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Influence of Carbohydrates on Phosphorylase Structure and Activity. I. Activation by Preincubation with Glycogen*

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ABSTRACT: Preincubation of phosphorylase *a* at 20° with glycogen results in an approximately 6-fold increase in maximal velocity with glucose 1-phosphate with no apparent alteration in the Michaelis constant. Activation does not appear related to formation of a better primer. The dependence of the extent of activation with protein concentration, temperature, and ionic strength suggests that activation is related to enzyme dissociation and is consistent with the conversion of a less active tetrameric form of phosphorylase *a* or *b* to a more active dimeric species. Activation is not related to priming capacity of polysaccharide since amylopectin or its β-amylase limit dextrin, or hydrolyzed amylose, were found to be more efficient activators than glycogen. Although hydrolyzed amylose was found to be a competitive inhibitor with respect to glycogen in the activity test, activation by hydrolyzed amylose is not inhibited by glycogen, a weaker activator. These data show that activation by amylose is not a simple consequence of binding at the primer site and suggest that activation is a result of binding at an additional or activation site on the enzyme.

Although it has been clearly established that glycogen acts as a primer for the *in vitro* synthesis of glycogen by muscle phosphorylase (Cori and Cori, 1939), the exact consequences of binding of polysaccharide on enzymic structure and activity have not been fully realized. The interaction of phosphorylase with polysaccharide appears to involve the nonreducing ends of the primer (Brown and Cori, 1961), and this interaction, although not absolutely specific for glycogen, is favored by highly branched polysaccharides (Swanson and Cori, 1948). Ultracentrifugal studies of Madsen and Cori (1958) showed that the nonreducing ends of one molecule of corn glycogen can interact with as many as 33 enzyme molecules. Further investigation by Selinger and Schramm (1963) indicated that glycogen and phosphorylase can form an insoluble complex similar to the type formed by the reaction of an antigen with an antibody. Recent light-scattering measurements support the view that phosphorylase has several combining sites for glycogen (Lowry *et al.*, 1964). In the preparation of liver phosphorylase (Sutherland and Wosilait, 1956) and phosphorylase *b* kinase (Krebs *et al.*, 1964), a strong association between phosphorylase and glycogen was also observed; this interaction is no doubt of some physiological significance since this interaction has been found to depend in part on the nutritional status of the animal (Tata, 1964; Sie *et al.*, 1964).

Kinetic studies from this laboratory indicated that the dimeric form of phosphorylase *a* is more active than the tetrameric form of the enzyme and that glycogen blocked the conversion of the more active species to the less active form (Wang and Graves, 1964). The study of the consequences of this interaction on enzymic structure and activity of the various molecular forms of phosphorylase has been initiated. The results reported herein show that phosphorylase may be activated by preincubation with glycogen and that activation is related to enzyme dissociation.

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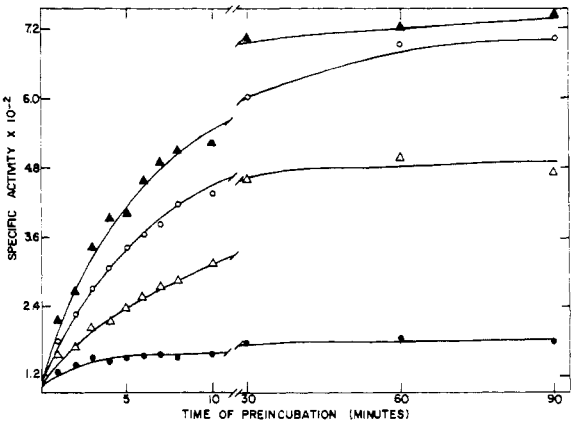

FIGURE 1: Activation of phosphorylase *a* by glycogen. Phosphorylase *a* (0.67 mg/ml) was preincubated in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8, at 20° (●); in buffer with 0.1% glycogen (△); in buffer with 0.5% glycogen (○); and in buffer with 1% glycogen (▲). At various intervals, 0.2-ml aliquots were removed and added to 0.2 ml of substrate, pH 6.8, at 20° containing 0.032 M glucose 1-phosphate and 2% glycogen.[2] The inorganic phosphate released was measured after 30 sec of reaction.

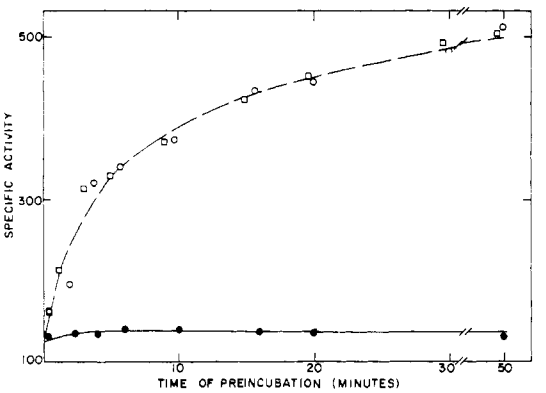

FIGURE 2: Activation by glycogen preincubated with phosphorylase *a*. Phosphorylase *a* (0.3 mg/ml) was preincubated in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8, at 15° (full line) and in buffer containing 2% glycogen (broken line). At various intervals aliquots were removed and tested for enzymic activity for 2 min. Glycogen (○) was first preincubated with enzyme (0.3 mg/ml) for 80 min, after which enzyme was inactivated by heating for 15 min in a boiling-water bath. Fresh enzyme was added at t_0 of this figure. Glycogen (□) was heated for 15 min, cooled, and added to enzyme at t_0.

Experimental Procedure

Materials. Cysteine hydrochloride, sodium glycerophosphate, potassium glucose 1-phosphate, and shellfish glycogen were obtained from Sigma Chemical Co. The glycogen was purified according to Sutherland and Wosilait (1956). Hydrolyzed amylose with a degree of polymerization (DP) of 50 D-glucose units, potato amylopectin, and its β-amylase limit dextrin were gifts from Dr. J. Robyt. Crystalline phosphorylase *b* was isolated by the procedure of Fischer and Krebs (1958). Phosphorylase *a* was prepared with the use of phosphorylase *b* kinase (Fischer and Krebs, 1962) and crystalline phosphorylase *b*. Third or fourth crystals of enzyme were treated with Norit A to remove firmly bound adenosine monophosphate (AMP).

Methods. Protein concentration was determined spectrophotometrically with the use of an absorbance index of 11.7 for a 1% solution of protein (Velick and Wicks, 1951). Phosphorylase activities were measured according to the procedure of Illingworth and Cori (1953).

Results

Activation of Phosphorylase a by Glycogen. The data illustrated in Figure 1 show that preincubation[1] of phosphorylase *a* with glycogen at 20° results in a marked increase of enzymic activity. This activation is highly dependent upon glycogen concentration and time of preincubation. At 90 min, a time for maximal activation, activation with 1% glycogen is approximately 1.5 times greater than that observed with 0.1% glycogen. These data tend to eliminate the possibility that activation occurs by some catalytic process.

Since Illingworth *et al.* (1961) have demonstrated that crystalline phosphorylase *a* is often contaminated with trace amounts of amylo-1,6-glucosidase, amylo-1,-4→1,6-transglucosidase, and α-amylase, the possibility that activation of phosphorylase *a* results from formation of a better primer during preincubation was tested. A solution containing phosphorylase *a* and glycogen was preincubated for 80 min and then heated to inactivate the enzyme. Activation by this solution of glycogen was compared with a boiled solution not preexposed to phosphorylase. The results of Figure 2 show that the courses of activation with the two solutions of glycogen are identical and, therefore, do not support the view of formation of a better primer during preincubation.

Relationship of Activation to Enzyme Dissociation. Since previous kinetic studies indicated that the tetrameric form of phosphorylase *a* can dissociate into a more active dimeric unit (Wang and Graves, 1964) and that glucose can induce enzyme dissociation and activation (Wang *et al.*, 1965), the hypothesis that activation by glycogen is due to enzyme dissociation has been tested.

[1] Preincubation, in this case, refers to incubation of enzyme with effectors prior to the measurement of enzymic activity. This designation will be used throughout this paper.

[2] No adjustment was made for the carry-over of glycogen from the preincubation solutions since the activity of phosphorylase is not altered by increase of glycogen concentration from 1 to 1.5% at 20°.

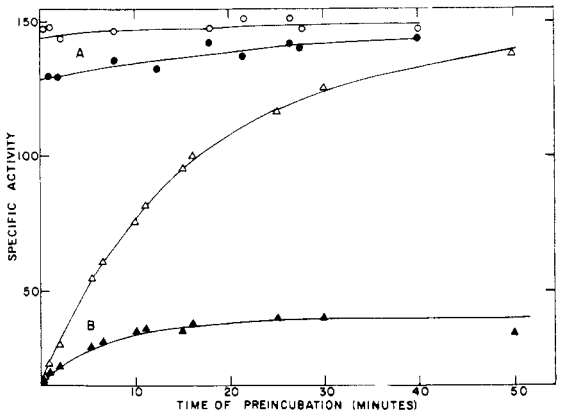

FIGURE 3: Effect of glycogen on phosphorylase *a* activity in 3.0 M NaCl. Phosphorylase *a* (6.0 mg/ml) was preincubated at 20° in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8, containing 3.0 M NaCl (enzyme A); and in buffer with 3.0 M NaCl and 10^{-3} M AMP (enzyme B). After 50 min (t_0 of this figure), enzymes were diluted 10-fold at 20°. Enzyme A was diluted in buffer with NaCl (●); enzyme A, in buffer, NaCl, and 2% glycogen (○); enzyme B, in buffer, NaCl, and AMP (▲); enzyme B, in buffer, NaCl, AMP, and 2% glycogen (△). At various intervals aliquots were removed and tested for enzymic activity at pH 6.8 at 20° (5 min) with substrate containing 3.0 M NaCl and 10^{-3} M AMP.

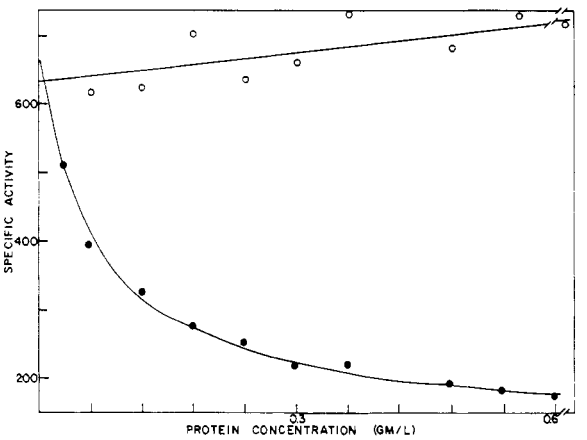

FIGURE 4: Effect of protein concentration of activation by glycogen. Phosphorylase *a* was diluted to various concentrations in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8, and preincubated at 20° (●); and in buffer with 2% glycogen (○). After 30 min, aliquots were removed for measurement of enzymic activity at 20°. The time of the assay varied from 30 sec to 5 min as the protein concentration varied approximately from 0.3 to 0.015 mg/ml, respectively.

The correlation of activation with enzyme dissociation by ultracentrifugation is technically impossible because of the high and heterogeneous molecular weight of glycogen. Since it has been shown that various parameters influence the state of aggregation of phosphorylase, a study of these effectors on activation by glycogen has been carried out.

In 3.0 M NaCl, phosphorylase *a* exists as a dimer; in 3.0 M NaCl with 10^{-3} M AMP, phosphorylase *a* exists as a tetramer (Wang and Graves, 1963). Figure 3 shows that preincubation of the tetrameric species with glycogen results in a marked activation (△ *vs.* ▲), whereas the dimeric form, although more active, appears essentially insensitive to preincubation with glycogen (○ *vs.* ●). It should also be noted that the activity of the tetramer after preincubation approaches the activity of the dimer, a result consistent with the hypothesis that glycogen induces enzyme dissociation

The effect of protein concentration on the extent of activation by glycogen is illustrated in Figure 4. The specific activity of phosphorylase *a* at 20° is highly dependent upon protein concentration and has been interpreted on the basis of conversion of a less active tetramer to a more active dimer upon enzyme dilution (Wang and Graves, 1964). If preincubation with glycogen induces enzyme dissociation, it might be expected that activation would only occur at protein concentrations where phosphorylase *a* existed as a tetramer. Figure 4 shows that activation by glycogen decreases with enzyme dilution and that equal specific activities of phosphorylase *a* are obtained by extrapolation to infinite dilution; these data support the view that glycogen does not activate the dimeric form.

The extent of activation was also found to be dependent upon temperature. Preincubation of enzyme (0.03 mg/ml) for 30 min in 2% glycogen resulted in 46% activation at 15°, 34% activation at 20°, and only 2% activation at 30°, a temperature routinely used for the measurement of enzymic activity.

To further investigate the relationship of enzymic activity to enzyme dissociation, a study of the catalytic characteristics of the various forms of phosphorylase *b* was made. Although phosphorylase *b*, in contrast to phosphorylase *a*, ordinarily exists as a dimer, ultracentrifugal studies have suggested that a tetrameric species can be induced by preincubation of the enzyme in Mg^{2+} and AMP at low temperatures (Kent *et al.*, 1958). Table I shows that preincubation of phosphorylase *b* with Mg^{2+} and AMP significantly alters enzymic activity at 13°, but not at 26°. No change in enzymic activity is apparent if enzyme is preincubated with Mg^{2+} or AMP. The fact that activity is reduced significantly only in the presence of Mg^{2+} and AMP at 13°, a condition which would be expected to favor the conversion of the dimeric form of phosphorylase *b* to a tetrameric species, suggests that the lower enzymic activity is due to enzyme association. The effect of preincubation with glycogen at 12° on the two molecular forms of phosphorylase *b* has been tested. The results illustrated in Figure 5 show that only phosphorylase *b* with Mg^{2+} and AMP (the tetrameric form) can be activated by glycogen and that the catalytic activity approaches that of the

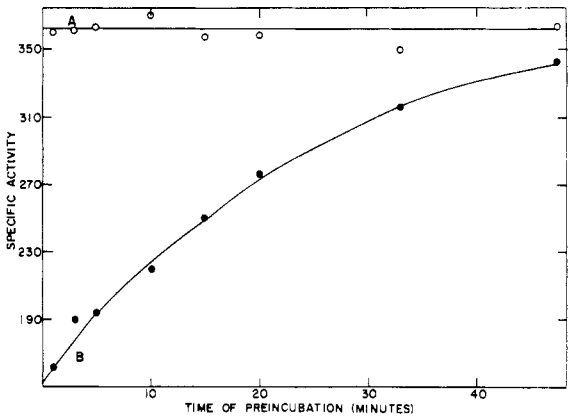

FIGURE 5: Activation of phosphorylase *b* by glycogen. Phosphorylase *b* (5 mg/ml) was preincubated at 12° in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8 (enzyme A); and in buffer containing 10^{-2} M Mg^{2+} and 10^{-3} M AMP (enzyme B). After 50 min enzyme A (○) and enzyme B (●) were diluted 10-fold in their respective buffers containing 2% glycogen (t_0 of this figure). Aliquots were removed at various intervals for measurements of enzymic activity (1 min) at 12°. All activities were tested with 10^{-3} M AMP and 10^{-2} M Mg^{2+}.

TABLE 1: Effect of Preincubation with Mg^{2+} and AMP on Catalytic Activity of Phosphorylase *b*.[a]

Additions	Specific Activity 13°	26°
None	397	1166
10^{-2} M Mg^{2+}	409	1190
10^{-3} M AMP	381	1159
10^{-3} M AMP and 10^{-2} M Mg^{2+}	179	1174

[a] Enzyme (0.4 mg/ml) was preincubated for 1 hr in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8, at 13 and 26° with the above additions prior to the measurement of enzymic activity. All activities were tested with 10^{-2} M Mg^{2+} and 10^{-3} M AMP.

dimeric form. Preincubation of enzyme in the absence of glycogen with Mg^{2+} and AMP does not activate phosphorylase *b* (Table I).

Kinetic Characteristics of Activated Phosphorylase a. To define more precisely the consequences of preincubation with glycogen on enzymic activity, the kinetic parameters, K_M and V_M, for glucose 1-phosphate were determined with enzyme activated by glycogen. Figure 6 shows that the K_M for glucose 1-phosphate is not altered by preincubation of phosphorylase *a* with glycogen, whereas V_M is increased approximately six times. Although the number of active centers of phosphorylase

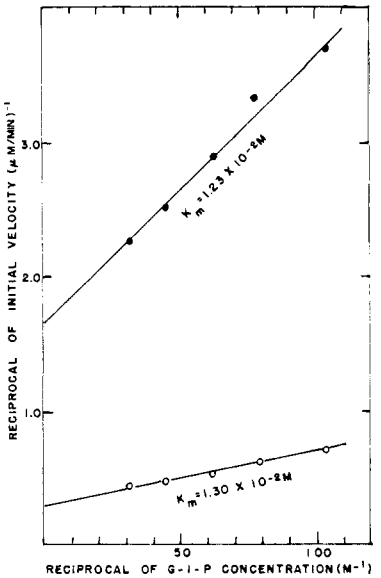

FIGURE 6: Effect of preincubation of phosphorylase *a* with glycogen on K_M and V_M for glucose 1-phosphate. Enzyme (0.5 mg/ml) was preincubated for 50 min at 20° in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8 (●); and in buffer with 2% glycogen (○). Phosphorylase activities were determined with substrates containing 1% glycogen and various concentrations of glucose 1-phosphate. Initial velocities were measured from phosphate progress curves by construction of the tangent t_0 with the use of a tangentimeter.

is not known, it appears unlikely that the large increase of V_M can be explained simply by an increase of available sites for glucose 1-phosphate after preincubation.

Action of Other Carbohydrates. Since the data of Swanson and Cori (1948) suggested that interaction at the primer site for muscle phosphorylase is highly specific and is favored by highly branched polysaccharides, various carbohydrates have been tested to determine whether the same structural requirements existed for activation. Preincubation of phosphorylase *a* at 20° with amylopectin, β-amylase limit dextrin of amylopectin, or hydrolyzed amylose results in an increase of enzymic activity.[3] The extents of activation by 0.5% solutions of these polysaccharides are essentially equal to activation obtained by preincubation of enzyme with glycogen (Table II). It should be noted, however, that the rates for activation were found to be very different and that there was no apparent relationship between activation and priming capacity. These data show that the structural requirements for activation are not nearly as specific as for priming and indicate that activation

[3] Although glucose has been found to activate phosphorylase *a*, no glucose could be detected in these polysaccharides by paper chromatography (French *et al.*, 1965). The rate of activation by amylopectin, hydrolyzed amylose, and β-amylase limit dextrin is such that activation would not be explained by contamination of glucose even if the entire fraction were free glucose.

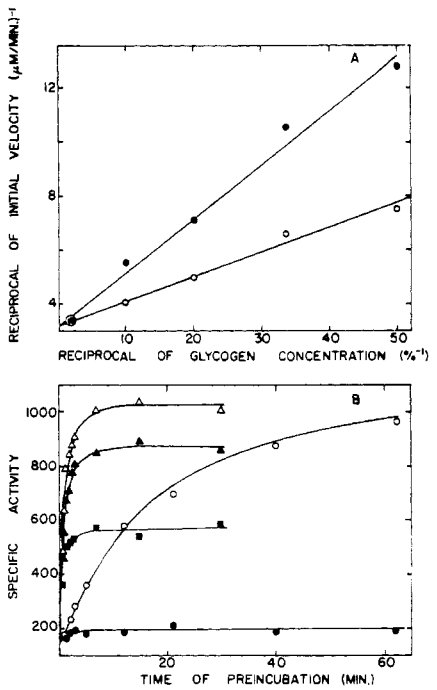

FIGURE 7: Effect of hydrolyzed amylose and glycogen on phosphorylase *a* activity. A: Effect of hydrolyzed amylose on phosphorylase *a* activity with glycogen. Phosphorylase *a* activity was measured at 30°, pH 6.8, with 0.016 M glucose 1-phosphate and various concentrations of glycogen without hydrolyzed amylose (○); and with 0.25% hydrolyzed amylose (●); initial velocities were determined as in Figure 6. B: Effect of glycogen on the activation by hydrolyzed amylose. Phosphorylase *a* (0.5 mg/ml) was preincubated in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8, at 20° (●); in buffer and 2.5% dimethyl sulfoxide with 2.5% glycogen (○); in buffer and 2.5% dimethyl sulfoxide with 0.25% hydrolyzed amylose (■); in buffer and 2.5% dimethyl sulfoxide with 0.5% hydrolyzed amylose (▲); and in buffer and 2.5% dimethyl sulfoxide with 2.5% glycogen and 0.5% hydrolyzed amylose (△). At various intervals, 0.2-ml aliquots were removed and tested for enzyme activity at pH 6.8 at 20° as in Figure 1.

and priming may occur at two different sites on the enzyme.

To test this hypothesis we studied the effect of hydrolyzed amylose on phosphorylase activity with glycogen and the effect of glycogen on activation by hydrolyzed amylose. Figure 7a shows that amylose is a competitive inhibitor with respect to glycogen in the activity test and that this inhibition is essentially overcome by 0.5% glycogen. Figure 7b shows that the initial rate of activation with 0.5% amylose is much greater than with 2.5% glycogen and that inclusion of glycogen in the hydrolyzed amylose solution does not inhibit activation by amylose. This lack of inhibition by glycogen at a concentration 2.5 times greater than that required to reverse inhibition by amylose (Figure 7a) further supports the view that activation and priming are results of different interactions of polysaccharide with enzyme.

TABLE II: Activation and Priming Capacity of Polysaccharides for Muscle Phosphorylase *a*.[a]

Polysaccharides	Extent of Activation (%)	Priming Capacity (%)	Time for Half-Activation (min)
Shellfish glycogen	100[b]	100[b]	10
Potato amylopectin	94	39	2
Hydrolyzed amylose[c] (DP ~50)	93	17	0.5
β-Amylase limit dextrin of amylopectin	99	11	2

[a] Enzyme (0.5 mg/ml) was preincubated with different polysaccharides (0.5%) at 20° in 0.03 M cysteine–0.04 M glycerophosphate, pH 6.8. For assessment of activation, aliquots were removed at various intervals and tested for enzymic activity at 20°. The assay mixture contained 0.016 M glucose 1-phosphate, 1% glycogen, and 0.25% polysaccharide from the preincubation solution. Priming capacity was tested at 20° with 0.5% solution of polysaccharides and 0.016 M glucose 1-phosphate. [b] Activation and priming capacity of these polysaccharides were compared by assuming 100% activation and priming capacity for glycogen. [c] To facilitate dissolution, dimethyl sulfoxide was present at a concentration of 2.5% in the preincubation solution (little or no activation of phosphorylase *a* has been detected by preincubation with 2.5% dimethyl sulfoxide).

Although the difference in the rate of activation of phosphorylase *a* by glycogen and by amylose may be due to the difference in extent of saturation of these polysaccharides, the fact that the extent of activation with 0.5% amylose is lower than with 2.5% glycogen or with the mixture of glycogen and amylose (Figure 7b) suggest that the lower rate of activation with glycogen is not due to incomplete saturation. Furthermore, with 0.25% amylose, a concentration far below saturation, the initial rate of activation is still much higher than obtained by glycogen. Although the nature of the interaction of phosphorylase with amylose and glycogen is uncertain, the difference in the rate of activation cannot be simply explained by the binding of polysaccharide at their nonreducing ends since 0.5% hydrolyzed amylose of DP 50 and 2.5% glycogen represent end groups of approximately 6×10^{-4} M and 1×10^{-2} M, respectively.

Discussion

Although it has been shown that the quaternary structure of proteins may be influenced by various small

molecules, little is known about the interaction of macromolecules with proteins containing subunits (Reithel, 1963). The present study shows that preincubation of phosphorylase with glycogen markedly alters its catalytic characteristics.

Although Illingworth and Cori (1953) recognized a slight stimulation of enzymic activity upon preincubation at 30°, the present work shows that this activation is very small compared with activation at lower temperatures and is dependent upon the state of aggregation of enzyme. The latter is based upon the fact that activation is only observed under experimental conditions where phosphorylase exists in a tetrameric form. For phosphorylase *a* at 20°, these conditions are low ionic strength solutions or high ionic strength solutions in the presence of 10^{-3} M AMP. For phosphorylase *b* these conditions are 10^{-3} M AMP and 10^{-2} M Mg^{2+} at low temperatures. Since the dimeric forms of phosphorylase cannot be activated by preincubation with glycogen and since the activity of the tetrameric forms approaches the activity of the dimeric species after preincubation, these data strongly suggest that activation is directly related to enzyme dissociation.

It has been suggested from previous kinetic data that (1) phosphorylase dissociates into a dimeric species upon dilution, and (2) the conversion of the dimeric species of phosphorylase *a* to the tetrameric form is effectively blocked by glycogen (Wang and Graves, 1964). It appears that activation may be described by eq 1–3 where T, D, and G represent a tetramer, a

$$T \rightleftharpoons 2D \qquad (1)$$

$$D + G \rightleftharpoons DG \qquad (2)$$

$$T + nG \rightleftharpoons TG_n \longrightarrow 2DG_{n/2} \qquad (3)$$

dimer, and glycogen, respectively. Equations 1 and 2 predict that activation occurs simply by a shift of equilibrium from tetramer to dimer. If activation is explained solely by eq 1 and 2, it would appear that the dependence of initial rate of activation upon glycogen concentration is due to a rate-limiting combination of glycogen with dimer. This seems unlikely, however, since the time for half-activation is in the order of minutes and the decay of activity of the dimeric species to a tetrameric form is instantaneously blocked by glycogen (Wang and Graves, 1964). This dependence could be explained by a combination of the tetrameric form with glycogen which facilitates enzyme dissociation (eq 3). Preliminary kinetic experiments show that the extent of activation and the initial rate of activation depend differently upon glycogen concentration (Wang and Graves, unpublished results) and further suggest that activation also depends on the third equation.

The activation of phosphorylase by various polysaccharides raises the question whether activation is a result of binding of polysaccharide at the primer site or is a consequence of a different interaction. The observation that activation is not related to priming capacity of the polysaccharide and the fact that activation by hydrolyzed amylose, a competitive inhibitor with respect to glycogen as a primer, is not inhibited by glycogen, a weaker activator, suggest that activation is not a simple consequence of binding at the primer site. The specific features of polysaccharide structure that are required for activation are not known and are presently under investigation in this laboratory.

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