Kinetic Mechanism of Rabbit Muscle Glycogen Phosphorylase α*

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SUMMARY

Isotope-exchange rates at chemical equilibrium were determined for the glycogen-α-D-glucopyranose 1-phosphate-Pi system in the presence of phosphorylase α. Exchange of 32P from α-D-glucopyranose 1-phosphate (glucose-1-P) into Pi and exchange of 14C from glucose-1-P into glycogen were followed simultaneously by the use of glucose-1-P containing both isotopes. Concentrations of glucose-1-P and Pi were varied together in their equilibrium ratio at constant glycogen concentration, and the concentration of glycogen was varied at fixed concentrations of phosphates. Exchange rates for the two isotopes were equal under all conditions (with the possible exception of measurements at the highest concentrations of the phosphates) and gave linear reciprocal plots. One exception was noted in which the reciprocal plot was concave upward at low concentrations of substrate, probably because of allosteric effects. The results support a rapid equilibrium mechanism.

Initial velocity of the reaction in the absence of product was determined by the use of an isotopic assay. The results were characteristic of a sequential mechanism.

Isotope-exchange rates were also determined under non-equilibrium conditions. Exchange of 32P from glucose-1-P into Pi was followed as a function of glycogen concentration at a fixed concentration of glucose-1-P and several fixed concentrations of Pi. The exchange was also followed as a function of glucose-1-P concentration at fixed glycogen concentration and several fixed Pi concentrations. Similar experiments were done while following the exchange from Pi into glucose-1-P. These experiments are equivalent to conventional product inhibition experiments. The results indicate that the two phosphates are noncompetitive inhibitors with respect to glycogen, but are competitive with respect to one another.

Our conclusion is that phosphorylase α has a rapid equilibrium Random Bi-Bi mechanism involving binary complexes of enzyme with glycogen, glucose-1-P, and Pi, and ternary complexes of enzyme with glycogen and glucose 1-P and with glycogen and Pi.

Numerous kinetic studies have been performed on glycogen phosphorylase of rabbit muscle (α-1,4-glucan: orthophosphate glucosyltransferase EC 2.4.1.1); nevertheless, the kinetic mechanism of this enzyme remains unknown. This is partially attributable to the fact that the catalyzed reaction is difficult to subject to ordinary kinetic analysis. Since polysaccharide is both substrate and product, it is impossible to vary its concentration in an initial velocity experiment without changing the concentration of product simultaneously. Product inhibition studies are subject to a similar ambiguity when polysaccharide is considered the product inhibitor; when Pi or α-D-glucopyranose 1-phosphate is the product inhibitor a back reaction occurs which obscures the inhibitory effect. Another source of difficulty lies in the allosteric properties of both phosphorylase b (2, 3) and a (4) which further complicate the kinetic analysis.

The most comprehensive studies of the steady-state kinetics of rabbit muscle phosphorylase were carried out by Lowry, Schula, and Passonneau (5, 6) who limited their work to initial velocity experiments. Their results with phosphorylase α are consistent with a sequential mechanism in which both polysaccharide and phosphate must bind to the enzyme before any products are liberated. This agrees with the findings of Cohn and Cori (7) and Illingworth et al. (8) who observed that phosphorylase a will not catalyze 32P exchange between P1 and glucose-1-P in the absence of polysaccharide. The kinetics of phosphorylase b are complicated by nonlinear reciprocal plots characteristic of allosteric enzymes and no conclusions could be drawn about its mechanism. Lowry et al. and many others have assumed, for purposes of calculation and discussion, that muscle phosphorylase has a rapid equilibrium Random Bi-Bi mechanism (9), although an ordered mechanism in which polysaccharide is the first substrate to bind and last product to dissociate is equally consistent with the evidence.

Maddiah and Madsen (10) have reported a careful study of the kinetics of rabbit liver phosphorylase by means of initial velocity and dead-end inhibition experiments, while Chao, Johnson, and Graves (11) have carried out a thorough investiga-

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1 This is referred to as glucose-1-P in the text.
tion of the kinetic mechanism of the maltodextrin phosphorylase of Escherichia coli by means of initial velocity, dead end inhibition, and equilibrium isotope-exchange experiments. Both groups found strong evidence for rapid equilibrium Random Bi-Bi mechanisms for their respective phosphorylases, although the isotope-exchange data of Chao et al. (11) was not completely consistent with a rapid equilibrium mechanism.

In the present work we have attempted to elucidate the kinetic mechanism of rabbit muscle phosphorylase a by means of equilibrium and nonequilibrium isotope-exchange rate measurements. The evidence clearly indicates a rapid equilibrium Random Bi-Bi mechanism for this enzyme. Following completion of our studies a publication by Engers, Bridge, and Madsen (12) appeared reporting the results of their kinetic experiments on phosphorylase b. Engers et al. came to the conclusion that phosphorylase b also has a rapid equilibrium Random Bi-Bi mechanism on the basis of equilibrium isotope-exchange studies and comparison of kinetic constants with enzyme-substrate dissociation constants determined in independent experiments.

**EXPERIMENTAL PROCEDURE**

**Materials**—Phosphorylase b was prepared from frozen rabbit skeletal muscle (Pel-Freez Biologicals, Inc., type I) by a modification (13) of the method of Fischer and Krebs (14). Phosphorylase a was prepared from three times recrystallized phosphorylase b by the method of Krebs and Fischer (15) using a crude preparation of phosphorylase b kinase (18). The procedure was modified by substituting gel filtration on Bio-Gel P-10 for dialysis of the ammonium sulfate precipitate of phosphorylase a. The phosphorylase a was recrystallized at least three times in the presence of 0.019 M glycerophosphate, 0.012 M dithiothreitol, and 0.1 M NaF.

AMP, ATP, glucose-1-P dipotassium salt, and shellfish glycogen were obtained from Sigma Chemical Company. The glycogen was freed of possible ionic contaminants, such as AMP, by passing a 4% solution through a column of AG 501-X8 mixed-bed resin (Bio-Rad, Richmond, California) and precipitating the glycogen with an equal volume of ethanol.

ATP; labeled phosphoric acid (carrier-free) was purchased from New England Nuclear Corporation and uniformly labeled with tritium by a method developed by Brown and Ochoa (19) using a crude preparation of liver pyruvate kinase and glucose-1-P, 2-deoxyglucose, and ATP as substrates. The preparation was recrystallized at least three times in the presence of 0.019 M glycerophosphate, 0.012 M dithiothreitol, and 0.1 M NaF.

AMP, ATP, glucose-1-P dipotassium salt, and shellfish glycogen were obtained from Sigma Chemical Company. The glycogen was freed of possible ionic contaminants, such as AMP, by passing a 4% solution through a column of AG 501-X8 mixed-bed resin (Bio-Rad, Richmond, California) and precipitating the glycogen with an equal volume of ethanol.

**Methods**—Concentration of phosphorylase a was determined spectrophotometrically at 279 nm using a molar absorption coefficient of 13.0. Glycogen concentration in kinetic runs is expressed as total concentration of glycolyl residues and is based upon dry weight of glycogen. All experiments were carried out at 30° and pH 6.8.

Initial velocity in the direction of glycogen synthesis was determined by estimating the release of 32P-phosphate from isotopic glucose-1-P. All rate measurements were done in 1.0-ml volumes containing final concentrations of 0.025 m potassium maleate, 0.10 mM EDTA, 7.0 mM cysteine, 0.10 mM AMP, 1 µg per ml of phosphorylase a, and glucose-1-P and glycogen as indicated. Each solution contained 5 x 10^6 cpm of [32P]glucose-1-P and sufficient potassium chloride to bring the ionic strength to 0.28 M (calculated using pK₅ 6.1 for glucose-1-P, 6.8 for maleate, and 7.2 for P₁). All components except glycogen were combined in a volume of 0.90 ml and equilibrated for 5 to 10 min before adding 0.100 ml of glycogen to start the reaction. After incubating for a time sufficient to allow up to 3% conversion of isotopic substrate, the reaction was stopped by adding 1 drop of concentrated ammonia and cooling in ice. When all samples were collected, 1 µmole of P₁ carrier was added and the P₁ was extracted by the method of Berenblum and Chain (17). One milliliter of acid molybdate (5% ammonium molybdate in 1 M H₂SO₄) was added and the solution was extracted with 5.00 ml of water-saturated benzene-isobutanol, 1:1. One milliliter of the organic phase was used for liquid scintillation counting.

Blanks were determined by omitting enzyme and treating the solution in the same manner. Total 32P was determined by adding 0.100 ml of the reaction mixture to 1 ml of 0.1 M H₂SO₄, heating at 80° for 30 min to hydrolyze the glucose-1-P, and extracting the P₁ as described.

Nonequilibrium isotope-exchange rates in the direction of glycogen synthesis were determined similarly to initial velocities, with the difference that the reaction mixtures contained varying concentrations of potassium phosphate in addition to the other components. Since the concentration of P₁ exceeded that which could be extracted as described above, it was necessary to remove 0.200-ml aliquots of the quenched solutions for treatment with 1 ml of acid molybdate and 3.00 ml of benzene-isobutanol. Because of the small aliquots taken, 4 times the concentration of isotopic substrate, the reaction was stopped by adding 1 drop of concentrated ammonia and cooling in ice. When all samples were collected, 1 µmole of P₁ carrier was added and the P₁ was extracted by the method of Berenblum and Chain (17). One milliliter of acid molybdate (5% ammonium molybdate in 1 M H₂SO₄) was added and the solution was extracted with 5.00 ml of water-saturated benzene-isobutanol, 1:1. One milliliter of the organic phase was taken for liquid scintillation counting.

Nonequilibrium isotope-exchange rates in the direction of glycogen breakdown were determined by estimating the synthesis of [32P]glucose-1-P from P₁-phosphate. The procedure was similar to that used for the synthetic reaction, except that potassium phosphate was substituted for glucose-1-P and 6 x 10^6 cpm of [32P]phosphoric acid was substituted for the isotopic glucose-1-P. Because of the high phosphate concentrations, 0.200-ml aliquots were taken for treatment with 1.00 ml of acid molybdate. The solutions were extracted twice with 4 ml of benzene-isobutanol, taking care to remove as much organic solvent as possible each time, and 0.200 ml of the aqueous phase was taken for liquid scintillation counting.

Nonequilibrium isotope-exchange rates in the direction of glycogen breakdown were determined similarly to the initial velocities with the difference that the reaction mixtures contained varying concentrations of nonisotopic glucose-1-P in addition to the other components. In all cases above, suitable blanks were run and total 32P was determined.

Equilibrium isotope-exchange rates were determined by measuring the initial rates of 14C and 32P exchange. All reactions were carried out at final concentrations of 0.020 m potassium maleate, 15 mM cysteine, 1 to 3 µg per ml of phosphorylase a, and AMP, glucose-1-P, P₁, and glycogen as indicated. P₁ and glucose-1-P were always present in the equilibrium ratio of 3.56:1, respectively. Sufficient potassium chloride was present to keep the ionic strength constant in a particular experiment, but experiments were carried out at different ionic strengths. All compon-
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30° for 5 min before adding 0.050 ml of a mixture of 14C- and 32P-labeled glucose-1-P. Four aliquots were taken at appropriate time intervals (the first immediately) and quenched by addition to 2.0 ml of 0.1 N ammonia. The radioactivity of 32P-phosphate was determined with 1.00 ml of the quenched aliquot as described above. Specific activity of glycogen was determined with the remaining solution. Phosphates were removed by passing the solution through a small column (0.6 x 5 cm) of AG 1-X8 chloride (200 to 400 mesh) resin (Bio-Rad, Richmond, California) and washing with 1 to 2 ml of water. A portion of the combined eluate was used for liquid scintillation counting while the remainder was used to determine the concentration of glycogen. High concentrations were determined by means of optical rotation at 323 nm. Low concentrations were determined in triplicate by making the solution 0.1 N in HCl and hydrolyzing in an autoclave at 125° for 45 min. Samples were contained in screw-capped test tubes with Teflon cap liners. After neutralizing the solutions with NaOH, the concentration of free glucose was determined with Glucomat reagent. In both methods, a stock solution of glycogen was used as a standard. The hydrolytic method yielded 92% of the theoretical amount of glucose based on dry weight of glycogen.

Total radioactivity of 14C and 32P in the doubly labeled glucose-1-P was determined by first hydrolyzing an aliquot of the glucose-1-P in the presence of an accurately known amount of carrier and then subjecting it to the procedures described above. The AG 1 eluate was analyzed directly with Glucostat reagent. In both methods, a stock solution of glycogen was used as a standard. The hydrolytic method yielded 92% of the theoretical amount of glucose based on dry weight of glycogen.

Results

The simplest types of kinetic mechanism that might apply to a polysaccharide phosphorylase have been considered by Chao et al. (11) in their studies on the maltodextrin phosphorylase of E. coli. Four mechanisms they enumerated are rapid equilibrium Random Bi-Bi; Ordered Bi-Bi, with polysaccharide the first substrate to bind and last product to dissociate; Ordered Bi-Bi, with polysaccharide the last substrate to bind and first product to dissociate; and Bi-Bi Ping Pong. The last two of these mechanisms may be eliminated as possibilities for phosphorylase a on the basis of work presently in the literature. Initial velocity experiments reported by Lowry et al. (5) exclude the last two mechanisms since both give rise to nonlinear reciprocal plots when polysaccharide is the variable substrate. Furthermore, the last mechanism gives linear reciprocal plots when phosphate is the varied substrate but increasing the concentration of polysaccharide increases the slope of the plot, opposite to what has been observed. Isotope-exchange experiments of Cohn and Cori (7) and Illingworth et al. (8) exclude a Ping Pong mechanism. A distinction between the first two mechanisms can be made on the basis of isotope-exchange rates in the presence of all reactants.

Initial Velocity—Measurements of reaction rates in this work were generally done at high ionic strength (0.25 M) so that the ionic strength could be kept constant while the glucose-1-P and P, were varied up to high concentrations. In this way substrate or product inhibition could be detected without interference from changing ionic strength. Since the most reliable values for the kinetic constants of phosphorylase a were determined at low ionic strength (5) we repeated some of these initial velocity experiments under our experimental conditions, both to arrive at more applicable values for the kinetic constants and to test our isotopic assay method. Results for the glycogen synthesis reaction in the presence of AMP are shown in Figs. 1 and 2, while data for the degradative reaction are shown in Figs. 3 and 4.

These results are qualitatively similar to those reported by Lowry et al. (5) although the values of the kinetic constants differ greatly in some cases. The pattern of lines intersecting at a point to the left of the vertical axis is characteristic of a sequential reaction mechanism and is represented by the initial velocity equation

\[ v = \frac{V_1AB}{K_{d0} + K_d A + K_s B + AB} \]  

Equation 1 refers to the synthetic reaction in which A is polysaccharide and B is glucose-1-P. A similar equation holds for the degradative reaction in which P, representing P, replaces B. Kinetic constants derived from the data are presented in Table I.

Duplicate experiments carried out at different times afforded kinetic constants which differed from one another by as much as a factor of 2, even though the standard errors were much smaller within each experiment. The basis of this variability may lie in the chemistry of the enzyme. It is known that phosphorylases
FIG. 1. Phosphorylase a activity as a function of glycogen concentration at fixed concentrations of glucose-1-P. AMP is 0.10 mM. Concentration of glucose-1-P: 10 mM, O; 4.0 mM, ●; 2.5 mM, □; 1.4 mM, ■; 1.0 mM, ♦; 0.60 mM, △.

FIG. 2. Phosphorylase a activity as a function of glucose-1-P concentration at fixed concentrations of glycogen. AMP is 0.10 mM. Glycogen concentration: 24.7 mM, O; 0.617 mM, ●; 0.420 mM, □; 0.308 mM, ■; 0.185 mM, ♦. The data is the same as in Fig. 1.

\( a^2 \) and \( b \) contain two extraordinarily reactive sulfhydryl groups in each identical subunit and that substitution of these sulfhydryl groups in phosphorylase b leads to large changes in the kinetic constants. Furthermore, it has been our experience that phosphorylase b rarely contains the full complement of reactive sulfhydryl groups and that the reactive sulfhydryl content declines with the age of the enzyme despite all precautions to maintain them in a reduced state. If these considerations are valid with respect to phosphorylase b they could explain the variability in the kinetic constants. No other investigators have reported similar variability; it may be that our conditions (high ionic strength or the absence of a protective protein such as bovine serum albumin) promote expression of the variability in some way.

If the data in Table I are applied to the Haldane relationship in Equation 5, the equilibrium constant is calculated to be 1.8, compared with a value of 3.56 from direct measurement. This is fair agreement in view of the observed variability of the kinetic constants. The other Haldane relationship in Equation 6 gives equivalent agreement since it is not independent. A more serious discrepancy exists with respect to the values of \( K_{a} \) determined in the synthetic and degradative reactions. The...
TABLE I

Kinetic constants for phosphorylase a from initial velocity experiments

The conditions are pH 6.8, 30°, ionic strength 0.28 M, 0.10 mM AMP. The limits shown are standard error. The constants are derived from data in Figs. 1 through 4.

<table>
<thead>
<tr>
<th>Glycogen synthesis</th>
<th>Glycogen degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a = 0.15 \pm 0.05$ mM</td>
<td>$K'_a = 1.7 \pm 0.4$ mM</td>
</tr>
<tr>
<td>$K_{ia} = 0.56 \pm 0.07$ mM</td>
<td>$K_{ia} = 2.0 \pm 0.6$ mM</td>
</tr>
<tr>
<td>$K_b = 2.7 \pm 0.2$ mM</td>
<td>$K_b = 4.0 \pm 0.8$ mM</td>
</tr>
<tr>
<td>$K_{ag} = 10.1 \pm 2.5$ mM</td>
<td>$K_{ag} = 4.7 \pm 2.0$ mM</td>
</tr>
<tr>
<td>$V_1 = 0.026 \pm 0.001$ m min⁻¹ mg⁻¹</td>
<td>$V_2 = 0.021 \pm 0.002$ m min⁻¹ mg⁻¹</td>
</tr>
</tbody>
</table>

*Calculated from Equation 3.
*Calculated from Equation 4.

**Fig. 6.** Equilibrium isotope-exchange rates as a function of glycogen concentration at a fixed concentration of glucose-1-P and P_i. AMP concentration is 1.0 mM, glucose-1-P is 0.099 mM, P_i is 0.352 mM, and ionic strength is 0.23 M. ¹⁴C exchange (glucose-1-P into glycogen), O; ³²P exchange (glucose-1-P into P_i), ●.

**Fig. 7.** Equilibrium isotope-exchange rates as a function of glycogen concentration at a fixed concentration of glucose-1-P and P_i. AMP concentration is 1.0 mM, glucose-1-P is 0.279 mM, P_i is 0.99 mM, and ionic strength is 0.17 M. ¹⁴C exchange (glucose-1-P into glycogen), O; ³²P exchange (glucose-1-P into P_i), ●.

The kinetic mechanism deduced in this work requires that $K_{ia}$ be the same regardless of the direction in which the reaction is followed; in fact, the constant differs significantly when determined in the two types of experiment. It is tempting to attribute this difference to the variability in the constants, but the difference is relatively large and the ranges of values observed in several experiments of each type do not overlap. No satisfactory explanation can be offered at the present time.

**Equilibrium Isotope Exchange**—Considerable information about the kinetic mechanism of a reaction can be obtained by determining the rates of isotope exchange among the reactants when the reaction is at chemical equilibrium. The theory has been developed in detail by Boyer (19) and Boyer and Silverstein (20). In the case of muscle phosphorylase there are only two exchange reactions that can be followed, exchange of ³²P between glucose-1-P and P_i and exchange of ¹⁴C between glucose-1-P and glycogen. Exchange of ³²P would be expected to follow a normal first-order approach to equilibrium but the ¹⁴C exchange should not follow simple kinetics because of the structural heterogeneity of the terminal chains of glycogen and because the redistribution of glucose residues that takes place under the influence of phosphorylase should “bury” labeled residues in the polyglucose chains where they are less readily available for exchange. Both predictions were verified experimentally; the ¹⁴C exchange rate falls off faster than the ³²P exchange rate which follows the expected first-order course. This problem was circumvented by measuring the initial rates of both exchange reactions and by limiting the extent of ¹⁴C exchange to no more than 5% of the calculated concentration of terminal glucose residues.

The result of an experiment in which glucose-1-P and P_i were varied together at constant glycogen and AMP concentration is shown in Fig. 5, while Figs. 6 and 7 illustrate the results obtained when the glycogen concentration was varied at two fixed concentrations of glucose-1-P and P_i in the presence of AMP. Similar experiments performed in the absence of AMP are shown in Figs. 8 to 10.
A striking feature of the data is the similarity of the rates for $^{14}$C and $^{32}$P exchange in all cases, with the possible exception of those measured at the highest concentration of phosphates (Fig. 5). Another important feature is the linearity of all the reciprocal plots at high substrate concentrations. The curve in Fig. 8 is nonlinear at low substrate concentration, probably because of allosteric effects. There is no evidence for inhibition of either of the exchange reactions at high concentrations of variable substrates.

These results strongly support a rapid equilibrium mechanism in which the rate-limiting step is interconversion of central complexes. Since all exchanges must go through the same rate-limiting step they will have the same rate under all conditions. An ordered mechanism in which polysaccharide is first substrate to bind and last product to dissociate requires that at high concentrations of phosphates $^{14}$C exchange be inhibited while the $^{32}$P exchange continues to rise to a maximum. The alternative ordered mechanism requires inhibition of both $^{14}$C and $^{32}$P exchange at high polysaccharide concentration. In either ordered mechanism the two exchange rates would not be equal except at low substrate concentrations.

If the kinetic mechanism is accepted to be rapid equilibrium, then the form of the initial velocity equations requires the existence of binary complexes of enzyme with glucose-1-P, P$_i$, and polysaccharide and ternary complexes of enzyme with glucose-1-P and polysaccharide and with P$_i$ and polysaccharide. The simplest random mechanism that can describe the phosphorylase reaction is shown in Fig. 11. This mechanism leads to the rate equation

$$v = \frac{V_1AB - V_1AP/K_{ap}}{K_{a0}K_b + K_{b0}A + K_{b}B + A + (K_{ip} + A)P/V_1K_{ap}}$$

where the Michaelis constants are dissociation constants of the designated ligand from a ternary complex and the inhibition constants are dissociation constants of the binary complexes. $K_{a0}$ and $K_{a}'$ are Michaelis constants for polysaccharide in the synthetiotic and degradative reactions respectively; only one $K_{a0}$ is required. Several relations between the constants are apparent.

$$K_{a0}K_b = K_{b0}K_a$$

$$K_{a0}K_p = K_{p0}K_a'$$

$$K_a = \frac{V_1K_p}{V_1K_{ip}}$$

$$K_a' = \frac{V_1K_p}{V_1K_{ip}K_{a0}}$$
In the rate equation for a rapid equilibrium mechanism the first numerator term over the denominator expresses the total reaction flux in the forward direction, while the second numerator term over the denominator expresses the flux in the reverse direction. The rate equation for isotope-exchange in the direction of glycogen synthesis under any concentration conditions can, therefore, be derived by simply dropping the second numerator term in Equation 2 (20, 21). For experiments at chemical equilibrium, P can be eliminated from the equation by substitution of $BB_{eq}$, an identical rate equation can be derived by the method of Boyer and Silverstein (20). Rearranging the resulting equation for analysis of data obtained at constant phosphates and varying polysaccharide concentration gives

$$\frac{1}{v} = \left(\frac{1}{V_1} + \frac{1}{V_2} + \frac{K_a}{BV_1}\right) + \frac{1}{A} \left(\frac{K_a}{V_1} + \frac{K_c}{V_2} + \frac{K_aK_c}{BV_1}\right)$$

while the corresponding equation for constant polysaccharide concentration and varying phosphates is

$$\frac{1}{v} = \left(\frac{1}{V_1} + \frac{1}{V_2} + \frac{K_a}{BV_1}\right) + \frac{K_c}{BV_1} \left(1 + \frac{K_a}{A}\right)$$

These equations qualitatively describe the equilibrium isotope-exchange data presented in this work. Since the equations are quite complex it is not very rewarding to test them quantitatively against the experimental results without having kinetic constants of high reliability; however, it is possible to compare the exchange rate at saturating substrate concentrations, $V_{exch}$, with the value predicted on the basis of $V_1$ and $V_2$, which have much greater reliability than the other kinetic constants. Fromm, Silverstein, and Boyer (22) have pointed out the relationship, $V_{exch} = V_1V_2/(V_1 + V_2)$, which holds for a rapid equilibrium Random Bi-Bi mechanism and this equation may be verified for the present case by inspection of Equations 7 and 8. Using the constants in Table I, $V_{exch} = 0.0117 \text{ M min}^{-1} \text{ mg}^{-1}$. The equilibrium isotope-exchange experiment in Fig. 5 is the only one from which $V_{exch}$ can be evaluated since it is the only one done at a high concentration of fixed substrate in the presence of AMP. The intercept gives $V_{exch} = 0.0112 \text{ M min}^{-1} \text{ mg}^{-1}$, in excellent agreement with the theoretical value.

**Nonequilibrium Isotope Exchange**—Isotope-exchange rates in this system are direct estimates of the total reaction flux in one direction or the other. Consequently, we may circumvent the problem arising from back reactions in product inhibition studies when glucose-1-P or P$_i$ are the inhibitors. The rate equation describing the initial velocity of $^{32}$P exchange from glucose-1-P into P$_i$ may be derived by dropping the second numerator term in Equation 2. This equation may then be rearranged to express the reciprocal exchange rate as a function of reciprocal glycogen concentration at a fixed concentration of glucose-1-P and various fixed concentrations of P$_i$.

$$\frac{1}{v} = \frac{1}{V_1} \left(1 + \frac{K_c}{B}\right) \left(1 + \frac{P}{K_p(1 + B/K_a)}\right)$$

or to express the reciprocal exchange rate as a function of reciprocal glucose-1-P concentration at a fixed concentration of glycogen and various fixed concentrations of P$_i$.

$$\frac{1}{v} = \frac{1}{V_1} \left(1 + \frac{K_a}{A}\right) + \frac{K_c}{BV_1} \left(1 + \frac{K_a}{A}\right)$$

Inorganic phosphate is seen to be a competitive inhibitor of glucose-1-P and a noncompetitive inhibitor of polysaccharide. A completely analogous pair of equations can be derived for inhibition of the degradative reaction by glucose-1-P. This differs from the usual case of a rapid equilibrium Random Bi-Bi mechanism in which all product inhibition is competitive, in the absence of "dead end" complexes. The difference is due to the fact that glycogen is both a substrate and a product, thus an AP term appears in the rate equation for isotope-exchange in the direction of glycogen synthesis and gives rise to an intercept effect in the reciprocal plot when glycogen is the varied substrate.

Figs. 12 to 14 present data for three of the four types of product inhibition experiments described, all in the presence of AMP. It is notable that in certain cases the net reaction flux
is opposite to the flux in the direction measured, sometimes by a large margin. These measurements confirm the prediction that the phosphates are competitive inhibitors with respect to one another, while both are noncompetitive with respect to polysaccharide.

Since the competitive nature of the inhibition by $P_1$ with respect to glucose-1-P is critical for the conclusions that will be drawn, it is necessary to have an objective criterion by which to judge whether the intersection point in the reciprocal plot is indeed on the vertical axis. This was done by determining the intercepts and slopes of the individual lines by means of a computer program described by Cleland (18) for a hyperbolic function. Table II shows the results of three similar experiments carried out at different concentrations of polysaccharide. It appears reasonable to conclude that the lines in each experiment have a common intercept on the vertical axis, within the precision of the experiments.

**DISCUSSION**

The foregoing experiments are consistent with a rapid equilibrium Random Bi-Bi mechanism and a rate equation of the form of Equation 2 for rabbit muscle phosphorylase a. One discrepancy is apparent in Fig. 5 where the $^3$H exchange is slightly slower than the $^4$P exchange at high concentrations of phosphates. It is possible that the difference is due to a systematic error at the high concentrations of phosphates used here. However, greater differences have been observed with other enzymes (11, 22) that appear to have rapid equilibrium random mechanisms. In these cases the effect is interpreted to indicate that interconversion of central complexes is not completely rate-limiting. Cleland (23) has stated that theoretical studies in his laboratory show that most random mechanisms will resemble rapid equilibrium mechanisms in initial velocity and product inhibition experiments; only isotope-exchange rates can accurately distinguish a true rapid equilibrium mechanism. None of our other data show significant differences in the exchange rates but none of these were collected at such high glucose-1-P and $P_1$ concentrations.

**Table II**

Intercepts and slopes for reciprocal plots from nonequilibrium isotope-exchange experiments

Glucose-1-P is the variable substrate, glycogen is present at a fixed concentration, and AMP is present at a concentration of 0.10 mM. Conditions are the same as in Table I. Limits shown are standard error.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$P_1$</th>
<th>Intercept</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 24.6 mm glycogen</td>
<td>0</td>
<td>30.7 ± 2.8</td>
<td>0.068 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20.4 ± 1.7</td>
<td>0.141 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.4 ± 0.4</td>
<td>0.191 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>19.9 ± 2.6</td>
<td>0.277 ± 0.020</td>
</tr>
<tr>
<td>2. 12.3 mm glycogen</td>
<td>0</td>
<td>72.9 ± 5.4</td>
<td>0.115 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>68.8 ± 5.4</td>
<td>0.225 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>76.9 ± 7.8</td>
<td>0.247 ± 0.037</td>
</tr>
<tr>
<td>3. 0.60 mm glycogen</td>
<td>0</td>
<td>35.2 ± 2.0</td>
<td>0.048 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36.3 ± 4.8</td>
<td>0.110 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>38.5 ± 9.5</td>
<td>0.139 ± 0.062</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>43.0 ± 1.1</td>
<td>0.275 ± 0.008</td>
</tr>
</tbody>
</table>

The apparent competitive relation between glucose-1-P and $P_1$ in the nonequilibrium isotope-exchange experiments is evidence for the lack of $ABP$ and $BP$ terms in the denominator of the rate equation. The presence of a $BP$ term is excluded by Experiment 3 in Table II, in which the competition is studied at low glycogen concentration. The presence of an $ABP$ term is excluded by all the experiments in Table II, since it would produce a nonequivalent effect regardless of the glycogen concentration. Similarly, an $ABP$ term would give rise to substrate inhibition in the equilibrium isotope-exchange experiments when phosphates are the varied substrates. A term containing $BP$ would have the same effect only at low glycogen concentrations, which were not studied. Absence of an $ABP$ term, which represents a quaternary complex of enzyme, glucose-1-P, $P_1$, and glycogen, and a $BP$ term, which represents a ternary complex of enzyme, glucose-1-P, and $P_1$, is a reasonable basis for concluding that there is only one kinetically significant site in each enzyme subunit capable of binding glucose-1-P or $P_1$ at the concentrations used in these experiments.

No evidence was found for substrate inhibition in any of our experiments, in agreement with the results of Lowry et al. (5). This excludes terms involving $A^2$, $B^2$, or $P^2$ and provides suggestive evidence that there is only one site for binding glucose-1-P or $P_1$. If separate sites for the two ligands existed it is likely that substrate inhibition would result from $P_1$ binding to the glucose-1-P site and possibly glucose-1-P binding to the $P_1$ site.

Alternate kinetic mechanisms for the phosphorylase reaction are rapid equilibrium Ordered Bi-Bi with dead end complexes. For example, the upper branches in Fig. 11 with dead end enzyme-glucose-1-P and enzyme-$P_1$ complexes would give a rate equation identical with Equation 2. Similarly, the lower branches in Fig. 11 with a dead end enzyme-glycogen complex would give the same rate equation. These mechanisms cannot be distinguished from a rapid equilibrium Random Bi-Bi mechanism by steady-state methods, as long as the rapid equilibrium condition holds.

Our kinetic model includes only one enzyme-glycogen complex although it seems logical to assume that two such com-
plexes can exist. A complex of enzyme and glycogen in which the latter is bound as a glucosyl acceptor (EA<sub>γ</sub>) should function in the synthetic reaction, whereas a complex with glycogen bound as a glucosyl donor (EA<sub>δ</sub>) should function in the degradative reaction. A rate equation based on two such complexes is identical with Equation 2. However, K<sub>ia</sub> now becomes a function of two intrinsic dissociation constants so that it represents the macroscopic dissociation constant of the total enzyme-glycogen complex. If K<sub>1</sub> and K<sub>2</sub> represent the intrinsic dissociation constants of the two enzyme-glycogen complexes, then K<sub>ia</sub> = K<sub>1</sub>K<sub>2</sub>/(K<sub>1</sub> + K<sub>2</sub>). Michaelis constants for glucose-1-P and P<sub>1</sub> also become macroscopic dissociation constants. These two models cannot be distinguished by steady-state kinetic methods, by equilibrium binding studies, or by combinations of the two. Dead end complexes, such as EA<sub>δ</sub>B or EA<sub>δ</sub>P, will complicate the interpretation of the kinetic constants in terms of intrinsic dissociation constants. In view of these observations we have used the simplest model which describes the data, the case involving only one enzyme-glycogen complex. It must be clearly understood that the kinetic constants are macroscopic dissociation constants and are not meant to represent intrinsic dissociation constants.<sup>3</sup>

Phosphorylase a has been shown to have allosteric properties but a low allosteric constant (4). Therefore, all of our experiments carried out in the presence of AMP would be expected to show no allosteric effects and relate only to the active form of the enzyme. In the absence of AMP and at low substrate concentrations we would expect to see sigmoid substrate saturation curves. The only case in which this is observed is the equilibrium isotope-exchange experiment shown in Fig. 8. The points in the reciprocal plot are linear at high concentration of phosphates but form a distinct upward concave curve at lower concentrations. Our conclusions are not affected by the allosteric properties since they are based upon results obtained under conditions where the enzyme is completely in the active form.

It is interesting to note that the most widely discussed models for allosteric enzymes, those of Monod, Wyman, and Changeux (24) and Koehland, Nemethy, and Filmer (25), assume rapid equilibrium mechanisms and thereby avoid some of the complications that can arise in mult-substrate reactions.

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