and glutamyl residues (Tower *et al.*, 1962). The availability of these peptidoglutaminases which selectively remove γ-amide of peptide-bound glutamine should be a distinct advantage in the characterization of peptides. Peptidoglutaminases may be able to eliminate a complete hydrolysis of protein or peptide for the determination of its glutamine content because of its specificity toward deamidation of peptide-bound glutamine.

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Isotopic Effects and Inhibition of Polysaccharide Phosphorylase by 1,5-Gluconolactone. Relationship to the Catalytic Mechanism*

Jan-I Tu, Gary R. Jacobson,† and Donald J. Graves‡

ABSTRACT: Reaction rates of acid hydrolysis and hydrolysis by alkaline phosphatase were compared to rates obtained with rabbit muscle phosphorylase *b* and *Escherichia coli* maltodextrin phosphorylase by using reaction mixtures containing 1-deuterio-α-D-glucose 1-phosphate or the normal isotope. Secondary isotope effects were observed consistent with a mechanism involving a transition state with considerable positive charge similar to that expected for a carbonium ion. Inhibition studies were reported with 1,5-gluconolactone, a compound that possesses a half-chair conformation similar to the oxonium ion. The inhibition is highly specific and competitive with glucose 1-phosphate and noncompetitive with respect to glycogen in the direction of synthesis. For glycogen degradation, 1,5-gluconolactone is noncompetitive either with respect to arsenate or with respect to glycogen. These data have been interpreted to mean that 1,5-gluconolactone competes for the glucosyl transfer site of polysaccharide phosphorylase and that the transition state involves formation of an enzyme glucosyl complex in which the glucosyl residue is in the half-chair conformation.

1,5-Gluconolactone affects the properties of the pyridoxal 5′-phosphate binding site. The possible interaction of 1,5-gluconolactone with pyridoxal 5′-phosphate at the active site is discussed.

It has been clearly established that in the phosphorolysis of sucrose by sucrose phosphorylase an intermediate enzyme glucosyl complex is formed (Doudoroff *et al.*, 1947; Silverstein *et al.*, 1967; Voet and Abeles, 1970). No evidence has yet been given for such an intermediate in the polysaccharide phosphorylase reaction. Early studies with polysaccharide

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phosphorylase showed that its reaction mechanism is different from that of sucrose phosphorylase since no isotopic exchange was seen between glucose 1-phosphate and [^{32}P]P_i in the absence of the second substrate (Cohn and Cori, 1948). Kinetic studies (Maddiah and Madsen, 1966; Lowry *et al.*, 1967; Chao *et al.*, 1969; Engers *et al.*, 1969; Gold *et al.*, 1970) show that polysaccharide phosphorylase from various sources has a rapid equilibrium random bi–bi mechanism. Sucrose phosphorylase in contrast has a bi–bi Ping-Pong mechanism (Silverstein *et al.*, 1967). The results with polysaccharide phosphorylase do not mean that an intermediate glucosyl complex is not possible; they do suggest, however, that if such a complex is formed in the reaction its formation does not release product in the absence of the second substrate or that it is not made until the second substrate is bound to the enzyme.

The polysaccharide phosphorylase reaction like the sucrose phosphorylase reaction occurs with cleavage of the C–O bond of α-D-glucose 1-phosphate (Cohn, 1949) and with retention of configuration. Since a frontal displacement at C_1 of glucose 1-phosphate by the hydroxyl group at C_4 of the nonreducing end of the polysaccharide appears unlikely to us because of steric considerations, we have attempted to give evidence for the formation of a glucosyl intermediate in the polysaccharide phosphorylase reaction.

This report includes data which show that a secondary isotope effect occurs in the reaction with 1-deuterio-α-D-glucose 1-phosphate and that potent competitive inhibition results with 1,5-gluconolactone. These data are taken as support for an enzyme–glucosyl complex in which the glucosyl residue has a structure which resembles that of a half-chair conformation.

Materials and Methods

Crystalline rabbit muscle phosphorylase *b* was prepared according to a procedure of Fischer and Krebs (1958), and was recrystallized three times before use. Maltodextrin phosphorylase from *Escherichia coli* was obtained by the method of Schwartz and Hofnung (1967). Bacterial alkaline phosphatase was purchased from Worthington. Yeast hexokinase, yeast glucose 6-phosphate dehydrogenase, shellfish glycogen, gluconic acid, and 1,5-gluconolactone were products of the Sigma Chemical Co. The glycogen was further purified according to the procedure of Sutherland and Wosilait (1956). Dipotassium glucose 1-phosphate was purchased from Calbiochem, and 1,5-mannonolactone was a gift from Dr. Dexter French. Dipotassium 1-deuterioglucose 1-phosphate was prepared as follows. α-D-glucose was deuterated at C-1 from 1,5-gluconolactone by the method of Isbell and Frush (1955) by using D_2O as the solvent. The pentaacetate of this deuterated sugar was made as described by Posternak (1957). Finally, the crystalline β-D-1-deuterioglucose pentaacetate was treated with anhydrous phosphoric acid as described by MacDonald (1966) except that the reaction was allowed to continue for 1 hr instead of 5 min to increase the yield of α anomer. The resulting crystalline salt gave 0.99 mole of acid-labile phosphate/372 g. Nuclear magnetic resonance examination of the deuterated salt showed almost complete suppression of the absorption peak characteristic of the C-1 portion of glucose 1-phosphate (~5.4 ppm on the δ scale) indicating that well over 90% of the product was deuterated at this position. The specific rotation, α_D^{20}, is +79° (*c* 1.0, water). This compares well to a specific rotation α_D^{20} +78° for α-D-glucose 1-phosphate (Wolfrom and Pletcher, 1941). Apophosphorylase *b* was prepared as described by Shaltiel *et al.* (1966).

Phosphorylase was assayed in the direction of glycogen synthesis by the method of Illingworth and Cori (1953). In some of the enzymic reactions and all reactions of glucose 1-phosphate in HCl, initial velocities were determined by removing aliquots of the reaction mixture at various timed intervals and measuring inorganic phosphate released (Fiske and Subbarow, 1925). Rates of arsenolysis of glycogen were evaluated by a procedure similar to Metzger *et al.* (1968) except that the reactions were carried out at pH 6.0. The liability of 1,5-gluconolactone at pH 6.0 at 30° was evaluated by converting the remaining lactone into the hydroxamate at various timed intervals using hydroxylamine as described by Lipmann and Tuttle (1945). Ultracentrifugal runs were performed in a Spinco Model E analytical ultracentrifuge at a rotor speed of 52,000 rpm. The temperature for all runs was maintained at 20 ± 1°. Sedimentation coefficients were determined with the aid of a Nikon Model 6C microcomparator. Enzyme concentration was determined spectrophotometrically at 280 mμ. An absorbancy index of 11.9 (Appleman *et al.*, 1963) was used for a 1% enzyme solution.

Results

Isotopic Experiments. The absence or presence of secondary isotope effects in solvolytic reactions has been used as a powerful probe into mechanism. In reactions in which carbonium ions are thought to be intermediates, ratio of rates, k_h/k_d of 1.14 have been observed (Halevi, 1963), whereas ratios of 1.00 have been seen in various displacement reactions. The acid-catalyzed hydrolysis of glucose 1-phosphate is believed to proceed through formation of a carbonium ion (Bunton *et al.*, 1958). To determine whether polysaccharide phosphorylase shows a mechanism similar to acid hydrolysis, 1-deuterio-α-D-glucose 1-phosphate was synthesized and tested for isotopic effects in the enzymatic and nonenzymatic systems.

The results illustrated in Figure 1A clearly illustrate such a secondary isotope effect between normal glucose 1-phosphate and glucose 1-phosphate deuterated on C-1 when both are subjected to acid hydrolysis at 24°. Similar experiments at temperatures varying between 48 and 10° indicated that the ratio k_h/k_d increases with decreasing temperature for acid hydrolysis. The effect is barely observable at 48° but at temperatures less than 24° it becomes slightly more pronounced than in Figure 1A. These data support the conclusions of Bunton *et al.* (1958) arrived at from different experimentation that acid hydrolysis of glucose 1-phosphate proceeds through formation of a carbonium ion.

Initial rate data for *E. coli* maltodextrin phosphorylase and rabbit muscle phosphorylase *b* on substrates containing normal and deuterated glucose 1-phosphate at 10° (Figure 1C,D) also show the secondary isotope effect observed in acid hydrolysis. Experiments on both enzymes between 24 and 0° failed to show the temperature effect observable for acid hydrolysis. In this instance little effect on the ratio k_h/k_d was seen over this temperature range. The results strongly suggest that the polysaccharide phosphorylase reaction, at least in regard to the two enzymes used in this study, proceeds through a transition state with some positive charge similar to that established for the acid-catalyzed hydrolysis.

The bacterial alkaline phosphatase reaction was carried out with substrates containing the normal and isotopic glucose 1-phosphates as an enzymic control experiment since this reaction is known to involve hydrolysis of the P–O bond, unlike the phosphorylase reaction (Cohn, 1949). For such a

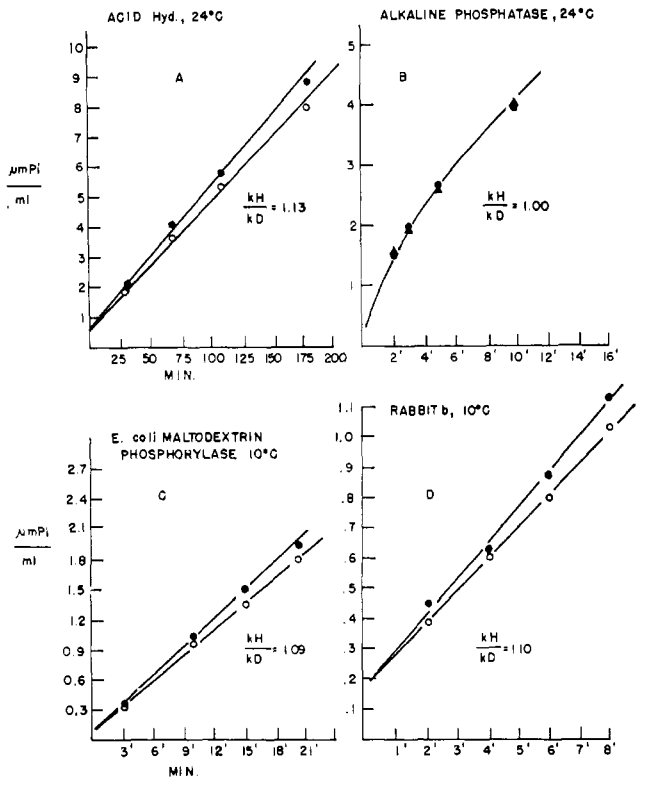


FIGURE 1: Studies of glucose 1-phosphate. (A) Acid hydrolysis of normal and deuterated glucose 1-phosphate. Hydrolysis carried out in 1.1 N HCl at 24°, glucose 1-phosphate concentration of 26 mmoles/ml, (●) normal glucose 1-phosphate, (○) 1-deuterioglucose 1-phosphate. (B) Hydrolysis of normal and deuterated glucose 1-phosphate by alkaline phosphatase. Experiment was carried out at 24°, pH 8.0, in 12.5 mM glycylglycine with a glucose 1-phosphate concentration of 8 mmoles/ml in reaction mixture, (●) normal glucose 1-phosphate, (▲) deuterated glucose 1-phosphate. (C) *E. coli* maltodextrin phosphorylase reaction with normal and deuterated glucose 1-phosphate. Reaction started by mixing of equal volumes of diluted enzyme and substrate at 10°. Enzyme: 0.5 mg/ml in 100 mM Tris–20 mM EDTA (pH 7.5). Substrate: 8 mM glucose 1-phosphate/ml and 1% C-7 dextrin/ml. (●) Normal glucose 1-phosphate substrate (○) deuterated glucose 1-phosphate substrate. (D) Rabbit skeletal muscle phosphorylase *b* reaction with normal and deuterated glucose 1-phosphate. Reaction started by mixing of equal volumes of diluted enzyme and substrate at 10°. Enzyme: 0.07 mg, ml in 40 mM glycerophosphate–40 mM β-mercaptoethanol (pH 6.8). Substrate: 22 mM glucose 1-phosphate, 1.3% glycogen, and 2.5 mM AMP. (●) Normal glucose 1-phosphate substrate, (○) deuterated glucose 1-phosphate substrate.

cleavage, no secondary isotope effect would be expected. Figure 1B shows the initial rate data for this experiment. As expected, no difference in the rate of hydrolysis between the normal and isotopic glucose 1-phosphate can be detected in this reaction.

Effect of 1,5-Gluconolactone. Substrate binding to enzyme is likely greater with the "activated" substrate than with the ground state form (French, 1957). The high degree of inhibition of proteases by the phosphofluoridates (Bernhard and Orgel, 1959) and glycosidases by gluconolactone (Leaback, 1968) has been suggested to be due to binding by these molecules in a structural form which imitates the transition state of the normal substrate. Structural analysis of 1,5-gluconolactone by X-ray crystallography (Hackert and Jacobson, 1969) shows that this compound possesses a half-chair conformation (Figure 2) similar to that proposed for the intermediate oxonium ion in the acid-catalyzed hydrolysis of the glucose 1-

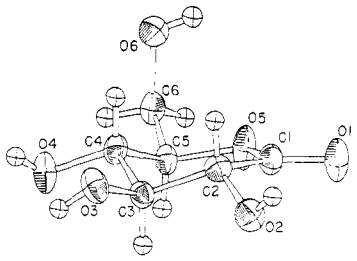


FIGURE 2: Conformation of 1,5-gluconolactone. C-1, C-2, O-1, and O-5 are essentially in one plane; the CH_2OH and OH groups occupy the most "equatorial" positions possible for a half-chair conformation of the ring (M. L. Hackert and R. A. Jacobson).

phosphate. Experiments, therefore, were undertaken to determine the strength and character of inhibition of polysaccharide phosphorylase by 1,5-gluconolactone.

In Figure 3, reciprocal plots are illustrated with respect to glucose 1-phosphate for muscle phosphorylase *b* and maltodextrin phosphorylase in the presence of 1,5-gluconolactone. The data show that inhibition is complex for both enzymes. The plots are nonlinear and show that 1,5-gluconolactone is a competitive inhibitor for glucose 1-phosphate. The insert of Figure 3A is from an experiment at high concentrations of glucose 1-phosphate and shows convincingly that the maximal velocities are identical with and without inhibitor. It is important to point out that this experiment was done with the second substrate, maltoheptaose, at its K_M value. If 1,5-gluconolactone binds both to the polysaccharide site used in the synthetic reaction and the glucose 1-phosphate site, noncompetitive inhibition would have been seen under these conditions. The insert of Figure 3B shows a plot of slopes (from concentrations of glucose 1-phosphate of 30 mM and above) *vs.* inhibitor concentration for phosphorylase *b*. The plot is linear and K_i of 1.0 mM was obtained which is six to eight times lower than the K_M for glucose 1-phosphate, which was calculated from the data of Figure 3. The fact that the 1,5-gluconolactone binds better to the enzyme than the substrate is what is expected for a transition state analog and agrees with the type of observations made on glycosidases with lactones as inhibitors (Leaback, 1968; Conchie *et al.*, 1967).

No inhibition of phosphorylase was observed by the same concentrations of D-gluconic acid at the same pH, ruling out any effect produced by hydrolysis of the 1,5-gluconolactone to gluconic acid. The results of Figure 4 show that hydrolysis of 1,5-gluconolactone does occur at pH 6.0 but that the extent of hydrolysis compared to the time used for enzyme assays (5 min) is small. Inclusion of glucose 1-phosphate at 0.1 M accelerates hydrolysis but again little occurs over the five-minute time period. Phorphorylase, itself, was also found not to affect the stability of 1,5-gluconolactone. Therefore, the data presented in Figure 3 are not complicated by hydrolysis of the inhibiting compound. In addition to the lack of effect of sodium gluconate no inhibition was observed with glucuronolactone, sodium glucuronate, 1,4-galactonolactone, 1,4-gulonolactone, and 1,5-mannonolactone illustrating the high degree of specificity of this inhibition.

Since 1,5-gluconolactone is a potent competitive inhibitor, with respect to glucose 1-phosphate, it was important to establish what type of inhibition exists with respect to the polysaccharide. Figure 5 shows that in the direction of glycogen synthesis, 1,5-gluconolactone is a noncompetitive in-

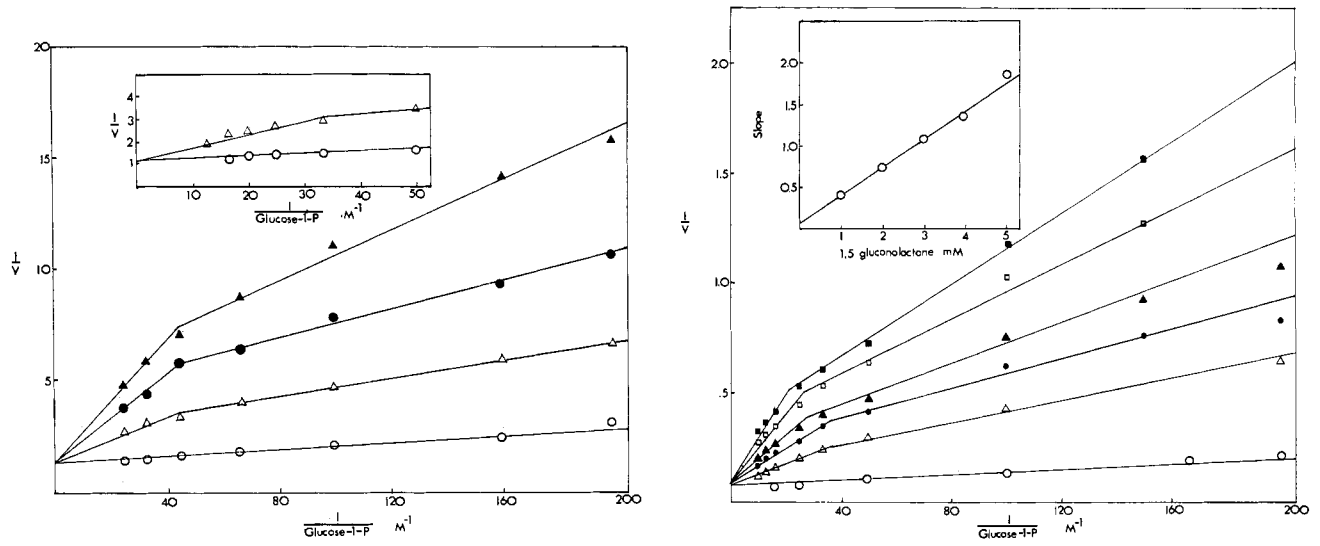


FIGURE 3: Kinetics of 1,5-gluconolactone inhibition, with respect to glucose 1-phosphate. (A, left) Double-reciprocal plot for *E. coli* phosphorylase at pH 6.0 in 40 mM Tris–40 mM maleate, 0.5% G-7 dextrin, and 0 (○), 1 (△), 2 (●), or 4 (▲) mM 1,5-gluconolactone. Insert: condition is same as described in part A except that the highest glucose 1-phosphate concentration is 80 mM instead of 40 mM. No additions (○), with 1 mM 1,5-gluconolactone (△). (B, right) Double-reciprocal plot for rabbit muscle phosphorylase *b* at pH 6.0 in 40 mM Tris–40 mM maleate, 1% glycogen, 1 mM AMP, and 0 (○), 1 (△), 2 (●), 3 (▲), 4 (□), and 5 (■) mM 1,5-gluconolactone. Insert: secondary plot of the slopes from part B. Initial velocity is expressed as absorbance at 660 nm/5 min.

hibitor. At concentrations up to 250 times the K_M value for glycogen (insert Figure 5), there is no indication of nonlinearity in the plot nor evidence that the same maximal velocity will be obtained with and without inhibitor.

The kinetics with respect to substrates in the direction of glycogen degradation in the presence of 1,5-gluconolactone are shown in Figures 6 and 7. Kinetic studies are not presented with inorganic phosphate because of the difficulty of using the coupling enzyme system of Helmreich and Cori (1964) at pH 6.0 in the presence of 1,5-gluconolactone. The problem of interference of 1,5-gluconolactone was circumvented by using arsenate as the substrate. Reactions were carried out at pH 6.0, after which the enzyme was removed by precipitation with $HClO_4$. The supernatant fluid was adjusted to pH 6.8 to allow complete hydrolysis of 1,5-gluconolactone to gluconic acid and then analyzed for free glucose according to Metzger *et al.* (1968).

Figure 6 shows that 1,5-gluconolactone is a noncompetitive inhibitor with respect to arsenate. Using concentrations of arsenate up to 0.17 M (approximately 30 times the K_M value), there is no evidence of the unusual kinetics seen with respect

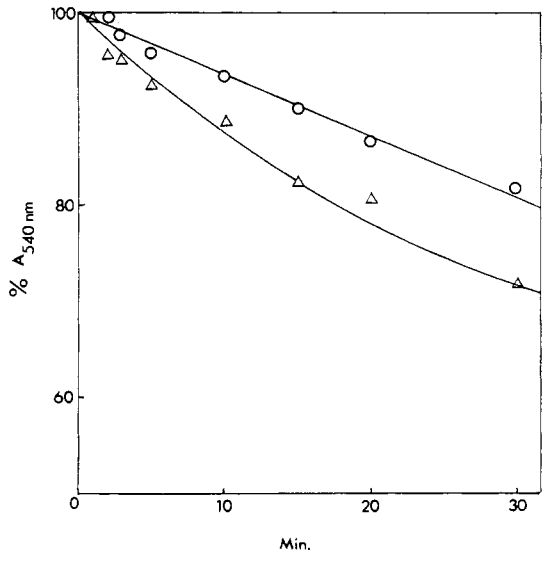


FIGURE 4: Lability of 1,5-gluconolactone. Reactions were carried out at 30°, pH 6.0 in 40 mM Tris–40 mM maleate–10 mM 1,5-gluconolactone, in the absence (○) or presence (△) of 100 mM glucose 1-phosphate. Lability is expressed as per cent absorbance decrement at 540 nm.

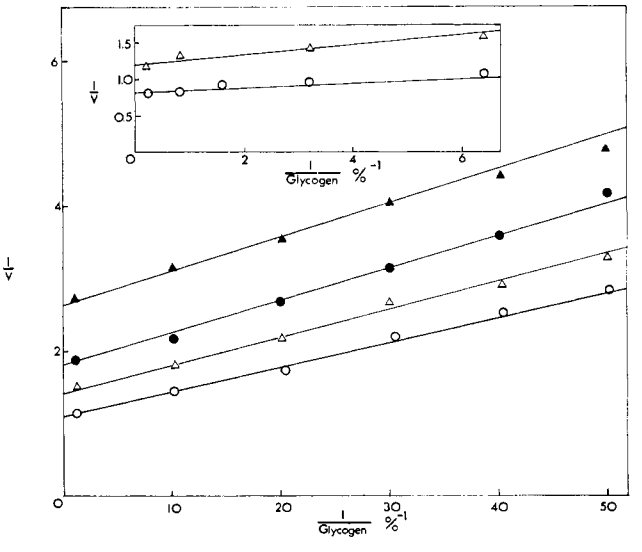


FIGURE 5: Kinetics of 1,5-gluconolactone inhibition with respect to glycogen in the direction of glycogen synthesis. Double-reciprocal plot for rabbit muscle phosphorylase *b* at pH 6.0 in 40 mM Tris–40 mM maleate, 16 mM glucose 1-phosphate, 1 mM AMP, and 0 (○), 0.5 (△), 1 (●), or 1.5 (▲) mM 1,5-gluconolactone. Insert: experimental condition is same as described above except the highest glycogen concentration is 5% instead of 1%. No additions (○), with 1 mM 1,5-gluconolactone (△).

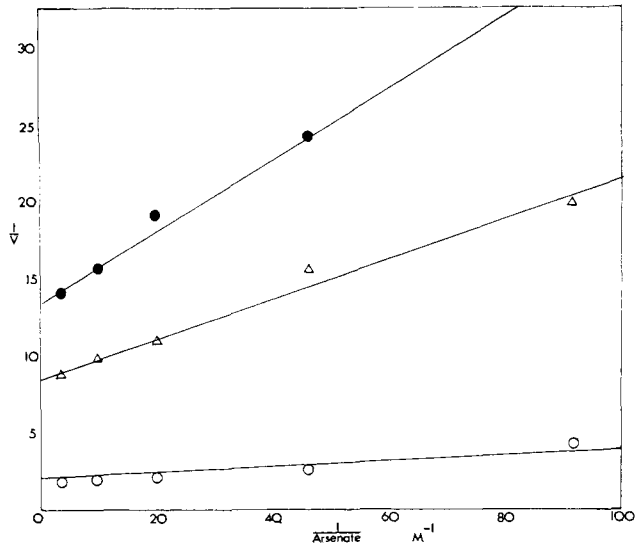


FIGURE 6: Kinetics of 1,5-gluconolactone inhibition with respect to arsenate in the direction of glycogen degradation. Double-reciprocal plot for rabbit muscle phosphorylase *b* at pH 6.0 in 40 mM glycerophosphate–30 mM β-mercaptoethanol, 5% glycogen, 1 mM AMP, and 0 (○), 0.15 (△), or 3 (●) mM 1,5-gluconolactone. Initial velocity is expressed as absorbance increment at 340 nm/min.

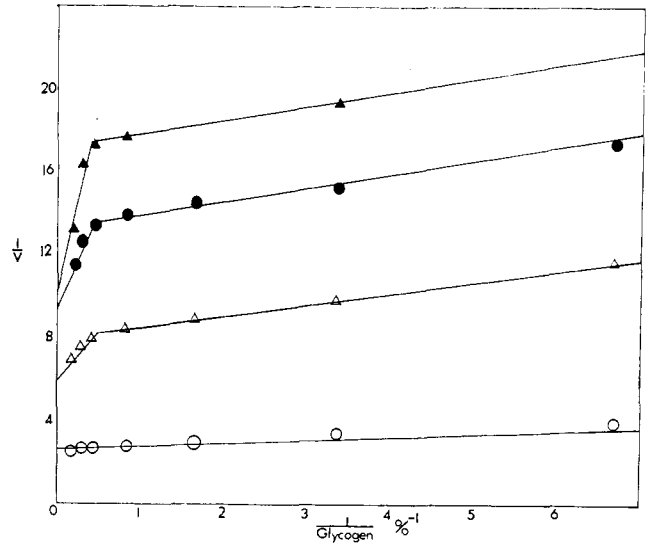


FIGURE 7: Kinetics of 1,5-gluconolactone inhibition with respect to glycogen in the direction of glycogen degradation. Double-reciprocal plot for rabbit muscle phosphorylase *b* at pH 6.0 in 40 mM glycerophosphate–30 mM β-mercaptoethanol, 160 mM arsenate, 1 mM AMP, and 0 (○), 0.15 (△), 0.3 (●), or 0.6 (▲) mM 1, 5-gluconolactone. Initial velocity is expressed as absorbance increment at 340 nm/min.

to the other anionic substrate, glucose 1-phosphate (Figure 3). In this experiment, the second substrate, glycogen, was used at concentration approximately 250 times its K_M value. In other experiments, not illustrated, similar results were obtained with glycogen at 1% (50 times the K_M value). These results suggest that 1,5-gluconolactone does not bind to the arsenate site.

An interesting aspect of the reaction catalyzed by polysaccharide phosphorylase is that polysaccharide serves as a reactant in both directions. It has been suggested that polysaccharide binds differently to the enzyme for phosphorolysis and synthesis (Chao *et al.*, 1969). For phosphorolysis, the nonreducing end of the polysaccharide chain is thought to occupy the site at which the glucosyl residue of glucose 1-phosphate resides for synthesis. Kinetics in the direction of glycogen degradation are shown in Figure 7 and can be compared to results obtained in the direction of synthesis (Figure 5). In both cases a wide range of glycogen concentrations were used and results are shown at high concentrations of polysaccharide (approximately 250 times the K_M value). The kinetics for this experiment and that illustrated in Figure 7 indicate noncompetitive inhibition. Thus, at an infinite concentration of glycogen, and a concentration of arsenate well above its K_M value (Figure 7) and at an infinite concentration of arsenate and a concentration of glycogen well over its K_M value (Figure 6), inhibition in the direction of glycogen degradation, in contrast to results seen in the direction of glycogen synthesis (Figure 3), never appears to be completely reversed.

The results with 1,5-gluconolactone indicate that there is a glucosyl intermediate in the polysaccharide phosphorylase reaction and can be interpreted that the glucosyl residue in the transition state exists in a conformation similar to that of a half-chair. If 1,5-gluconolactone does imitate the transition state of the substrate rather than the ground state, it was thought that binding of 1,5-gluconolactone to the enzyme might show some changes in properties of the pyridoxal 5′-phosphate binding site. Figures 8 and 9 present the findings of experiments designed to test the effect of 1,5-gluconolactone on the resolution of native phosphorylase *b* and the reconstitution of apophosphorylase *b*. In both cases, it is clear that low levels of 1,5-gluconolactone markedly retard these processes although the substrates, glycogen and glucose 1-phosphate, have no appreciable effect on these rates (Hedrick *et al.*, 1966, 1969). The concept that the pyridoxal 5′-phosphate binding site is affected by binding of 1,5-gluconolactone was also seen by the fact that pyridoxal 5′-phosphate in phosphorylase could not be reduced by $NaBH_4$ in 1.5 M NaCl at low temperatures. As it had been previously shown that the 330-nm band for

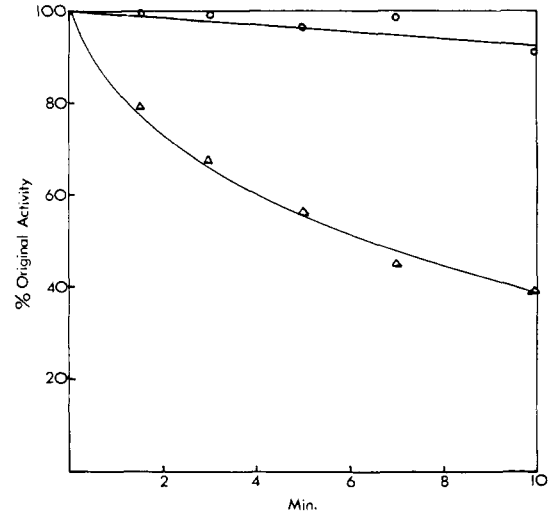


FIGURE 8: Effect of 1,5-gluconolactone on resolution of rabbit muscle phosphorylase *b*. Resolution was carried out at pH 6.0, 0° with 2.5 mg/ml of enzyme in 0.4 M imidazole–0.1 M cysteine-HCl. Aliquots were removed at indicated timed intervals and diluted 50-fold with 40 mM glycerophosphate–30 mM β-mercaptoethanol buffer, for measurement of enzymatic activity. No additions (○), with 10 mM 1,5-gluconolactone in the resolution mixture (△).

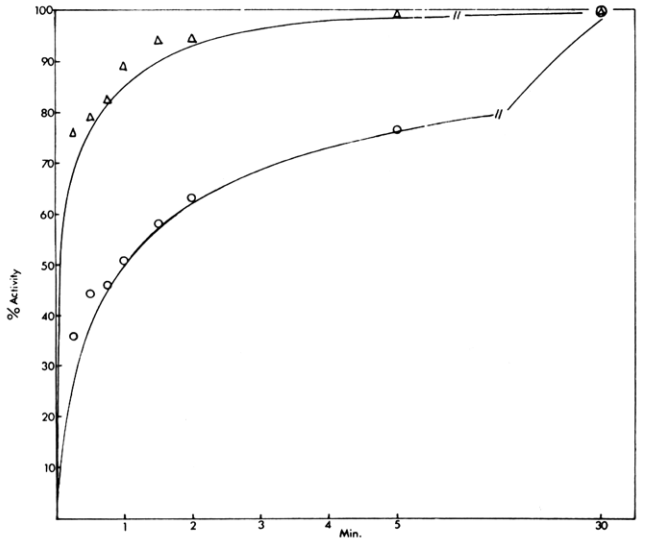


FIGURE 9: Effect of 1,5-gluconolactone on reconstitution of rabbit muscle phosphorylase *b*. Reconstitution was carried out at pH 6.0, 30° with 5 mg/ml of enzyme, in 40 mM glycerophosphate–30 mM β-mercaptoethanol. Aliquots were removed at indicated timed intervals and diluted 75-fold with ice-cold buffer to stop the reaction. Enzymatic assay was at 30°. No additions (○), with 10 mM 1,5-gluconolactone in the reconstitution mixture (△).

pyridoxal 5′-phosphate possesses optical activity, experiments were done to determine whether 1,5-gluconolactone would alter the asymmetry of this site. In this case, as was observed early with experiments with substrates (Johnson and Graves, 1966), 1,5-gluconolactone had no effect on the optical activity enzyme bound pyridoxal 5′-phosphate.

Since rabbit muscle phosphorylase *b* is known to exist in different states of aggregation, ultracentrifugal analysis was carried out to determine if the unusual effect of 1,5-gluconolactone on the kinetics of phosphorylase *b* was related to some change in enzyme conformation. As shown in Figure 10A, the enzyme in the presence and absence of 1,5-gluconolactone sedimented in both cases as single components with $s_{20,w}$ values of 8.1 S. The findings presented in Figure 10B show that

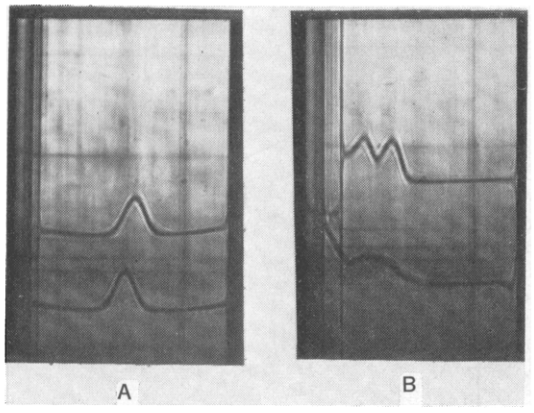
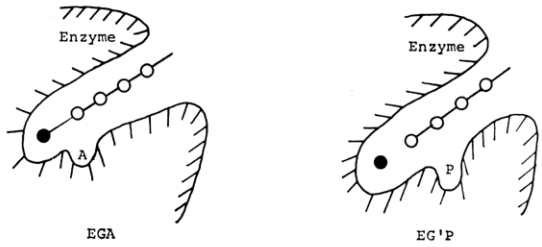


FIGURE 10: Effect of 1,5-gluconolactone on ultracentrifugation patterns of rabbit muscle phosphorylase *b*. Enzyme was centrifuged at 52,000 rpm at 20 ± 1° in 40 mM glycerophosphate–30 mM β-mercaptoethanol. (A) Upper curve: enzyme (5 mg/ml), no additions. Lower curve: enzyme (5 mg/ml), with 20 mM 1,5-gluconolactone. (B) Upper curve: enzyme (4.5 mg/ml) with 1 mM AMP and 100 mM glucose 1-phosphate. Lower curve: enzyme (4.5 mg/ml) with 1 mM AMP, 100 mM glucose 1-phosphate, and 20 mM 1,5-gluconolactone. Direction of sedimentation is to the left.

SCHEME I: Schematic Representation of the Complexes for Enzyme–Glucosyl Polymer–Arsenate and Enzyme–Glucosyl Polymer–Glucose 1-Phosphate.



in the presence of glucose 1-phosphate, AMP, and 1,5-gluconolactone the sedimentation patterns are changed. In the absence of 1,5-gluconolactone (Figure 10B, top) two distinct sedimenting species are present with $s_{20,w}$ values of 4.8 and 9.9 S. Further work is required to determine what molecular forms are represented by these sedimentation patterns. In the presence of 1,5-gluconolactone (Figure 10B, bottom) the polydisperse nature of the enzyme is quite apparent from the considerably broadened sedimentation pattern. Under this condition, the $s_{20,w}$ was determined to be 11.5 S. The different ultracentrifugal patterns indicate that 1,5-gluconolactone affects the conformation of the enzyme. This phenomenon might be important in explaining the nonlinear kinetics displayed in Figures 3 and 7.

Discussion

The possibility that the reaction catalyzed by polysaccharide phosphorylase involves a glucosyl intermediate was investigated in this work. A secondary isotope effect was seen by using 1-deuterio-α-D-glucose 1-phosphate with *E. coli* maltodextrin phosphorylase and rabbit muscle phosphorylase *b*. The extent of this effect was somewhat less than seen with the acid hydrolysis of glucose 1-phosphate which is known to proceed through a carbonium ion mechanism (Bunton *et al.*, 1958). A similar secondary isotope effect has been reported on the reaction catalyzed by lysozyme (Dahlquist *et al.*, 1968) and fumarase (Schmidt *et al.*, 1969), and it has been concluded that both of these reactions have a transition state with considerable positive charge consistent with a mechanism which involves some carbonium ion character. A similar conclusion can be reached for the polysaccharide phosphorylase reaction.

The possibility that the polysaccharide phosphorylase reaction involves a transition state which resembles that of an oxonium ion was further investigated by experiments with 1,5-gluconolactone. This compound was of particular interest because it has been shown to possess a half-chair structure similar to that of the oxonium ion. Therefore, if 1,5-gluconolactone imitates the transition state, it ought to be a strong inhibitor. Kinetic experiments presented in the direction of glycogen synthesis and degradation show that inhibition is highly specific and potent. To facilitate the explanation for the kinetic results, Scheme I is included and shows the binding of polysaccharide for degradation (EGA complex) and for synthesis (EG′P complex). The dark circle in both complexes represents a glucosyl transfer site. If inhibition by 1,5-gluconolactone is attributed to binding at this site, the scheme for synthesis predicts that at infinite glucose 1-phosphate no combination of inhibitor can occur, *i.e.*, the inhibition is competitive. At infinite glycogen concentration, inhibitor can still bind to enzyme and inhibition, therefore, should not be com-

petitive with respect to glycogen. These results were seen in the kinetic experiments (Figures 3 and 5). From Figure 3, a K_I for 1,5-gluconolactone was calculated and found to be six to eight times lower than the K_m for glucose 1-phosphate. An inhibition constant lower than the substrate's Michaelis constant is expected for an inhibitor that effectively mimmicks that transition state. Transition-state analogs have been found for such diverse reactions as the adenosine deaminase reaction (Wang *et al.*, 1963) and Δ^5-3-keto steroid isomerase reaction (Evans and Wolfenden, 1970). In both cases the inhibiting compound binds more tightly to the enzyme than the substrate.

In the direction of glycogen degradation, certain dead-end inhibitor complexes are possible. These could not be formed in the other direction because the glucosyl moiety of glucose 1-phosphate and 1,5-gluconolactone are considered to occupy the same site on the enzyme. Since glycogen can bind to the enzymes as EG and EG′ complexes, and inhibitor can combine with the EG′ complex, inhibition with glycogen in the direction of degradation should not be competitive. The combination of 1,5-gluconolactone with an enzyme aresenate complex also means that inhibition should not be competitive with respect to arsenate. These results were seen in Figures 6 and 7. Thus, the kinetic experiments are in agreement with a model in which 1,5-gluconolactone binds at the glucosyl transfer site.

The exact reason for the nonlinear kinetics exhibited with respect to glucose 1-phosphate and glycogen in the direction of degradation remains to be established. Ultracentrifugation showed that in the presence of 0.1 M glucose 1-phosphate, 1 mM AMP, and 1,5-gluconolactone rabbit muscle phosphorylase *b* is polydisperse. Nonlinear kinetics would be expected for the case in which two or more enzyme conformational states are present if (a) these species have different kinetic properties and (b) the equilibrium between these forms is slowly adjusted in comparison to the catalytic rate. An explanation of negative cooperativity (Conway and Koshland, 1968) might explain the data with rabbit muscle phosphorylase *b*, but it is difficult to reconcile the data with maltodextrin phosphorylase on this basis since this enzyme is believed to be a monomer (Schwartz and Hofnung, 1967). Another possibility is that if under the experimental conditions used, polysaccharide phosphorylase changes from the rapid equilibrium random bi–bi kinetic mechanism to a steady-state mechanism, nonlinear reciprocal plots would be found.

All polyglucose phosphorylases are known to catalyze the phosphorolysis of an α-1,4-polyglucose chain with retention of configuration contain pyridoxal 5′-phosphate (R. H. Abeles, unpublished results); therefore, pyridoxal 5′-phosphate is not required per se for the phosphorolysis of glucosidic bonds to give retention of configuration. The function of pyridoxal 5′-phosphate in α-glucan phosphorylases is yet to be established. If pyridoxal 5′-phosphate is at the active site and participates directly in the catalytic process, it might be expected that a transition-state inhibitor could influence the properties of the pyridoxal 5′-phosphate binding site. 1,5-gluconolactone as reported here was shown to (1) block the resolution of pyridoxal 5′-phosphate from native phosphorylase *b*, (2) retard the reconstitution of apophosphorylase *b* with pyridoxal 5′-phosphate, and (3) block reduction of enzyme-bound coenzyme with $NaBH_4$. No or little effect of substrates have been seen on these properties. It is possible that observed differences between substrates and 1,5-gluconolactone can be related to the steady-state concentrations of various enzyme forms. Because substrates react to form products, it is not unreasonable to believe that concentration of various enzyme species would be different than that in the presence of a transition-state analog. Thus, the use of analogs such as 1,5-gluconolactone could trap a particular enzyme form and provide interesting information about the active site which could not be obtained by using substrates alone. The action of 1,5-gluconolactone might be interpreted as direct interaction of the inhibitor at the pyridoxal 5′-phosphate site. This is the simplest explanation of the data although it is still possible that these results could be brought about by a conformational change due to binding of 1,5-gluconolactone at a site distinct from the coenzyme site.

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Glucose 6-Phosphate Dependent and Independent Forms of Yeast Glycogen Synthetase. Their Properties and Interconversions*

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ABSTRACT: Preparations of glycogen synthetase in the glucose 6-phosphate dependent (D) and independent (I) forms were obtained, respectively, from cells in the logarithmic and stationary phase of growth of a haploid strain of *Saccharomyces cerevisiae*. Some kinetic properties of the two forms were examined. The I form has a partial requirement for glucose 6-phosphate, but the corresponding K_m is 30 times smaller than that of the D form. The latter is much more strongly inhibited by adenosine triphosphate, at low concentrations of glucose 6-phosphate, than the I form. Inverse plots of the rate against uridine diphosphate glucose concentration yield two intersecting lines with both forms, and the two K_m values are similar in either case. The kinetic properties of glycogen synthetase from mutant GS 1-36, which does not accumulate glycogen during growth, appear to be very similar to, if not identical with, those of the D form from the parent strain. Storage of preparations of the D form at −20° resulted, in some cases, in a conversion into a glucose 6-phosphate independent (pseudo-I) form. The pseudo-I form was refractory to inhibition by ATP. The original properties of the D form were restored by incubation with high concentrations of β-mercaptoethanol. Incubation of glycogen synthetase in the I form with a cell-free extract from lyophilized yeast resulted in a decrease of the "ratio of independence" (activity without glucose 6-phosphate × 100/activity with 10 mM glucose 6-phosphate). ATP and Mg^{2+} were required for the conversion. After this treatment, glycogen synthetase showed the expected properties of the D form. Another extract, obtained from yeast spheroplasts, catalyzed the opposite effect, that is, an increase in the ratio of independence. The reaction was stimulated by Mg^{2+} and inhibited by fluoride. By allowing the increase in ratio of independence to progress to a certain extent and then adding ATP and fluoride, it was possible to reverse the effect in the spheroplast extract. The total activity of glycogen synthetase remained constant through all the changes in ratio of independence. It is proposed that the observed effects correspond to interconversions between the I and D forms of glycogen synthetase. Both interconverting systems are present in extracts from logarithmic phase and stationary phase cells, either of the wild-type strain or of the "glycogenless" mutant GS 1-36. Therefore, the factors which trigger unidirectional conversions between the I and D forms *in vivo* remain unknown.

The existence, in muscle and liver, of two forms of glycogen synthetase (UDP-glucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11), which differ in their requirement for glucose-6-P,[1] was documented several years ago by Larner and his coworkers (Larner, 1966). Recently, the two forms were also detected in *Neurospora* (Téllez-Iñón *et al.*, 1969). In earlier studies of the yeast synthetase we found evidence for the presence by only one form, which showed a relatively small stimulation of glucose-6-P, in the absence of allosteric inhibitors (Rothman and Cabib, 1967a). However, recent results (Rothman-Denes and Cabib, 1970) indicate that the response of yeast glycogen synthetase to glucose-6-P depends on the stage of growth at which the cells are harvested. Thus, early and middle logarithmic phase cells yield enzyme in the glucose-6-P-dependent (D) form, whereas the glucose-6-P-independent (I) form predominates in early stationary phase cells. These results were obtained with a haploid strain of *Saccharomyces cerevisiae*, αS288C.

These findings opened the possibility of studying the

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[1] Abbreviations used are: glucose-6-P, glucose 6-phosphate; D, glucose 6-phosphate dependent form of glycogen synthetase; I, glucose 6-phosphate independent form of glycogen synthetase; UDP-glucose, uridine diphosphate glucose; *RI*, ratio of independence (for definition, see text).