The Effect of Temperature on the Allosteric Transitions of Rabbit Skeletal Muscle Phosphorylase *b**

Lewis L. Kastenschmidt,† Jeannine Kastenschmidt, and Ernst Helmreich‡

ABSTRACT: The allosteric transitions of rabbit muscle phosphorylase *b* were analyzed in the temperature range from 29 to 4° by means of kinetic and binding studies. The binding of 5′-adenosine monophosphate to phosphorylase *b* was studied using a Sephadex gel filtration technique. A computer was programmed to calculate best fits to experimental curves using equations for the two-state model of Monod *et al.* (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88). The results indicated values of L for the ratio T:R of phosphorylase *b* in the absence of ligands ranging from 6100 at 29° to 11 at 4°. The values for L' for the ratio T:R in the presence of the substrate anion, glucose 1-phosphate, ranged from 52 at 29° to 0.05 at 4°. At low temperatures phosphorylase dimer *b* associates. The aggregated (tetrameric) forms of phosphorylase *b* bind 5′-adenosine monophosphate tighter. K_{equil} of [tetramer *b*]/[dimer *b*]^2 was determined by light scattering and by ultracentrifugal sedimentation velocity measurements. The association–dissociation equilibrium was taken into consideration in the theoretical treatment of the data. Aside from substrate anions various other anions and divalent cations function as allosteric effectors and promote the T to R transition of phosphorylase *b*. The interaction of the glycerol phosphate buffer anion with phosphorylase *b* was taken into account in the theoretical treatment. Mathematical analysis of kinetic data revealed an activity of phosphorylase *b* at 1×10^{-5} M 5′-adenosine monophosphate that was too low considering that phosphorylase *b* is a K system. The evidence suggested less than theoretically expected heterotropic cooperativity of glucose 1-phosphate and 5′-adenosine monophosphate in sodium glycerol phosphate buffer. In order to account for the deviations from theory a scheme is proposed that assumes that phosphorylase *b* in sodium glycerol phosphate buffer may exist in three states. State T binds little to glucose 1-phosphate and not at all to 5′-adenosine monophosphate. State R as compared with state T binds exclusively to 5′-adenosine monophosphate and preferentially to anionic substrates (*i.e.*, glucose 1-phosphate). State R′ as compared with state R binds more tightly to 5′-adenosine monophosphate but less tightly to glucose 1-phosphate. The existence of two R states of phosphorylase *b* and *a* is also deduced from a study of the temperature dependency of the allosteric equilibria and of the K_{asen} values for 5′-adenosine monophosphate. In the case of phosphorylase *b*, the transition from R to R′ correlates well with the shift to tetrameric structures (or aggregates of higher order) at high enzyme concentrations (13–18 mg/ml) and at temperatures below 13°. It is suggested therefore that phosphorylase dimer *b* in state R′ readily associates in the presence of 5′-adenosine monophosphate at low temperatures. This is in contrast to phosphorylase *a* where the transition from R to R′ occurs around 23° and where the dimeric forms R and the corresponding tetrameric form R (or for that matter, the dimer R′ and the tetramer R′), have similar binding properties for 5′-adenosine monophosphate. Dimer *a* and tetramer *a* can each undergo transitions from one form, R, to another form, R′, with temperature. Hence association of dimer *a* to tetramer *a* linked to the binding of 5′-adenosine monophosphate does not seem to play a major role, whereas the dimer *b* → tetramer *b* association is strongly dependent upon 5′-adenosine monophosphate. The vastly different values for the allosteric T:R equilibrium of phosphorylase *b* where L is 2100 at 23° and of phosphorylase *a* where L is 3–13 at this temperature may well explain the equally large differences in the requirements of the unphosphorylated (*b*) and the phosphorylated (*a*) enzyme for 5′-adenosine monophosphate for activity.

Thus from this point of view the biological role of the phosphorylase *b* ⇌ *a* interconversion may be pictured as the interconversion between two enzymes one of which (phosphorylase *b*) is in the resting state (*i.e.*, in the absence of ligands) exclusively in the inactive state T whereas the other (phosphorylase *a*) which catalyzes the same reaction is present to a considerable extent even in the absence of allosteric ligands in the active state R.

This report intends to clarify further the mechanism of allosteric transitions of crystalline rabbit skeletal muscle phosphorylase *b* (Helmreich and Cori, 1964a; Helmreich, 1967; Kastenschmidt *et al.*, 1967, 1968;

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† National Science Foundation Postdoctoral Fellow. Present address: Muscle Biology Laboratories, The University of Wisconsin, Madison, Wis. 53706.

‡ Present address: The Department of Physiological Chemistry, The University of Würzburg, Würzburg 87, Germany. Reprint requests should be mailed to this address.

Buc, 1967; Buc and Buc, 1967; Madsen and Shechosky, 1967; Avramovic and Madsen, 1968). Certain features of the allosteric properties of rabbit muscle phosphorylase *a* and frog skeletal muscle phosphorylase *a* have recently been discussed (Helmreich and Cori, 1964b; Helmreich *et al.*, 1967; Metzger *et al.*, 1968).

The concerted transition model of Monod *et al.* (1965) serves as theoretical framework for the interpretation of the results.[1] One of the attractions of this model is that it can explain homotropic interactions (*i.e.*, interactions between the binding sites for the same stereospecific ligand) and heterotropic interactions (*i.e.*, interactions between the binding sites for different stereospecific ligands).

There is ample evidence from kinetic measurements for homo- and heterotropic interactions in the case of rabbit skeletal muscle phosphorylase *b* (Helmreich and Cori, 1964a; Madsen, 1964). The kinetic evidence has now been verified in a more direct way by binding studies using 5′-AMP as ligand. The evidence suggests that phosphorylase *b* can exist in sodium glycerol-P buffer under the conditions specified in three conformational states: R and R′, the tight binding forms, and T, the loose binding form. Each state has different binding properties for the modifier 5′-AMP, for the substrate glucose-1-P, and for the inhibitor glucose-6-P.[2] The tendency of the phosphorylase *b* subunits to form aggregates at low temperatures allowed a study of the association of phosphorylase *b* linked to the binding of 5′-AMP. The aggregated forms of phosphorylase *b* bind 5′-AMP more tightly. It was assumed therefore that the polymeric forms of phosphorylase *b* have the R′ conformation.

These observations are interpreted in the form of a scheme for the allosteric transitions of muscle phosphorylase *b*. The results are compared with those obtained recently for phosphorylase *a* (Helmreich *et al.*, 1967). The different allosteric properties of muscle phosphorylases *a* and *b* are discussed with respect to the biological role of the phosphorylase *b* $\rightleftharpoons$ *a* interconversion in muscle.

Methods

Experimental Procedures. The procedures and the materials used for kinetic and binding measurements are described in Kastenschmidt *et al.* (1968). Additional information is given in the legends to tables and figures.

Light-Scattering Experiments. These experiments were carried out with a Brice-Phoenix photometer equipped with temperature control, as described by Frieden (1962). Cells with an inside diameter of 10 mm were used. The calibration of the light-scattering instrument was carried out with crystalline rabbit skeletal muscle phosphorylase *b*, free of 5′-AMP. The same concentration of protein, the same buffer system, and the same temperature were used for calibration as in the actual experiments.

Ultracentrifugal Analyses. Sedimentation velocity experiments were carried out as described previously (Kastenschmidt *et al.*, 1968). The rotor speed was 59,780 rpm. Pictures shown were taken 27–33 min after the rotor had reached full speed.

Treatment of the Data. KINETIC DATA. Initial rates were first plotted in the form of v/s *vs.* v as proposed by Frieden (1967). The linear portions of the slopes were then fitted by the method of least squares and a value for the apparent V_{max} was obtained from the intercept of the lines with the abscissa. The linear portion of the slope represents a hyperbolic substrate saturation curve. Expressed in terms of the Monod model this limiting slope represents $(-1/K_{dissn})((1 + Lc^n)/(1 + Lc^{n-1}))$. This is explained by Frieden (1967). In this equation, L describes the ratio of two conformational states of the enzyme at equilibrium in the absence of ligand: $L = T_0/R_0$; c represents the ratio of the binding affinities of the two states (R and T) for the substrate, glucose-1-P: $c = K_{dissn\,(R)}/K_{dissn\,(T)}$; n is the number of ligand binding sites.

The above expression reduces to $-1/K_{dissn}$ when $c \ll 1$ or when L is close to zero. At saturating concentrations of 5′-AMP, L approaches zero and the K_{dissn} constant for glucose-1-P represents the affinity of the R (the tighter binding) conformational form of phosphorylase *b* for glucose-1-P. This value of K_{dissn} for glucose-1-P was utilized in analyzing the data in terms of the saturation function of Monod *et al.* (1965).

The equation for the fractional saturation of a protein according to Monod *et al.* (1965) is

$$\bar{Y} = \frac{\alpha(1 + \alpha)^{n-1} + Lc\alpha\,(1 + c\alpha)^{n-1}}{(1 + \alpha)^n + L(1 + c\alpha)^n} \qquad (1)$$

The term α refers to the normalized concentration of free ligand: $\alpha = C$(glucose-1-P)$/K_{dissn}$(glucose-1-P (R)). The saturation function is a binding function. It can, however, be applied to initial velocity data by making additional assumptions which have been stated by Frieden (1967). In this case $\bar{Y}$ may then be replaced by the fraction v_0/nV_{max} as discussed by Frieden (1967). V_{max} in this case refers to the maximal velocity expressed per protomer.

Mathematically it is possible to incorporate heterotropic interactions in the saturation equation (eq 1) by substituting L' for L (Rubin and Changeux, 1966). This follows because L' is related to L: $L' = L[((1 + \gamma e)/(1 + \gamma))((1 + \beta f)/(1 + \beta))]^n$. In this formulation γ refers to the normalized 5′-AMP concentration and e refers to the ratio of the binding constants of the R and T states for 5′-AMP: $\gamma = C$(5′-AMP)$/K_{dissn}$ (5′-AMP (R)) and e is K_{dissn} (5′-AMP (R)$/K_{dissn}$ (5′-AMP (T)). Since experimental observations indicated that the glycerol-P buffer anion should also be considered an allosteric effector, the corresponding terms were included in the equations: β refers to the normalized con-

[1] The nomenclature introduced by Monod *et al.* (1965) is used. For further information this and other papers by Monod and colleagues may be consulted. Additional references may be found in the paper by Helmreich *et al.* (1967).

[2] Glucose-1-P, glucose-6-P, and glycerol-P are α-D-glucose 1-phosphate, α-D-glucose 6-phosphate, and DL-β-glycerol phosphate.

centration of glycerol-P and f is the ratio of the binding constants of the two conformational forms. $\beta = C$-(glycerol-P)/K_{dissn} (glycerol-P (R)) and $f = K_{dissn}$ (glycerol-P (R))/K_{dissn} (glycerol-P (T)). Equation 1, modified by including these terms, was used for the analysis of the kinetic data.

The data were plotted in the form of Y/α *vs.* α and were compared with the theoretical curves plotted in the same form. The values for K_{dissn} and V_{max} derived from the limiting slope and from the intercept of the v/s *vs.* v plots, respectively, were used for the theoretical curves.

The R function gives the fraction of the enzyme which is in the R conformational state. This is defined by the expression

$$R = \frac{\Sigma R}{\Sigma R + \Sigma T} = \frac{(1 + \alpha)^n}{(1 + \alpha)^n + L(1 + \alpha c)^n}$$

(*cf.* Rubin and Changeux, 1966)

The difference between the values of R in the presence and in the absence of a given ligand represents the allosteric range, Q (Monod *et al.*, 1965; Rubin and Changeux, 1966; Blangy *et al.*, 1968). This range was calculated for a series of L' values using the equation

$$Q = R_{\alpha \to \infty} - R_{\alpha = 0} = \frac{L'(1 + c^n)}{(L' + 1)(L'c^n + 1)}$$

BINDING DATA. The data were plotted in the form of r/c *vs.* r (Scatchard, 1949; Klotz, 1953; see also Helmreich *et al.*, 1967), where $r/C(5'\text{-AMP}) = K_{assn}(n - r)$; r represents the average number of 5′-AMP molecules bound to each molecule of phosphorylase b, n is the apparent number of binding sites on each molecule of phosphorylase b, and C(5′AMP) is the concentration of free 5′-AMP. In the case that all binding sites are equivalent (*i.e.*, in the absence of homo- or heterotropic cooperativity) straight lines should be obtained in binding plots.

The saturation equation (see eq 1) of Monod *et al.* (1965) can be used to treat nonlinear binding data provided that no change in the quaternary structure of the enzyme occurs.

Ultracentrifugal analyses and light-scattering measurements indicated however that at high concentrations of phosphorylase b as are used in binding experiments and at low temperatures dimer b associates to tetramer b. The aggregated form of phosphorylase b was designated as R′. A term for the K_{equil} = tetramer b (R′)/dimer b (R)^2 had therefore to be included in the theoretical treatment. This equation was kindly provided by Dr. Carl Frieden.

$$\bar{Y} = \frac{\gamma(1 + \gamma) + L\gamma e(1 + \gamma e) + 2K_{equil}E_F d\gamma(1 + d\gamma)^3}{(1 + \gamma)^2 + L(1 + e\gamma)^2 + 2K_{equil}E_F(1 + d\gamma)^4} \quad (2)$$

The terms used in the treatment of the binding data are: $\gamma = C(5'\text{-AMP})/K_{dissn}(5'\text{-AMP (R)})$; $e = K_{dissn}(5'\text{-AMP (R)})/K_{dissn}(5'\text{-AMP (T)})$; $d = K_{dissn}(5'\text{-AMP (R)})/K_{dissn}$ (5′AMP (R′)). Equation 2 can be solved for E_F, *i.e.*, the concentration of unliganded enzyme in the R state. E_F is related to E_0, the total enzyme concentration, by the expression

$$E_F = \frac{-[(1 + \gamma)^2 + L(1 + e\gamma)^2] + \sqrt{[(1 + \gamma)^2 + L(1 + e\gamma)^2]^2 + 8E_0K_{equil}(1 + d\gamma)^4}}{4K_{equil}(1 + d\gamma)^4}$$

This term was substituted in eq 2 which was used for the analysis of the binding data. At higher temperatures (23 and 29°), the equilibrium is shifted toward dimer b. Thus under these conditions K_{equil} approaches 0. Therefore eq 2 becomes the $\bar{Y}$ saturation function given in eq 1.

As in the case of eq 1, the terms for the heterotropic ligands glucose-1-P and the glycerol-P buffer anion were also included in eq 2. In this case glucose-1-P and glycerol-P anions are heterotropic ligands. In the series of experiments with Mg^{2+}, the divalent cation was treated like glucose-1-P. Under all these conditions L' is related to L

$$L' = L\left(\frac{1 + \alpha c}{1 + \alpha} \times \frac{1 + \beta f}{1 + \beta}\right)^n$$

The R function for the binding data is defined by $R = (1 + \gamma)^n/((1 + \gamma)^n + L(1 + e\gamma)^n)$ for the case where no association to tetrameric forms occurred. When association of the enzyme occurred, the saturation function as defined by eq 2 was used and the R function now becomes $R = ((1 + \gamma)^2 + 2K_{equil}E_F(1 + d\gamma)^4)/((1 + \gamma)^2 + L(1 + e\gamma)^2 + 2K_{equil}E_F(1 + d\gamma)^4)$. The fraction of phosphorylase b present in the tetrameric state was calculated as follows. Fraction tetramer = $2K_{equil}E_F(1 + d\gamma)^4/((1 + \gamma)^2 + L(1 + e\gamma)^2 + 2K_{equil}E_F(1 + d\gamma)^4)$. The Q function for the kinetic data was already defined. For the binding data

$$Q = R_{\gamma \to \infty} - R_{\gamma = 0} = \frac{L'(1 - e^n)}{(L' + 1)(L'e^n + 1)}$$

Q_{max} gives the maximal limit of Q for the range of 5′-AMP concentrations where interaction is maximal.

Numerical Values. For the kinetic measurements the following numerical values were used. The values of V_{max} and of K_{dissn} (glucose-1-P) were obtained from the data in Figure 1. V_{max} was 53.4 μmoles of product formed/min per mg of enzyme, K_{dissn} (glucose-1-P(R)) was 2.2×10^{-3} M and c was 0.06. However for experiments at a 5′-AMP concentration of 0.01 mM, a K_{dissn} value for glucose-1-P of 5.5×10^{-3} M had to be used in order to obtain a good correlation between experimental and theoretical curves. It is assumed that the high K_{dissn} value represents K_{dissn} for glucose-1-P and a somewhat different form (R′) of phosphorylase b. This is explained later. Binding plots of phosphorylase b and 5′-AMP are curvilinear. Two methods were used for the calculation of K_{assn} (5′-AMP) ($K_{assn} = 1/K_{dissn}$); K_{dissn} can

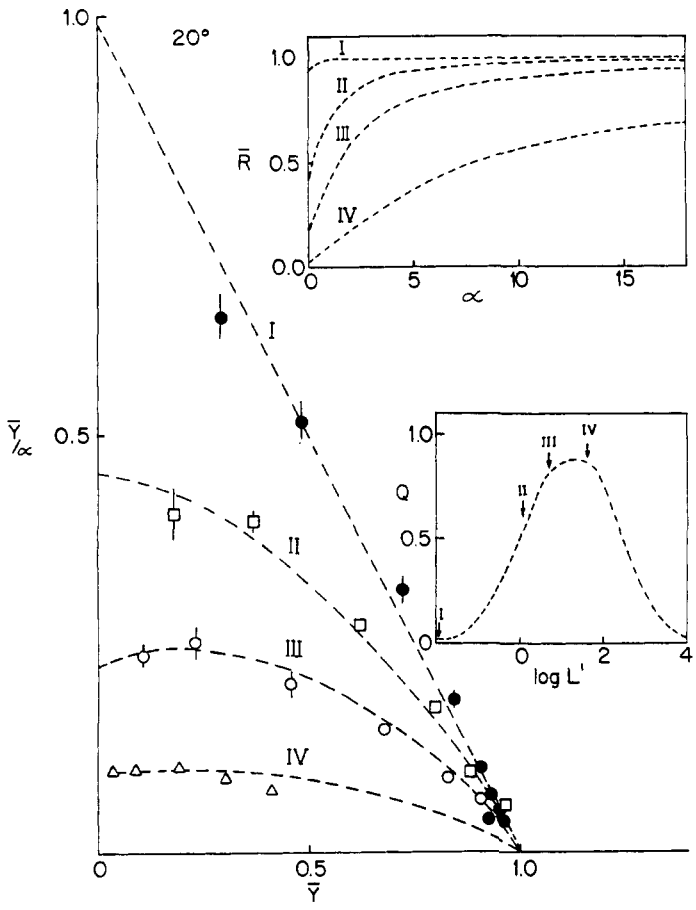


FIGURE 1: Allosteric behavior of phosphorylase *b*. Kinetic experiments. Initial velocity measurements were carried out in the direction of glycogen synthesis at a constant level of glycogen and at varying concentrations of glucose-1-P and 5′-AMP. Phosphorylase *b* (about 10–20 μg/ml) was incubated at 20° in 5 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (*pH 6.8*). The buffer solution was kept at constant ionic strength, $\Gamma/2 = 0.13$, by addition of KCl. It contained 0.5% glycogen (corresponding to 2.5×10^{-3} M in terms of glucosyl groups at the nonreducing end of the chains and from 1 to 60 mM glucose-1-P). The concentrations of 5′-AMP were 1 mM for the experiments in curve I (●), 0.10 mM for curve II (□), and 0.05 and 0.01 mM for curves III (○) and IV (△), respectively. The P_i released during the reaction was measured by the method of Fiske and Subbarow (1925). The average of four experiments is shown for each experimental point and the standard error of the mean is indicated by vertical bars. The broken lines represent calculated curves. The method of plotting and the equation used are given in the text. The minimum–maximum deviations of calculated and theoretical curves ranged from 0.4 to 1.8% and the least-squared deviations from the mean expressed in per cent ranged from 0.3 to 1.2% for curves I–IV.

In the inserts are given derivations of the data. The $\bar{R}$ function gives the fraction of the enzyme present in the R state for experiments in curves I–IV. The cooperativity function Q gives the change in the allosteric equilibrium as a function of glucose-1-P concentrations at the different levels of 5′-AMP studied. The Q values for curves I–IV are indicated by the arrows in the Q function plot.

be estimated by drawing a tangent to the linear part of the curves. The other method is to convert the binding curves into straight lines by plotting the data in the form of $\log r/(n - r)$ *vs.* $\log C$(5′-AMP). The lines were drawn by the method of least squares. The concentration required for half-maximal saturation of binding sites can then be estimated by extrapolation to the abscissa ($\log C$(AMP)) from the intercept of the lines with the 0 axis (on the $\log r/(n - r)$ ordinate). The slope also defines the interaction coefficient of Brown and Hill (1922). Agreement between the values of K_{diss_n} (5′-AMP) estimated by the two different approaches will depend upon the relationships of the range of maximal interaction with the range of 5′-AMP concentrations. In the experiments reported here this agreement is good. The value for K_{diss_n} (5′-AMP (R)) was determined from binding measurements at 4° and in the presence of 50 mM glucose-1-P. It was 1.5×10^{-5} M. The value for e was 0.001. The same values for K_{diss_n} (5′-AMP (R)) and for e were used in kinetic and binding experiments. However in the case of the kinetic experiments at 0.01 mM 5′-AMP concentrations it was assumed that e should represent the ratio of the affinities of the states R′:T for 5′-AMP Since in binding experiments a value of 5 was used for d (the ratio of the binding constants, K_{diss_n}, for the states R′:R for 5′-AMP), a value of e of 0.0002 was used in the kinetic experiments at 0.01 mM 5′-AMP. This value of e represents the ratio of the binding constants, K_{diss_n} (5′-AMP), for the R′ and T states. α and γ and K_{equil} = (tetramer R′)/(dimer R)^2 are variables. K_{equil} was estimated from light scattering and from sedimentation velocity measurements. The effect of the glycerol-P buffer anion was taken into consideration in kinetic and binding experiments. It was estimated that β, C(glycerol-P)/K_{diss_n} (glycerol-P (R)), should be about 50 mM/20 mM = 2.5. The value of f was assumed to be equal to $c = 0.06$. The number of binding sites, n, is 2 for dimer b (T, R, and R′) and 4 for tetramer b (R′) for each ligand including glycerol-P and Mg^{2+}. However since no data on the binding of glycerol-P or of Mg^{2+} to phosphorylase b are available, the assumption that the number of binding sites for all these ligands is the same is not verified experimentally in the case of glycerol-P and Mg^{2+}. A molecular weight of 185,000 daltons for phosphoryl-

ase dimer *b* was used for calculations. It was assumed that phosphorylase *b* has one binding site for each ligand per protomer of 92,500 daltons (Seery *et al.*, 1967; DeVincenzi and Hedrick, 1967; Buc and Buc, 1967; Metzger *et al.*, 1968).

The general approach was to program a computer for the calculation of an average value of L on the basis of the above equations. The IBM Computer System 360 screened for the best values of L in steps of 0.1–1% of the upper limit of L which was estimated from preliminary calculations with an Olivetti Programma 101 computer. As criteria for the fit of theoretical to experimental curves the minimum–maximum deviation and the minimum-squared deviation (least squares) were used. The agreement between calculated and experimental curves was good over the range of experimental observations with the exceptions noted.

Results

Kinetic Measurements. The results of kinetic measurements are shown in Figure 1. It is implicit in the theoretical treatment that phosphorylase *b* is a "K" system. Evidence to support this assumption was reported (Helmreich and Cori, 1964a). If this were an ideal case one would predict that phosphorylase *b* should be fully active in the absence of modifier provided the enzyme can be saturated with substrates in the absence of 5′-AMP. The fit to the experimental points in the kinetic experiments in Figure 1 was good for the range of 5′-AMP concentration from 1 to 0.05 mM. However at 5′-AMP concentrations < 0.05 mM (data for 0.01 mM are shown in Figure 1), the curves did not fit the experimental points. This is expressed in a very large deviation of the experimental and calculated value for L (>300 *vs.* 29) (see Table I). The calculated curve could be made to fit the experimental curve at 0.01 mM 5′-AMP if a K_{dissn} value for glucose-1-P was substituted into the equation which was 2.5 times larger than the actual K_{dissn} value derived from kinetic measurements at high concentrations of 5′-AMP (2.2×10^{-3} M *vs.* 5.5×10^{-3} M).[3]

One should be cautious in interpreting initial velocity measurements at low substrate concentrations, since the rate could fall off because of substrate depletion. This possibility was carefully considered in the present experiments and appropriate precautions were taken. The change in rate due to approach to equilibrium of the phosphorylase reaction was taken into account and the first-order velocity constants were determined as described by Cori *et al.* (1943). There was no change in the rate constants even at the lowest 5′-AMP and glucose-1-P concentrations for the time period in which the reaction was measured. Although we are aware that this may not suffice to exclude completely the possibility that the actual rate determined was too low because of limitations due to the assay, we wish to point out that the treatment of the data gives further assurance as to the validity of the conclusions derived from these experiments. The computer fitting and the treatment of Frieden (1967) which we have used weigh in essence the initial velocity values obtained at all concentrations of substrate in an equal manner, since the whole curve and not only the part of the curve which pertains to the low substrate concentrations is scrutinized by the computer for best fit. We can then be reasonably certain that, in the limitations of any kinetic assay, the too-low rate observed at low 5′-AMP concentrations is not due to substrate depletion and most likely represents less than theoretically expected heterotropic cooperativity between the unlike binding sites for 5′-AMP and glucose-1-P.

Madsen and Shechosky (1967) also observed that phosphorylase *b* has little activity under comparable conditions (sodium glycerol-P buffer, pH 6.8) in the absence of 5′-AMP and at high levels of substrates. Without 5′-AMP these authors observed a rate which was only 0.4% of the rate in the presence of saturating concentrations of 5′-AMP. The highest activity we have observed in the absence of 5′-AMP was 4.7% of the rate in the presence of 5′-AMP at 20°. Our results as well as those of Madsen and Shechosky (1967) do not agree with the finding of Buc and Buc (1967). The latter authors state that phosphorylase *b* has close to 100% maximal activity in the absence of 5′-AMP at 28°. The experiments of Buc and Buc were carried out in glycylglycine buffer with 0.12 M P_i and in the presence of Mg^{2+} ions. Initial rates were determined by phosphorolysis of

TABLE I: A Comparison of Experimental and Calculated Values for L'.[a]

Concn of Free 5′-AMP (mM)	L' (Exptl)	L' (Calcd)
1.00	0.01	0.02
0.10	1.35	1.35
0.05	4.90	4.20
0.01	36.5 (>300.00)	29.0

[a] The experimental values of L' are derived from the kinetic experiments shown in Figure 1. The equation and the numerical values used for the calculation of the value of L' are given in the text. L was 125. In the experiment at 0.01 mM 5′-AMP e was 0.0002 (R'/T) and K_{dissn} (glucose-1-P) was 5.5×10^{-3} M (see text). The value in parentheses gives the value of L' which was obtained if e was 0.001 and K_{dissn} (glucose-1-P) was 2.2×10^{-3} M. This is an estimate only since experimental and calculated curves deviated greatly in this case.

[3] The computer analysis of the kinetic experiment at low concentrations of 5′-AMP (0.01 mM) indicated that experimental and theoretical curves could best be fitted in either of two ways. One could increase the value of c from 0.06 to 0.273 using a value for K_{dissn} of 2.2×10^{-3} M for glucose-1-P and the R form. The other possibility was to use a K_{dissn} value for glucose-1-P and the hypothetical form R′ of 5.5×10^{-3} M and keeping the value of c at 0.06. The computer could not distinguish between these possibilities.

TABLE II: Binding of 5′-AMP to Phosphorylase *b* in Glycylglycine and Glycerol-P Buffer (pH 6.8).[a]

Additions (mM)	Glycylglycine Buffer K_{dissn} (5′-AMP) (M × 10^4)	Glycerol-P Buffer K_{dissn} (5′-AMP) (M × 10^4)
None	50.0	3.7
P_i (20)	2.9	5.0
K_2SO_4 (20)	8.1	4.6
Glucose-1-P (20)	0.8	1.7
Glucose-1-P (50)		0.7
Glucose-1-P (20) + P_i (20)	1.2	
Glucose-6-P (20)	36.0	21.0

[a] Binding experiments were carried out using a Sephadex gel filtration technique as described by Kastenschmidt *et al.* (1968). The concentration of enzyme ranged from 13 to 18 mg per ml. The temperature was 23°. Only one concentration of 5′-AMP (1×10^{-4} M) was used. In some instances the entries represent the average of two experiments. Either 10 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8) or 50 mM glycylglycine buffer (pH 6.8) with the above additions were used. These and all other binding measurements were carried out at a constant ionic strength of $\Gamma/2 = 0.13$ in order to make the results obtained with high concentrations of charged ions comparable with the results obtained in the absence of these charged ligands. This was done by adding KCl and keeping the buffer ion concentration constant.

TABLE III: The Effect of Divalent Cations on the Binding of 5′-AMP to Phosphorylase *b*.[a]

Additions (mM)	K_{dissn} (5′-AMP) (M × 10^4)
None	3.7
$SrCl_2$ (20)	1.6
$MgCl_2$ (10)	2.2
$MgCl_2$ (20)	1.2
$MgCl_2$ (30)	1.3
$MnCl_2$ (20)	0.8
$CaCl_2$ (20)	0.5
Glucose-1-P (50)	0.8
Glucose-1-P (50) + $MgCl_2$ (20)[b]	0.8 (0.3)

[a] The binding experiments were carried out in 10 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8) at 23°. The ionic strength was kept constant at $\Gamma/2 = 0.13$ with the exception noted (see legend to Table II). The concentration of enzyme ranged from 12.2 to 15.2 mg per ml and the concentration of 5′-AMP was 1.0×10^{-4} M. The entries represent the results of one or two experiments. The value in parentheses gives the expected K_{dissn} value for 5′-AMP in the presence of glucose-1-P and $MgCl_2$ assuming that the effect of these ions are additive (see text). [b] Ionic strength was $\Gamma/2 = 0.18$.

glycogen. The discrepancy between these data suggested a drastic effect of the buffer ions on the allosteric transitions of phosphorylase *b*. This was studied by binding experiments.

Effects of Anions and Cations on the Binding of 5′-AMP. The binding affinity of phosphorylase *b* for 5′-AMP in glycylglycine buffer is much lower than in glycerol-P buffer (Table II). This suggests that the allosteric equilibrium is shifted toward the T state in glycylglycine and toward the R state in sodium glycerol-P buffer. A low binding affinity of phosphorylase *a* for 5′-AMP in imidazole-acetate as compared with sodium glycerol-P buffer was reported (Helmreich *et al.*, 1967). The experiments in Table II are comparable with respect to temperature, pH, and ionic strength to the experiments shown in Figure 1; however the enzyme concentration was at least 500 times higher in binding experiments. It is of interest that aside from P_i the sulfate anion which is not a substrate has an effect in glycylglycine buffer, but neither P_i nor sulfate increased the binding of 5′-AMP to phosphorylase *b* in sodium glycerol-P buffer. In fact they rather decreased the binding affinity of the enzyme for the nucleotide. P_i also did not increase the binding affinity of phosphorylase *a* for 5′-AMP in sodium glycerol-P buffer (Helmreich *et al.*, 1967). It may also be noted that in glycylglycine buffer a combination of P_i and glucose-1-P is not more effective than each substrate alone. Thus the effects of the substrates are not additive. Although glucose-1-P is the only anionic substrate which has an effect on the allosteric equilibrium of phosphorylases *b* and *a* in sodium glycerol-P buffer, it is apparent from the data in Table II that glucose-1-P at 20 mM concentrations (*i.e.*, about ten times the K_m value as determined from the experiments in Figure 1) is not maximally effective as heterotropic ligand in sodium glycerol-P buffer and under the conditions of these binding experiments. An increase in glucose-1-P concentrations from 20 to 50 mM lowers the K_{dissn} value of 5′-AMP and phosphorylase *b* from 1.7 to 0.7×10^{-4} M. The same effect obtained with 50 mM concentrations is observed with 20 mM glucose-1-P in glycylglycine buffer. It is difficult to test higher than 50 mM concentrations of glucose-1-P because of the strong effect of ionic strength on the allosteric equilibria (*cf.* Helmreich *et al.*, 1967). At high ionic strength the equilibrium is shifted to the T form. However glucose-6-P, a negative modifier, is highly effective in glycerol-P buffer. This is expected since the allosteric equilibrium is shifted toward the R state in glycerol-P buffer. Accordingly the value for *L*, *i.e.*, the ratio T:R of phosphorylase *b*, should be considerably larger at this temperature (23°) or at any temperature in glycylglycine than in glycerol-P buffer.

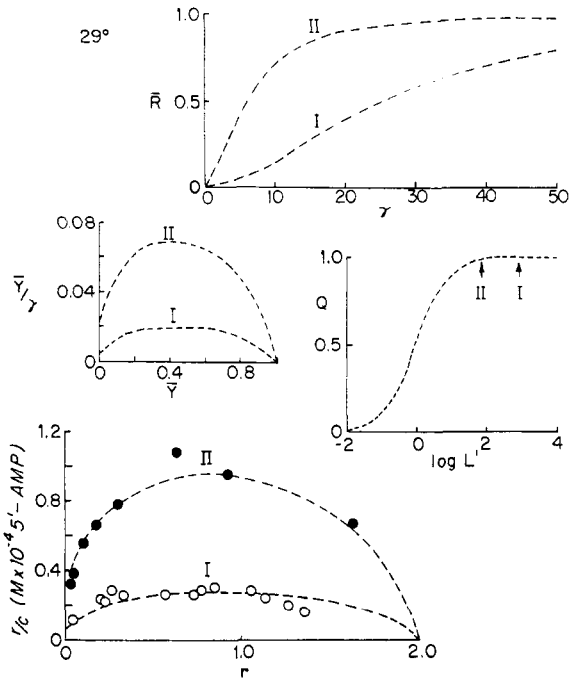


FIGURE 2: Allosteric behavior of phosphorylase *b*. The binding of 5′-AMP to phosphorylase *b* at 29°. The range of the concentrations of phosphorylase *b* was from 13.0 to 19.3 mg per ml and from 14.1 to 19.6 mg per ml and the range of 5′-AMP concentrations was from 5×10^{-5} to 9×10^{-4} M and from 8×10^{-6} to 3×10^{-4} M for curves I and II, respectively. The experiments were carried out in 50 mM sodium glycerol-P buffer (pH 6.8), $\Gamma/2 = 0.13$, with the additions listed in the legend to Table II. Curve I (○): no glucose-1-P; curve II (●): 50 mM glucose-1-P. The broken lines represent curves calculated by the computer on the basis of the equation and the numerical parameters given in the text. The arrows in the *Q* plot indicate the change of the allosteric equilibrium as a function of 5′-AMP concentrations in the absence or presence of glucose-1-P (curves I and II, respectively). The minimum–maximum deviation of experimental points and theoretical curves ranged for curves I and II from 10.2 to 12.6% and the minimum-squared deviations (expressed in per cent) ranged from 6.2 to 7%.

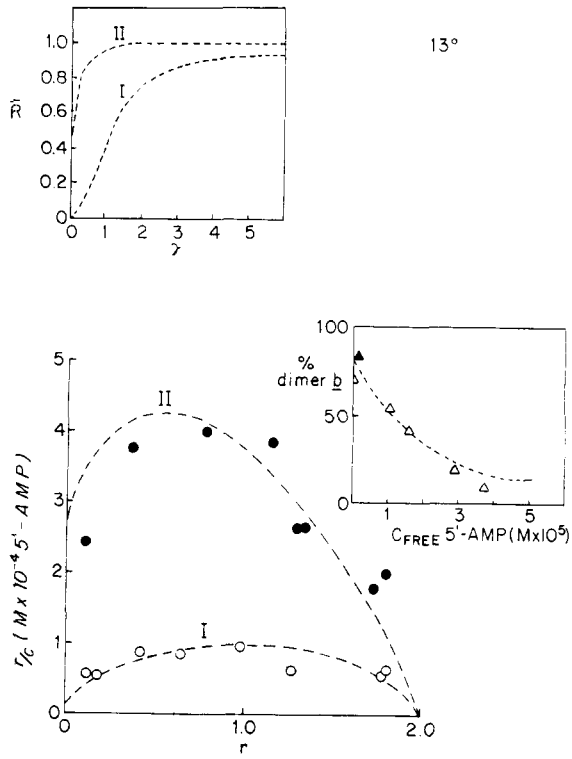


FIGURE 3: The binding of 5′-AMP to phosphorylase *b* at 13° The range of the concentrations of phosphorylase *b* was from 12.1 to 16.5 mg per ml and from 11.8 to 14.0 mg per ml and the range of 5′-AMP concentrations was from 2×10^{-5} to 3×10^{-4} M and from 5×10^{-6} to 1×10^{-4} M for curves I and II, respectively. Curve I (○): no glucose-1-P; curve II (●): 50 mM glucose-1-P. The broken lines are calculated by the computer on the basis of the equation and the numerical values given in the text. Minimum–maximum deviations ranged from 7.6 to 5.6% and the minimum squared deviations (expressed in per cent) ranged from 4.0 to 3.0% for curves I and II, respectively. In the insert are given data for the tetramer/dimer equilibrium at this temperature and in the presence of 50 mM glucose-1-P. Per cent dimer *b* is plotted against the concentration of free 5′-AMP. The concentration of free 5′-AMP in light-scattering experiments was calculated from the total amount of 5′-AMP added and the dissociation constant for 5′-AMP at 13°. (△) Values from light-scattering experiments. (▲) Value from sedimentation velocity measurement. K_{equil} was 3.7×10^4. Broken lines represent a theoretical curve calculated on the basis of the equation for fraction tetramer given in the text.

A possible explanation for the observation in Figure 1 is that phosphorylase *b* is not fully active in sodium glycerol-P buffer in the absence of 5′-AMP (or at concentrations of 5′-AMP $< 5 \times 10^{-5}$ M) because the active form of the enzyme may not be fully saturated with glucose-1-P under these conditions. It could be that glycerol-P anions and glucose-1-P anions compete for the anion substrate binding site of this form because both anions bind preferentially to the R (or active) state of phosphorylase. Conversely one would expect that glycylglycine or imidazole cations which bind preferentially to the T state should not interfere with binding of ligands to the R states.

The results support the contention that in the case of the phosphorylase system buffer ions should be considered as true allosteric effectors. The theoretical treatment takes the allosteric effect of the glycerol-P anion into consideration.

Aside from buffer ions (and other factors: temperature, ionic strength, pH, etc.) divalent metal cations strongly affect the binding of 5′-AMP to phosphorylase *b*. As shown in Table III, divalent cations have effects similar to those of substrate anions. They are equally if not more effective than glucose-1-P. Mg^{2+} ions also tighten the binding of 5′-AMP to phosphorylase *a* (Helmreich *et al.*, 1967). As in the case of P_i and glucose-1-P in glycylglycine buffer, the effects of Mg^{2+} ions and glucose-1-P are not additive in sodium glycerol-P buffer. This makes it quite unlikely that Mg^{2+} increases the binding of 5′-AMP to phosphorylase through the formation of a metal nucleotide chelate. It is more likely that divalent cations and anionic ligands bind preferentially to that conformational state of phosphorylase *b* that has a greater affinity for the modifier 5′-AMP. These observations are of interest in view of the findings of Krebs and Fischer (1962) and of Madsen (1965) who have shown that Mg^{2+} and Ca^{2+} decrease

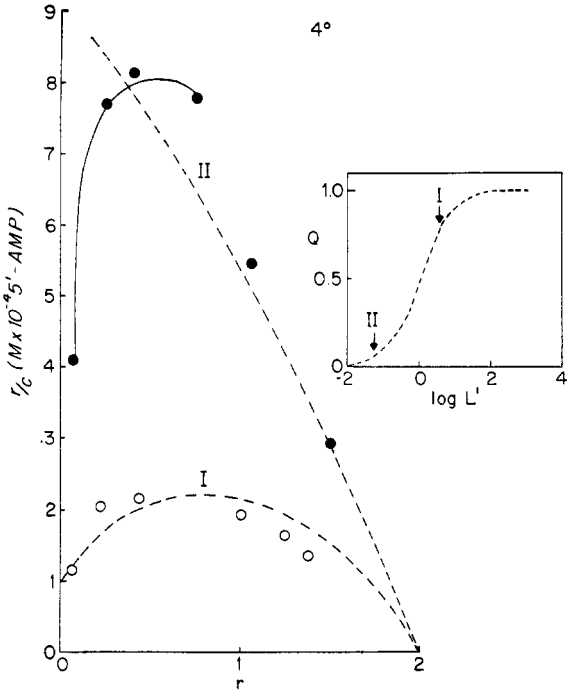


FIGURE 4: The binding of 5′-AMP to phosphorylase *b* at 4°. The range of the concentrations of phosphorylase *b* was from 6.5 to 7.8 mg per ml and that of 5′-AMP from 1.5×10^{-6} to 1×10^{-4} M. Curve I (○): no glucose-1-P; curve II (●): 50 mM glucose-1-P. The broken lines are curves calculated by the computer on the basis of the equations and the numerical values given in the text. In the insert is given the Q function. For curve I the minimum–maximum deviation of experimental and theoretical binding curves was 6.9% and the minimum squared deviation (expressed in per cent) was 4.0%. For curve II the minimum–maximum deviation of experimental and theoretical binding curves was 6.9% and the minimum-squared deviation was 4.0%. For curve I the fit was good over the range of 5′-AMP concentrations from 1.0×10^{-4} to 5.0×10^{-6} M and for curve II for the range of 5′-AMP concentrations from 5.3×10^{-5} to 5.0×10^{-6} M. The deviation of experimental and calculated curve II at 5′-AMP concentrations <0.01 mM is discussed in the text.

the concentration of 5′-AMP required for half-maximal activation of phosphorylase *b*. This may be explained by assuming that these cations are likewise allosteric effectors which promote T → R transitions. A biological role of the potent allosteric effector Ca^{2+} in the activation of phosphorylase should be kept in mind. Additional data for Mg^{2+} are included in Table IV.

The Effect of Temperature on the Binding of 5′-AMP. Figures 2–4 and Table IV illustrate measurements of binding of 5′-AMP to phosphorylase *b*. Experiments at 29, 13, and 4° are shown here but measurements identical in number and experimental design were performed at 23°. The latter results are included in Table IV.

Addition of the substrate anion glucose-1-P results in heterotropic cooperativity and the range of maximum interaction is now shifted to lower concentrations of 5′-AMP. For example, at 29° and in the presence of glucose-1-P, maximal interaction ($Q_{max} \rightarrow 1$) occurred between 0.008 and 0.06 mM 5′-AMP as compared with 0.05–0.3 mM 5′-AMP in the absence of the heterotropic ligand, glucose-1-P. Accordingly, the amount of the enzyme present in the R state increased in the range of lower concentrations of modifier. This is illustrated in the inserts in Figures 2 and 3 where changes of $\bar{R}$ are related to γ and changes in the cooperative site–site interactions, Q, to L'. In binding experiments at 29 and 23° in the presence or absence of glucose-1-P the agreement between experiment and theory was satisfactory (see Figure 2). At 13° the range of maximal interaction was further shifted by the heterotropic ligand, glucose-1-P, toward lower concentrations of 5′-AMP (see Figure 3 and Table IV). Lowering the temperature from 13 to 4° and adding glucose-1-P further shift the equilibrium toward the tight binding forms of phosphorylase *b*. Correlation of theory with experiment was satisfactory (see Figures 3 and 4). This was also the case when glucose-1-P was replaced by Mg^{2+} as heterotropic effector (see Table IV). However at 4° in the presence of glucose-1-P and at concentrations of 5′-AMP $< 9.5 \times 10^{-6}$ M the binding affinity of phosphorylase *b* for the modifier suddenly falls and the experimental points deviate greatly from the theoretical curve (curve II, Figure 4). An interpretation is given later.

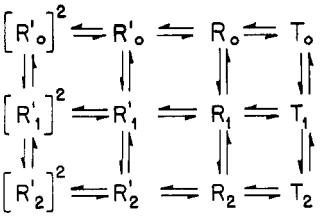


FIGURE 5: A model for the allosteric transitions of phosphorylase *b*. The scheme is explained in the text. This scheme applies only to the experimental conditions described in this paper.

Effect of Temperature on the Tetramer b ⇌ Dimer b Equilibrium. At the higher temperatures, 29 and 23°, the tetramer *b* ⇋ dimer *b* equilibrium is shifted to the right. At these temperatures and under the conditions of the binding experiments phosphorylase *b* (15 mg/ml) is present mainly in dimeric forms over the whole range of 5′-AMP concentrations (see Table V). At lower temperatures, 13 and 4°, phosphorylase *b* exists as a mixture of dimer *b*, tetramer *b*, and possibly aggregates of a higher order. Phosphorylase dimer *b* associates to the tetrameric form at 13° at concentrations of 5′-AMP $> 3 \times 10^{-5}$ M, but in the presence of glucose-1-P tetramer *b* is formed at concentrations $> 9 \times 10^{-6}$ M 5′-AMP. This is expressed in the values for K_{equil} (Table IV). Ultracentrifugal analyses indicated at least two molecular species in varying proportions with s_{20} values in the range of 8 and 12 S (Table V). The [tetramer *b*] ⇌ [dimer *b*]^2 equilibrium is, aside from other factors, for example, temperature and protein concentration, strongly dependent upon 5′-AMP concentrations (see Table V). K_{equil} = [tetramer *b*]/[dimer *b*]^2 was determined from light-scattering measurements under conditions approximating those of binding measurements. Light-scattering measurements for 13° are shown in the insert of Figure 3. Additional information was obtained from ultracentrifugal sedimentation velocity measurements (Table V). The values of K_{equil} should be considered as estimates

TABLE IV: Allosteric Properties of Phosphorylase *b*.[a]

Temp (°C)	Range of 5′-AMP Concn (mM)	Additions (mM)	L_0	L'	K_{equil} ($\times 10^3$)	Q_{max}
29	0.04–0.9	None	5280–6090	600–750	0	1.0
	0.008–0.32	Glucose-1-P (50)	5280–6090	52–60	0	1.0
23	0.02–1.0	None	2100	250	0	1.0
	0.01–0.3	Glucose-1-P (50)	2131	21	0	1.0
13	0.02–0.3	None	84–106	23–29	3	1.0
	0.010–0.2	Mg^{2+} (20)	(100–200)	8	20	1.0
	0.005–0.1	Glucose-1-P (50)	85	2.0	37	1.0
4	0.04–0.10	None	11.0–14.6	3–4	4	1.0
	0.0015–0.05	Glucose-1-P (50)	5.1–20.3	0.05–0.2	52[b]	1.0

[a] The data are derived from the binding experiments shown in Figures 2–4 and from similar experiments at 23 and 13° with the addition of Mg ions. The values in parentheses for Mg^{2+} are based on assumptions rather than on actually determined constants. The minimum–maximum deviations for the experiments at 23° in the presence and the absence of glucose-1-P ranged from 1 to 10.1% and the minimum-squared deviations (expressed in per cent) ranged from 0.55 to 6.3%. [b] This value agrees with the K_{equil} value expected from the thermodynamic relationship ($\Delta F = -RT \ln K_{equil}$) and from the experimentally determined K_{equil} at 13° in the presence of glucose-1-P.

because of the well-known limitations in the analysis of associating–dissociating systems. The binding measurements over the whole range of temperatures from 29 to 4° were compared with theoretical curves derived from eq 2. Strong homotropic cooperativity of the binding sites for 5′-AMP is apparent at all temperatures. Q_{max} approached 1 indicating that the interaction reached the theoretical limit for the available number of binding sites in the range of 5′-AMP concentrations where interaction is maximal (see Table IV). This applied to dimer *b*, tetramer *b*, or a mixture of both. Thus tetramer *b* molecules may be considered operationally as fully cooperative dimers with no detectable interaction between the dimeric structural units in a tetramer.

Interpretation

The scheme (Figure 5) presents the allosteric transitions of rabbit muscle phosphorylase *b* as concerted T → R transitions according to the general model of Monod *et al.* (1965) and to that of Buc and Buc (1967) for phosphorylase *b*. One significant difference between these models and the scheme proposed here will be noted. Our scheme includes a third state. It is assumed that dimer *b* (R) can further tautomerize to a form R′ and that at high protein concentrations and at low temperatures R′ readily associates to $(R')_2$. The form R′ as compared to R has the unusual properties that it binds more tightly to 5′-AMP (d = 5!) but less tightly to glucose-1-P (K_{diss} (glucose-1-P) is 5.5×10^{-3} M for R′ but 2.2×10^{-3} M for R). Postulating this additional tautomeric form R′ is another way of describing the weak heterotropic cooperativity of glucose-1-P in sodium glycerol-P buffer. It is easy to see that 5′-AMP and glucose-1-P have quantitatively different effects on the allosteric equilibria because the former ligand is bound exclusively (e = 0.001 or 0.0002) to R or R′, respectively, whereas the latter ligand is bound only preferentially to R (c = 0.06) and even less to R′ (c = 0.27).

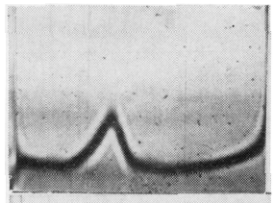
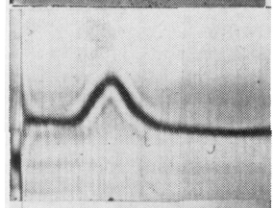
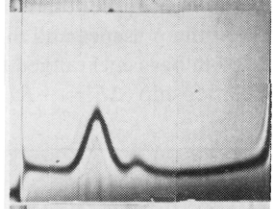
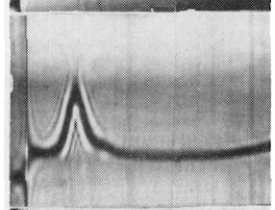
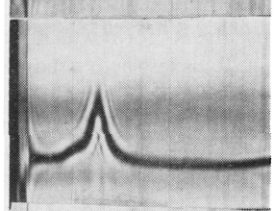
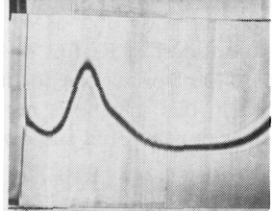
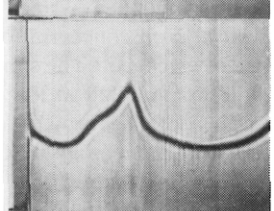
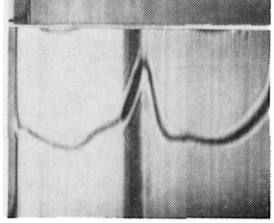
We visualize that the species which determine the rate in kinetic experiments are the R forms of dimer *b*. At concentrations of 5′-AMP too low to saturate R, the activity declines because the R′ form which remains becomes rate determining. R′ is less active than R because of its even lower affinity for anionic substrates (glucose-1-P) in sodium glycerol-P buffer. This explains the lower than theoretically expected activity (*i.e.*, about one-half of V_{max}) of phosphorylase dimer *b* at 0.01 mM 5′-AMP (*cf.* Figure 1). If the concentration of 5′-AMP falls further (below the saturation level of R′ for 5′-AMP) the equilibrium shifts toward the T or inactive form.

The role of glycogen in this scheme is not clear at present. Glycogen, for example, could stabilize the active dimeric form R.

Binding experiments (*cf.* Figures 2–4 and Tables II and III) were carried out at high concentrations of enzyme and in the absence of glycogen.[4] The simplest version of the scheme would assume that at low temperatures (13–4°) only dimers *b* of the form R′ can associate and form tetramers (R′). In analogy to kinetic experiments with dimer *b* it may be assumed that the transition from R to R′ and the association reaction is strongly dependent upon the concentration of

[4] Quantitative measurements of binding of a small molecule (5′-AMP) to a protein are difficult to do with a Sephadex gel filtration technique in the presence of a macromolecule of the size and of the polydispersity of glycogen. From the measurements which we have done with glycogen and phosphorylase *b* at 29° it appeared that the polysaccharide is a remarkably weak allosteric effector. This is in contrast to phosphorylase *a* where glycogen significantly tightens the binding of 5′-AMP (Helmreich *et al.*, 1967). These differences may be related to the different tetramer ⇌ dimer equilibria of the phosphorylases *b* and *a* (*cf.* Wang *et al.*, 1965; Metzger *et al.*, 1967, 1968).

TABLE V: The Temperature-Dependent Association of Phosphorylase Dimer *b*.

Plate[a]	Conditions	Centrifugation Time (min)	$s_{20} \times 10^{13}$ (S)	% of Total
	29° Enzyme (15 mg/ml) 50 mM glucose-1-P 3×10^{-4} M AMP	29.7	9.3	100
	23° Enzyme (16.5 mg/ml) 50 mM Glucose-1-P 3×10^{-4} M AMP	28.7	9.9	100
	12.5° Enzyme (20 mg/ml) No AMP	28.1	8.4 12.8	90 10
	13° Enzyme (10.2 mg/ml) 0.2×10^{-4} M AMP	32.1	8.0 12.1	93 7
	12.6° Enzyme (10.2 mg/ml) 3×10^{-4} M AMP	28.0	88.2 11.8	9 91
	4° Enzyme (8 mg/ml) 50 mM glucose-1-P No AMP	21.1	9.4 12.7	80 20
	4° Enzyme (8 mg/ml) 50 mM glucose-1-P 0.2×10^{-4} M AMP	28.1	9.3 12.1	39 61
	4° Enzyme (8 mg/ml) 50 mM Glucose-1-P 0.4×10^{-4} M AMP	33.4	8.6 12.0 14.5	20 75 5

[a] All ultracentrifugal sedimentation velocity measurements were carried out in 50 mM sodium glycerol-P buffer containing 2 mM EDTA–1 mM 2-mercaptoethanol (pH 6.8) and at constant ionic strength of $\Gamma/2 = 0.13$. In the case of addition of glucose-1-P the buffer anion was replaced by the substrate anion. Time of centrifugation was calculated for 20°. It is the interval between the time after the rotor had reached full speed and the time the picture which is shown was taken. Sedimentation was from left to right.

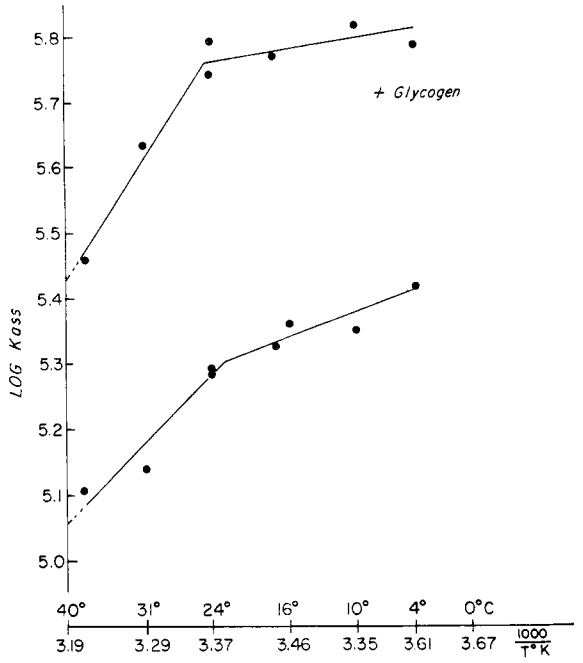


FIGURE 6: Changes of K_{assn} (5′-AMP) for phosphorylase *a* with temperature. Log K_{assn} (5′-AMP) is plotted *vs.* $1/T$ °K. Upper curve: 1% glycogen. The straight lines were drawn by the method of least squares. Lower curve: no glycogen. The concentration of phosphorylase *a* was 1 mg/ml and the concentrations of 5′-AMP ranged from 9×10^{-7} to 3×10^{-5} M. The experiments were carried out in sodium glycerol-P buffer, pH 6.8, $\Gamma/2 = 0.14$. Binding of radioactive 5′-AMP was measured by an equilibrium dialysis method. The experimental conditions are described in a preceding publication (Helmreich *et al.*, 1967).

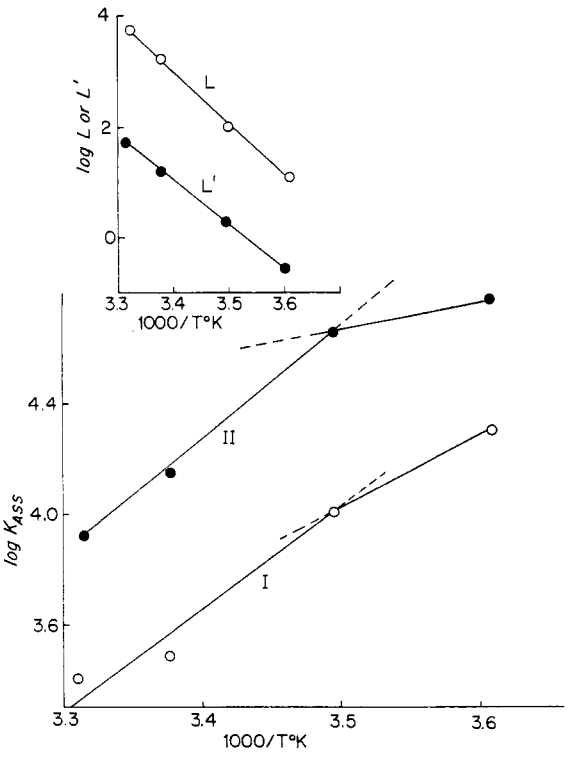


FIGURE 7: Changes of K_{assn} (5′-AMP) and of L and L' for phosphorylase *b* with temperature. Log K_{assn} (5′-AMP) is plotted *vs.* $1/T$ °K. Curve I (○): no glucose-1-P; curve II (●): 50 mM glucose-1-P. The values for K_{assn} (5′-AMP) and of L and L' were derived from the binding experiments in Figures 2–4 and Table V. In the insert are plotted log L (curve I, ○) and log L' (curve II, ●) *vs.* $1/T$ °K. The values are from Table IV.

5′-AMP whereas the transition from T to R is dependent upon 5′-AMP and anionic ligands as well. Moreover R′, the form that binds 5′-AMP most tightly, would be expected to accumulate at equilibrium (*i.e.*, in binding experiments) only at low temperatures (and in the presence of glucose-1-P), where the equilibrium is already shifted nearly completely from T to R ($L = 0.05$; see Table V). When the concentration of 5′-AMP is lowered from 9.5×10^{-6} to 1.5×10^{-6} M a sudden shift occurs from tetrameric forms (R′) to dimeric forms despite the presence of glucose-1-P. These dimeric forms bind 5′-AMP poorly and may be designated as T forms (see curve II, Figure 4). Although the relationships between the hypothetical dimeric state R′ observed at low enzyme and 5′-AMP concentrations in kinetic experiments and the hypothetical tetrameric state R′ observed at low temperatures and at high enzyme concentrations in binding experiments are conjectural, it is apparent nevertheless that in both instances 5′-AMP cannot be substituted for by glucose-1-P. Stated differently: the heterotropic cooperativity of glucose-1-P is too weak in sodium glycerol-P buffer to counteract effectively the shift in the equilibrium from the forms R′ toward the T form (or forms) when the concentration of 5′-AMP is lowered further. It is assumed here that the T forms as compared to the R′ forms do not bind appreciably to 5′-AMP ($e = 0.0002$) and that T forms are inactive and cannot associate under these conditions.

This scheme would seem to offer a reasonable explanation for our experimental observations in sodium glycerol-P buffer. In order to explain why the tetramer *b* is a fully cooperative dimer *b* we speculate that the additional surface contacts established in the tetrameric structure (R′) (or in higher aggregates) of phosphorylase *b* (R′) are weak and contribute little to cooperativity (see Buc and Buc, 1967; Kastenschmidt *et al.*, 1968).

Aside from the evidence presented above and elsewhere (Helmreich *et al.*, 1967) the experiments shown in Figures 6 and 7 are relevant to the scheme under discussion. Figure 6 is a van't Hoff plot of binding measurements with rabbit muscle phosphorylase *a*. We are obligated to Dr. Maria C. Michaelides for this information. Each point represents a value for K_{assn} (5′-AMP) that is derived from a tangent to the linear part of a binding curve over a range of 5′-AMP concentrations from 2×10^{-7} to 3×10^{-4} M. Hence the K_{assn} values for 5′-AMP in Figure 6 represent the binding affinity of the tightest binding forms (*i.e.*, R forms) present at equilibrium, at each of the temperatures indicated. If the values for log K_{assn} (5′-AMP) are plotted against $1/T$ °K lines are obtained which are nearly parallel and which have similar breaks with inflection points in the same temperature range (around 23°). This applies to exper-

iments in the presence of glycogen, where only the dimeric R species, and to experiments in the absence of glycogen, where only the tetrameric R species, of phosphorylase *a* are present (*cf.* Helmreich *et al.*, 1967; Metzger *et al.*, 1967, 1968). A similar series of experiments with phosphorylase *b* is shown in Figure 7, where a van't Hoff plot of the K_{assn} values derived from the binding plots in Figures 2–4 and Table V is shown. It may be noted that the lines in the presence and absence of glucose-1-P have breaks. The transition occurs around 13°. In the absence of glucose-1-P the discontinuity in the lines is much less pronounced.

As described in the legend to Figure 7 the same mode of calculation was used to obtain the values of K_{assn} (5′-AMP) for phosphorylase *b* as was used in the experiments with phosphorylase *a*. Therefore the break in these lines suggests the existence of two forms (R) of phosphorylases *b* and *a* with somewhat different ligand binding, catalytic, and thermodynamic properties. The enthalpy changes for the binding of 5′-AMP to phosphorylase *a* are about 0.9 and 2.2 kcal for experiments with and without glycogen, respectively, and for the temperature range from 23 to 4°. For temperatures greater than 23°, the enthalpy changes are about 9.1 and 5.9 kcal for experiments with and without glycogen, respectively (for additional information, see Helmreich and Cori, 1964b; Helmreich *et al.*, 1967; and Metzger *et al.*, 1968). With phosphorylase *b*, the enthalpy changes for the binding of 5′-AMP to the R forms are about 18.8 and 16.9 kcal for temperatures from 29 to 13° with or without glucose-1-P, respectively, but considerably smaller for temperatures from 13 to 4°: $-\Delta H = 3.8$ kcal for experiments with glucose-1-P and 12.2 kcal for experiments without glucose-1-P. It is important to note that the T to R equilibrium is dependent upon temperature in the usual fashion as is indicated by the straight lines without breaks in the *L* and *L′* plots over the same range of temperatures (29–4°) (insert to Figure 7). Thus the breaks in the van't Hoff plots suggest another allosteric equilibrium that has an unusual temperature dependence and differs from the T $\rightleftharpoons$ R equilibrium. This we believe is the allosteric equilibrium between R and R′.[5]

We are aware that our interpretation of the allosteric transitions of muscle phosphorylase is incomplete because other possibilities which might explain the data were not considered (including the possibility of hybrid states, Koshland *et al.*, 1966). In order to decide that and to see whether the differences in the allosteric transitions of phosphorylase *b* in glycerol-P and in glycylglycine buffer are solely due to the interaction of the enzyme with anionic *vs.* cationic buffer ions, Dr. Henri Buc and E. H. have initiated kinetic experiments using the temperature-jump relaxation method of Eigen and de Maeyer (1963). These experiments are carried out in collaboration with Dr. H. Winkler in Dr. M. Eigen's laboratory in Göttingen. The results of the kinetic study will be presented jointly at a later time. The results of the kinetic work may allow us perhaps to design a more definitive model for the allosteric transitions of rabbit skeletal muscle phosphorylase *b*. This model must also take into account the recent important findings of Valentine and Chignell (1968) that on the basis of electron microscopic evidence the subunits of muscle phosphorylase are similar but not identical.

Discussion

Information presented here and in the report of Buc and Buc (1967) points to differences in the allosteric transitions of phosphorylase *b* which seem to depend mainly on the nature of the buffer ions. These differences are most pronounced with respect to the heterotropic cooperativity of the substrate anions. For instance phosphate anions are ineffective in sodium glycerol-P buffer but very effective in glycylglycine buffer. The latter buffer was used by Buc and Buc (1967). This indicates that there are other effectors aside from reactive ligands that profoundly change the allosteric equilibrium of muscle phosphorylase. These are, among other factors (ionic strength, pH, and temperature), various anions and cations. Effects of some of these ligands are quantitatively quite comparable with the effects of stereospecific substrates and the modifier. We have therefore considered these ions as allosteric ligands in their own right. The effect of the glycerol-P anion on muscle phosphorylase is reminiscent of the effect recently reported by Benesch *et al.* (1968) of the 2′,3′-diphosphoglycerate anion on hemoglobin. It is at present a matter of speculation what role these ionic effectors may play in the control of phosphorylase activity in the living cell.

In a preceding paper (Helmreich *et al.*, 1967) a general scheme of allosteric transitions of rabbit skeletal muscle phosphorylase *a* was presented. It was shown that the affinities of the tetrameric and dimeric forms (R) of phosphorylase *a* for 5′-AMP are quite similar. Therefore, it was concluded that association and dissociation linked to the binding of 5′-AMP are not likely to play important roles in the allosteric transitions of muscle phosphorylase *a*. Phosphorylase *b* differs in this respect, because the allosteric T $\rightarrow$ R transitions are complicated by the tendency of the phosphorylase *b* subunits to associate at low temperatures to aggregated forms that bind 5′-AMP tighter. Graves *et al.* (1965) showed first that phosphorylase *b* is inactivated on exposure to cold and that inactivation involves aggregation. It may be noted that the scheme proposed by us assumes that the decline in activity on exposure to cold is due to the formation of the aggregated species R′. This form is less active because of its low affinity for glucose-1-P. Some aspects of subunit interactions of phosphorylase *b* including the effect of temperature and their relationship to the allosteric properties of this enzyme have recently been discussed (Kastenschmidt *et al.*, 1968).

Inactivation of phosphorylase *b* and aggregation on

[5] The striking dependency of the cooperative binding of 5′-AMP upon temperature has also been observed in glycylglycine buffer (Drs. H. and M. H. Buc, personal communication). In their experiments the change in 5′-AMP binding and a change in viscosity in the temperature range from 5 to 30° matched each other. The viscosity changes are indicative of a melting of the protein structure.

exposure to cold and linked to the binding of 5′-AMP may not be of regulatory significance in homoiothermic animals. However one could speculate in quite general terms that the sensitivity to cold of muscle phosphorylase and of other allosterically regulated mammalian enzymes (*e.g.*, the mitochondrial chicken liver pyruvate carboxylase which is activated by acyl-CoA derivatives (Scrutton and Utter, 1965) and the rabbit muscle pyruvate kinase which is cation activated (Kayne and Suelter, 1968; for other examples see Massey *et al.*, 1966) may reflect an evolutionary change related to homoiothermy. This is supported by the differences in the effect of temperature on activity and on the tetramer $\rightleftharpoons$ dimer equilibria of mammalian and frog muscle phosphorylase. This was recently discussed (Metzger *et al.*, 1968).

It now appears in the light of recent evidence presented here and elsewhere (Helmreich *et al.*, 1967) that the phosphorylase *b* to *a* interconversion involves transition from one allosteric system (phosphorylase *b*) with an *L* value of ~2100 to an allosteric system (phosphorylase *a*) with an *L* value of 3–13 (at 23°) (*cf.* Helmreich *et al.*, 1967). It is therefore of interest to discuss the possible biological significance of the phosphorylase $b \rightleftharpoons a$ interconversion (Danforth *et al.*, 1962; Danforth and Helmreich, 1964) in terms of the very different allosteric properties of the two muscle phosphorylases. Although it is admittedly hazardous at present to speculate on the state of an allosteric enzyme *in vivo*, phosphorylase *b* might reasonably be assumed to be inactive in the resting muscle. Furthermore changes in the tissue concentrations of any of the specific allosteric ligands which promote the T → R transitions of phosphorylase *b* *in vitro* do not correlate well with the actual increase in the rate of glycogenolysis *in vivo* in the electrically stimulated and in the epinephrine-treated muscle (Karpatkin *et al.*, 1964; Helmreich and Cori, 1965, 1966). Although there is no doubt that phosphorylase *b* can effectively catalyze glycogen breakdown as shown by the experiments of Danforth and Lyon (1964) with the I strain of mice lacking phosphorylase *b* kinase, it seems that there are conditions where the most effective means by which a muscle in the active state can furnish active phosphorylase is by conversion of phosphorylase *b* which is present exclusively in the inactive T state to phosphorylase *a* which is present to a considerable extent in the R or active state even in the absence of reactive ligands. The partially phosphorylated hybrids formed in the interconversion which were described by Hurd *et al.* (1966) would add additional control properties since one would expect these hybrids to have control properties different from phosphorylases *b* and *a*.

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Added in Proof

Editorial comments and discussions with several colleagues prompted us to give the derivation of eq 2 used for binding experiments with 5′-AMP. The same terms as defined in the paper are used. The binding of 5′-AMP to tetramer T can be neglected and the binding of 5′-AMP to tetramer R′ at equilibrium may be described as follows

$$R_0' + \text{AMP} \rightleftharpoons R_1'$$

$$R_1' + \text{AMP} \rightleftharpoons R_2'$$

$$R_2' + \text{AMP} \rightleftharpoons R_3'$$

$$R_3' + \text{AMP} \rightleftharpoons R_4'$$

$$R_1' = R_0'[4(\text{AMP})]/K_{\text{dissn}R_0'} \longrightarrow 4R_0'd\gamma$$

$$R_2' = R_0'\{[4(\text{AMP})]/K_{\text{dissn}R_0'}\}[{}^3/_2(\text{AMP})/K_{\text{dissn}R_0'}] \longrightarrow 6R_0'(d\gamma)^2$$

$$R_3' = R_0'\{[4(\text{AMP})]/K_{\text{dissn}R_0'}\}[{}^3/_2(\text{AMP})/K_{\text{dissn}R_0'}] \times [{}^2/_3(\text{AMP})/K_{\text{dissn}R_0'}] \longrightarrow 4R_0'(d\gamma)^3$$

$$R_4' = R_0'\{[4(\text{AMP})]/K_{\text{dissn}R_0'}\}[{}^3/_2(\text{AMP})/K_{\text{dissn}R_0'}] \times [{}^2/_3(\text{AMP})/K_{\text{dissn}R_0'}][{}^1/_4(\text{AMP})/K_{\text{dissn}R_0'}] \longrightarrow R_0'(d\gamma)^4$$

The binding of 5′-AMP to dimer R or dimer T can be described accordingly.

The $\bar{Y}$ function gives the relationship of sites bound to 5′-AMP per total number of available AMP binding sites.

$$\bar{Y} = \frac{(R_1 + 2R_2) + (T_1 + 2T_2) + (R_1' + 2R_2' + 3R_3' + 4R_4')}{n(R_0 + R_1 + R_2) + n(T_0 + T_1 + T_2) + 2n(R_0' + R_1' + R_2' + R_3' + R_4')}$$

By substituting the above factors for the *R*, *R′*, and *T* terms we obtain

$$\bar{Y} = \frac{2R_0(\gamma + \gamma^2) + 2T_0(e\gamma + (e\gamma)^2) + 4R_0'(d\gamma + 3(d\gamma)^2 + 3(d\gamma)^3 + (d\gamma)^4)}{2R_0(1 + 2\gamma + \gamma^2) + 2T_0(1 + 2e\gamma + (e\gamma)^2) + 4R_0'(1 + 4d\gamma + 6(d\gamma)^2 + 4(d\gamma)^3 + (d\gamma)^4)}$$

The equilibrium $R_0' \rightarrow 2R_0$ may be described by L_0^*: $L_0^* = (R_0)^2/R_0'$; K_{equil} was defined as $K_{\text{equil}} = R_0'/(R_0)^2$ (see Table IV); thus it follows that L_0^* and K_{equil} are related to each other because $R_0' = (R_0)^2 \cdot K_{\text{equil}}$. Substituting $(R_0)^2K_{\text{equil}}$ for R_0' and L_0R_0 for T_0 we obtain eq 2 in the form given in the paper. It also follows that $L_0^* = (R_0)^2/(R_0)^2K_{\text{equil}}$ or that $L_0^* = 1/K_{\text{equil}}$.

The L_0^* value may be calculated from the data for K_{equil} given in Table IV. The L_0^* values are in the range

from 1.9×10^{-5} to 3.3×10^{-4} depending on conditions and temperature. This indicates that at low temperatures under the conditions of the experiments in Table IV the concentration of R_0' is $\gg (R_0)^2$ and that at equilibrium the concentration of tetramer (R′) is $\gg$ dimer (R). This was predicted and is stated in the interpretation of the results.

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