Effects of Substrates and a Substrate Analog on the Binding of 5′-Adenylic Acid to Muscle Phosphorylase *a**

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ABSTRACT: The method of dialysis equilibrium was used for measurements of the binding of radioactive adenosine 5′-monophosphate (5′-AMP) to phosphorylase *a*. Two forms of phosphorylase *a* were distinguished, a form designated as T which binds 5′-AMP poorly and a form designated as R which binds 5′-AMP strongly. The binding of the nucleotide to phosphorylase *a* can be modified by a variety of factors: substrates, a substrate analog (glucose), aging of the enzyme, changes in ionic strength and in the composition of the buffers. Increased or decreased ligand affinity of phosphorylase *a* is thought to reflect a displacement of the T $\rightleftharpoons$ R equilibrium. Among the substrates, glycogen and glucose 1-phosphate (glucose-1-P) are mainly responsible for a shift in this equilibrium toward the R state, whereas P_i alone is not effective in glycerophosphate buffer, but has some effect in imidazole buffer. There is an upper limit for the binding of 5′-AMP to phosphorylase *a* with a value for K_{diss} of 1×10^{-6} M. This limit is approached at low ionic strength without any additions or high ionic strength in the presence of substrates. A lower limit of the binding affinity of the enzyme for 5′-AMP is approached when glucose is added in amounts which inhibit the enzyme. Glucose cannot be displaced by P_i or glycogen, but when glucose-1-P is added in equimolar concentration, high binding affinity of the enzyme for 5′-AMP is restored. In kinetic experiments the K_m value of each of the three substrates of phosphorylase *a* was decreased by the addition of 5′-AMP. Another effect of addition of 5′-AMP was to convert sigmoid substrate saturation curves in the presence of glucose to hyperbolic saturation curves. A computer programmed to calculate best fits to experimental curves indicated large differences in the values for L', the ratio of T:R of the protein, for curves with glucose plus 5′-AMP as compared to curves with glucose alone. In the absence of any ligands the value L_0 of the ratio T:R was in the limits from 3 to 13. These results allow the description of phosphorylase *a* as an allosteric enzyme of a class where the T form has more affinity for the inhibitor glucose and the R form has greater affinity for the activator 5′-AMP and for substrates. Morever, the enzyme exists in the absence of reactive ligands to a considerable extent (8% or more) in the R or active form. In general, the binding and kinetic data in conjunction with ultracentrifugal analysis show that both the tetramer *a* and the dimer *a* can undergo T $\rightleftharpoons$ R transitions.

The role of adenylic acid in the activation of phosphorylase has been studied recently in several laboratories (Helmreich and Cori, 1964a,b; Lowry *et al.*, 1964b, 1967; Madsen, 1964; Madsen and Shechosky, 1967; Morgan and Parmeggiani, 1964; Ullman *et al.*, 1964; Buc, 1967). Results of kinetic studies suggested that the modifier, 5′-AMP,[1] activates rabbit skeletal muscle phosphorylases *b* and *a* by increasing the affinity of the enzyme for each of its substrates (glycogen, P_i, and glucose-1-P). These effects are reciprocal since increasing the concentrations of substrates over a wide range increases *pari passu* the affinity of the enzyme for 5′-AMP. On the basis of the Monod model one would propose that 5′-AMP promotes an allosteric transition from a form of the enzyme with poor affinity to a form with high affinity for substrates (Monod *et al.*, 1965). This paper provides further evidence in support of this view. It is difficult to study kinetically the mechanism of allosteric transitions with an enzyme catalyzing a two-substrate reaction because it is not possible to study the effect of one substrate in the absence of the other. A different approach was therefore chosen. This involved the measurement of binding of 5′-AMP to phosphorylase in the presence and absence of substrates using the method of dialysis equilibrium. Phosphorylase *a* was used in this work. Recently, the binding of 5′-AMP to phosphorylase *b* has been measured using the Sephadex gel filtration technique (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data).

The present data show that interactions do occur between substrate binding sites and the nucleotide binding site. However, these effects are strongly modified by the nature of the buffer ions, the ionic strength, and glucose, a substrate analog. It may be visualized that phosphorylase *a* exists at high ionic strength (*i.e.*,

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[1] Abbreviations are: AMP, adenosine monophosphate; glucose-1-P, glucose 1-phosphate.

$\Gamma/2 > 0.1$ as in its natural environment) in a conformationally restrained form with low affinity for 5′-AMP. Using the nomenclature proposed by Monod *et al.* (1965) this form is designated as the tight or T conformation. Addition of substrates promotes transition to a more relaxed conformation with high affinity for 5′-AMP. This form will be referred to as the R form. In glycerophosphate buffer glycogen and glucose-1-P played a primary role in bringing about the T → R transition. The orthophosphate anion seemed only to cooperate with glycogen presumably through the formation of glucose-1-P and had no effect alone. In imidazole buffer an effect of P_i alone could be demonstrated.

Methods

The following experimental procedure was designed with the aim of obtaining data with an over-all precision comparable to that of kinetic measurements.

Binding of 5′-AMP. Nucleotide binding was studied by dialysis equilibrium using radioactive 5′-AMP as ligand. For these experiments, Plexiglass containers custom made by United Engineering Co., St. Louis, were used. Each of the two chambers separated by a semipermeable membrane had a volume slightly greater than 1 ml. The sealed containers were tested for leaks by submerging them in water at a pressure of $^1/_{500}$ atm. Two types of dialysis membranes were used: Nojax casings and Metricel protein-enrichment membranes with a pore size of 75 A. Circles of 1.25-in. diameters were cut out of Nojax casings. The protein-enrichment membranes are commercially available in suitable sizes (1-in. diameter). The Nojax membranes were thoroughly washed for a day with several changes of distilled water under continuous stirring. The protein-enrichment membranes did not require such prolonged washing. Both types of membranes were then soaked for at least 6 hr at room temperature in the buffer used for the experiments. After inserting the membrane between the chambers and attaching both parts with screws, 1 ml of a buffer solution containing radioactive 5′-AMP was delivered into one chamber with a pipet with polyethylene tubing attached to its tip. The concentration of 5′-AMP was varied over a 30-fold range from 9×10^{-7} to 3×10^{-5} M. This was done by keeping the amount of radioactive AMP constant but changing the amount of nonradioactive AMP. Since the other chamber was filled with the same volume of the buffer solutions containing the enzyme but no 5′-AMP, the actual concentration of 5′-AMP in contact with the enzyme was reduced by one-half. The enzyme concentration ranged from 1.0 to 0.9 mg/ml with the exceptions stated. The use of radioactive ligands of high specific activity made possible the use of relatively small amounts of protein.

The buffer solutions differed in concentration and composition in different series of experiments. The pH was kept constant at 6.8 with the exception of the experiments with Tris-acetate buffer (pH 7.5). All solutions contained from 0.2 to 2 mM Na_2EDTA and from 0.1 to 1 mM thiol reagents in the form of 2-mercaptoethanol or dithiothreitol. When a nondiffusible substance, *i.e.*, glycogen, was used, it was added in equal concentrations to both chambers. Additional information is given in the legends to tables and figures. After the chambers were filled and the containers sealed tightly, they were mounted on a wheel which could accommodate 24 containers. The aluminum wheel was turned by an electric motor with a constant speed of 3 rpm.[2] Dialysis was carried out at room temperature (23–24°). The time allowed for equilibration was in no case less than 18 hr. At this time the concentrations of radioactive nucleotide were equal in both chambers as shown in control experiments without the enzyme. Loss of 5′-AMP by adsorption to the membrane and to the walls of the Plexiglass chamber was also estimated and was found to be negligible (less than 1%). The influence of the "Donnan effect" on the equilibration of the nucleotide in the presence of enzyme protein was studied with [^{14}C]2′,3′-AMP. This nucleotide does not bind specifically to rabbit skeletal muscle phosphorylase (Madsen and Cori, 1957). For each series of experiments, especially those with low ionic strength solutions ($\Gamma/2 < 0.1$), identical controls were set up with [^{14}C]2′,3′-AMP instead of [^{14}C]5′-AMP. In every instance, the same amount of radioactivity was found in both chambers at the end of dialysis. This was also the case in a control experiment with [^{14}C]5′-AMP and phosphorylase *a* which had been heat inactivated by incubation at 60° for 5 min.

All experiments were run simultaneously in duplicate or triplicate. After equilibrium was attained, two aliquots (0.05 ml) were removed from each chamber and delivered into 20-ml glass counting vials.[3] Hyamine 10X (1 ml) was added and the contents were mixed thoroughly. Following addition of 10 ml of a toluene solution containing the scintillator (Bray, 1960), the vials were counted in a Packard Tri-Carb liquid scintillation spectrometer Model 4000. Experimental samples were at least 100 times background.

Analyses of aliquots from both chambers allow one to check the reliability of the method. Measurements based on aliquots from the "outside" chamber, that is, the one without the enzyme, indicate changes in the concentrations of free 5′-AMP only, whereas the "inside" chamber with the enzyme gives the concentration of free plus bound 5′-AMP. If no loss occurs during dialysis, calculations based on the analyses of the outside chamber must give the same results as those of the inside chamber.

Calculations. Apparent association constants (K_{ass}) or dissociation constants ($K_{diss} = 1/K_{ass}$) and the apparent number of binding sites (n) may be calculated using a mass law binding expression in the following form

$$r/C_{AMP} = K_{ass}(n - r) \quad (1)$$

[2] This apparatus was custom made by United Engineering Co., St. Louis, Mo.

[3] Packard Instrument Co., La Grange, Ill.

where r represents the average number of 5′-AMP molecules bound to each molecule of phosphorylase a, n is the apparent number of available equivalent binding sites on each molecule of phosphorylase a, and C_{AMP} is the concentration of unbound 5′-AMP. A discussion of the assumptions underlying this type of treatment may be found in several review articles (Scatchard, 1949; Klotz, 1953).

Plots of r/C_{AMP} *vs.* r are shown in Figure 1. The intercept on the abscissa is equal to n and the slope is equal to K_{ass}. The best straight line was drawn using the method of least squares. In addition, several series of experiments were further analyzed using the Sips distribution function (Sips, 1948; Karush, 1962). Rearranging eq 1 and taking logs of both sides yields

$$\log r/(n - r) = a \log C_{AMP} + a \log K \qquad (2)$$

The slope constant (a) is introduced and is usually referred to as the heterogeneity index. If $a = 1$, all binding sites are equal and have the same association constant. Thus, eq 1 is valid. However, in some cases where the association constants show considerable dispersion about the average value, $a \neq 1$. For several series of experiments the value of "a" was calculated from a plot of log $r/(n - r)$ against log C_{AMP} using the method of least squares to draw the best fit straight line. The fit of the data to the linear regression was computed as a correlation coefficient which ranged from 0.868 to 0.999. In 14 replicate experiments using the same enzyme preparation the standard deviation of the K_{ass} values from their mean was ±8.2%. Calculations were based on an average molecular weight of 495,000 for phosphorylase a.[4] Use was made of computer facilities for the evaluation of the data. Calculations were performed with the IBM computer, Model 7072.

Ultracentrifugal Analysis. Analyses of phosphorylase a in the ultracentrifuge were carried out with the Spinco Model E ultracentrifuge. We are greatly indebted to Miss C. Lowry for carrying out these measurements.

Enzyme Activity and Kinetics. Routine assays were carried out at 30° with 0.002% phosphorylase a and 25 mM glucose-1-P, 1% glycogen, and 3 mM 5′-AMP in the glycero-P buffer described below. Enzyme stability was tested by activity measurements before and after dialysis. No change in activity occurred on prolonged dialysis including experiments in imidazole–acetate buffer. The effect of glucose on the kinetics of the phosphorylase a reaction was determined as follows. When glucose-1-P was the variable, incubations were carried out at 30° with 0.2% glycogen with and

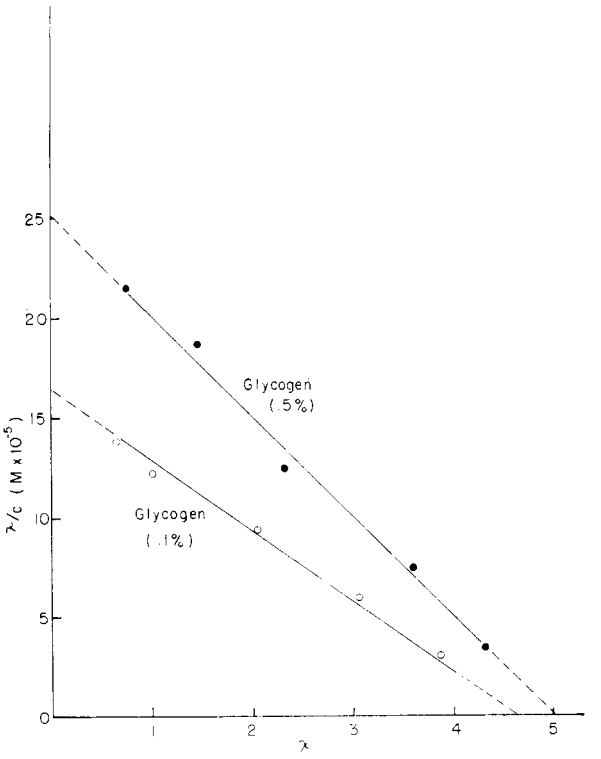

FIGURE 1: A plot of the binding of 5′-AMP to phosphorylase a according to an equation given in the text.

without 1 mM 5′-AMP and 50 mM glucose. In one experiment used for computer analysis the concentration of 5-AMP was 0.02 mM. The buffer was 25 mM sodium β-glycerophosphate (pH 6.8), 0.5 mM Na_2-EDTA, and 1 mM mercaptoethanol. At different times during the early and nearly linear part of the reaction aliquots were withdrawn and delivered into acid ammonium molybdate solution. P_i was determined according to Fiske and Subbarow (1925). With P_i glycogen or 5′-AMP as variable ligands, the phosphorylase reaction was measured in a coupled assay system in the direction: glycogen + P_i → glucose 1-P → glucose-6-P, with the aid of phosphoglucomutase, glucose-6-P dehydrogenase, and TPN. The assay conditions were as described previously except that the temperature was 30° and the pH was 7.5 (Helmreich and Cori, 1964a). The buffer was 45 mM Tris-acetate. Glucose when added was 50 mM. When the concentration of one component was varied the concentration of the other reactants was kept constant (glycogen, P_i, and 5′-AMP at 0.2%, 20 and 1 mM, respectively). In some experiments bovine serum albumin (0.1%) was added. Linear initial rates of the reaction were monitored with a Photovolt recorder using an automatic sample changer attachment for the Zeiss PMQ II spectrophotometer. Velocities are expressed as micromoles of product formed per milligram of phosphorylase a per minute.

Materials. Two different [^{14}C]5′-AMP preparations were used: [^{14}C]5′-AMP uniformly labeled and [8-

[4] Recent determinations of the molecular weight of rabbit skeletal muscle phosphorylase a by sedimentation equilibrium yielded a value of 370,000 (Fischer *et al.*, 1967). Independent measurements made in this laboratory with frog skeletal muscle and with rabbit skeletal muscle phosphorylase a gave similar values (B. E. Metzger, L. Glaser, and E. Helmreich, unpublished data).

^{14}C]5′-AMP. Both preparations were obtained from Schwarz BioResearch, Inc. They had a specific activity of 150 and 21 mc/mmole, respectively. Uniformly labeled [^{14}C]5′-AMP was further purified by chromatography on Dowex AG 1-X8 followed by gradient elution with formate. After removal of formate by evaporation *in vacuo*, the dry nucleotide preparation was taken up in water and kept frozen. [^{14}C]2′,3′-AMP was also a product of the Schwarz Co. Its specific activity was 44 mc/mmole. Nonradioactive 5′-AMP was a preparation of the Sigma Chemical Co. These preparations were used without further purification. Rabbit liver glycogen was purchased from Mann Research Laboratories, Inc. It was freed of 5′-AMP by dialysis in the cold for 18 hr against two changes of 0.8 mM acetate (pH 4.0) containing Dowex 1 (acetate). Following dialysis glycogen was precipitated by addition of two-thirds volume of 95% ethyl alcohol and NaCl to a final concentration of 2 mM. The glycogen precipitate was washed twice with absolute alcohol, once with ethyl ether, and dried. It was taken up in water and stored frozen.

"Nojax" dialysis tubing was a product of Union Carbide Co., Food Products Division, Chicago, Ill., and Metricel-type protein-enrichment membranes, lot no. 1480, were products of the Gelman Instrument Co., Ann Arbor, Mich.

Sugar phosphates, TPN, and Sephadex G-25 were obtained from the Sigma Co. Norit A was from Pfanstiehl Laboratories, Inc. Na_2EDTA was a product of Geigy Chemical Co. Dithiothreitol (2,3-dihydroxy-1,4-dithiolbutane) and Dowex AG 1-X8 were purchased from Calbiochem, Los Angeles. Hyamine 10X, the scintillators, 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, were products of the Packard Instrument Co., LaGrange, Ill. Imidazole was obtained from Eastman Kodak Co. It was purified by treatment with charcoal as described by Lowry *et al.* (1964a). All other chemicals were analytical grade. Double-distilled water was used throughout.

Enzymes. Glucose-6-P dehydrogenase and phosphoglucomutase were products of Boehringer & Sons. These enzymes were dialyzed prior to use as described previously (Helmreich and Cori, 1964a). Phosphorylase *a* was prepared from phosphorylase *b* with purified phosphorylase *b* kinase, ATP, and Mg^{2+}. Phosphorylase *b* was prepared from frozen rabbit meat following the procedure of Fischer and Krebs (1958). Phosphorylase *b* kinase was prepared according to a published procedure (Danforth and Helmreich, 1964). The phosphorylase *a* preparations used in the present experiments were at least four-times recrystallized. They were homogeneous in the ultracentrifuge with a s_{20} value of 13.4 S. Phosphorylase *a* was freed of 5′-AMP by chromatography on Sephadex G-25. With later preparations, the enzyme was passed over a Norit A column, instead of passing it over Sephadex G-25. The absorbancy ratio 260:280 mμ was 0.6 for the former preparations and somewhat lower for the charcoal-treated preparations. The activity of different freshly prepared samples of phosphorylase *a* varied between 47 and 73 μmoles of P_i formed per mg of enzyme per min under the conditions of the routine assay described above.

Phosphorylase *a* crystal suspensions were kept in 50 mM glycero-P buffer (pH 6.8), 1 mM Na_2EDTA, and 0.1 mM dithiothreitol at 4° under toluene vapor. For use, aliquots were removed and the enzyme crystals were separated by centrifugation. The supernatant fluid was removed and the crystals were washed once with the buffer used for the experiment, then taken up in the same buffer solution and diluted. Protein concentrations were determined by absorbance measurements at 280 mμ using an extinction coefficient of 11.7 ($1\% \times cm^{-1}$) (Velick and Wicks, 1951). In some cases, the enzyme concentration was determined by the method of Lowry *et al.* (1951) using a calibration curve for phosphorylase *a* against bovine serum albumin as reference standard.

Results

Interactions between Substrate and Nucleotide Binding Sites. Parameters for nucleotide binding were evaluated graphically as described under Methods. A plot obtained recently with a charcoal-treated preparation of phosphorylase *a* is presented in Figure 1. Based on a molecular weight of 495,000 the lines extrapolate to an intercept on the abscissa of $n > 4$ (Figure 1). In an initial series of experiments in which the charcoal treatment was not used, the value of n was about 4, which is too low on the basis of the newly determined molecular weight of phosphorylase *a* of 370,000 (see footnote 4). The recent determinations indicate that 4 moles of 5′-AMP are bound to each 370,000 g of phosphorylase *a*.[5]

Analysis of the binding data (except those with glucose) on the basis of the Sips distribution function gave for 56 different sets of experiments a mean value for the heterogeneity index "*a*" of 0.988 ± 0.039 (standard error of the mean). This indicates that under the conditions studied and in the range of 5′-AMP concentrations used, all four 5′-AMP binding sites of a population of phosphorylase *a* molecules are equivalent and possess equal affinities for the nucleotide.

In the experiments in Table I, the composition of the buffer, the ionic strength, the concentration of Mg^{2+} ions, pH, and temperature were the same as used in kinetic experiments reported previously from this laboratory (Helmreich and Cori, 1964a,b). However, the concentration of enzyme used in binding studies was of necessity 300 times greater than that used in the kinetic experiments. The values for K_{diss} in Table I suggested that the binding of 5′-AMP to phosphorylase *a* was rather sensitive to changes in

[5] In the presence of glucose the tetramer *a* dissociates into two dimers and in some other cases there is uncertainty how much of the enzyme is present as tetramer and as dimer. For this reason the values of n are omitted from the tables. The values of the apparent dissociation constants are not affected by the numerical values assigned to the molecular weight.

TABLE I: The Binding of 5′-AMP to Phosphorylase *a* under Conditions Similar to Those of Kinetic Experiments.[a]

Additions	K_{diss} (M × 10^6)
None	1.6
Glycogen (1%)	1.2
P_i (50 mM)	4.0
Glucose-1-P (50 mM)	2.3[b]
Glycogen + P_i or glycogen + glucose-1-P (50 mM)	1.4

[a] Experiments were carried out in Tris-acetate (45 mM), Mg-Ac (10 mM), 2-mercaptoethanol (1 mM), and Na_2EDTA (2 mM); Γ/2 = 0.08; pH 7.5. The concentration of phosphorylase *a* was 0.9 mg/ml. [b] Amylose chains were formed during long periods of equilibration.

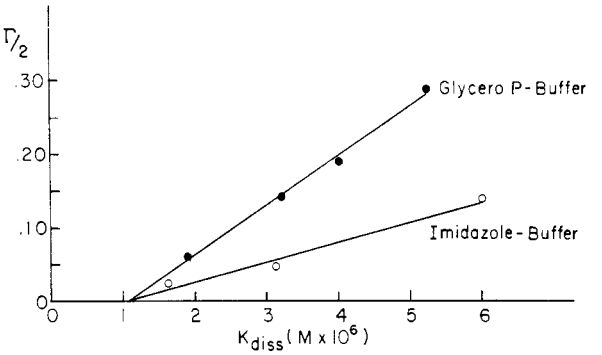

FIGURE 2: A plot of ionic strength of buffer *vs.* K_{diss} of 5′-AMP. The point at Γ/2 = 0.19 was obtained in phosphate rather than glycerophosphate buffer.

ionic strength. Thus, addition of inorganic phosphate (50 mM) which increased the ionic strength from 0.08 to 0.188 caused a 2.5-fold decrease in the affinity of the enzyme for 5′-AMP. At low ionic strength glycogen alone had little effect on binding, but when P_i was added, the adverse effect of high ionic strength on binding could be overcome by glycogen.[6] The effect of glucose-1-P alone was uncertain since amylose chains were formed which probably acted in a manner similar to that of glycogen. It seemed likely that at low ionic strength the intrinsic binding affinity of the enzyme for nucleotide approaches a limit probably set by the conformation of the protein.

Effect of Ionic Strength and Buffer Ions. In order to find out what this limit might be, available data were plotted as shown in Figure 2. It can be seen that K_{diss} is linearly related to ionic strength and that both the glycerophosphate series and the imidazole series extrapolate to a K_{diss} of 1.1 × 10^{-6} M at zero ionic strength. A value of K_{diss} of 1.85 × 10^{-6} M obtained previously in glycerophosphate buffer by Madsen and Cori (1957) with an ultracentrifugal separation method fits the glycerophosphate curve. In addition, the nature of the buffer ion also plays a role. Imidazole at the same ionic strength has a stronger effect on K_{diss} than glycerophosphate (Figure 2).[7] In the experiments in Table II and in all subsequent experiments the ionic strength was kept constant by decreasing the concentration of buffer in proportion to the addition of phosphate or other charged ligands.

Under these conditions P_i alone (10 mM) in glycerophosphate buffer failed to increase the binding of 5′-AMP to the enzyme and in combination with glycogen did not reinforce the effect of the latter, except for a slight effect when P_i was combined with a high (1%) concentration of glycogen (Table II). This is perhaps not unexpected since 0.1 M glycerophosphate might well compete with P_i for binding sites on the enzyme. In fact, glycerophosphate has been shown to inhibit the enzymatic reaction about 66% at the above concentration (Cori *et al.*, 1943). In imidazole buffer, on the other hand, even 1 mM P_i caused a decrease in K_{diss}, and the effect became greater with higher concentrations

TABLE II: Effect of Substrates on Binding of 5′-AMP to Phosphorylase *a* at High Ionic Strength.[a]

Additions	Freshly Prepared Enzyme K_{diss} (M × 10^6)	Aged Enzyme K_{diss} (M × 10^6)
None	5.2	6.5
P_i (10 mM)	6.4	
Glycogen (1%)	1.6	2.3
Glycogen (1%) + P_i (10 mM)	1.1	2.6
Glycogen (0.1%)	2.2	4.4
Glycogen (0.1%) + P_i (10 mM)	2.7	
Glycogen (0.02%)	5.0	
Glycogen (0.02%) + P_i (10 mM)	5.8	

[a] The experiments were carried out in disodium β-glycerophosphate buffer (Γ/2 = 0.29, pH 6.8), 2-mercaptoethanol (1 mM), and Na_2EDTA (2 mM).

[6] Under the conditions of these experiments (*i.e.*, a long period of equilibration) the phosphorylase reaction reached equilibrium with either P_i or glucose-1-P and glycogen. Identical results were therefore expected (and actually observed) with either of the two phosphates and glycogen.

[7] This is in agreement with an observation of Shaltiel *et al.* (1966) that the removal of pyridoxal 5-phosphate from phosphorylase *b* is greatly facilitated by imidazole buffer because, as these authors express it, imidazole has a stronger "deforming" effect on the enzyme than other buffers.

TABLE III: Effect of Orthophosphate on Binding of 5′-AMP to Phosphorylase *a* in Imidazole Buffer.[a]

Additions	$\Gamma/2 = 0.14$ K_{diss} (M × 10^6)	$\Gamma/2 = 0.045$ K_{diss} (M × 10^6)
None	6.0	3.1
Mg-Ac (10 mM)	4.6	
P_i (1 mM)	4.6	
P_i (10 mM)		2.0
P_i (50 mM)	3.6	
Glycogen (0.05%)	4.4	
Glycogen (0.05%) + P_i (1 mM)	2.9	
Glycogen (0.1%)	3.9	2.0
Glycogen (0.1%) + P_i (10 mM)		1.6
Glycogen (0.1%) + glucose-1-P (50 mM)	3.0	

[a] The experiments were carried out in imidazole-acetate buffer (pH 6.8) containing 0.2 mM dithiothreitol and 0.2 mM Na_2EDTA. The concentration of enzyme was 0.9 mg/ml.

of P_i (Table III). Furthermore, the effects of P_i and glycogen were additive. Thus, P_i alone (1 mM) caused a decrease in K_{diss} from 6.0 to 4.6 × 10^{-6} M, *i.e.*, of 1.4, glycogen alone (0.05%) of 1.6, and both combined of 3.1. An effect of P_i alone or in combination with glycogen is also seen in imidazole buffer of low ionic strength (Table III). In kinetic measurements in Tris buffer it was not possible to study the effects of P_i and glycogen on the affinity of the enzyme for 5′-AMP independently from each other. The results in imidazole buffer suggest that P_i alone does have a specific effect on the conformation of the enzyme under certain conditions.[8]

Glycogen, on the other hand, increases binding of 5′-AMP under all conditions tested and its effect is concentration dependent (Figure 3). For curves I and II in Figure 3, one finds that a half-maximal increase in K_{ass} is obtained at about 0.085–0.1% glycogen. This is somewhat higher than expected from kinetic data, which yield K_m values of 0.01–0.02% glycogen, but is of the same order of magnitude as the values for glycogen binding obtained by the ultracentrifugal separation method (Madsen and Cori, 1958). The discrepancy probably arises from the fact that when the enzyme becomes attached to the glycogen surface, the number of available glycogen end groups becomes reduced by steric hindrance (Madsen and Cori, 1958; Metzger *et al.*, 1967).

The observation recorded in Table III that Mg^{2+} ions increase the binding of 5′-AMP is of interest in view of the inclusion of these ions in reaction mixtures for coupled assay of phosphorylase activity (Helmreich and Cori, 1964a). Madsen (1965) concluded from kinetic measurements involving competition between 5′-AMP and ATP that divalent cations (Mg^{2+} and Ca^{2+}) increase the binding of 5′-AMP to phosphorylase *b*. Further work is needed to study the nature of the interaction of Mg^{2+} ions with phosphorylase.

"Aged" Phosphorylase a. It has been reported that aging alters the requirement of phosphorylase *a* for 5′-AMP for activity (*cf.* Helmreich and Cori, 1964a). Crystalline phosphorylase *a* eluted from a DEAE-cellulose column or phosphorylase *a* freshly prepared from phosphorylase *b* by the action of phosphorylase *b* kinase and then passed over Sephadex G-25 do not show any stimulation of activity by 5′-AMP when tested at high substrate concentration. After storage of the crystal suspension under toluene vapor at 4° for varying periods of time, generally several months, the activity ratio, −5′-AMP:+5′-AMP, falls from 1.0 to 0.7 and in some cases even lower. The specific activity of such aged phosphorylase *a* preparations is from 90 to 60% of the freshly prepared enzyme. In a recent study with soluble antibody fragments, it was shown that aged preparations were more susceptible to inhibition than fresh preparations and that the interaction of antibody with enzyme in the former case (but not in the latter) was of the "cooperative" type with a value of *n* for the Hill equation of 2 (Michaelides and Helmreich, 1966). In view of these findings it is of interest that the aged preparations bind 5′-AMP less tightly than the fresh preparations. This is illustrated in Figure 3. The values for K_{ass} obtained with the aged preparation at different concentrations of glycogen are lower than the corresponding ones with fresh preparations. Additional data are shown in Table II, where comparative values for K_{diss} are recorded for a fresh enzyme and for the same enzyme after aging 6 months. In all cases, the aged enzyme has less affinity for 5′-AMP than the fresh enzyme.

Effect of Glucose on Binding of 5′-AMP. In previous experiments (Cori *et al.*, 1943) glucose was found to be a specific inhibitor of phosphorylase. This inhibition could be counteracted by 5′-AMP and in the absence of 5′-AMP by glucose-1-P. In the latter case, plots of $1/v$ *vs.* $1/S$ were concave upwards, whereas plots of $1/v$ *vs.* $1/(S)^2$ were linear, suggesting in the light of present knowledge a cooperative interaction between homologous binding sites (homotropic interactions) (Monod *et al.*, 1965).

It seemed likely that a negative modifier, like glucose, would be useful in a study of binding of 5′-AMP, because it might give a greater spread of K_{diss} values. So far the maximum spread of values observed (*cf.* Figure 2) was from 1 to 6 × 10^{-6} M, where the lower value was assumed to be one limit set by the conformation of the enzyme. The question was what the

[8] On comparing phosphorylase *a* activity in glycerophosphate and imidazole buffer of the same ionic strength one finds that enzyme activity is inhibited 50% in the latter buffer.

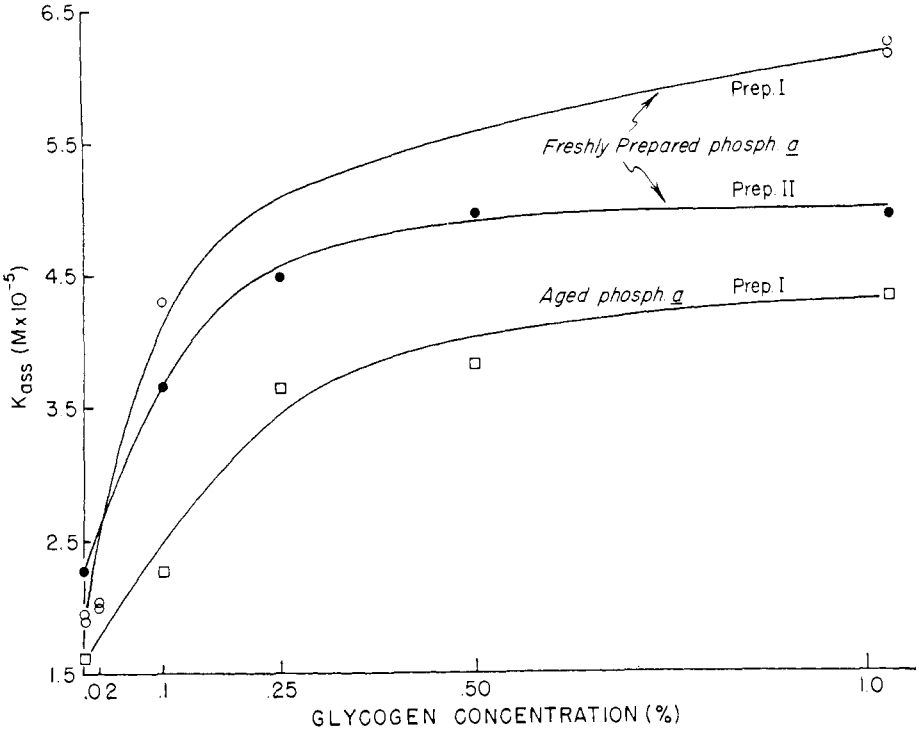

FIGURE 3: A plot of K_{ass} (equivalent to $1/K_{diss}$) *vs.* concentration of glycogen. The experimental conditions are given in Table II. The freshly prepared enzyme I and the aged enzyme are the same as those used in Table II. Enzyme II is a different freshly prepared phosphorylase *a*.

other limit might be. Another aim was to carry out a more detailed study of the kinetics of glucose inhibition as a basis of comparison with the binding data. The results of kinetic measurements are presented in Figures 4–7 and are summarized in Table IV.

In agreement with previous results with phosphorylase *b* (Helmreich and Cori, 1964a), addition of 5′-AMP quite generally results in a decrease in the K_m values of each of the three substrates, glucose-1-P (Figure 4), P_i (Figure 5), and glycogen (Figure 6). With glucose in the absence of 5′-AMP each of the three substrates yields sigmoidal saturation curves (see part B of Figures 4–6). It will be evident that glucose in the absence of 5′-AMP has very large effects on the concentration of each of the three substrates required for half-saturation and relatively little effect on V_{max}. When now 5′-AMP is added, the inhibitory effect of glucose is largely overcome and the double-reciprocal plots assume a linear form. In this case, it is possible to evaluate kinetic parameters as follows, based on the assumption that glucose and glucose-1-P compete for the same site. In Table IV, second line, the slope 2.8/60.0 is equal to $(K_m/V_{max})(1 + I/K_i)$. Taking the K_m of glucose-1-P in the presence of 5′-AMP as 1.8 mM (fourth line in Table IV), the K_i for glucose becomes 90.0 mM. If we now apply the same calculation to line one of Table IV, taking 5.3 mM as the K_m of glucose-1-P in the absence of 5′-AMP, K_i becomes 7.9 mM. A further analysis will be given in the discussion (*cf.* Figure 7).

A kinetic experiment with glucose as the inhibitor and 5′-AMP as the variable is shown in Figure 8. The data have been plotted as per cent of the maximal rate *vs.* the concentration of 5′-AMP rather than as a double-reciprocal plot which would require that the activity without adenylic acid be deducted from each rate measurement. The estimated K_m value is 2.0×10^{-6} M. This is of the same order as the K_m values for 5′-AMP obtained in the absence of glucose. It is assumed that in these experiments the effect of 5′-AMP in overcoming glucose inhibition is exerted indirectly by increasing the binding affinity for substrate. A Hill plot (Brown and Hill, 1922) of the data of Figure 8 for 5′-AMP activation in the presence of glucose gave a slope of $n > 1$ at concentrations of 5′-AMP below 1.66×10^{-6} M whereas at higher concentrations the slope changed to $n = 1$. This suggests that 5′-AMP increases the binding affinity for substrates by shifting the equilibrium toward the R or more active form of the enzyme. Glucose would then act as a substrate analog which is bound to one or the other or possibly both substrate binding sites. Furthermore, the sigmoidal activity response of the enzyme in the presence of glucose suggests that glucose combines preferentially with the T or less active form of the enzyme where it could "fit" a substrate binding

TABLE IV: Kinetic Constants for Phosphorylase *a*.[a]

Variable Ligand	Constant Ligands	Glucose (50 mM)	App K_m Values (mM)	V_{max} (μmoles/mg per min)	Value of *n* from Hill Plots[b]
Glucose-1-P	Glycogen	+	39	60	1.5
Glucose-1-P	Glycogen + 5′-AMP[c]	+	2.8 (2.4)	60	1.1 (1.3)
Glucose-1-P	Glycogen	−	5.3	57	
Glucose-1-P	Glycogen + 5′-AMP	−	1.8	65	
Orthophosphate	Glycogen	+	54	8.3	1.2
Orthophosphate	Glycogen + 5′-AMP	+	1.6	8.3	0.9
Orthophosphate	Glycogen[d]	−	1.4 (1.8)	4.7 (14.8)	
Orthophosphate	Glycogen + 5′-AMP[d]	−	0.6 (0.8)	6.4 (14.8)	
Glycogen	Orthophosphate	+	0.36	6.7	1.3
Glycogen	Orthophosphate + 5′-AMP	+	0.06	6.7	1.0
Glycogen	Orthophosphate	−	0.04	8.8	
Glycogen	Orthophosphate + 5′-AMP	−	0.01	9.1	
5′-AMP	Orthophosphate + glycogen	+	0.002[e]	5.7	>1
5′-AMP	Orthophosphate + glycogen	−	0.001	6.8	1.0

[a] The experimental conditions are described under Methods. The V_{max} values for substrates in the presence of glucose and without 5′-AMP were estimated by drawing tangents to the lines in $1/v$ *vs.* $1/S$ plots as indicated in Figures 4–6. The concentration of 5′-AMP required to give half-maximal activation above the activity without 5′-AMP was estimated from Figure 8. The apparent K_m values in the presence of glucose and without 5′-AMP were estimated from Hill plots (*cf.* Figure 7). The concentrations of reactants and the experimental conditions are given in Methods. [b] Log $v/(V_{max} - v)$ was plotted against log concentration of ligand which yields *n* as the slope. [c] The values in parentheses refer to an experiment with 0.02 mM 5′-AMP (*cf.* Figure 7). [d] The values in parentheses were obtained in glycerophosphate buffer at pH 6.8 which makes the conditions more comparable to those obtaining in the experiments with glucose-1-P as variable ligand (*cf.* Figure 5A). Moreover, V_{max} for the reaction, glycogen → glucose-1-P, is now about one-fourth of that in the reverse direction, as expected for the equilibrium of the phosphorylase reaction. [e] The value for the aged preparation of phosphorylase *a* was 0.003 mM.

site or might even combine with a separate inhibitor site. The inhibition would then be overcome by substrates and activator combining preferentially with the R form of the enzyme. The difficulties in interpreting kinetic data have prompted the following experiments.

First the question was investigated whether glucose competes with glycogen for the same site on the enzyme. This was done with the centrifugal separation technique of Madsen and Cori (1958) using the same sample of phytoglycogen they had used. Phosphorylase *a* (1 mg) was incubated in 1 ml of a solution containing 4 mg of phytoglycogen, 50 μmoles of glycerophosphate (pH 6.8, $\Gamma/2 = 0.14$), 0.5 μmole of 2-mercaptoethanol, and 0.1 μmole of Na_2EDTA for 2 hr at room temperature. In some experiments, 50 μmoles of glucose alone or together with 1 μmole of 5′-AMP were included in the solution. Following preincubation the samples were centrifuged for 20 min at 35,000*g* in a RC2B Sorvall centrifuge at 22–23°. An aliquot (0.35 ml) of the supernatant solution was withdrawn for analysis of protein by the method of Lowry *et al.* (1957). Corrections for a small glucose blank in the Lowry protein method were made. In control experiments with either phytoglycogen alone (molecular weight *ca.* 20 million) or enzyme alone practically all of the glycogen but none of the enzyme was sedimented. When both were present, about 75% of the enzyme was carried down with the glycogen. It was found that glucose displaced only about 10% of the bound protein from glycogen. This makes it unlikely that glucose competes with glycogen for the same site on the enzyme.

Binding experiments with 5′-AMP in the presence of glucose and with two different buffers are shown in Table V. It can be seen that the effect of glucose is

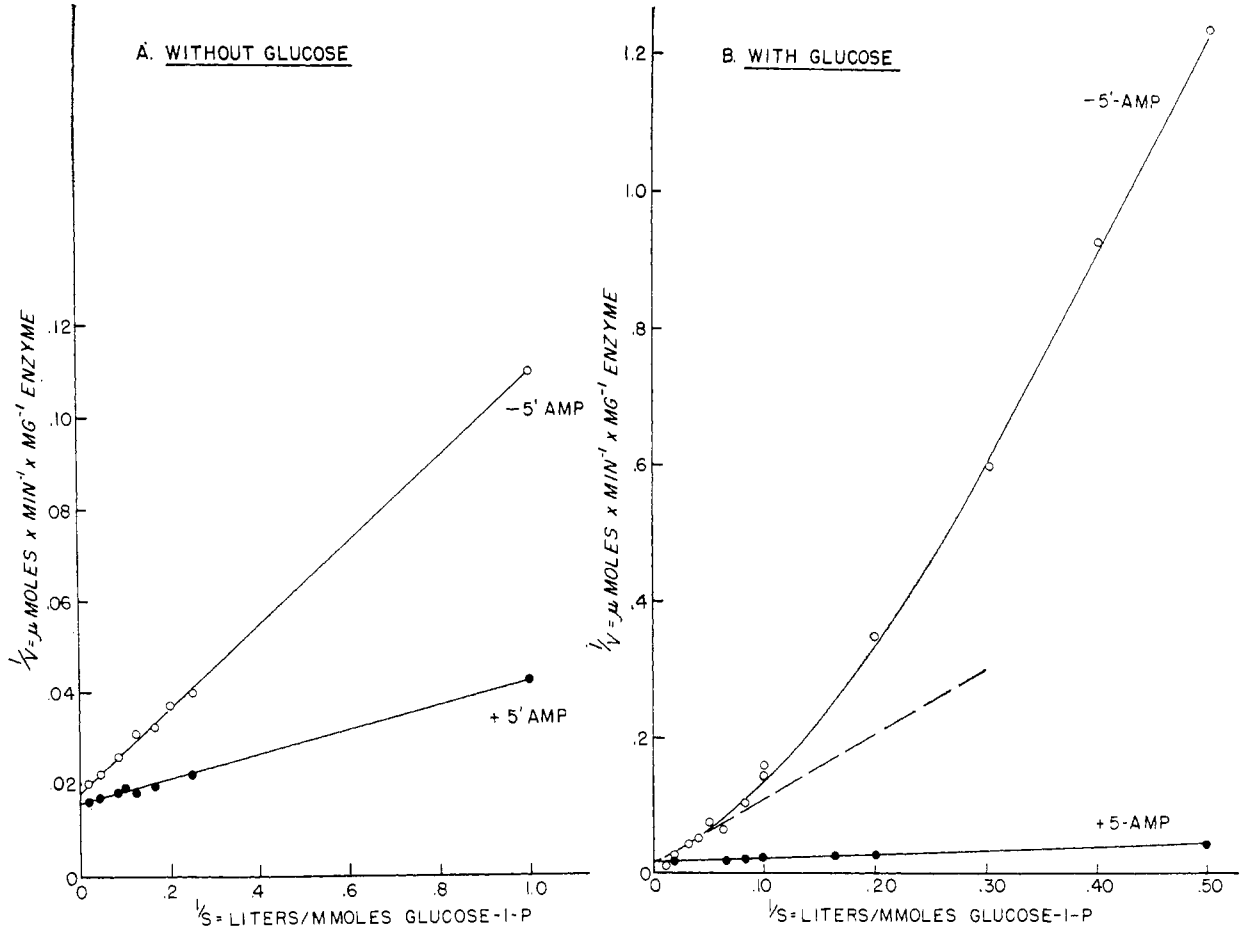

FIGURE 4: Saturation kinetics of phosphorylase *a* with glucose-1-P in the absence and presence of 50 mM glucose without and with 1mM 5′-AMP. The experimental conditions are described under Methods. The kinetic constants are given in Table IV. They were derived from best fit straight lines drawn by the method of least squares. Because of the sigmoidal activity response of the enzyme in the presence of glucose and the absence of 5′-AMP V_{max} was estimated from the tangent to the curve and the apparent K_m values from Hill plots (*cf.* Figure 7).

specific, since galactose in the same concentration has no effect. The affinity of phosphorylase *a* for 5′-AMP is greatly reduced in the presence of glucose when substrates are omitted. In fact, when glycerophosphate buffer was replaced by imidazole buffer of the same ionic strength ($\Gamma/2 = 0.14$), K_{diss} in the presence of glucose became too large for accurate determination by dialysis equilibrium. This effect of imidazole has been commented on before. It now becomes evident that neither glycogen alone nor P_i alone can reverse the effect of glucose on the binding of 5′-AMP. However, when glucose-1-P is either generated from glycogen plus P_i or added as such, the glucose inhibition of 5′-AMP binding is completely overcome (Table V).[9] It was felt, however, that the formation of amylose chains which occurs during long incubation of enzyme with glucose-1-P (Illingworth *et al.*, 1961) made somewhat uncertain the conclusion that glucose combines only with the glucose-1-P site. A better experiment was therefore designed making use of the Sephadex gel filtration technique where the enzyme is in contact with substrate for only 30 min. The data are shown in Table VI.[10] It can be seen that glucose-1-P counteracts the effect of glucose on the binding of 5′-AMP to phosphorylase *a* whereas P_i does not. It is, moroever, of interest that glucose-1-P (in contrast to P_i) also increases the binding of 5′-AMP in glycerophosphate buffer. These differential effects of the two anion substrates are rather unusual. They suggest that the glucose part of glucose-1-P is specifically bound at the active site, as is also suggested by the specificity of glucose inhibition. P_i, on the other hand, may not be bound

[9] In kinetic experiments (*cf.* Figure 8 and Table IV) the effect of glucose on the affinity of the enzyme for 5′-AMP is much smaller, because glucose-1-P which is generated by the enzymatic reaction remains at a finite concentration in spite of the presence of auxiliary enzymes which remove it.

[10] We are indebted to Dr. and Mrs. L. L. Kastenschmidt who kindly performed these experiments.

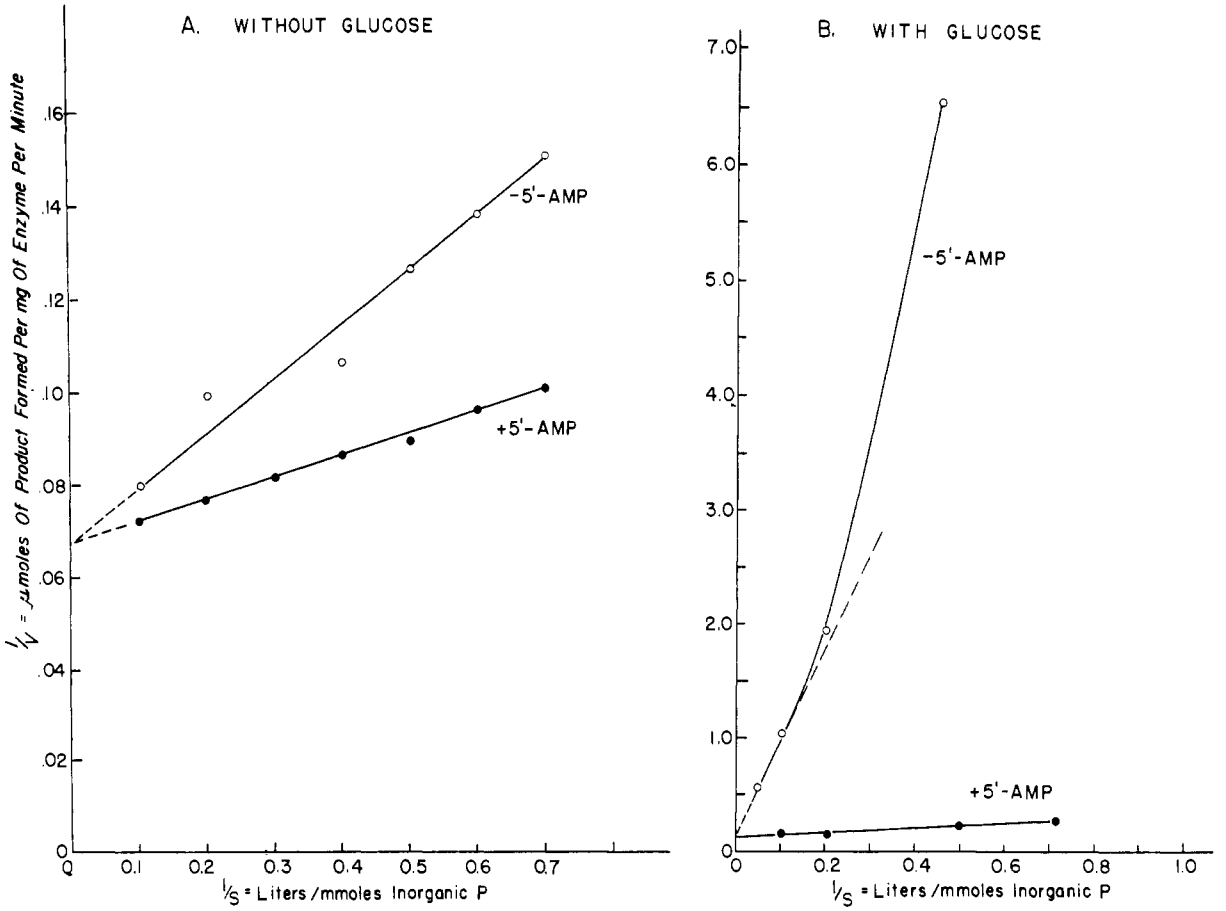

FIGURE 5: Saturation kinetics of phosphorylase *a* with P_i (*cf.* legend to Figure 4). The numerical values of part A are given in parentheses in Table IV. The conditions of these experiments are described in the legend to Table IV.

effectively to the active site unless glycogen is also present, at least in glycerophosphate buffer. More data on the effect of glucose-1-P on the binding of 5′-AMP to phosphorylases *a* and *b* will be reported in a forthcoming publication (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich in preparation).

Tetramer a $\rightleftharpoons$ *Dimer a Transition.* Under certain conditions, experiments with phosphorylase *a* are complicated by a dissociation of the tetrameric form of the enzyme to a dimeric form. Some of the conditions which favor this dissociation are dilution of the enzyme and increase in temperature above 25° (Wang and Graves, 1964). The dimeric form is catalytically more active than the tetrameric form and is stabilized by glycogen (Wang and Graves, 1964; Wang *et al.*, 1965a; Metzger *et al.*, 1967). These effects play no role in the kinetic experiments here reported, since they were conducted at 30° and at such high dilution of the enzyme that practically all of it would be present in the dimeric form.

Table VII contains data on the sedimentation behavior of reaction mixtures closely resembling those used in the binding experiments. In glycerophosphate buffer and in imidazole buffer in a range of ionic strength from $\Gamma/2 = 0.14$ to 0.025 the enzyme under the stated conditions of concentration and temperature is mainly present as a tetramer (expt 1–3). In Tris buffer of low ionic strength containing Mg^{2+} ions (expt 4), corresponding to the experiments in Table I, the s_{20} value of 12.1 S was low for tetramer *a* and the peak was broad and asymmetrical, suggesting that a mixture of tetramer and dimer was present. When 50 mM glucose was added to the enzyme in either glycerophosphate or imidazole buffer of ionic strength 0.14, two distinct peaks were present, the major one corresponding to dimer *a*. This confirms similar observations made by Wang *et al.* (1965b). At lower ionic strength in imidazole buffer the enzyme was almost completely dissociated into dimer when glucose was present. It made no difference in the sedimentation behavior whether the enzyme was preincubated for 1 hr with glucose or was spun immediately after the addition of glucose. Addition of an amount of glucose-1-P equimolar to that of glucose or of a high concentration of 5′-AMP (1 mM) reversed the dissociation (expt 5–6 and 9–10). This was also reported by Wang *et al.* (1965b). Addition of glucose-1-P or glucose-6-P alone did not have an effect on the

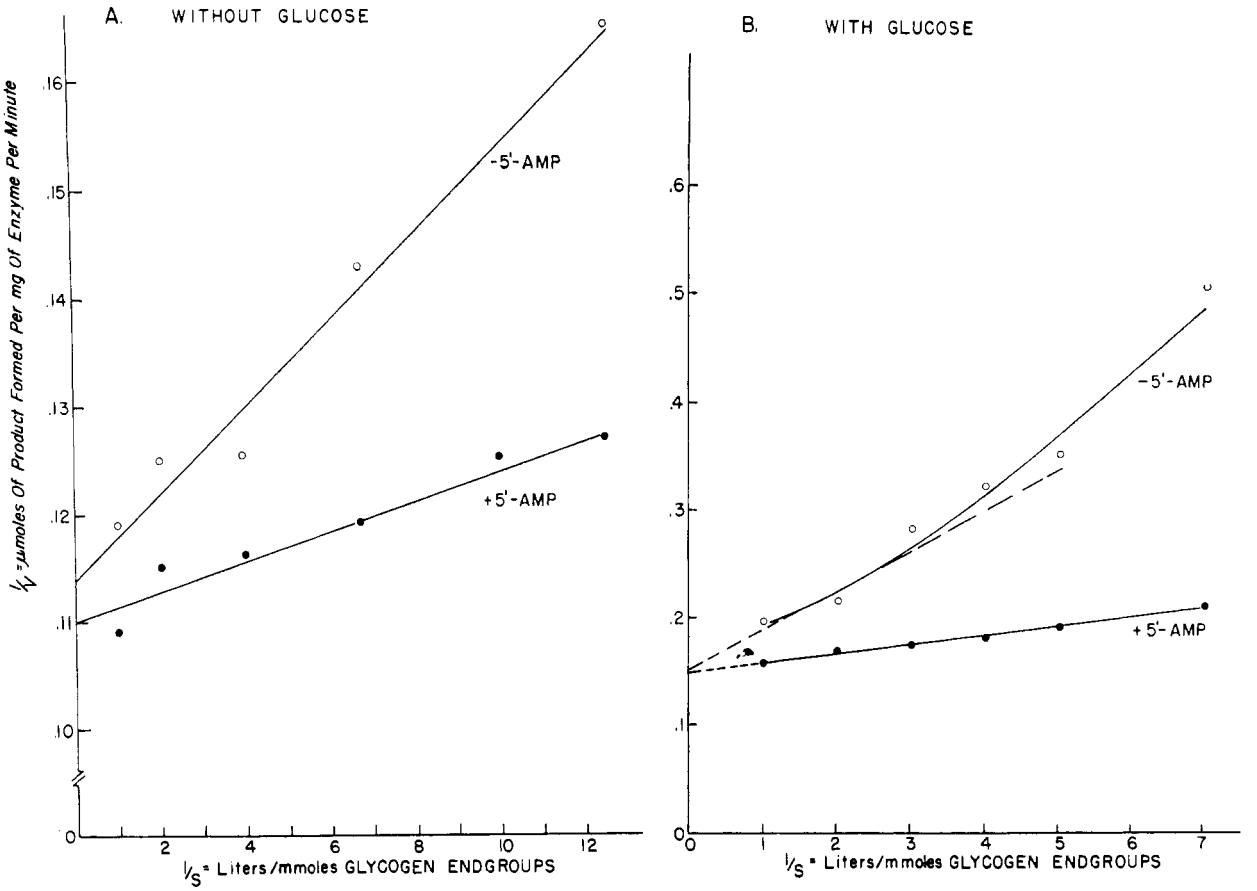

FIGURE 6: Saturation kinetics of phosphorylase *a* with glycogen. The concentration of glycogen is expressed in terms of millimolar concentration of glucosyl residues at the nonreducing end of the glycogen chains (*cf.* legend to Figure 4).

sedimentation pattern of the enzyme (expt 7 and 12). It should be noted here that glucose-6-P has little if any inhibitory effect on phosphorylase *a*, in contrast to its effect on phosphorylase *b* (Morgan and Parmeggiani, 1964).

Discussion

A general scheme of allosteric transitions of phosphorylase *a*, based on observations recorded in this paper, is shown in Figure 9. A change in ligand affinity is interpreted as a displacement of the spontaneous T $\rightleftharpoons$ R equilibrium of the enzyme (Monod *et al.*, 1965). Thus, an increase in ionic strength over the range shown in Figure 2 decreases the affinity of the enzyme for 5′-AMP five- to sixfold under conditions where according to Table VII most of the enzyme is present in the tetrameric form. This is interpreted as a shift in the tetramer equilibrium of the T and R forms as illustrated. Addition of glycogen or of glucose-1-P counteracts the adverse effect of high ionic strength on affinity of the enzyme for 5′-AMP, whereas P_i is without effect in glycerophosphate buffer but has some effect in imidazole buffer. The dimeric form of phosphorylase *a* is present whenever glycogen is added (Wang and Graves, 1964; Wang *et al.*, 1965a; Metzger *et al.*, 1967) In the case of addition of glucose-1-P alone the tetrameric form remains (Table VII) and since glycogen and glucose-1-P are about equally effective in decreasing K_{diss} for 5′-AMP at high ionic strength, it seems probable that the affinity of the tetrameric and dimeric form R of phosphorylase *a* for 5′-AMP is similar. Therefore, association or dissociation linked to the binding of 5′-AMP is not likely to play an important role in the allosteric transitions of phosphorylase *a*. This is also supported by the close agreement between the K_m values for 5′-AMP determined under conditions where the enzyme is present as dimer and the K_{diss} values obtained from binding measurements on the tetrameric form (R) of phosphorylase *a*.

The dimer formed in the presence of glucose is represented as inactive in Figure 9 because of its low binding affinity for 5′-AMP. Reassociation to tetramer takes place on addition of glucose-1-P which competes with glucose and restores binding affinity of the enzyme for 5′-AMP. Neither glycogen nor P_i shows this effect (Tables V and VI). Addition of 5′-AMP in high concentration also leads to reassociation of the dimer formed in the presence of glucose (Table VII).

In kinetic experiments (the situation represented by

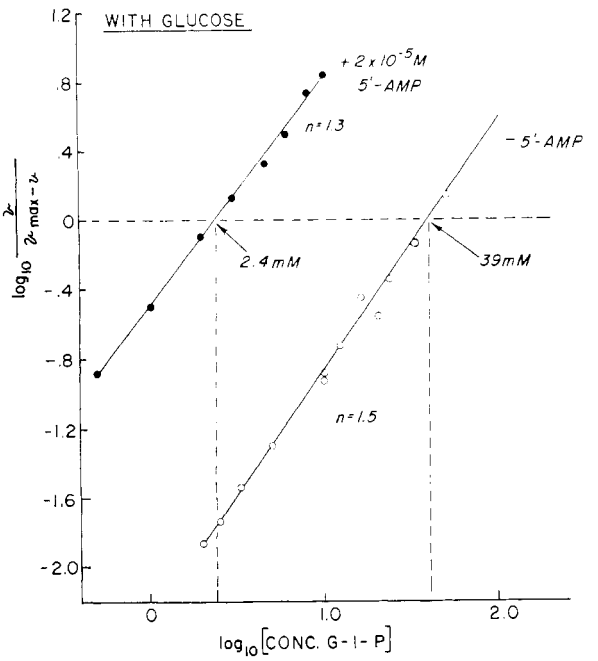

FIGURE 7: Hill plot of the saturation of phosphorylase *a* with glucose-1-P in the presence of glucose with and without 5′-AMP. The experiment without 5′-AMP is shown in Figure 4B. The values for the experiment with 5′-AMP are given in parentheses in Table IV. The 5′-AMP concentration was 0.02 mM and that of glucose 50 mM. The lines were drawn according to the method of least squares.

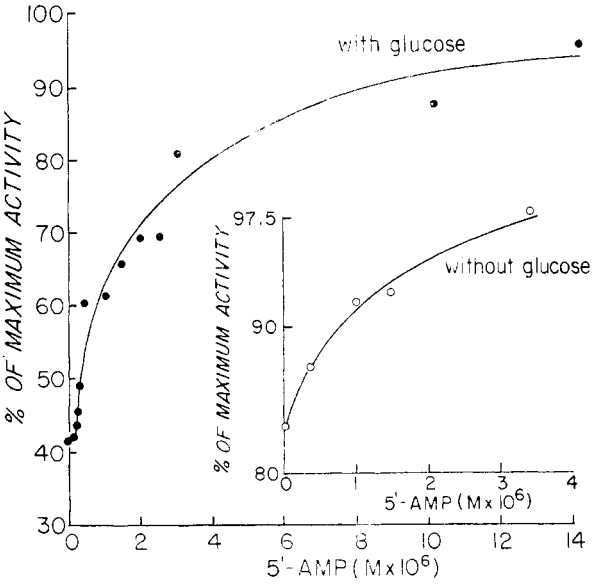

FIGURE 8: Activation of phosphorylase *a* by 5′-AMP in the absence and presence of 50 mM glucose.

the arrows at the bottom of Figure 9), 5′-AMP has a very strong effect in counteracting glucose inhibition. Another effect of 5′-AMP in kinetic experiments is a change in the shape of double-reciprocal plots from concave upwards in the presence of glucose to linear when 5′-AMP is added. Although numerical values for the affinities of the different ligands for the T and R states of the enzyme have only been obtained in the case of 5′-AMP, it is possible to estimate the affinities for substrates and glucose in the manner described below where it is assumed that a negative modifier such as glucose will have greater affinity for the T than for the R state and *vice versa* for the case of the positive modifier 5′-AMP and substrates.

Cases of the Monod model in which the T and R states of the enzyme have nonexclusive ligand binding characteristics have recently been treated mathematically by Rubin and Changeux (1966). The equation

$$\bar{Y} = \frac{\dfrac{\alpha}{1+\alpha} + L_0 c\alpha \dfrac{(1+c\alpha)^{n-1}}{(1+\alpha)^n}\left(\dfrac{1+d\beta}{1+\beta}\right)^n\left(\dfrac{1+e\gamma}{1+\gamma}\right)^n}{1 + L_0\left(\dfrac{1+c\alpha}{1+\alpha}\right)^n\left(\dfrac{1+d\beta}{1+\beta}\right)^n\left(\dfrac{1+e\gamma}{1+\gamma}\right)^n}$$

shown is derived from a general treatment given by Rubin and Changeux.[11]

$\bar{Y}$ is v/V_{max}; L_0 is the ratio T:R of the protein in the absence of specific ligands; n is the number of identical binding sites for each type of ligand; c, d, and e are the ratios of dissociation constants, K_R/K_T, for substrate, inhibitor, and activator, respectively; α, β, and γ are the concentrations of substrate, inhibitor, and activator relative to the respective dissociation constants for the R form of the protein, that is, α = [substrate]/K_{diss} substrate (R). It can be seen in the above equation that if L_0 is zero, $\bar{Y}$ becomes $\alpha/(1+\alpha)$, which corresponds to a Michaelis–Menten type of saturation curve.

The above equation can be rearranged to

$$L_0 = \frac{\dfrac{\alpha}{1+\alpha} - \bar{Y}}{\left(\bar{Y} - \dfrac{c\alpha}{1+c\alpha}\right)\left(\dfrac{1+c\alpha}{1+\alpha}\right)^n\left(\dfrac{1+d\beta}{1+\beta}\right)^n\left(\dfrac{1+e\gamma}{1+\gamma}\right)^n}$$

A computer program was set up for the calculation of an average value of L_0 and of c by the method of minimum–maximum deviations from the experimental curves. The lines shown in the Hill plot (Figure 7) were replotted in the form of $\bar{Y}$ *vs.* α. A K_m for glucose-1-P of 1.8 mM (Table IV) was used for the calculation of α. The computer was programmed to find the value of c for a range from 0.001 to 0.13 and the value of L_0 for a range from 0 to 2500 which gave the best fit to the experimental curve B in Figure 10 for a range of α from 0.25 to 35 in steps of 0.25. The numerical values listed below were used in the computer program:

[11] We are indebted to Dr. Carl Frieden for valuable advice in deriving this equation.

TABLE V: Effect of Glucose on Binding of 5′-AMP to Phosphorylase *a*.[a]

Additions	Glycero-P Buffer K_{diss} (M × 10^6)	Imidazole-Acetate Buffer K_{diss} (M × 10^6)
None	3.2	6.0
Galactose (50 mM)	3.2	
Glucose (50 mM)	11.3	>50
Glucose + glycogen (0.1%)		31.3
Glucose + glycogen (1%)	12.8	
Glucose + P_i (50 mM)	17.6	
Glucose + P_i (50 mM) + glycogen (1%)	1.7	
Glucose + P_i (10 mM) + glycogen (0.1%)		6.6
Glucose + glucose-1-P (50 mM)	1.5	

[a] The ionic strength ($\Gamma/2$ = 0.14) was the same for both buffers, which also contained 0.1 mM dithiothreitol and 0.2 mM Na_2EDTA.

TABLE VI: Effect of Glucose-1-P on Binding of 5′-AMP to Phosphorylase *a*.[a]

Additions (mM)	$\Gamma/2$ = 0.14 K_{diss} (M × 10^6)	$\Gamma/2$ = 0.28 K_{diss} (M × 10^6)
None	4.2	5.5
P_i (50)		7.3
Glucose-1-P (50)	0.8	2.9
Glucose (50)	10.7	
Glucose-1-P (50) + glucose (50)	1.8	

[a] Binding of 5′-AMP was measured with a Sephadex gel filtration technique the details of which are described in another paper (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, in preparation). Glycerophosphate buffers of two ionic strengths were used as indicated. The concentration of 5′-AMP was 6×10^{-6} M and that of the enzyme 3.5–4.8 mg/ml.

n = 2; α = variable; β = 0.33 (based on a K_i of glucose of 150 mM (Figure 7)); d = 19 (this value is derived from the ratio of the apparent K_i values of glucose in the presence of 2×10^{-5} M 5′-AMP and in its absence); γ = 20 (based on a K_m value of 5′-AMP of 1×10^{-6} M (Table IV)); e = 0.08 (this is the value determined experimentally in glycerophosphate buffer in the presence of glucose (*cf.* Table V)). The results recorded by the computer were L_0 = 13 and c = 0.01. The calculated curve (Figure 10B) showed a maximum deviation of about 5% at an α of 30. Thus, the fit to the experimental curve was good up to about 65% saturation of the enzyme. Using a value of c = 0.01 and all the above values the computer found a value of L_0 of 3 for curve A in Figure 10 with a maximum deviation from the theoretical curve of about 3% which occurred at an α of 6. In this case the fit to the experimental curve was good up to about 85% saturation of the enzyme.

The spread of L_0 values from 3 to 13 could be due to the fact that different enzyme preparations were used for the experiments in curves A and B of Figure 10. Although one cannot be certain that K_m or K_i are in all cases equivalent to K_{diss} (R), the use of the same constants should give at least values for the two curves which are comparable. L', the ratio of T:R in the presence of specific ligands, is related to L_0 by the following equation (Rubin and Changeux, 1966)

$$L' = L_0\left(\frac{1+\beta d}{1+\beta}\right)^n\left(\frac{1+\gamma e}{1+\gamma}\right)^n$$

In the present case n = 2 and the first term refers to glucose and the second term to 5′-AMP as ligands. For the curve with glucose and 5′-AMP, L' = 1.4 and for the curve with glucose alone, L' = 390.

The results of the computer analysis lead to the following conclusions. Phosphorylase *a* can be described as an allosteric enzyme with preferential binding of 5′-AMP and of substrates to the R form and of the inhibitor glucose to the T form of the enzyme. Moreover, the enzyme exists in the absence of reactive ligands to a considerable extent in the R or active form (L_0 = *ca.* 8). This could explain why phosphorylase *a* can be maximally catalytically active in the absence of 5′-AMP, under conditions where phosphorylase *b* is not. In fact, recent observations show that phosphorylase *b* in the absence of specific ligands exists mainly in the T form ($L_0 \geq 500$; L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data; Helmreich, 1967). Otherwise, the predominantly dimeric phosphorylase *b* exhibits allosteric properties very similar to those described here for phosphorylase *a*. It therefore seems probable that the T $\rightleftharpoons$ R transitions of phosphorylase (*a* or *b*) involve the same two, presumably symmetrical subunits in the tetrameric and the dimeric state. This idea is also supported by the fact that the binding forces which hold the dimer together are much stronger than those involved in tetramer formation.

Some previous observations deserve comment in the light of the present findings. It was shown that the nearly complete inhibition of phosphorylase *a* or *b* by univalent antibody could be overcome by glycogen plus 5′-AMP but only if they were added prior to the addition of antibody (Michaelides *et al.*, 1964). This suggests that two conformational states of the enzyme could be distinguished by the antibody and that it

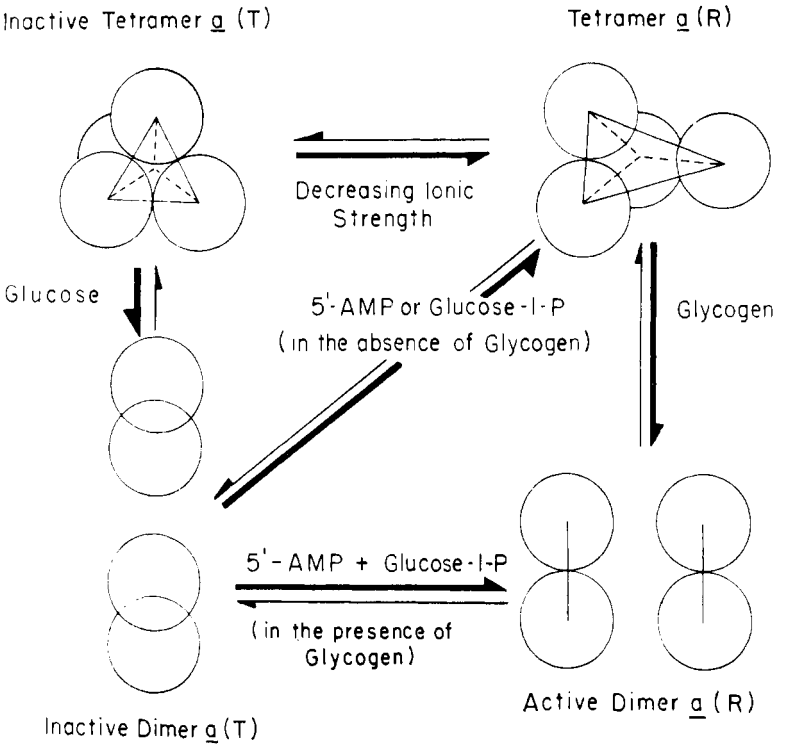

FIGURE 9: The T $\rightleftharpoons$ R transitions of phosphorylase *a*. Only the conditions studied in this paper are considered.

TABLE VII: Sedimentation Velocity Measurements on Phosphorylase *a*.[a]

Expt	Buffer	Ionic Strength	Enzyme Concn (mg/ml)	Temp (°C)	Additions (mM)	$s_{20} \times 10^{13}$ (S) Major Peak	$s_{20} \times 10^{13}$ (S) Minor Peak
1	GP	0.14	3.0	19		13.4	
2	Im	0.14	1.2	24		12.7	
3	Im	0.025	0.97	24		13.8	
4	Tris	0.08	0.90	24		12.1	(8.8)
5	GP	0.14	0.95	22	Glucose (50)	8.4	12.6
6	GP	0.14	0.95	22	Glucose (50) + G-1-P (50)	12.6	
7	GP	0.14	0.95	22	G-1-P (50)	13.2	
8	Im	0.14	1.20	24	Glucose (50)	8.3	12.0
9	Im	0.07	1.0	21	Glucose (50)	7.7	
10	Im	0.07	1.0	24	Glucose (50) + AMP (1)	13.4	
11	Im	0.025	0.97	24	Glucose (50)	9.0	
12	Im	0.14	1.0	22	G-6-P (50)	12.8	

[a] The composition of the buffer, including 7×10^{-6} M 5′-AMP, the enzyme concentration, and the temperature were similar to those used in the binding experiments. A 12-mm cell and a rotor speed of 59,780 was used in all cases. GP refers to glycerophosphate and Im to imidazole.

reacted, like other inhibitors, preferentially with the T form of the enzyme. Interestingly enough, the combination of P_i and 5′-AMP did not afford much protection against antibody inhibition, and glycogen alone or 5′-AMP alone were also relatively ineffective. There is a parallel here to the ineffectiveness of P_i in overcoming the adverse effect of high ionic strength on the binding of 5′-AMP to the enzyme.

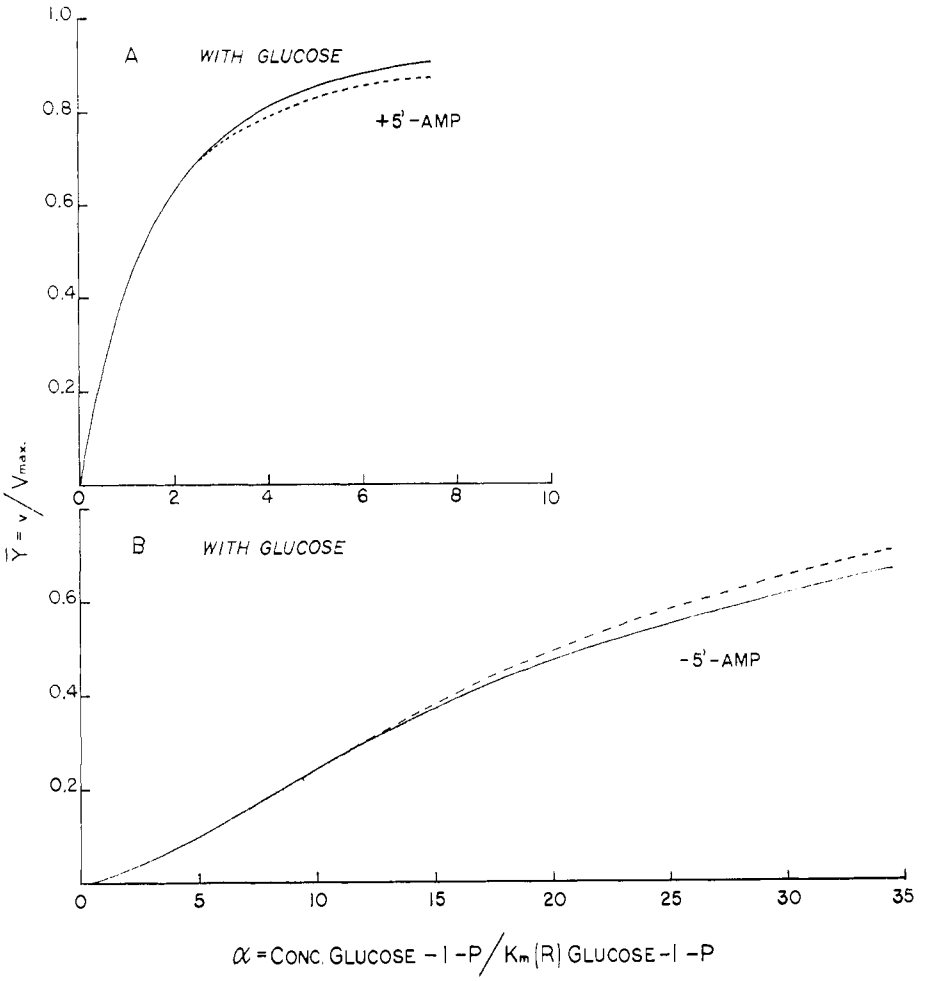

FIGURE 10: The effect of glucose and of 5'-AMP on the saturation of phosphorylase *a* with glucose-1-P. The data from Figure 8 were replotted as $\bar{Y}$ *vs.* α; (——) experimental curve; (- - - -) calculated curve. (A) With 50 mM glucose plus 0.02 mM 5'-AMP. (B) With glucose alone. The calculated curve was drawn on the basis of the equation and the constants given in the text.

Some new information has been gained about the binding sites of phosphorylase. It is known that the maltosidic chain of branched or linear polymers must be at least four glucose units long to become effective as acceptor of glucose units from glucose-1-P. It is also known that the binding site for α-glucose-1-P is highly specific, since no other sugar phosphate is known to react (Brown and Cori, 1961). Inhibition by glucose is also specific, the α form being more inhibitory than the β form (Cori and Cori, 1940). It was not known, however, at which site glucose exerted its inhibitory effect. The present experiments show that glucose-1-P is the only substrate which can overcome the glucose inhibition of binding of 5'-AMP. This mutual antagonism suggests that the glucose moiety, either free or combined with phosphate, undergoes specific binding at the active site. The specific binding site for P_i is somewhat of a puzzle. It does not overlap sufficiently with that of glucose-1-P, since P_i does not antagonize glucose inhibition in binding experiments with 5'-AMP and P_i is not very effective in promoting allosteric transitions, unless glycogen is also present. A compulsory order of binding is a possibility, but so far this has not been demonstrated with certainty.

In conclusion it should be pointed out that although the results presented here are entirely compatible with the basic premises of the Monod model, they are not sufficiently discriminatory to exclude other possible explanations for the allosteric properties of phosphorylase.

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