melilotate hydroxylase. Stopped-flow spectrophotometry has revealed no isotope effect in the formation or decay of the oxygenated intermediate which is observed when reduced melilotate hydroxylase reacts with molecular oxygen. Steady-state analysis has corroborated this result, and in addition shows that there is no isotope effect in the reductive cycle of the enzyme mechanism. This analysis does reveal a reproducible 8% decrease in V_{max} for the enzyme when using deuterated melilotate. These observations are compatible with the thesis that the above intermediate is an oxygenated form of the reduced flavine prosthetic group and that the last step of the proposed mechanism is rapid and involves a primary isotope effect. The existence of the NIH shift mechanism has been studied using combined gas chromatography–mass spectrometry. No evidence could be obtained for intramolecular migration of deuterium during the hydroxylation reaction. However, the small amount of migration expected when phenols are hydroxylated precludes elimination of the NIH shift as a possibility.

Melilotate hydroxylase is a flavoprotein which catalyzes the conversion of melilotate (2-hydroxyphenylpropionate) to 2,3-dihydroxyphenylpropionate (Levy and Frost, 1966). In the course of a detailed kinetic study of this enzyme, an intermediate enzyme form was observed by stopped-flow spectrophotometry when the reduced enzyme was reacted with molecular oxygen in the presence of melilotate (Strickland and Massey, 1973b). The transitory nature of this intermediate precluded more than its spectrophotometric characterization at that time. It was proposed that the intermediate is a complex between reduced enzyme, oxygen, and melilotate, with a covalent bond between the flavine prosthetic group and oxygen.



There did exist a reasonable alternative explanation for the absorption spectrum observed in these experiments. Conceivably, an *o*-quinone form of the product complexed

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104. *Received November 1, 1974.* This research was supported by Grants No. GM-11106 and GM-00187 from the U.S. Public Health Service. The mass spectral facility is supported by Grant RR-004080 from the U.S. Public Health Service.

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