STUDIES ON THE MECHANISM OF ALLOSTERIC INTERACTIONS OF GLYCOGEN PHOSPHORYLASE

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ABSTRACT

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ABSTRACT

The kinetic and structural properties of glycogen phosphorylases *a* and *b* have been studied in the presence of various effectors. The allosteric transitions of these two enzyme forms were analysed, and compared with the predictions of the theoretical model proposed by Monod, Wyman, and Changeux.

Thirty-three nucleotides and related compounds have been investigated as effectors to further understand the mechanism of AMP activation of phosphorylase *b*. All nucleoside-5'-monophosphates and 2' deoxyadenosine-5'-monophosphate activated the enzyme at values ranging from 10% to 60% of the activation by AMP. This activation was dependent upon the glucose-1-P concentration. Other deoxynucleotides, cyclic-2'3'-AMP, cyclic-3'5'-AMP, 2'-AMP, 3'-AMP, nucleosides and bases did not activate the enzyme. All effectors tested were competitive inhibitors with respect to AMP. The mode of activation and binding to the enzyme by each compound was analysed kinetically in terms of their respective K_a's and K_i's. These studies revealed a high degree of enzyme specificity for AMP. The 5'-monophosphate group was absolutely required for activation and the hydroxyl group at position 2' of the ribose moiety contributed to both the activation and the binding. The amino group at position 6 and the imidazole moiety of the purine ring appeared to be important for binding.

In order to investigate the difference between the mechanism of phosphorylase b activation by AMP and the other nucleotides, IMP was selected for a detailed study of nucleotide interaction with the enzyme. In contrast to AMP, IMP affected the V_{max} but not the affinity of the enzyme for the substrate, glucose-1-P. The plots of initial rate (v_i) against glucose-1-P concentration for IMP activation were sigmoidal. Hill coefficients of 2.0 were observed at all levels of IMP indicating that the homotropic cooperativity of the substrate was independent of nucleotide concentration. The plots of v_i against IMP concentration were sigmoidal at low glucose-1-P concentrations but hyperbolic at high concentrations. All 5'-nucleotides tested exhibited kinetic properties similar to IMP. The activation of phosphorylase b by nucleotides was enhanced by sodium fluoride (NaF), and the polycationic molecules: spermine, and protamine. In the presence of 0.3 M NaF the kinetics were normalized and the nucleotide affinity increased. Under these conditions the K_m values of both AMP and IMP were now independent of substrate concentration; only the V_{max} changed with increasing glucose-1-P. The kinetic data suggested that the mechanism of nucleotide activation resulted in a two-stage allosteric transition of the enzyme, viz. a change in both catalytic efficiency and enzyme affinity for glucose-1-P. The proposed mechanism, an extension of Koshland's "induced-fit" theory, received strong support from the following physical observations. AMP, IMP, and glucose-1-P showed varying degrees of protection against cold inactivation and the removal of pyridoxal phosphate from the enzyme. While AMP caused partial dimerization of phosphorylase b, other 5'-nucleotides dimerized the enzyme only in the presence of glucose-1-P.

Limited tryptic attack of phosphorylase a or b results in the

release of a specific hexapeptide from the enzyme to produce the dimer, phosphorylase b'. The effect of nucleotides on this modified enzyme form were examined. Similar to the activation of phosphorylase b, only the nucleoside-5'-monophosphates and dAMP activated phosphorylase b'. This activation was insensitive to the addition of polyamines. However, with the exception of AMP, activation of phosphorylase b' by nucleotides was stimulated in the presence of NaF.

In addition to the study of the allosteric mechanism of phosphorylase b activation by nucleotides, the activation and dissociation of tetrameric phosphorylase a to a dimer upon prior incubation with glucose and AMP has been studied in relation to the allosteric transitions of this enzyme. The dependence on glucose concentration of both the initial rate and the extent of activation were characteristic of homotropic interactions, having Hill coefficients of 2.9 and 1.5, respectively. The initial rate as well as the extent of phosphorylase a activation was also dependent upon AMP concentration. The maximum extent of AMP activation of the enzyme was much lower than that of glucose activation. In the presence of 0.005 M to 0.1 M glucose, AMP caused inactivation rather than activation. The final enzyme activity obtained at saturating AMP concentration was dependent upon the glucose concentration. At 0.2 M glucose, AMP had no effect on the enzyme activity. The reversal of glucose dissociation by AMP showed a similar dependence upon glucose concentration. Both glucose and AMP affected the rate of inactivation of phosphorylase a in the presence of trypsin or p-hyroxymercuribenzoate (HMB). Although 0.2 M glucose enhanced the rate of phosphorylase a inactivation by trypsin, this effect was completely reversed by AMP. Glucose at 0.2 M retarded the initial rate

of phosphorylase a inactivation by HMB. Although AMP by itself provided slight protection of the enzyme against HMB inactivation, it enhanced the inactivation rate when 0.2 M glucose was present. These results supported the view that enzyme dissociation was a result of conformational changes of the enzyme subunits. The effect of modifier concentration on phosphorylase a activation could be explained by the model proposed by Monod, Wyman, and Changeux if three conformational states were postulated for the enzyme. This model, however, could not readily account for the effect of modifiers on the rate of enzyme inactivation by HMB and trypsin. These results suggested that the allosteric transition mechanism of phosphorylase a, like that of phosphorylase b, could be best explained by a direct application of Koshland's "induced-fit" theory.

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LIST OF ABBREVIATIONS

AMP	Adenosine-5'-monophosphate
2'-AMP	Adenosine-2'-monophosphate
3'-AMP	Adenosine-3'-monophosphate
cyclic-2'3'-AMP	Adenosine-2'3'-phosphate
cyclic-3'5'-AMP	Adenosine-3'5'-phosphate
dAMP	2'-deoxyadenosine-5'-monophosphate
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
CMP	Cytidine-5'-monophosphate
dCMP	2'-deoxycytidine-5'-monophosphate
NAD	Nicotinamide adenine dinucleotide
EDTA	Etylenediaminetetraacetate
GMP	Guanosine-5'-monophosphate
dGMP	2'-deoxyguanosine-5'-monophosphate
GTP	Guanosine-5'-triphosphate
Glucose-1-P G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
HMB	p-hydroxymercuribenzoate
IMP	Inosine-5'-monophosphate
5'-NMP	Nucleoside-5'-monophosphate
PCMB	p-chloromercuribenzoate
P_i	Inorganic phosphate
PLP	Pyridoxal phosphate
rpm	Revolutions per minute
d-Ribose	2-deoxy ribose

Ribose-5-P	Ribose-5-phosphate
$s_{20,w}$	Sedimentation coefficient corrected to water at 20°
dUMP	2'-deoxyuridine-5'-monophosphate
XMP	Xanthosine-5'-monophosphate
dXMP	2'-deoxyxanthosine-5'-monophosphate

I. INTRODUCTION

1. INTRODUCTION

Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) is present in rabbit skeletal muscle in two molecular forms: dimeric phosphorylase b, which is obligatorily dependent upon AMP for activity, and phosphorylase a, a tetramer active in the absence of this nucleotide (1). The exact role of AMP in the activation of phosphorylase b has not yet been established. Although phosphorylase b is inactive in the absence of AMP, this nucleotide does not participate as an intermediate in the reaction. Cohn and Cori (2) have shown that the 5'-monophosphate of AMP does not exchange with the substrates, glucose-1-P or P_i, during enzymic action. Although both phosphorylases a and b contain one AMP binding site per subunit (3), phosphorylase a has a higher affinity for AMP than has phosphorylase b (3,4). Since the quarternary structure of phosphorylase b can be affected by AMP (5), it has been suggested that activation results from a conformational change of the enzyme (6). Based on this assumption, Monod, Changeux, and Jacob (6) have used this enzyme to illustrate the general mechanism of allosteric transitions.

Glycogen phosphorylase a can be dissociated into dimeric units upon prior incubation in glucose (7). This dissociation of phosphorylase a tetramer to a dimer has been correlated with an increase in the specific activity of the enzyme. The effect of glucose can be reversed by glucose-1-P and AMP. Prior incubation of tetrameric phosphorylase a with AMP also induced the formation of a more active dimer (7,8). The fact that glucose and AMP are not related in structure suggested that the interaction of these ligands with phosphorylase a has characteristics common to most allosteric enzymes.

a. Purpose and scope of the study

The purpose of this work was to investigate the activation of glycogen phosphorylases a and b by their respective effectors in relation to the allosteric transitions of these two enzyme forms.

Both phosphorylases a and b can exist in equilibrium between a tetrameric and a dimeric species (5,9). Activation of phosphorylase b either by enzyme-catalysed phosphate incorporation or by AMP binding results in an enhanced association of the dimeric form of the enzyme into a tetramer, suggesting that there is a correlation between the appearance of catalytic activity and enzyme association. However, since at the low enzyme concentrations used for measurement of catalytic activity both phosphorylases a and b exist as dimeric species irrespective of the presence of activators (7,8,10), association alone can not account for enzyme activity. Conceivably, activation of glycogen phosphorylase could arise from conformational alterations in the enzyme upon binding with the activators. Such a change in conformation may result in an alteration in the associative properties of the enzyme. An examination of the allosteric transition mechanism of glycogen phosphorylase will form the major part of this thesis and will be confined to the following lines of enquiry:

1) The allosteric mechanism of phosphorylase b activation by nucleotides.
2) The relationship between structural change and allosteric transition of phosphorylase a.

b. General approach to the problem

The first part of this thesis is concerned with studies on the

allosteric activation of glycogen phosphorylase *b* by nucleotides. The effect of AMP on the kinetic and structural properties of phosphorylase *b* has been studied extensively (11-14). From these studies, the suggestion that AMP causes conformational changes of phosphorylase *b* (6,10) has received strong support. Among other nucleotides examined, only IMP was found to have a slight activating effect which could be greatly enhanced by the protamine salmine, and polyamines (15,16). The mechanism of IMP activation has not yet been reported. Previous studies of phosphorylase *b* activation by nucleotides were carried out with a single, low concentration of glucose-1-P (0.016 M). Dixon and Webb (17) have pointed out the advantages in enzyme specificity studies of an analysis of the effects of substrate structure on both the V_{max} and K_m, as opposed to the common practice of only determining the initial rates with a series of related compounds at a constant concentration. Therefore, in the present investigation, the effect of over thirty structural analogues of AMP on phosphorylase *b* activity was studied at different concentrations of glucose-1-P. In addition, the kinetics of interaction of these compounds with glucose-1-P and AMP was studied. From the kinetic data the activation and inhibition constants were calculated wherever possible. Based on the relative values of these kinetic constants, an attempt was made to elucidate the functional groups in the AMP molecule required for the binding to and the activation of the enzyme, as well as the possible nature of the binding forces. IMP was selected as a model compound for a detailed study of the allosteric mechanism of phosphorylase *b* activation. In addition to the above inquiries into the nature of the mechanism of nucleotide activation, the effect of various ionic species on nucleotide activation of phosphorylase *b* was investigated. From the

kinetic data a model was proposed to explain the allosteric transitions in phosphorylase _b_, and various physical criteria were used to test the predictions of this model.

The recent work of Graves and coworkers (7,8,18) has shown that incubation of glycogen phosphorylase _a_ with AMP, glucose, or certain polysaccharides results in a time-dependent activation of this enzyme which can be correlated with an enhanced dissociation of phosphorylase _a_ tetramer into dimer. In this thesis, the activation and dissociation of phosphorylase _a_ has been reinvestigated in relation to the allosteric transitions of this enzyme. The dependence of both initial rate and final extent of phosphorylase _a_ activation upon glucose or AMP concentration was examined. In addition, the kinetic data were correlated with the effect of these modifiers on the rate of phosphorylase _a_ inactivation by trypsin or HMB. A model was proposed to explain the allosteric transitions of this enzyme.

c. Organization of Thesis

The body of the thesis is divided into four main sections: Review of the Literature, Experimental Procedure, Results, and Discussion. The literature review includes, in addition to the properties of glycogen phosphorylase, certain aspects of current knowledge about allosteric enzymes. The result section is divided into two parts, dealing with the experiments performed with phosphorylase _b_ and _a_, respectively. In the discussion the two forms of the enzyme are treated separately.

II. REVIEW OF THE LITERATURE

II. REVIEW OF THE LITERATURE

Since 1960 numerous excellent reviews and articles by a number of workers (1, 19-22) have been published describing the properties of rabbit skeletal muscle glycogen phosphorylases _a_ and _b_ and the factors that regulate their interconversions. Therefore, no attempt will be made to review all of the evidence contributing to our present knowledge of this enzyme but certain facts concerning phosphorylase pertinent to this thesis will be mentioned. The main emphasis will be placed on the allosteric properties of this enzyme reported in recent literature.

A. Allosteric Proteins

It is advantageous before discussing the properties of glycogen phosphorylase to first describe the current concepts regarding allosteric proteins and the basis of their allosteric effects.

1. Nomenclature

Many enzymes acting at critical metabolic steps are selectively endowed with specific functions of regulation and coordination enabling a constant balance to be maintained between the various catabolic and biosynthetic reactions that occur in living organisms. The biological activity of these enzymes can be either activated or inhibited by specific metabolites which are structurally dissimilar to the substrates or products of the reaction. Monod, Changeux, and Jacob (6) have proposed that such regulatory proteins be named _allosteric enzymes_ and the controlling metabolites, the _allosteric effectors_. It appears that these enzymes possess specific binding sites for the allosteric effectors. These sites, termed _allosteric sites_, are topologically distinct from

the active site which binds the substrate. The term ligand will include activators, substrates, and inhibitors. The binding of the allosteric effector to the enzyme is assumed to bring about a reversible alteration of the molecular structure of the protein modifying the kinetic properties of the active site. Such a molecular alteration is termed an allosteric transition. As a matter of historical importance, Monod *et al* (6) have cited muscle glycogen phosphorylase as the first allosteric enzyme to be studied and analysed in detail (1, 23, 24).

2. Properties of allosteric enzymes

The general properties of allosteric enzymes are as follows:

1) For many regulatory enzymes the plot of initial velocity against substrate concentration yields a sigmoid curve as opposed to the hyperbolic saturation curve obtained for non-regulatory enzymes obeying the Michaelis-Menten relationship. Sigmoid curves indicate that at least two molecules of substrate interact with the enzyme and the binding of one molecule in some way facilitates the binding of the next molecule.

2) The majority of allosteric enzymes thus far studied have been shown to be composed of subunits.

3) Allosteric enzymes can exhibit homotropic and heterotropic interactions, i.e. interactions occurring between identical or different ligands, respectively.

4) Few allosteric enzymes exhibit only heterotropic effects, cooperative homotropic effects being almost always observed with at least one of the ligands of the system.

5) Conditions or treatments which alter the heterotropic interactions also simultaneously alter the homotropic interactions.

In general, the plot of initial velocity versus substrate concentration for an allosteric enzyme yields a sigmoid saturation curve given by the equation

$$v = \frac{VS^{n}}{K + S^{n}} \qquad \text{(I)}$$

which by suitable manipulation yields (6,25) the empirical Hill equation (26, 27),

$$\log [v/(V-v)] = n \log S - \log K \qquad \text{(II)}$$

where v is the reaction velocity; V, the maximal velocity; and K a constant. Thus a plot of log [v/(V-v)] as a function of log S should give a straight line of slope *n*. This slope is a function of the number of interacting substrate binding sites per enzyme molecule and of the strength of site-site interaction. When these interactions are strong the slope will be numerically equal to *n*, the number of binding sites. As the intersite interactions are weakened the slope will decrease to a value of 1.0.

3. Theoretical models for allosteric transition

a. The Monod, Wyman, and Changeux model. -- Monod, Wyman, and Changeux (28) have postulated a theoretical model to explain the regulatory mechanism of allosteric enzymes which places emphasis on the subunit structure of these enzymes as the important characteristic in accounting for their unique kinetic behavior. The proposed model was based on the following assumptions:

1) Allosteric proteins are oligomers, molecules composed of

two or more identical subunits (protomers), in which the symmetry is conserved during allosteric transitions.

2) Each protomer contains a single active site specific for the substrate and a separate allosteric site for each activator or inhibitor.

3) The oligomer can exist in at least two different conformational states, designated as a "relaxed" state (R) and a "constrained" state (T), that differ in affinity for the various ligands. These two states exist in thermodynamic equilibrium in the absence of substrates or effectors.

4) For each conformational state all homologous sites have identical intrinsic affinities for the binding of their respective ligands.

Monod _et al_ (28) have assumed that the R state has significant affinity only for the substrate and activator, whereas the T state has affinity exclusively for the inhibitor. Each ligand acts by displacing the equilibrium in favor of the conformation state having the greater affinity for that ligand.

Since the activating and inhibitory effects in any enzyme system are measured in terms of variations in the Michaelis constant (K_m) and maximum velocity (V_{max}), Monod _et al_ (28) have classified allosteric enzymes as either a K or V class system. A K class system is the case in which the effectors will affect the K_m but not the V_{max}, whereas, for a V class system the exact opposite applies. For a system of class K, both the effectors and substrate have differential affinities for the T and R

states. It was predicted that if the effector affects the K_m of the substrate then the latter must exhibit cooperative homotropic interactions. If the assumption is made that the substrate has a preferential affinity for the R state and the free enzyme exists largely in the T state, then substrate addition will tend to pull the equilibrium in the direction of the R state and the resultant saturation curve will be sigmoid. Thus, the model can account for homotropic effects. The activator at high concentrations, having high affinity for the R state and low affinity for the T state, will displace the equilibrium entirely to the R state. Under this condition, the substrate will not show cooperativity since it also preferentially binds to the R state. At low substrate concentrations, the activator will show pronounced cooperativity because it can differentially bind to both states. The inhibitor, binding exclusively to the T state, will exhibit cooperativity at high substrate concentrations. This model can therefore explain homotropic and heterotropic effects for K systems.

For a V class system, it is assumed that the substrate has equal affinity for both the R and T states but only the R state can lead to product formation. The initial velocity versus substrate concentration plot will be hyperbolic. Depending on whether the effector has maximum affinity for the predominant state or the minor state, it will behave as either a positive effector (activator) or a negative effector (inhibitor). Cooperativity will be exhibited only if the effector preferentially binds to the less predominant enzyme form.

Several inconsistencies have been reported in the recent literature suggesting that the Monod, Wyman, and Changeux model (28) may not

be universally applicable to all allosteric enzymes. Although positive and negative heterotropic effects can be accounted for by this model, it can only account for positive homotropic effects. Recently, Koshland and coworkers have presented evidence for negative homotropic interactions (29-31) for the enzymes glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (29) and CTP synthetase from _E. Coli_ (31). Atkinson _et al_ (32) have pointed out that the substrate interactions of phosphofructokinase from yeast (32), liver fluke (33), and rabbit muscle (34), and NAD-specific isocitrate dehydrogenase from yeast (25, 35) and rat muscle are not affected by positive or negative effectors. Sanwal and coworkers have shown that their kinetic data for the allosteric enzymes NAD-specific isocitrate dehydrogenase from either _Neurospora_ or _Aspergillus_ (36-40) and threonine deaminase from _Salmonella_ (41) can be interpreted, using steady-state kinetic theory, on the basis of the presence of two different substrate binding sites in which one is an allosteric site. Several enzymes, including phosphorylase _b_, exist which are not V system enzymes, yet show heterotropic interactions between substrate and effector and no homotropic interaction of the substrate (11, 42-44).

b. _Model based on the induced-fit theory._ -- Koshland (29, 45-47) has applied his induced-fit hypothesis (48) to explain the allosteric and cooperative properties of proteins. In essence, the induced-fit theory proposes that there is a flexible interaction between ligand and protein which may induce a new conformation of the protein subunit. This alteration may in turn affect the shape and stability of neighboring subunits dependent upon the strength of the subunit interactions. If the interaction strength is weak, the alteration in one

subunit may not affect its neighbors and Michaelis-Menten kinetics will be observed. If the subunit interactions are strong, a change induced in one subunit will cause partial or equal changes in the neighboring subunits. Koshland predicted that these changes occur sequentially with ligand addition so that hybrid conformational states will be observed.

Cooperativity is explained on the assumption that each subunit can exist in two conformations, only one of which binds ligand to a significant extent. The binding of additional ligands will cause a shift in conformations to the preferred state with a resultant change in the strength of subunit interactions. Activators may bind at a specific allosteric activator site producing a conformational change that leads to an increase in affinity of the enzyme for the substrate at the catalytic site. Inhibitors may compete with the substrate for the same binding site or may bind at a specific allosteric inhibitor site decreasing substrate affinity at the catalytic site.

Atkinson et al (25) have extended the induced-fit hypothesis of Koshland (48) to explain the complicated kinetic behavior of yeast NAD-specific isocitrate dehydrogenase. They observed complex relationships between substrate and effectors with this enzyme which could be explained by a model which assumes progressive changes in ligand site interactions. In this model binding of ligand at one site can either increase or decrease the affinity of ligands at other sites, so that both positive and negative homotropic and heterotropic effects can be predicted. Employing the empirical Hill equation (equation II), Atkinson was able to construct a mathematical model and estimate the number of cooperative effector binding sites and substrate binding sites. Based

on Michaelis-Menten assumptions, it was first proposed that there was a rapid equilibrium between enzyme and substrate, and the formation of the final enzyme-substrate complex leading to product formation was the rate-limiting step. Based on their model, it was proposed from kinetic data that NAD-specific isocitrate dehydrogenase possesses two catalytic sites, two isocitrate regulatory sites, and two AMP regulatory sites.

c. Evaluation of the models. -- The two types of models lead to similar predictions in most respects. However, the models differ in their predictions with regard to the nature and number of conformations that an enzyme can assume. The concerted transition model of Monod, Wyman, and Changeux (28) assumes spontaneous equilibrium between predetermined conformations with ligand binding having no effect on conformation whereas the sequential model of Koshland and collaborators (25, 29, 45-47) assumes that the conformational change is caused by the binding of ligand. The concerted model assumes strong interactions between neighboring subunits, thereby assuring that all subunits within a given molecule will undergo conformational changes in a concerted manner. On the other hand, the model of Koshland *et al* (29, 45-47) is more flexible in that it permits a wide range of interactions from strongly positive through negligible to negative ones. This model can therefore easily account for the association-dissociation phenomena observed with several enzymes. The concerted model is essentially a two conformation model. The Koshland model assumes many final conformational states (as well as intermediate hybrid states), since the structure stabilized involves a complementary interaction of ligand and protein rather than the stabilization of a predetermined structure. For example, Koshland *et al* (45)

have proposed two conformations for each of the four subunits of hemoglobin, with the number of possibilities for the whole molecule depending on the interaction pattern assumed. The models differ most markedly in their predictions regarding the kinetic properties of effector addition. In the Monod _et al_ model (28) an activator reduces the site-site interactions resulting in a decrease in homotropic interactions and hence the reaction order, whereas, for the Koshland _et al_ model (45) the activator facilitates substrate binding by altering the enzyme conformation without necessary having an effect on the order of the reaction.

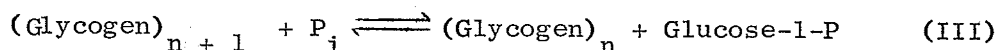
A value judgement of the acceptability of either model would be premature. The properties of some enzymes can be best explained by the concerted transition model (28) whereas others require the sequential model (45). However, compatibility of any theory with kinetic data does not necessary validate that theory. There is no reason to assume that any one allosteric model must be applicable to all regulatory enzymes. In all probability, different allosteric enzymes may follow a variety of mechanisms which have been selected through evolution to tailor the kinetic properties of the enzyme to the needs of the organism.

B. Glycogen Phosphorylase

1. Catalytic properties

Glucose-1-P is a key intermediate in both the synthesis and breakdown of glycogen. Therefore, it is evident that some form of cellular regulation is essential to direct the flow of metabolites in accordance with the physiological demand of the organism for glycogen. An enzyme that could perform this function was first reported by the Cori's (23, 49-52), who demonstrated that animal tissues contained a phosphorylase

which catalysed the phosphorolysis of α-1,4-glucosidic bonds from glycogen as glucose-1-P (4, 51, 53, 54):



$$(\text{Glycogen})_{n+1} + P_i \rightleftharpoons (\text{Glycogen})_n + \text{Glucose-1-P} \qquad \text{(III)}$$

This reaction was shown to be freely reversible having an equilibrium constant (K_{eq}) *in vitro* close to 3 at pH 7.0. Since glycogen is both a product and a reactant the value of the K_{eq} is determined solely by the ratio of P_i to glucose-1-P. However, the relatively high P_i: glucose-1-P ratios known to exist in most animal tissues (55) probably does not allow the phosphorylase reaction to proceed in the synthetic direction *in vivo*.

Crystalline phosphorylase was obtained from rabbit muscle by Cori and Green (24, 56, 57) and was reported to exist in two forms designated as *a* and *b*. Phosphorylase *b* has an absolute requirement upon AMP for activity whereas phosphorylase *a* is 70-80% active in the absence of this nucleotide (24, 58). The conversion of phosphorylase *b* to phosphorylase *a* occurs in the presence of Mg^{+2}, ATP, and the enzyme phosphorylase *b* kinase (59-62). The conversion of phosphorylase *b* to *a* is associated with the release of P_i (60, 63) and is catalysed by a specific phosphorylase phosphatase (64, 65).

2. Molecular properties

During the interconversion reactions of the two forms of phosphorylase, changes in the molecular weight of the enzyme molecule were observed (60, 66, 67). A doubling of the molecular weight on the conversion of phosphorylase *b* to *a* was demonstrated by sedimentation velocity analysis (66) and light scattering studies (68). The molecular weight of phosphorylase *b* was reported to be 242,000 with an $s_{20,w}$ value of

8.2 S, whereas phosphorylase a has a molecular weight of 495,000 with an $s_{20,w}$ value of 13.2 S.* Modification of the cysteinyl residues of phosphorylase with PCMB dissociated the enzyme into subunits of molecular weight 125,000, phosphorylase b into two nonphosphorylated subunits and phosphorylase a into four phosphorylated subunits (68, 72, 73). The dissociation could be reversed by the addition of an excess of cysteine. If it is assumed that the 125,000 molecular weight subunit is the monomeric unit of phosphorylase then phosphorylase a can be considered as a tetramer and phosphorylase b a dimer.

The phosphorylase molecule contains a multiplicity of binding sites, a characteristic now found for most regulatory enzymes. The number of such sites per mole of phosphorylase a and b supports their respective tetramer-dimer structures. There are two and four AMP binding sites for phosphorylase b and a respectively (3), four moles of PLP per mole of phosphorylase a and two moles of PLP per mole of phosphorylase b (74, 75). Furthermore, in the conversion of two moles of phosphorylase b to one mole of phosphorylase a four phosphates are introduced into the enzyme (61).

Although PLP is an essential component of all phosphorylases examined thus far (19, 74-76), its role in enzyme catalysis has not been determined. The removal of PLP under various conditions (21, 75-77) results in the conversion of the two phosphorylase forms to inactive apoenzymes. It appears that PLP, which is covalently bound to the ε-amino group of a lysine residue (21, 78, 79), is involved in maintaining the conformational stability of the enzyme since the removal of this

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* The molecular weights of the two forms of phosphorylase have been recently reinvestigated (14, 69-71). Phosphorylase a was calculated to have a molecular weight of 367,000 and phosphorylase b a molecular weight of 177,000.

prosthetic group results in the partial reversible dissociation of the tetramer into dimeric and monomeric units.

In 1965, Graves _et al_ (80) reported a phenomenon which gave added evidence to support the proposed role of PLP in maintaining phosphorylase conformation. When phosphorylase _b_ was stored at temperatures below 15° at pH 6.0, enzymic activity was lost and heterogeneous material could be detected when analysed in the ultracentrifuge. Inactivation could be reversed on warming. Cold inactivation caused an increase and a shift in the absorption maximum of the protein-bound PLP to shorter wavelengths. No loss of PLP occurred and cold inactivation could be slowed or prevented by glycogen, AMP, ATP, PLP, and organic solvents. In contrast, phosphorylase _a_ was sensitive to cold only in solutions of high ionic strength. Analyses of the data suggested that cold inactivation was a result of a conformational change in the enzyme that resulted in the exposure of PLP to a more polar environment.

The structure of the phosphate-binding site was determined from P^{32}-labelled phosphorylase _a_. Limited tryptic attack of tetrameric phosphorylase _a_ (or dimeric _b_) liberated all the incorporated phosphate attached to a specific hexapeptide with the sequence

-Lys-Gln-Ileu-Ser(P)-Val-Arg-

resulting in the formation of the dimer, phosphorylase _b_' (79, 81). Very little is known about the function of the phosphoserine residues in phosphorylase _a_. The phosphate groups probably do not participate directly in enzyme catalysis, since there is no exchange of these phosphate groups with P_i in the phosphorylase reaction (61), nor with the phosphate of glucose-1-P, AMP or PLP. Although phosphorylation of

phosphorylase b dimer leads to phosphorylase a tetramer (61), the exact relationship between phosphorylation and dimerization is not clear. Chelation of the four phosphoryl groups by a metal introduced in the phosphorylase b to a conversion has been excluded (61). The possibility that the formation of a phosphodiester bridge or a pyrophosphate bond results in dimerization appears remote since tryptic attack of phosphorylase a liberates all the protein-bound phosphate as a monoester with serine (81). Furthermore, the AMP binding site must be in a region of the enzyme other than that occupied by the hexapeptide since phosphorylase b' is enzymatically active in the presence of this nucleotide (67). The specific hexapeptide at the site of phosphorylation is highly positively charged. It has been suggested that the introduction of the negatively charged phosphate groups results in the neutralization of this positive site allowing an interaction between interpeptide regions that were previously electrostatically repulsed (79, 81). In addition, under certain conditions phosphorylase b can also exist as a tetramer in the presence of AMP suggesting that there may be a correlation between enzyme association and catalytic activity (5, 74, 82). Phosphorylase b' exhibits no tendency to associate into a tetramer under similar conditions.

A contribution to our knowledge of the groups of phosphorylase a important for the stabilization of the tetrameric form of this enzyme was made by Graves and coworkers. In 1963, Wang and Graves (9) showed that in the presence of 3 M NaCl at pH 7.4 phosphorylase a was completely dissociated into a form having a molecular weight of 258,000, whereas phosphorylase b was unaffected. Phosphorylase a dissociation could be prevented by AMP. This study suggested that the quarternary structure

of phosphorylase _a_ was indeed stabilized by electrostatic forces. It was later demonstrated that the phosphorylase _a_ dimer was more active than the phosphorylase _a_ tetramer and could be stabilized by the addition of glycogen (7, 8, 18). Dilution of the enzyme and increase in temperature also favored the dissociation of phosphorylase _a_ tetramer into dimer (8). These data strongly suggested that activation was directly related to enzyme dissociation. The work of Graves and collaborators strongly supported the notion that the appearance of enzymic activity that occurs in the conversion of phosphorylase _b_ to _a_ is more directly related to the phosphorylation of the enzyme than to molecular weight alterations.

3. Allosteric properties

Phosphorylase _b_ has many of the properties of an allosteric protein as predicted by the theoretical model of Monod _et al_ (28). The enzyme is composed of two identical subunits and its activity is greatly influenced by metabolites that are structurally unrelated to either substrates or products. Activation of phosphorylase _b_ by AMP is subject to modulation by the competitive inhibitors, ATP and glucose-6-P (11, 83). In addition, the metabolites 6-phosphogluconate, NADH, GTP, malate (84) and UDPG (84, 85) have been observed to inhibit phosphorylase _b_ activity, whereas phosphoenolpyruvate stimulated its activity (84). In contrast, only UDPG and malate were found to inhibit phosphorylase _a_ activity (84). This enzyme form is also inhibited by glucose a competitive inhibitor with respect to glucose-1-P, and by phlorizin, a non-competitive inhibitor (1, 4). The inhibition by both effectors is counteracted by the addition of AMP.

In recent years, the effect of AMP and other metabolites on the kinetic and structural properties of phosphorylase *b* has been studied extensively in an attempt to determine the allosteric transition mechanism of phosphorylase. It has been shown that the activator, AMP, and the substrate, glucose-1-P, have reciprocal effects on each other's apparent K_m value (11, 12). Similarly, an increase in the concentration of AMP decreased the K_m of P_i for phosphorylase *b*, while an increase in P_i concentration decreased the K_m for AMP (12, 86). A similar reciprocal interaction was also observed between AMP and glycogen. Phosphorylase *a* reacted in the same way; an increase in the activator concentration resulted in a decrease in the K_m for P_i without much change in the extrapolated maximum velocity (V_{max}) in a double reciprocal plot (86, 87). In contrast to the catalytic properties of phosphorylase *b*, the apparent K_m's of phosphorylase *b'* for substrates and activator were essentially independent of the concentrations of glycogen, P_i, and AMP (88).

Madsen (11) has investigated the kinetics of ATP inhibition of phosphorylase *b*. The substrate glucose-1-P was synergistic with respect to AMP and antagonized the inhibitory effects of ATP and glucose-6-P. In the presence of ATP a sigmoidal saturation curve was obtained for glucose-1-P, analysis of which indicated that there was cooperative interaction of at least 2 moles of glucose-1-P per mole of enzyme. Madsen and Schechosky (12) have compared the kinetic data for the inhibition of phosphorylase *b* with the predictions of the allosteric model postulated by Monod *et al* (28). The substrate, P_i, exhibited increased homotropic cooperativity as the AMP concentration was decreased, becoming pronounced

in the presence of glucose-6-P and ATP. AMP showed homotropic cooperative interactions which were not affected by a decrease in the substrate concentration; the Hill coefficients having *n* values of 1.4. ATP exhibited homotropic cooperativity which increased as the concentration of P_i or AMP decreased. The inhibitors also appeared to increase the homotropic cooperativity of AMP (12). An important assumption of the Monod, Wyman, and Changeux model (28) is that each ligand binds exclusively to only one of the two postulated (R or T) conformational states of the enzyme. Therefore, from the above data the substrate and activator could favor the active R state while the inhibitors favor the inactive T state. These data, with the exceptions of the homotropic cooperativity of AMP or ATP, are in qualitative agreement with the model proposed by Monod *et al*. Sealock and Graves (82) have also observed a sigmoidal saturation curve for AMP (*n* = 1.5) which did not appear to be a function of glucose-1-P concentration. Similar results will be presented in this thesis.

Applying the allosteric model of Monod *et al* (28), Buc (13, 14) has made quantitative predictions from the kinetic data of AMP-P_i interaction with phosphorylase *b* which appear to be in agreement with the conclusions of Madsen and coworkers (11, 12). Buc first assumed that "concerted transitions" exist between the conformational states of the enzyme. Phosphorylase *b* was predicted to exist in two conformational states, R and T. The R state showed exclusive affinity for AMP and P_i whereas the T state had little or no affinity for these effectors. The transition was fully concerted with no hybrid states being formed. The kinetic data classified phosphorylase *b* as an enzyme of the K class (28). This is in contrast to the kinetic results of AMP activation reported by Madsen (11),

and Sealock and Graves (82) which indicate that phosphorylase _b_ belongs to a mixed V and K class enzyme system. Buc (13, 14) also reported evidence that suggested phosphorylase _b_ lost its requirement for AMP in the presence of high concentrations of P_i. However, Engers and Madsen (89) have observed little phosphorylase _b_ activity with P_i in the absence of AMP and in fact observed that high concentrations of the substrate inhibited AMP activation.

While the work reported in this thesis was in progress, Kastenschmidt _et al_ (90) have presented a complicated model for the allosteric transitions of phosphorylase _b_ which attempts to explain the peculiar kinetic behavior of AMP (11, 12). The independent cooperative binding of AMP and glucose-1-P has been explained by the "concerted transition" model (28) by postulating, in addition to the low affinity T state, two high affinity states R and R', the latter binding AMP more strongly than the former. The binding of glucose-1-P is non-exclusive, having highest affinity for the R state, whereas AMP binds exclusively to the R state. Therefore, the concentration of activator determines the equilibrium between R and R', the latter forming at low AMP concentrations.

Thus, kinetic evidence reported by several investigators (11-14,90) appears to support the prediction that phosphorylase _b_ exists in an equilibrium between two conformational states which can be shifted by the binding of various effectors. This assumption received support from physical evidence. Helmreich and Cori (87) have interpreted the large variation in the apparent K_m of the enzyme for its substrates and for AMP and also variations in the V_{max} of the enzyme with changes in pH and temperature as reflecting the ease with which phosphorylase can

undergo conformational alterations. Ullman *et al* (10) have shown that the activation of phosphorylase *b* by AMP increased the capacity of the enzyme to bind the dye, bromthymol blue. The binding of the dye to phosphorylase *a* was not influenced by AMP addition. Glucose-1-P and AMP protected phosphorylase *b* against inactivation by isocyanate or PCMB (91). Chemical modification by these agents caused conformational changes leading to enzyme dissociation. AMP also protects phosphorylase against the action of trypsin or phosphorylase phosphatase (5) and against thermal inactivation (92). Hedrick (93) has also shown by optical rotatory dispersion that AMP can effect the conformation of phosphorylase *b*. All of this evidence strongly suggests that AMP and possibly some substrates do cause conformational changes in phosphorylase.

The kinetic properties of phosphorylase *a* have been studied less extensively than those of phosphorylase *b*. Lowry et al (94) have shown that phosphorylase *a* at low concentrations of glycogen, P_i, or glucose-1-P is extremely sensitive to activation by AMP. Under these conditions, the kinetics of the interaction of substrate and AMP with phosphorylase *a* are similar to those for phosphorylase *b*. Recently, Helmreich, Michaelides, and Cori (95) have carried out detailed studies on the interaction of phosphorylase *a* with glucose and AMP. Using the equilibrium dialysis method, AMP was found to have high affinity for the postulated, active R state, the binding of which was affected by substrates, glucose, and ionic strength. Glucose had high affinity for the inactive T state. The binding of AMP was decreased by inhibitory amounts of glucose, which could be counteracted by glucose-1-P. The sigmoidal saturation curves in the presence of glucose were converted

to hyperbolic saturation curves by the addition of AMP. Glycogen and glucose-1-P promoted the transition of the T to R state whereas P_i, alone, was ineffective. Both the tetrameric and dimeric forms of phosphorylase a could undergo T⇌R transitions, the enzyme existing in the T conformation at high ionic strength. The free enzyme was reported to exist in the active R form, since no homotropic interaction was observed in the binding of AMP to the enzyme.

4. Mechanism of AMP activation

The exact role of AMP in the activation of phosphorylase has not yet been elucidated. With the isolation of phosphorylase a (56), active in the absence of AMP, the concept of this nucleotide as an obligatory cofactor for the phosphorolysis of glycogen was abandoned. In fact, under certain conditions phosphorylase a is also sensitive to activation by AMP (94). The dissociation constant of AMP for phosphorylase a has a value of 2×10^{-6} M whereas for phosphorylase b it is of the order of 6×10^{-5} M. Thus, there is about a 30-fold difference in the tightness of binding between phosphorylase a and b. The adenine nucleotides ADP, ATP (4, 51), 2'-AMP, 3'-AMP (21, 96), and all other nucleotides with the exception of IMP were observed to be ineffective in the activation of either phosphorylase b or a. IMP showed a slight activating effect on phosphorylase b that was increased in the presence of protamine (15), a compound which also increased the affinity of phosphorylase b for AMP about 10 fold but had no effect on phosphorylase b' (15, 16). Mott and Bieber (97) have confirmed these results. They observed increased phosphorylase b activation in the presence of salmine for AMP, adenosine-5'-phosphoramidate, IMP, GMP, UMP, CMP, and a few structural analogues of

AMP. These data showed the critical importance of the 5'-monophosphate group in nucleotide activation of phosphorylase b. Recently, Okazaki et al (98) have reported a detailed study of the effects of 38 structural analogues of AMP on phosphorylase b activity. Their results indicated that the 5'-monophosphate group was essential for activation whereas the 2'hydroxyl group of the ribose moiety contributed to both binding and activation. In addition, the amino group at C-6 and the nitrogen atom at position 1 of the purine ring were effective in binding. Similar conclusions will be presented in this thesis as part of a study of the mechanism of allosteric transitions of phosphorylase b.

III. EXPERIMENTAL PROCEDURE

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A. Materials

1. Chemicals

Cysteine hydrochloride, sodium glycerophosphate, glucose, glucose-1-P, shellfish glycogen, and p-hydroxymercuribenzoate were purchased from Sigma Chemical Company. Glycogen was purified with Norit A according to the procedure of Sutherland and Wosilait (99).

Reagent grade sodium fluoride was purchased from Baker Chemical Company. Spermine tetrahydrochloride was obtained from Mann Research Laboratories. Protamine sulfate, trypsin (bovine pancreas), and egg white trypsin inhibitor were obtained from Calbiochem Company.

Adenine, adenosine, cytidine, guanosine, inosine, thymidine, uridine, xanthosine, pyrimidine, purine, purine riboside, ribose, d-ribose, ribose-5-P, AMP, 2'-AMP, 3'-AMP, cyclic-2'3'-AMP, cyclic-3'5'-AMP, CMP, IMP, TMP, UMP, XMP, dAMP, dCMP, dXMP, dUMP, adenosine-5'-phosphoramidate, adenosine-5'-nicotinate, and diadenosine-5'-pyrophosphate were products of Sigma Chemical Company. GMP and dGMP were purchased from Calbiochem.

The possibility of contamination by AMP and other nucleotides of the structural analogues of AMP was tested by ascending paper chromatography on Whatman No. 1 paper. Solvent systems were water-saturated ammonium sulfate-isopropanol-0.1 M, pH 7.2, sodium phosphate buffer (79:2:19) for most of the nucleotides, and isopropanol-acetic acid-1% aqueous ammonium sulfate (45:35:20) for dAMP. While 1 μg of AMP could be detected under an ultraviolet light, no absorbing material was observed at the AMP position when 520 μg of the other nucleotides were chromatographed. Furthermore, a complete separation was achieved when a mixture

of 520 μg of a nucleotide and 1 μg of AMP was chromatographed. Therefore, the level of AMP contamination in the nucleotides, if any, was less than 0.2%.

2. Enzyme Preparations

Crystalline rabbit muscle glycogen phosphorylase *b* was prepared from commercial frozen rabbit muscle (Pel-Freeze Biologicals, Inc., Rogers, Arkansas) by the method of Fischer and Krebs (100,101). Crystalline phosphorylase *a* was prepared from phosphorylase *b* with partially purified phosphorylase *b* kinase (102). The enzymes were recrystallized four or five times before use and treated with Norit A to remove nucleotides and other impurities (102). For ultracentrifugation and tryptic digestion experiments the enzyme was dialysed against 0.04 M sodium glycerophosphate-0.03 M cysteine buffer (pH 7.0) in order to remove Mg^{+2} ions used in the crystallization of phosphorylase *b*. Phosphorylase *b'* was prepared by controlled tryptic hydrolysis of phosphorylase *a* (103). The hydrolysis was stopped by addition of trypsin inhibitor and the enzyme was used without prior removal of trypsin and trypsin inhibitor.

B. Methods

Phosphorylase concentrations were determined spectrophotometrically with the use of an absorbancy index of 11.7 for a 1% solution of protein (104,105).

1. Kinetics of phosphorylase activation

Enzyme activities were measured at 30° in the direction of glycogen synthesis in the presence of the modifier or combination of modifiers to be studied by a method similar to the procedure of

Illingworth and Cori (106). When polyamines were added to the enzyme solution, the stopping reagent contained 1.0 M potassium chloride in addition to the molybdate and sulfuric acid. The potassium chloride prevented precipitation of the enzyme in the presence of the polyamines and had a negligible effect on the colorimetric assays.

For phosphorylase a activation experiments by various modifiers, the enzyme (0.4 to 0.5 mg per ml) was incubated in 0.03 M cysteine-0.04 M glycerophosphate buffer, pH 6.8, which contained the modifier or combination of modifiers to be studied. The activation was determined either (a) by following the change of enzyme activity with time, defined as the rate of activation, or (b) by determining the enzyme activity after prolonged incubation to achieve complete activation, defined as the extent of the activation. In all experiments assays were conducted in a refrigerated water bath at 20°; in addition, the room temperature was controlled to within 2° of the assay temperature to minimize any change of temperature that might occur during the sampling of enzyme solutions. While the activity unit in the assay system of Illingworth and Cori (106) was proportional to a first order kinetic constant, the reaction follows second order kinetics in the presence of glucose (4). Since glucose was an essential component in our system, higher concentrations of glucose-1-P were used, 0.032 M or greater. Under these conditions, the activity of the enzyme was directly proportional to the rate of phosphate production over short periods of reaction time. The enzyme activity in this work was expressed, therefore, as the rate of phosphate production.

2. Determination of Kinetic Parameters

Kinetic data were processed, wherever applicable, using an

Olivetti Programma 101 desk computer according to a program modified slightly from that provided by Cleland (107), which provides values for K_m, V_{max}, their standard errors of deviation, slopes and intercepts. After preliminary plots were made of the data in the reciprocal form (plots of 1/v versus 1/S), iterative least-square fits were made to equation (IV) when the reciprocal plots were believed to be straight lines.

$$v = \frac{VS}{K + S} \qquad \text{(IV)}$$

Replots of slopes against inhibitor concentration were used to determine the inhibition constants (K_i's) of the inhibitors of AMP activation of phosphorylase b and to determine the nature of the inhibition.

Since AMP was found to be the most effective activator of phosphorylase b tested, maximal activation (A_{max}) was expressed as the percentage of maximal activities for each analogue as compared with that for AMP at identical near-saturating concentrations. Calculation of the activation constants (K_a's) was difficult. Double reciprocal plots of (1/v) against either (1/A) or $(1/A)^2$, where A represents the concentration of the activator, failed to give a straight line. A rough estimation of the apparent K_a was calculated at 100 mM glucose-1-P (saturating) from a Hill plot (26) of log [v/(V - v)] against log (A). The apparent K_a was obtained from the log of the concentration of the activator giving half-maximal activation where log [v/(V - v)] equals 1.0. In the presence of 0.3 M NaF, double reciprocal plots of (1/v) against (1/A) were linear enabling direct calculation of the Ka's.

For purposes of simplifying the comparison of the activation effects and the binding strengths among the analogues tested, the values

K_a', and K_i', are expressed as the ratio of K_a and K_i for each analogue to the K_a for AMP. Since the K_a and A_{max} for the activators were variable, depending on the preparations and the enzyme concentration used, these two kinetic parameters were routinely determined for AMP with each analysis.

3. Tryptic Digestion Experiments

Phosphorylase a (2.4 mg per ml) was incubated at 20° with a modifier or combination of modifiers prior to the addition of trypsin (20 μg per ml). At appropriate time intervals, aliquots of the reaction mixture were removed and diluted in buffer containing trypsin inhibitor (60 μg per ml). Enzyme activity was measured at 30° for 5 minutes.

4. HMB Inactivation Experiments

Phosphorylase was dialyzed against three changes of 0.04 M glycerophosphate-0.001 M EDTA buffer, pH 7.0, over a period of 24 hours to free the enzyme from cysteine. The enzyme was then incubated for 50 minutes with the modifiers prior to the addition of HMB. At various intervals, aliquots of HMB incubated enzyme were added directly to the substrate for activity measurements. The interaction of HMB with sulfhydryl groups was followed by the increase in absorbance at 250 mμ as described by Boyer (108). The enzyme solution without HMB present was used as a blank. The absorbance of the sulfhydryl reagent in the buffer was subtracted from our results.

5. Ultracentrifugal Experiments

Ultracentrifugal runs were prepared on a Spinco model E analytical ultracentrifuge equipped with Schlieren optics employing a 12-mm single sector cell. All experiments were performed at a

temperature of 20 ± 1° and a rotor speed of 59,780 rpm with photographs taken at 8 minute intervals. Protein concentrations ranging from 3-6 mg per ml in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8 were routinely used. Sedimentation coefficients were determined with the aid of a Nikon model 6C microcomparator and were corrected for viscosity and for the density of the buffer to water at 20°.

6. Cold Inactivation

Phosphorylase b was incubated at 0° in 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, containing 0.1 M NaCl in the presence of modifier or combination of modifiers to be studied according to the method of Graves, Sealock and Wang (80).

7. Resolution of Phosphorylase b

Resolution of the enzyme was carried out at 0° in the presence of modifier or combination of modifiers in 0.4 M imidazole-0.1 M cysteine adjusted to pH 6.5 with citric acid similar to the procedure of Shaltiel, Hedrick, and Fischer (109).

IV. RESULTS

IV. RESULTS

A. Studies on Nucleotide Activation of Glycogen Phosphorylase *b*

1. Effects of AMP analogues on phosphorylase *b* activity

Glycogen phosphorylase *b* has an absolute requirement for the activator AMP (23, 24) the enzyme being inactive in the absence of this nucleotide. Cori, Colowick, and Cori (23) observed that only IMP at a concentration of 1 x 10^{-3} M could activate phosphorylase *b* to about 10% of maximum AMP activation. This activation, therefore, was considered to be specific for AMP since other nucleotides were found to be inactive. Similar results using structural analogues of AMP were recently reported by Mott and Bieber (97). The glucose-1-P concentration in these studies was that employed in the standard phosphorylase assay, 16 mM (106). In the present study, significant activation has been observed by a number of nucleotides in the presence of 100 mM glucose-1-P. The data in Table I represents the mean values of 5 surveys, reproducible to within $\pm$ 5%, carried out with phosphorylase *b* in the presence of thirty-two nucleotides and related structural analogues at 16 mM and 100 mM glucose-1-P. The concentration of effectors studied was 17.5 mM. Activation is expressed as the percent activation relative to AMP (A_{max}) since this nucleotide was the most effective compound tested. Values of 5% or less were considered to be negligible since it was difficult to establish the absolute purity of the nucleotides (see Experimental Procedure). For simplicity in analysing their activation effects, the structural analogues of AMP are classified into three main groups in which modifications occur (a) in the 5'-phosphate moiety, (b) in the adenine ring, and (c) in the ribose and base moieties. All nucleoside-5'-monophosphates, dAMP, and TMP

TABLE I

EFFECTS OF STRUCTURAL ANALOGUES OF AMP
ON PHOSPHORYLASE b ACTIVITY

Compound (17.5 mM)	A_{max}[a] 16 mM G-1-P	A_{max}[a] 100 mM G-1-P
	%	%
Control		
AMP	100	100
Modifications in 5'-phosphate		
Adenosine	0	0
Adenosine-2'-phosphate	0	0
Adenosine-3'-phosphate	0	0
Adenosine-2'3'-phosphate	0	0
Adenosine-3'5'-phosphate	0	0
Adenosine-5'-phosphoramidate	30	57
Adenosine-5'-nicotinate	0	0
Diadenosine-5'-pyrophosphate	0	0
Modifications in adenine ring		
CMP	18	36
GMP	6	46
IMP	20	64
UMP	23	45
XMP	13	16
Modifications in ribose and base		
Ribose	0	0
Ribose-5-P	0	0
d-Ribose	0	0
Pyrimidine, purine	0	0
Purine riboside	0	0
Adenine	0	0
Cytidine, guanosine, inosine	0	0
Thymidine, uridine, xanthosine	0	0
TMP	5	10
2'-dAMP	13	55
2'-dCMP	0	0
2'-dGMP	0	0
2'-dUMP	0	0
2'-dXMP	0	0

[a] Percentage of maximal activation (see Experimental Procedure).

activated the enzyme at values ranging from 10% to 60% of AMP activation. Other 2'-deoxynucleotides, 2'-AMP, 3'-AMP, cyclic-2'3'-AMP, cyclic-3'5'-AMP, bases or other analogues did not result in enzyme activation. These results suggested that both the 5'-phosphate and the 2'-hydroxyl groups on the ribose moiety of the nucleotide play roles in the activation of phosphorylase b. The enzyme appeared to show no preference for purine or pyrimidine-5'-monophosphates since UMP and CMP were as effective activators at the concentration tested as was GMP.

2. Dependency of IMP activation upon glucose-1-P concentration

In order to understand the difference between the mechanism of phosphorylase b activation by AMP and the other nucleotides, IMP was selected for a detailed study of the kinetics of interaction between activator and substrate sites on the enzyme. The effects of other nucleotides were then compared with the kinetics of IMP activation. The effects of increasing concentrations of IMP and AMP on the initial velocity of phosphorylase b were compared at three different levels of glucose-1-P (Figure 1). Adenylic acid was a more effective activator than IMP. At all glucose-1-P concentrations used, the V_{max} values obtained with AMP were higher than those in the presence of IMP. The V_{max} of IMP activation was, however, more dependent upon glucose-1-P concentration than was AMP. At a glucose-1-P concentration of 83 mM, maximum AMP activation was 1.5 times that at low glucose-1-P while maximum IMP activation was 10 times that at 16 mM glucose-1-P. When compared to maximal AMP activation, maximum IMP activation was 13%, 43%, and 77% that of AMP activation at substrate concentrations of 15.6, 41.5, and 83.3 mM, respectively.

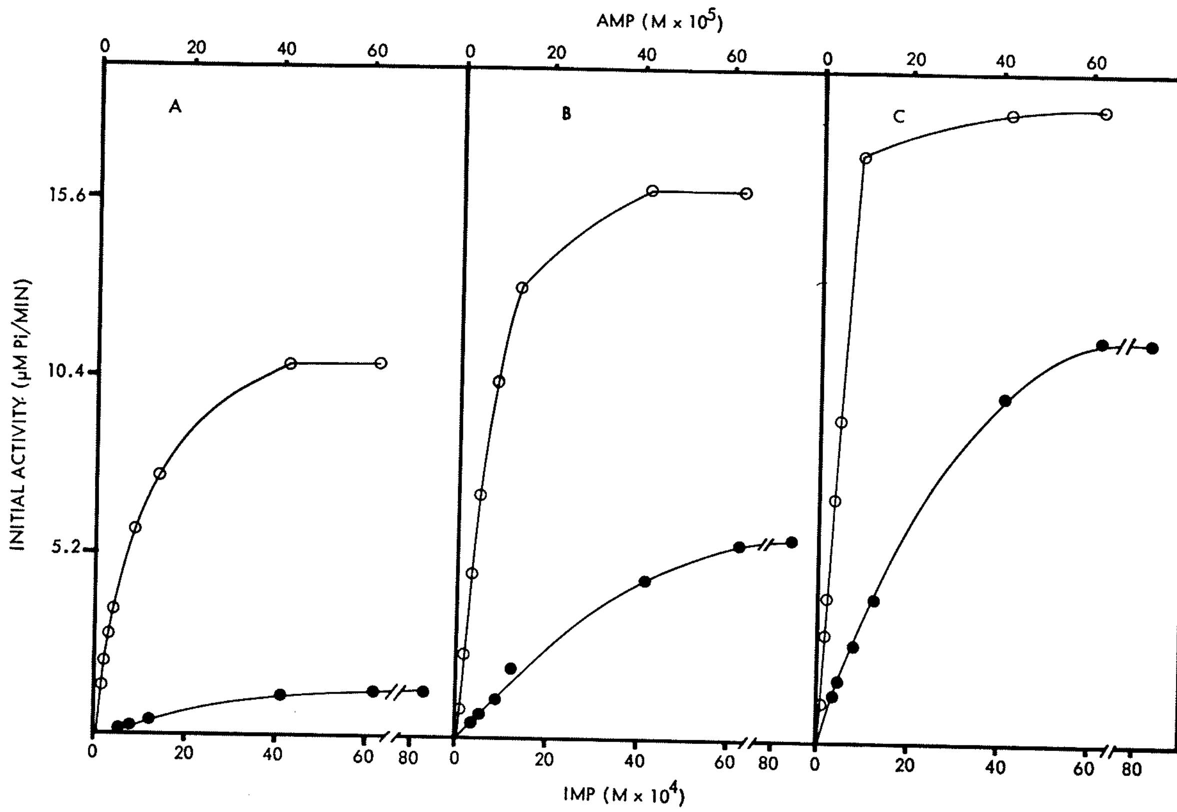


FIGURE 1

The initial velocity of phosphorylase b as a function of IMP (●) or AMP (○) concentration at glucose-1-P levels of: A, 15.6 mM; B, 41.6 mM; C, 83.3 mM.

3. Inhibition of AMP activation by IMP

Since IMP and AMP are related in structure, it was assumed that they may compete for the same binding site on phosphorylase b. The effect of IMP on enzyme activation at various concentrations of AMP was in agreement with this assumption. Figure 2 depicts the double reciprocal plot when AMP was varied against fixed concentrations of IMP. At a glucose-1-P concentration of 16 mM, IMP was essentially a competitive inhibitor with respect to AMP. In the absence of IMP, the kinetics of AMP activation did not obey the Michaelis-Menten relationship over a wide range of nucleotide concentration (82, 86). In Figure 2, a linear plot has been, however, approximated for convenience in estimation of the K_i of IMP. The K_i calculated from Figure 2 (inset) was 9×10^{-4} M.

4. Inhibition of AMP activation by AMP analogues

Other structural analogues of AMP that are listed in Table I were also found to be competitive inhibitors of AMP activation. In Figure 3, double reciprocal plots at 16 mM glucose-1-P of three activators (dAMP, CMP, and XMP), and three non-activating compounds (adenine, cyclic-2'3'-AMP, and dCMP) show linear competitive inhibition with respect to AMP. All effectors tested gave similar competitive kinetics. Replots of the slopes versus inhibitor concentrations were linear, enabling calculations of the inhibitor constants (K_i's) listed in Table II (see Experimental Procedure). For purposes of comparison with previous data, the inhibitors of AMP activation are divided into three major categories similar to the classification in Table I.

The relative magnitude of the K_i values should reflect the binding strength of the inhibitors to phosphorylase b, a low K_i value

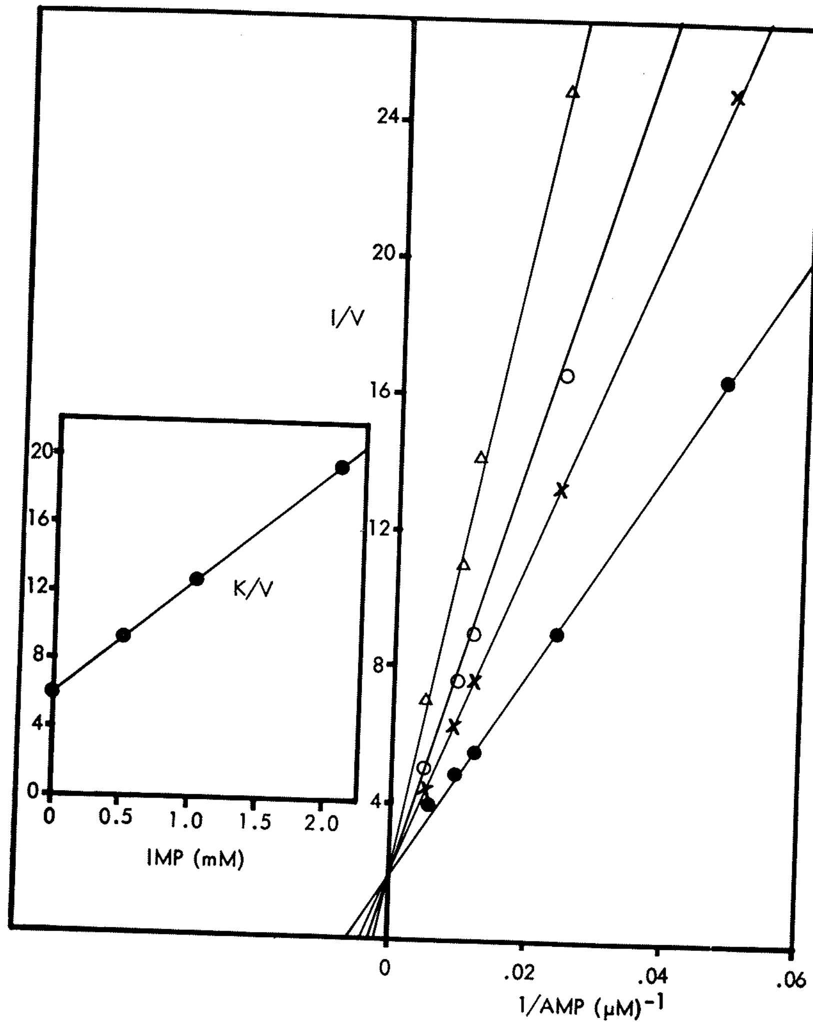


FIGURE 2

Velocity of phosphorylase b activity as a function of AMP concentration at 16 mM glucose-1-P and the following levels of IMP: ●, 0; **X**, 5.2 x 10^{-4} M; O, 10.4 x 10^{-4} M; △, 20.8 x 10^{-4} M. Inset: replot of the slopes from the reciprocal plots as a function of IMP concentration. The velocity (v) in this and all other Lineweaver-Burk plots is expressed as the change in absorbance ($A_{660\ m\mu}$) per 10 minutes at 30°.

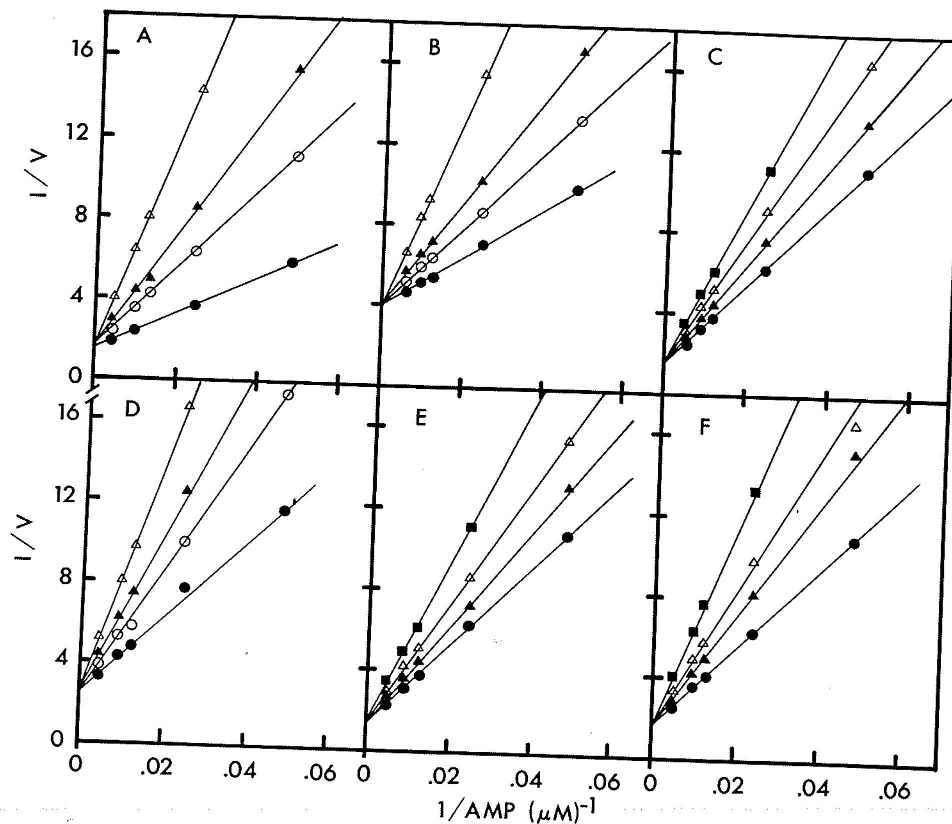


FIGURE 3

Inhibition of phosphorylase b by structural analogues of AMP. Double reciprocal plots of the initial velocity as a function of AMP concentration at 16 mM glucose-1-P. Frame A is inhibition by dAMP; B, CMP; C, XMP; D, adenine; E, cyclic-2'3'-AMP; F, dCMP. Inhibitor concentrations are: (●) 0; (○) 5.2 x 10^{-4} M; (▲) 10.4 x 10^{-4} M; (△) 20.8 x 10^{-4} M; (■) 41.6 x 10^{-4} M. Inhibitor constants (K_i's) were determined from replots of slopes as a function of inhibitor concentration as in Figure 2, inset. Different enzyme preparations were used for the 6 experiments.

indicating strong binding and a high degree of stereospecificity for the enzyme. Among the weak activators, the K_i values increased in the following order: dAMP, CMP, IMP, TMP, XMP, and UMP. Among the compounds which did not activate, adenine, 3'-AMP, and 2'-AMP bound the strongest to the enzyme whereas the cyclic AMP's were weak inhibitors of AMP activation. Deoxy CMP was a weaker inhibitor than was CMP. Among the free bases only adenine and inosine inhibited the enzyme. It, therefore, appeared that the purine ring could also bind to phosphorylase _b_.

For purposes of comparison with Figure 3, the kinetics of nucleotide interaction with respect to glucose-1-P at a fixed level of AMP (8.33×10^{-5} M) are depicted for 3'-AMP, cyclic-3'5'-AMP, and IMP (Figure 4). This low level of AMP, close to its K_a value, was chosen for an obvious reason. Since AMP has a higher affinity for the regulatory site than have the other nucleotide activators of phosphorylase _b_, a higher concentration of AMP would decrease the extent of nucleotide inhibition with respect to the glucose-1-P binding site making kinetic analysis difficult. As the concentrations of the nucleotides increased from 0 to 20.8×10^{-4} M the plots of initial activity against glucose-1-P concentration shifted from normal Michaelis-Menten kinetics to sigmoidal, indicating that in the presence of nucleotides there may be strong homotropic interactions between the glucose-1-P binding sites.

5. Interaction of IMP with glucose-1-P binding

The kinetics of glucose-1-P interaction with phosphorylase _b_ in the presence of IMP were investigated. Figure 5 depicts the plot of initial velocity against glucose-1-P concentration at fixed levels of the

TABLE II

INHIBITION CONSTANTS OF AMP ANALOGUES[a]
FOR PHOSPHORYLASE b

Compound	K_i	$K_{i'}$ [b]
	10^{-4} M	
Control		
AMP	0.5[c]	1.0
Modifications in 5'-phosphate		
Adenosine-2'-phosphate	14.0	28.0
Adenosine-3'-phosphate	10.5	21.0
Adenosine-2'3'-phosphate	41.0	82.0
Adenosine-3'5'-phosphate	21.0	42.0
Modifications in adenine ring		
CMP	9.0	18.0
GMP	11.0	22.0
IMP	9.0	18.0
UMP	78.0	156.0
XMP	41.0	82.0
Modifications in ribose and base		
Ribose	d	--
Ribose-5-P	d	--
d-Ribose	d	--
Pyrimidine	d	--
Purine	22.0	44.0
Purine riboside	32.0	64.0
Adenine	10.8	21.6
Cytidine	150.0	300.0
Inosine	21.5	43.0
Thymidine	55.0	110.0
Uridine	d	--
Xanthosine	d	--
TMP	11.5	23.0
2'-dAMP	5.0	10.0
2'-dCMP	29.0	58.0
2'-dXMP	23.0	46.0

[a] Experimental conditions as in Figure 2.

[b] Relative inhibition constant (see Experimental Procedure). Average value of 3 experiments.

[c] The K_a value of AMP.

[d] No inhibition at 41.6 x 10^{-4} M concentration of analogue.

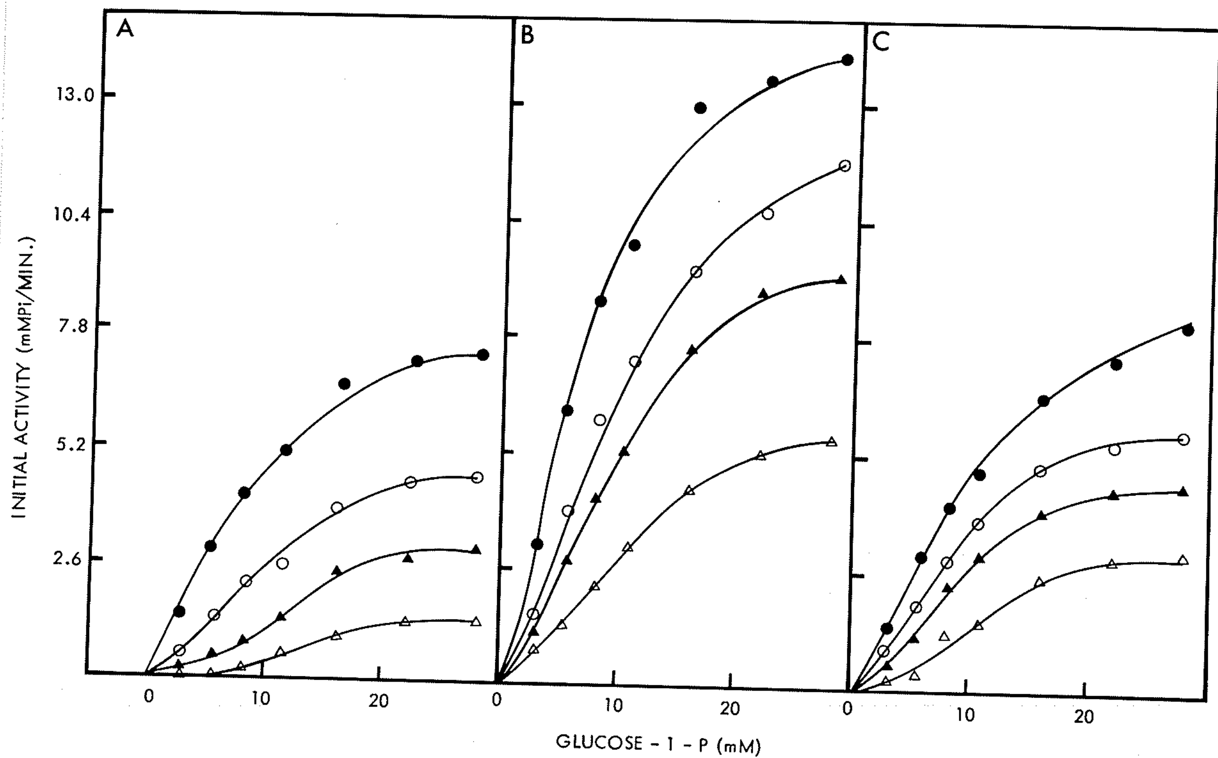


FIGURE 4

The initial velocity of phosphorylase b as a function of glucose-1-P concentration at 8.33 x 10^{-5} M AMP in the presence of: A, 3'-AMP; B, cyclic-3'5'-AMP; C, IMP. Analogue concentrations were: (●) 0; (○) 5.2 x 10^{-4} M; (▲) 10.4 x 10^{-4} M; (△) 20.8 x 10^{-4} M. Different enzyme preparations were used for the 3 experiments.

activator, IMP. The initial rate of IMP activation in the presence of substrate was sigmoidal, even at high activator concentrations, suggesting that the binding of glucose-1-P to phosphorylase b exhibited homotropic cooperative interaction. An increase in IMP concentration from 8.3 x 10^{-5} M to 41.6 x 10^{-4} M did not decrease the homotropic interaction of glucose-1-P. Although the maximum velocity of the enzyme with respect to glucose-1-P increased with increasing IMP concentration, little change in the substrate concentration required for half-V_{max} could be observed. At all IMP concentrations, 32 mM to 37 mM glucose-1-P was required to achieve half-V_{max}. When the data in Figure 5 were arranged in the form of a Hill plot (see inset), the dependence of the interaction strength for glucose-1-P upon IMP concentration could be examined. An n value approximating 2 was obtained for the substrate interaction irrespective of the activator concentration. Thus it appeared that the homotropic interactions of glucose-1-P were independent of IMP concentration.

6. Interaction of AMP with glucose-1-P binding

For purposes of comparison with Figure 5, the dependence of enzyme activation upon AMP concentration with respect to glucose-1-P is illustrated in Figure 6. Although plots of the initial velocity against glucose-1-P were sigmoidal at low concentrations of AMP, an increase in the activator concentration appeared to normalize glucose-1-P kinetics. This was verified in the form of a Hill plot (Figure 6, inset) in which a change in the n value from 1.6 to 1.2 could be observed as the AMP concentration was increased from 5.2 x 10^{-5} M to 208 x 10^{-5} M. Figure 6 also illustrates that the V_{max} with respect to glucose-1-P and

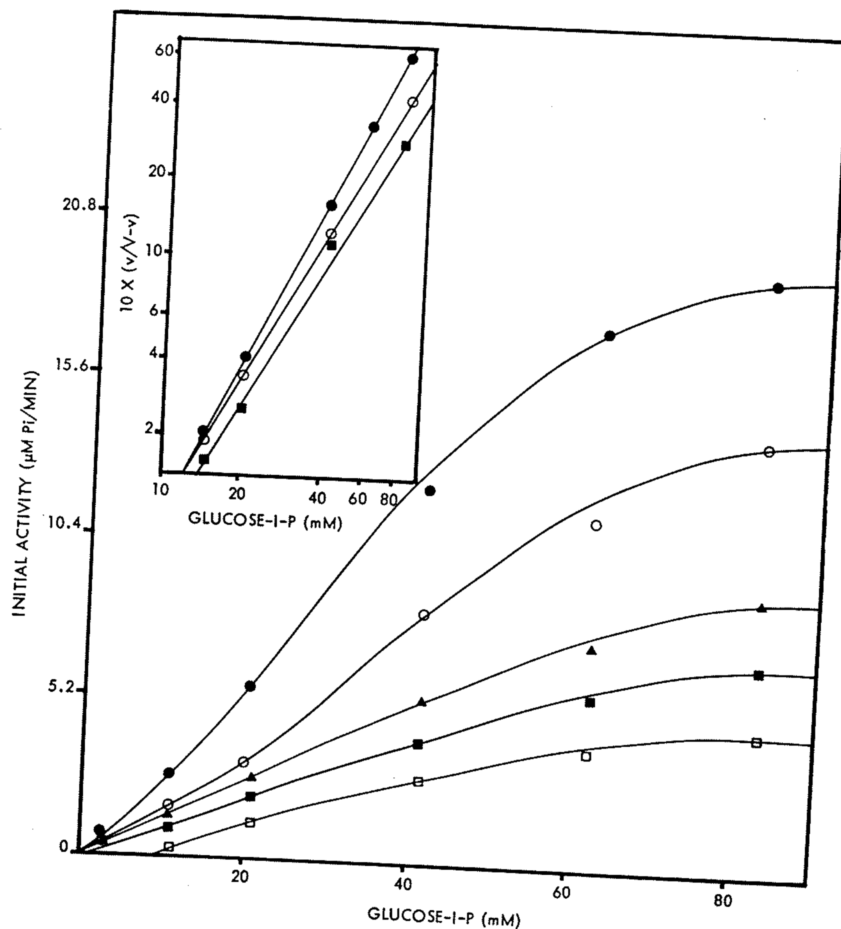


FIGURE 5

The initial velocity of phosphorylase b as a function of glucose-1-P concentration at IMP concentrations of: ●, 41.6 x 10^{-4} M; ○, 16.6 x 10^{-4} M; ▲, 8.3 x 10^{-4} M; ■, 3.33 x 10^{-4} M; □, 0.833 x 10^{-4} M. Inset: Hill plot with glucose-1-P varied, showing the effect of the following concentrations of IMP: ●, 41.6 x 10^{-4} M; n = 2.15; ○, 16.6 x 10^{-4} M; n = 2.0; ■, 3.33 x 10^{-4} M; n = 2.0.

the substrate concentration at half-V_{max} were both dependent upon AMP concentration. As the AMP concentration increased from 5.2×10^{-5} M to 208×10^{-5} M, the glucose-1-P concentration required for half-V_{max} decreased from 9.0 mM to 4.5 mM.

7. Interaction of glucose-1-P with nucleotide binding

The effect of glucose-1-P on the kinetics of IMP activation is shown in Figure 7. An increase in substrate concentration from 10.4 mM to 83.3 mM resulted in an increase in the maximum activation by IMP without having an effect on the IMP concentration required for half-maximum activation. At all glucose-1-P concentrations, an average of 14.6 mM IMP was required to achieve half-V_{max}. Madsen (11, 12) has reported that the affinity of phosphorylase *b* towards AMP was strongly dependent upon glucose-1-P concentration. Figure 7 (inset) also shows that IMP exhibited homotropic cooperative interactions at low substrate concentrations. As the glucose-1-P concentration was increased, the homotropic interaction was nearly eliminated. A decrease in the *n* value from 2.0 to 1.2 could be observed as glucose-1-P concentration increased from 10.4 mM to 83.3 mM.

Recently, Madsen and Shechovsky (12), as well as Sealock and Graves (82) have observed that although AMP exhibited homotropic cooperative interactions with phosphorylase *b*, this interaction was independent of substrate concentration. To verify this further, in the present study the kinetics of phosphorylase *b* with respect to AMP over a wide range of glucose-1-P concentrations has been examined. A change in the concentration of glucose-1-P from 2.7 mM to 25 mM did not result in any change of the *n* values for AMP interaction as determined from a

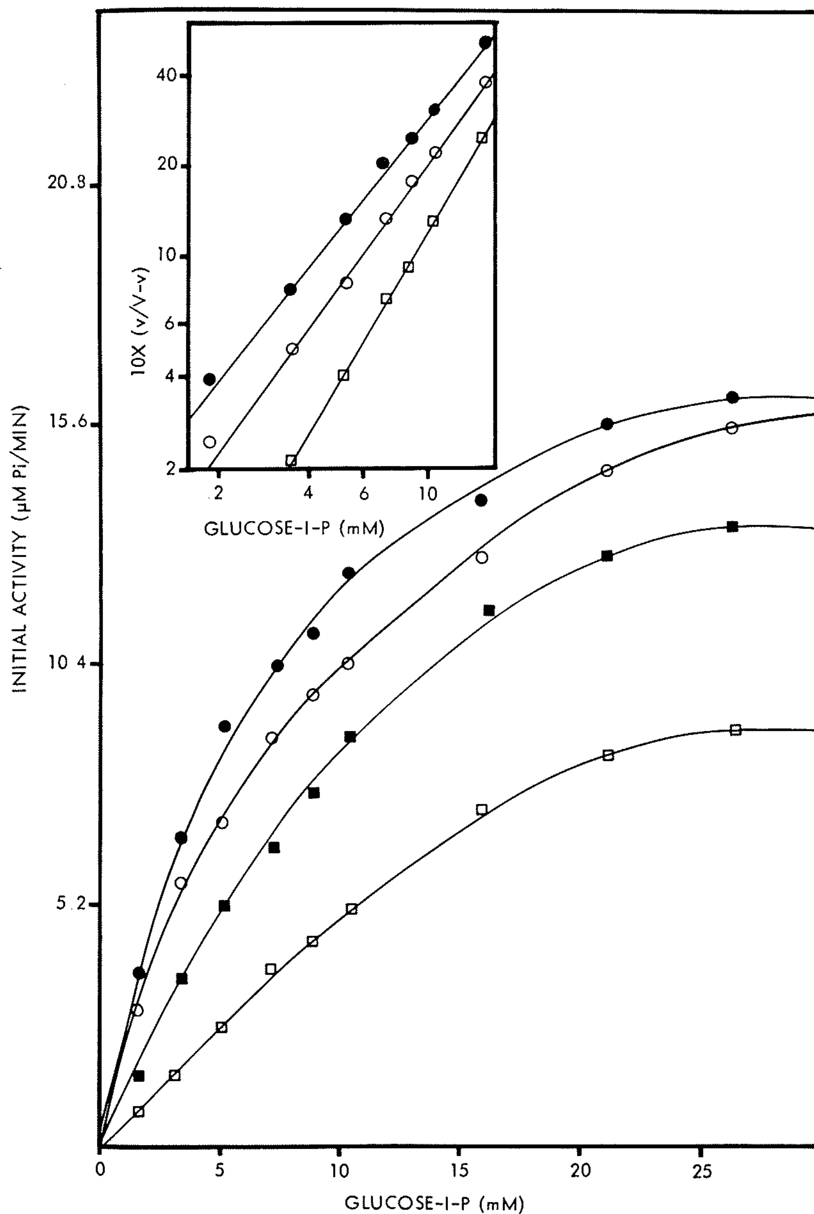


FIGURE 6

The initial velocity of phosphorylase $\underline{b}$ as a function of glucose-1-P concentration and the following levels of AMP: ● , 20.8 x 10^{-4} M; ○ , 20.8 x 10^{-5} M; ■ , 10.4 x 10^{-5} M; □, 5.2 x 10^{-5} M. Inset: Hill plot with glucose-1-P varied, showing the effect of the following concentrations of AMP: ● , 20.8 x 10^{-4} M; $\underline{n}$ = 1.2; ○ , 20.8 x 10^{-5} M; $\underline{n}$ = 1.4; □ , 5.2 x 10^{-5} M; $\underline{n}$ = 1.6.

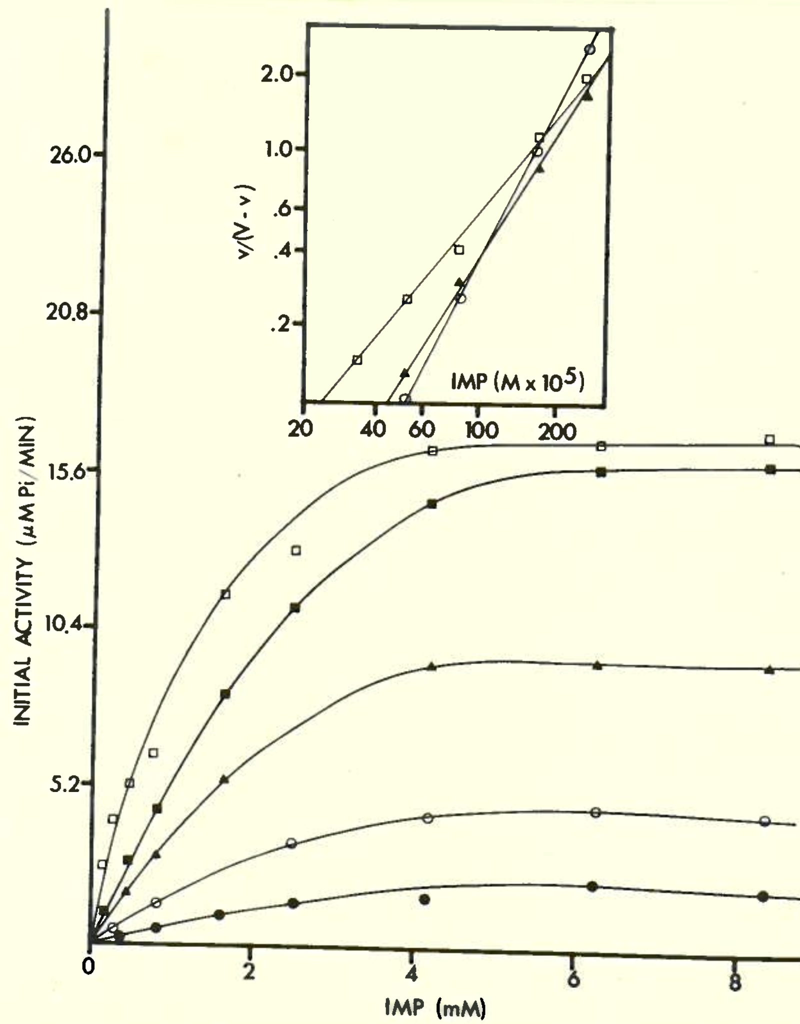


FIGURE 7

The activation of phosphorylase b by IMP at glucose-1-P concentrations of: ●, 10.4 mM; ○, 20.8 mM; ▲, 41.6 mM; ■, 62.5 mM; □, 83.3 mM. Inset: Hill plot with varied IMP, showing the effect of the following concentrations of glucose-1-P: ○, 10.4 mM; $\underline{n}$ = 2.0; ▲, 41.6 mM; $\underline{n}$ = 1.7; □, 83.3 mM; $\underline{n}$ = 1.2.

Hill plot, all slopes having values of approximately 1.4 in this experiment.

Thus, although it appeared that the same site is used for the binding of AMP and IMP, the allosteric mechanism of IMP activation differs from that of AMP activation. In the light of this difference, the kinetics of phosphorylase b activation by the other activators listed in Table I were investigated.

8. Interaction of AMP analogues with glucose-1-P binding

Figure 8 (upper) represents the kinetics of three activators (dAMP, CMP, and UMP) in which the initial velocities are plotted against glucose-1-P concentrations at fixed levels of the activators. These kinetic plots are sigmoidal even at high concentrations of activator, suggesting that the activators have no effect on the cooperativity of glucose-1-P binding to phosphorylase b. All activators tested gave similar allosteric kinetics in the presence of substrate. The sigmoidal kinetics of dAMP activation were not as pronounced as those of the other activators, but resembled the kinetics of AMP activation at high levels (see Figure 6). Although the V_{max} of the enzyme with respect to glucose-1-P increased with increasing activator concentration, little change in the substrate concentration required for half-maximum activation could be observed. The mean Km values for glucose-1-P calculated from Figure 8 (upper) were 14 mM at all concentrations of the activator dAMP, and 10.7 mM and 12.3 mM in the presence of the activators CMP and UMP, respectively. When the data in Figure 8 (upper) were arranged in the form of a Hill plot (Figure 9), the dependence of the interaction strength for glucose-1-P upon the nucleotide concentration could be examined. With

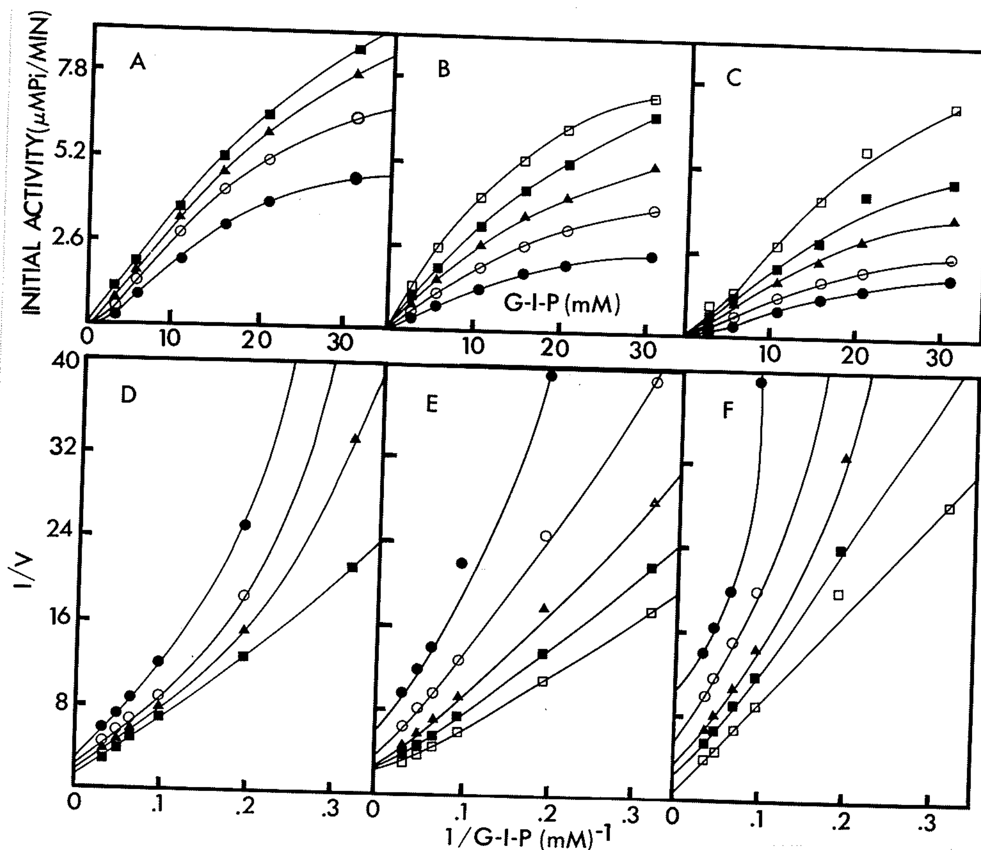


FIGURE 8

The kinetics of phosphorylase b activation by several structural analogues of AMP. Upper curves: the initial velocity of phosphorylase b as a function of glucose-1-P concentration and the following levels of activators: (●) 3.13×10^{-4} M; (○) 6.25×10^{-4} M; (▲) 12.5×10^{-4} M; (■) 20.8×10^{-4} M; (□) 41.6×10^{-4} M. The activators are: A, dAMP; B, CMP; C, UMP. Different enzyme preparations were used for the 3 experiments. Lower curves: D, E, and F are double reciprocal plots of the data from A, B, and C, respectively.

the exception of dAMP, increasing the nucleotide concentration had only a slight effect on the n value for substrate interaction. While an increase in CMP concentration from 3.13 x 10^{-4} M to 6.25 x 10^{-4} M decreased the n value from 1.95 to 1.6, no change in the latter value was observed as the activator concentration was raised further to 41.6 x 10^{-4} M. At low UMP concentrations an n value approximating 2.0 was obtained for substrate interaction. As the concentration of the activator was increased from 6.25 x 10^{-4} M to 41.6 x 10^{-4} M the slope vacillated about a mean value for n of 1.7. All activators tested, with the exception of IMP (see Figure 5, inset), gave n values which showed a similar slight declension on increasing the modifier concentration. However, the decrease in the n value for dAMP appeared to be more pronounced. That is, a change in the n value from 1.77 to 1.30 was observed as the dAMP concentration was increased from 3.13 x 10^{-4} M to 20.8 x 10^{-4} M. In this respect, the interaction of dAMP with glucose-1-P resembled that of AMP (see Figure 6, inset). This is to be expected since these two compounds are nearly identical in structure. However, unlike AMP, the substrate concentration at half-V_{max} remained independent of dAMP concentration.

The double reciprocal plots for dAMP, CMP, and UMP activation (Figure 8, lower) were non-linear (parabolic) making it difficult to accurately determine the activation constants (K_a's) for the activators using conventional methods. In this case, a rough estimation of the apparent K_a values was obtained for the activators at a saturating concentration of glucose-1-P (100 mM) from the corresponding Hill plots (see Experimental Procedure). Although the apparent K_a's were variable, dependent upon

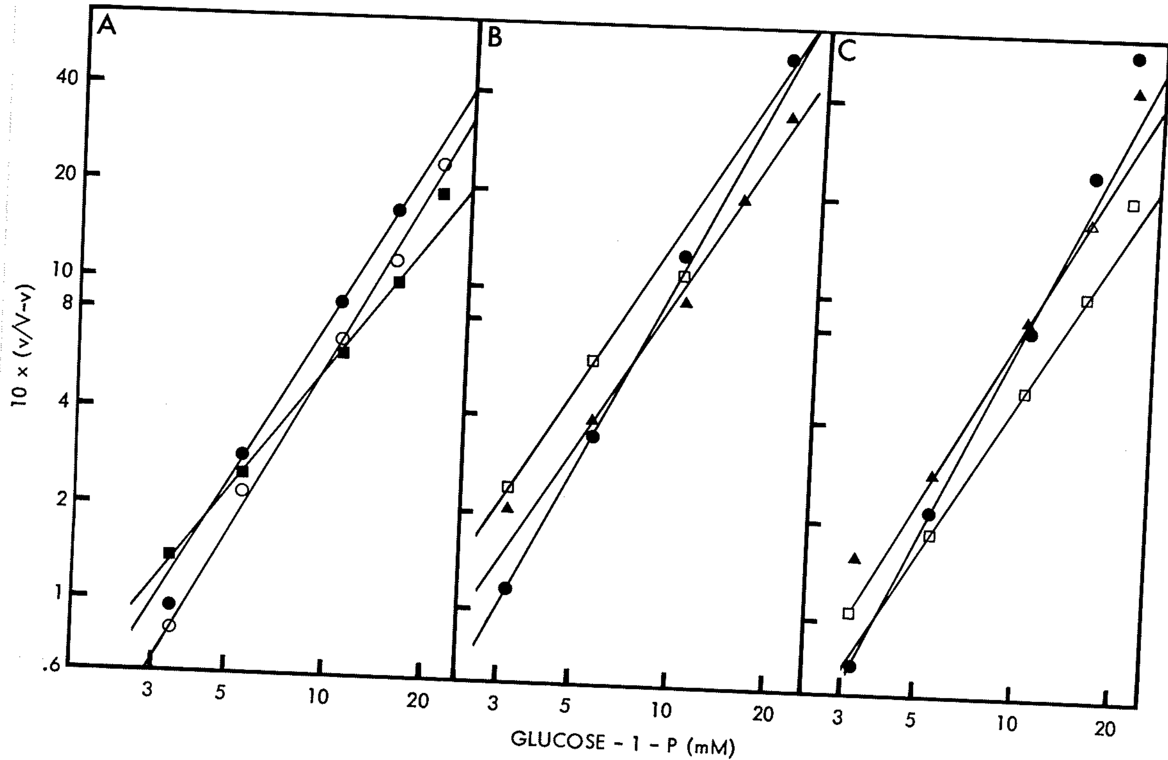


FIGURE 9

Hill plot of data from Figure 8, upper curves, with glucose-1-P varied showing the effects of increasing activator concentrations. Frame A, dAMP: (●) 3.13 x 10^{-4} M; n = 1.77; (○) 6.25 x 10^{-4} M; n = 1.8; (■) 20.8 x 10^{-4} M; n = 1.3. Frame B, CMP: (●) 3.13 x 10^{-4} M; n = 1.95; (▲) 12.5 x 10^{-4} M; n = 1.6; (□) 41.6 x 10^{-4} M; n = 1.6. Frame C, UMP: (●) 3.13 x 10^{-4} M; n = 2.1; (▲) 12.5 x 10^{-4} M; n = 1.82; (□) 41.6 x 10^{-4} M; n = 1.67. The apparent V_{max} values were determined from the double reciprocal plots (Figure 8, lower curves) extrapolated to the ordinate intercept.

the enzyme concentration and preparation, the relative activation constant (K_a's) for each activator was reproducible. The apparent K_a values, the relative activation constants, and the V_{max} values obtained at 100 mM glucose-1-P are listed in Table III.

9. Effect of fluoride and polycations on kinetics of phosphorylase *b*

Sealock and Graves (82) have reported that NaF can enhance the AMP affinity of phosphorylase *b*. In addition, various polycationic molecules have been shown to enhance the enzyme affinity toward AMP (15, 16). Furthermore, spermine and protamine can cause significant activation of phosphorylase *b* by IMP (15, 16). Therefore, the effects of these ionic species on the activation of the enzyme by many of the compounds listed in Table I was investigated. The data in Table IV represents the mean of three surveys, reproducible to within $\pm$ 9%, carried out with phosphorylase *b* (16 mM glucose-1-P) in the presence of various activators and non-activating compounds at concentrations of 5×10^{-3} M. This value was selected so that any enhanced activation effect of the ionic species would be pronounced. The A_{max} values of the activators were increased over the values for the native enzyme by a factor of 2 to 3 in the presence of NaF, protamine, and spermine. The only exception was XMP, whose activation of phosphorylase *b* was not enhanced in the presence of the polycations.

In Figure 10, the dependence of initial velocity of glycogen phosphorylase *b* upon AMP and IMP concentrations in the presence of NaF has been determined at three different levels of glucose-1-P. Maximum IMP activation of the enzyme was stimulated by 0.3 M NaF from control values of 13%, 43%, and 77% (see Figure 1) to 50%, 53%, and 87% of AMP

TABLE III

ACTIVATION CONSTANTS AND MAXIMUM VELOCITY OF AMP ANALOGUES FOR PHOSPHORYLASE b

Compound	K_a [a]	$K_{a'}$ [b]	V_{max} [c]
	10^{-4} M		
AMP	0.5	1.0	122
Adenosine-5'-phosphoramidate	33.0	66.0	74
2'-dAMP	10.0	20.0	56
CMP	30.0	60.0	49
GMP	15.0	30.0	47
IMP	12.5	25.0	81
TMP	39.5	79.0	24
UMP	29.0	58.0	52
XMP	35.0	70.0	24

[a] Apparent K_a calculated at 100 mM glucose-1-P (see Experimental Procedure). Average of 3 determinations.

[b] Relative activation constant (see Experimental Procedure).

[c] Expressed as μ Moles P_i released/minute/mg of protein. Activator concentrations ranged from 2.08×10^{-4} M to 83.3×10^{-4} M except that AMP concentrations were 10-fold lower (10^{-5} M). A change in activator concentration from 63×10^{-4} M to 83.3×10^{-4} M (10-fold lower for AMP) did not increase the V_{max} by more than 12%. Thus, the V_{max} values could be determined visually from plots of initial velocity versus activator concentration.

TABLE IV

EFFECTS OF NaF AND POLYAMINES ON REACTION OF AMP ANALOGUES WITH PHOSPHORYLASE b

Compound (5 mM)	No Addition	0.3 M NaF	Protamine (80 mg/ml)	Spermine ($5x10^{-3}$ M)
	A_{max}[a]	A_{max}	A_{max}	A_{max}
	%	%	%	%
AMP	100	100	100	100
Adenine	0	0	0	0
Adenosine	0	0	0	0
Adenosine-2'-phosphate	0	0	0	0
Adenosine-3'-phosphate	0	0	0	0
Adenosine-2'3'-phosphate ...	0	0	0	0
Adenosine-3'5'-phosphate ...	0	0	0	0
2'-dAMP	21	68	59	62
2'-dCMP	0	0	0	0
CMP	17	55	39	34
GMP	14	71	46	53
IMP	35	86	75	87
TMP	9	26	18	22
UMP	18	62	58	53
XMP	7	17	9	7

[a] Percentage of maximal activation (see Experimental Procedure).

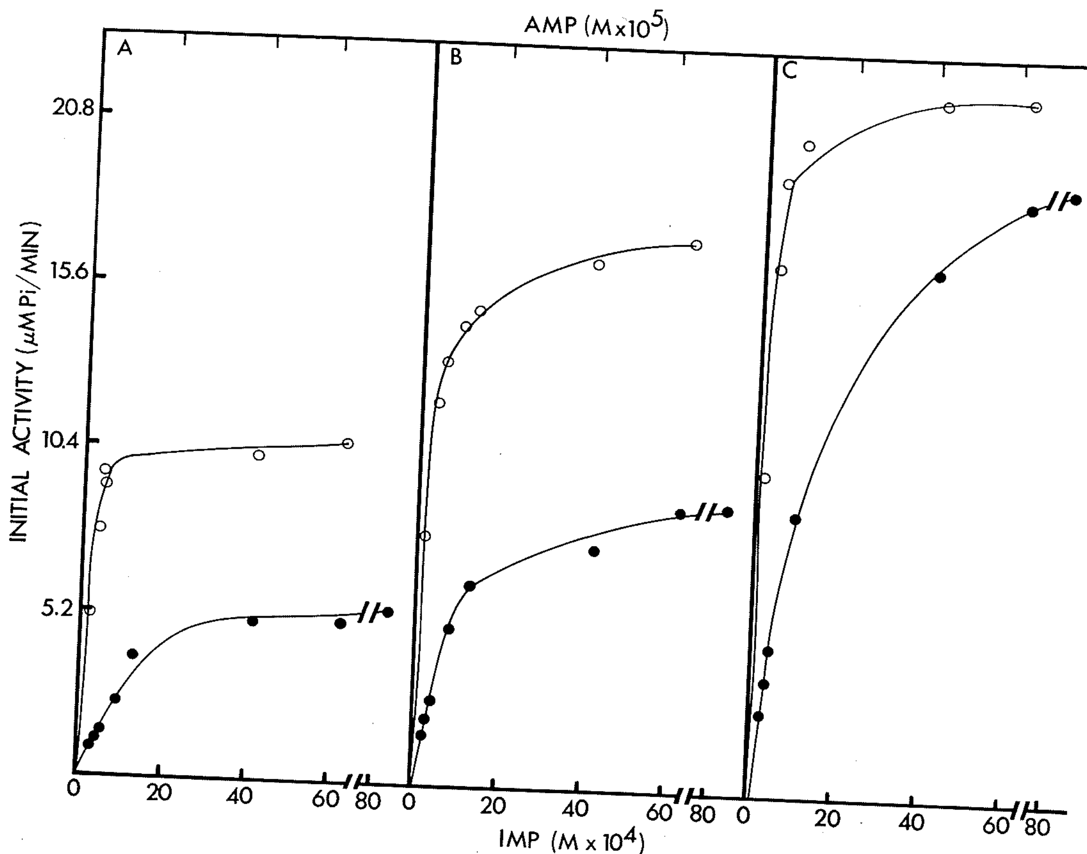


FIGURE 10

The initial velocity of phosphorylase b in the presence of 0.3M sodium fluoride as a function of IMP (●) or AMP (○) concentration at glucose-1-P levels of: A, 15.6 mM; B, 41.6 mM; C, 83.3 mM.

activation in the presence of glucose-1-P concentrations of 15.6 mM, 41.6 mM, and 83.3 mM, respectively.

The kinetic properties of the enzyme in the presence of NaF are illustrated in Figure 11. Lineweaver-Burk plots with respect to either substrate or IMP concentration were linear, suggesting that homotropic cooperative interactions have been "desensitized" by fluoride anions. While the maximum velocity of the enzyme with respect to glucose -1-P was dependent on IMP concentration, little effect of this nucleotide on the K_m was observed. The K_m values for glucose-1-P calculated from Figure 11A ranged from 28 mM to 36 mM, with a mean value of 32 mM. Increasing the glucose-1-P concentration slightly increased the K_m of IMP from 1.64 to 10^{-4} M to 1.75 x 10^{-4} M (Figure 11B). Figure 12 shows that, in addition to eliminating the homotropic interactions of AMP (82), NaF also affected the heterotropic interaction between AMP and glucose-1-P. The K_m values of substrate (Figure 12A) or AMP (Figure 12B) were independent of either nucleotide or glucose-1-P concentration, respectively. Thus, the reciprocal effects of the substrate and activator on the affinity of the enzyme for the other appeared to be eliminated in the presence of NaF.

The kinetics of activation of all activators tested were normalized in the presence of NaF. The linear Lineweaver-Burk plots enabled direct calculation of the K_a values for the activators from replots of the slope against activator concentration. These values, together with the apparent K_a values for the activation of the enzyme in the absence of NaF, are listed in Table V. NaF decreased the K_a's for all nucleoside-5'-monophosphates by approximately 5 fold.

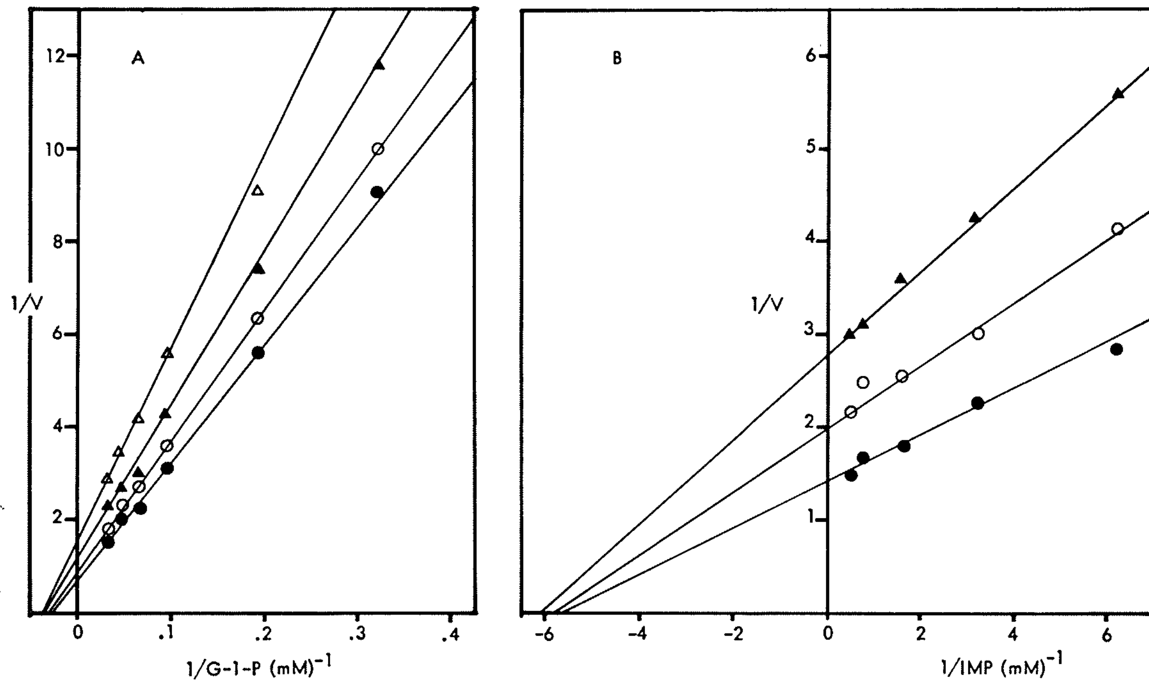


FIGURE 11

The kinetics of IMP activation in the presence of 0.3 M sodium fluoride. A, velocity of phosphorylase b activation as a function of glucose-1-P concentration at IMP concentrations of: ●, 12.5 x 10^{-4} M; ○, 6.25 x 10^{-4} M; ▲, 3.13 x 10^{-4} M; △, 1.67 x 10^{-4} M. B, velocity of phosphorylase b activation as a function of IMP concentration at glucose-1-P levels of: ●, 31.3 mM; ○, 15.6 mM; ▲, 10.4 mM.

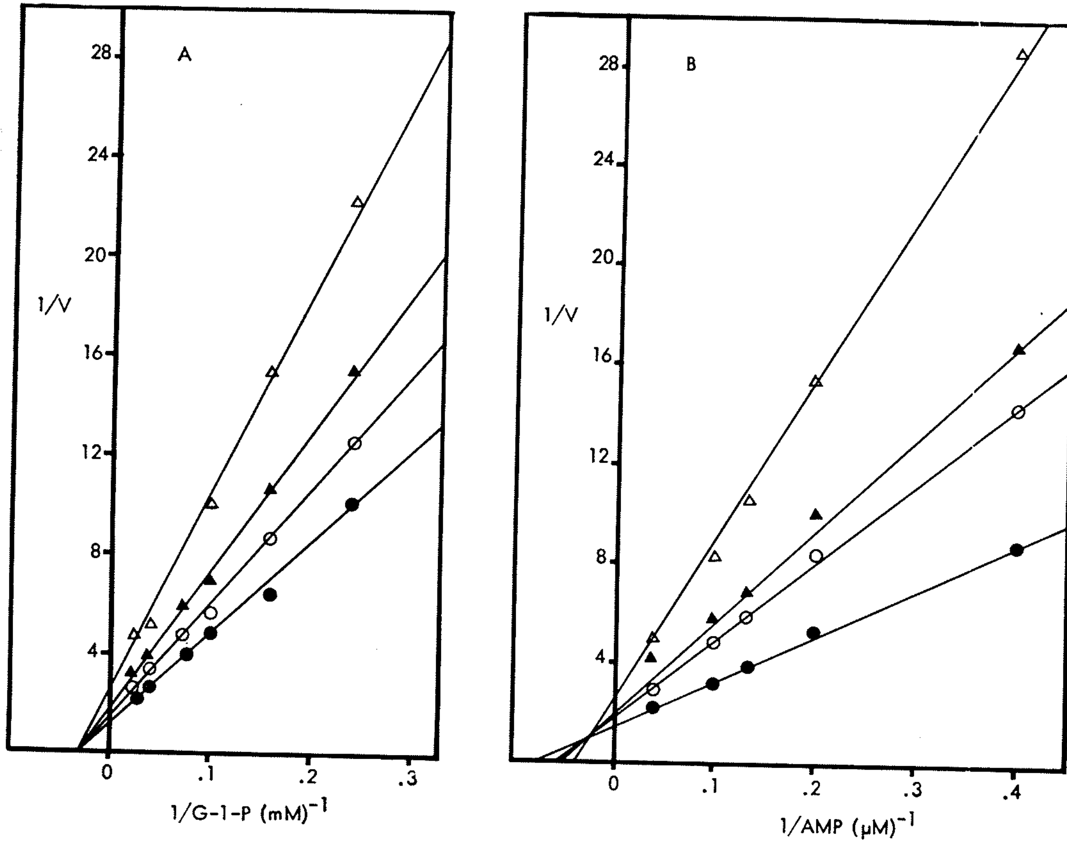


FIGURE 12

The kinetics of AMP activation in the presence of 0.3 M sodium fluoride. A, velocity of phosphorylase b activation as a function of glucose-1-P concentration at AMP concentrations of: ●, 25 x 10^{-6} M; ○, 10 x 10^{-6} M; ▲, 7.5 x 10^{-6} M; △, 5.0 x 10^{-6} M. B, velocity of phosphorylase b activation as a function of AMP concentration at glucose-1-P levels of: ●, 25 mM; ○, 12.5 mM; ▲, 10 mM; △, 6.25 mM.

TABLE V

EFFECT OF NaF ON THE ACTIVATION CONSTANTS OF AMP ANALOGUES FOR PHOSPHORYLASE *b*

Compound	No Addition		0.3 M NaF	
	K_a[a]	$K_{a'}$[b]	K_a[c]	$K_{a'}$[b]
	10^{-4} M		10^{-4} M	
AMP	0.5	1.0	0.05	1.0
Adenosine-5'-phosphoramidate ..	33.0	66.0	8.80	176.0
2'-dAMP	10.0	20.0	2.00	40.0
CMP	30.0	60.0	1.73	34.6
GMP	15.0	30.0	5.30	106.0
IMP	12.5	25.0	2.40	48.0
TMP	39.5	79.0	9.50	190.0
UMP	29.0	58.0	3.23	64.6
XMP	35.0	70.0	8.30	166.0

[a] See Footnote to Table III.

[b] Relative activation constant (see Experimental Procedure).

[c] Average value of 3 experiments. Determined from slope and intercept of a double reciprocal plot.

10. Effect of AMP analogues on activity of phosphorylase *b'*

Fischer *et al* (81) have reported that limited tryptic hydrolysis of phosphorylase *a* resulted in the release of a specific phosphohexa-peptide and concomitant formation of phosphorylase *b'*. Since Graves and coworkers (88) have recently shown that the allosteric behavior of phosphorylase *b'* differed from that of phosphorylase *b*, the effects of several structural analogues of AMP on the activity of phosphorylase *b'* were investigated. Table VI represents the mean values of three surveys, reproducible to within ± 7%, showing the effect of AMP analogues on phosphorylase *b'* activity at two different concentrations of glucose-1-P. There was no apparent differences between the activation properties of phosphorylases *b'* and *b*, only the nucleoside-5'-monophosphates and dAMP activated these two enzyme forms. The activation of phosphorylase *b'* by these compounds was still dependent upon the concentration of glucose -1-P. Studies comparing the effects of these activators on the kinetic properties of phosphorylase *b'* with those obtained previously for phos-phorylase *b* were not undertaken since it was not possible to obtain a homogeneous preparation of phosphorylase *b'*.

11. Effect of fluoride and polycations on phosphorylase *b'* activation

Since the kinetics of the AMP activation of phosphorylase *b'* were not affected by NaF (88) and the activation of this enzyme by either AMP or IMP was insensitive to the addition of either spermine or protamine (15, 16), surveys were carried out on the effect of the presence of these ionic species on the activation by AMP analogues. The data in Table VII

TABLE VI

EFFECTS OF STRUCTURAL ANALOGUES OF AMP
ON PHOSPHORYLASE *b'* ACTIVITY

Compound (17.5 mM)	A_{max}[a]	
	16 mM G-1-P	100 mM G-1-P
	%	%
Control		
AMP	100	100
Modifications in 5'-phosphate		
Adenosine	0	0
Adenosine-2'-phosphate	0	0
Adenosine-3'-phosphate	0	0
Adenosine-2'3'-phosphate	0	0
Adenosine-3'5'-phosphate	0	0
Modifications in adenine ring		
CMP	22	60
GMP	4	31
IMP	18	36
UMP	19	24
XMP	8	36
Modifications in ribose and base		
Ribose, ribose-5-P	0	0
Pyrimidine, purine	0	0
Purine riboside	0	0
Adenine	0	0
Cytidine, inosine	0	0
Thymidine, uridine	0	0
TMP	5	17
2'-dAMP	12	31
2'-dCMP, 2'-dGMP, 2'-dUMP	0	0

[a] Percentage of maximal activation (see Experimental Procedure).

are mean values of three surveys carried out with different samples of phosphorylase b'. The standard deviations were less than ± 8%. As observed in the case of the activation of phosphorylase b' by IMP (15, 16), the activation by the majority of the other activators was only slightly affected by addition of protamine or spermine. However, the anion of NaF stimulated the nucleotide activation of phosphorylase b'. This stimulation resembled that obtained by increasing the level of glucose-1-P (Table VI). In contrast, Graves and coworkers (88) have recently reported that AMP activation of phosphorylase b' was not affected by NaF. This has been confirmed in the present study; in fact, AMP activation appeared to be slightly inhibited from the control value in the presence of the anion.

12. Effect of AMP analogues on association of phosphorylase b

In an attempt to correlate the difference between IMP and AMP activation with differences in enzyme structure that may be induced by these nucleotides, the physical properties of the enzyme were examined. Appleman (5) has reported that dimeric phosphorylase b can partially associate into a tetrameric species in the presence of AMP. In contrast, IMP did not cause any alteration in the ultracentrifugal pattern of phosphorylase b. Figure 13 shows that phosphorylase b, in the presence of 10 mM IMP (Figure 13C, upper) or 0.1 M glucose-1-P (Figure 13B, lower) sedimented as a homogeneous protein with an $s_{20,w}$ value of 8.3 S. However, when both 0.1 M glucose-1-P and 0.01 M IMP were present together (Figure 13C, lower), a new, fast sedimenting, component appeared. The sedimentation constant (12.3 S) of this component corresponded closely to that of the tetrameric species of this enzyme. Glucose-1-P could

TABLE VII

EFFECTS OF NaF AND POLYAMINES ON REACTION OF AMP ANALOGUES WITH PHOSPHORYLASE b'

Compound (5 mM)	No Addition	0.3 M NaF	Protamine (80 mg/ml)	Spermine ($5x10^{-3}$ M)
	A_{max}[a]	A_{max}	A_{max}	A_{max}
	%	%	%	%
AMP	100	100	100	100
Adenosine-2'-phosphate	0	0	0	0
Adenosine-3'-phosphate	0	0	0	0
Adenosine-2'3'-phosphate	0	0	0	0
Adenosine-3'5'-phosphate	0	0	0	0
2'-dAMP	20	44	18	27
2'-dCMP	0	0	0	0
CMP	19	48	19	23
GMP	12	15	7	9
IMP	26	65	29	27
TMP	6	28	15	18
UMP	18	61	31	22
XMP	9	29	9	7

[a] Percentage of maximal activation (see Experimental Procedure).

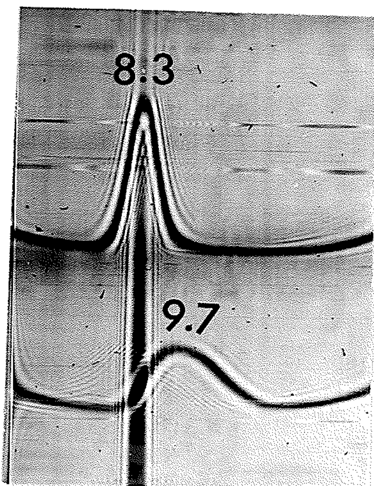
also enhance the association of phosphorylase b by AMP. In the presence of 1 mM AMP and 0.1 M glucose-1-P essentially all of the enzyme existed as the fast moving species (Figure 13B, upper). Inorganic phosphate, a substrate for the enzyme in the direction of glycogen breakdown, also induced the association of phosphorylase b in the presence of IMP (Figure 13D, lower), similar to the effect of glucose-1-P. No association, however, was observed in the presence of phosphate alone (Figure 13D, upper). Thus, as judged by ultracentrifugation, the effect of inorganic phosphate was similar to that of glucose-1-P and it could not replace AMP or other activators in promoting the association of phosphorylase b.

Evidence, similar to that observed for the association of phosphorylase b in the presence of IMP, was obtained for the other activators of phosphorylase b (Figure 14). At concentrations of 10 mM, the activators adenosine-5'-phosphoramidate, CMP, GMP, TMP, UMP, and XMP and the non-activating dUMP induced varying degrees of phosphorylase b association, the enzyme having $s_{20,w}$ values that ranged from 8.9 S to 11.6 S in the presence of 0.1 M glucose-1-P. The weakest activators TMP and XMP showed only slight association of phosphorylase b. In contrast, phosphorylase b sedimented as a homogeneous protein with an $s_{20,w}$ value of approximately 8.3 S in the presence of both 0.1 M glucose-1-P and 10 mM concentrations of 2'-AMP, 3'-AMP, cyclic-3'5'-AMP, dCMP, and dGMP. The fact that the majority of the non-activating nucleotides did not cause association of phosphorylase b in the presence or absence of glucose-1-P suggests that enzyme association is probably related to nucleotide activation.

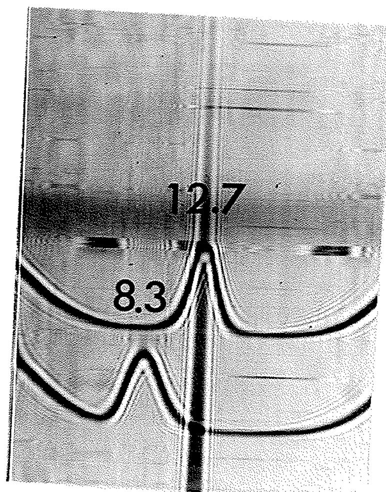
The effect of NaF on phosphorylase b association in the presence of the activator IMP was also investigated (Figure 15). Although NaF

FIGURE 13

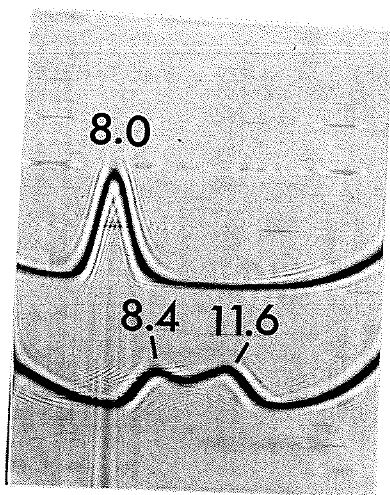
Effect of AMP and IMP on the association of phosphorylase *b*. Ultracentrifugation at 20° of phosphorylase *b* (5 mg per ml) in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8. *Upper curves*: *A*, control; *B*, 10^{-3} M AMP + 0.1 M glucose-1-P; *C*, 10^{-2} M IMP; *D*, 0.1 M P_i. *Lower curves*: *A*, 10^{-3} M AMP; *B*, 0.1 M glucose-1-P; *C*, 10^{-2} M IMP + glucose-1-P; *D*, 10^{-2} M IMP + 0.1 M P_i. Direction of sedimentation was from left to right.



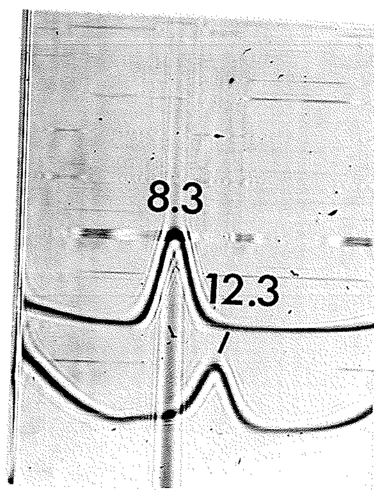
A



B



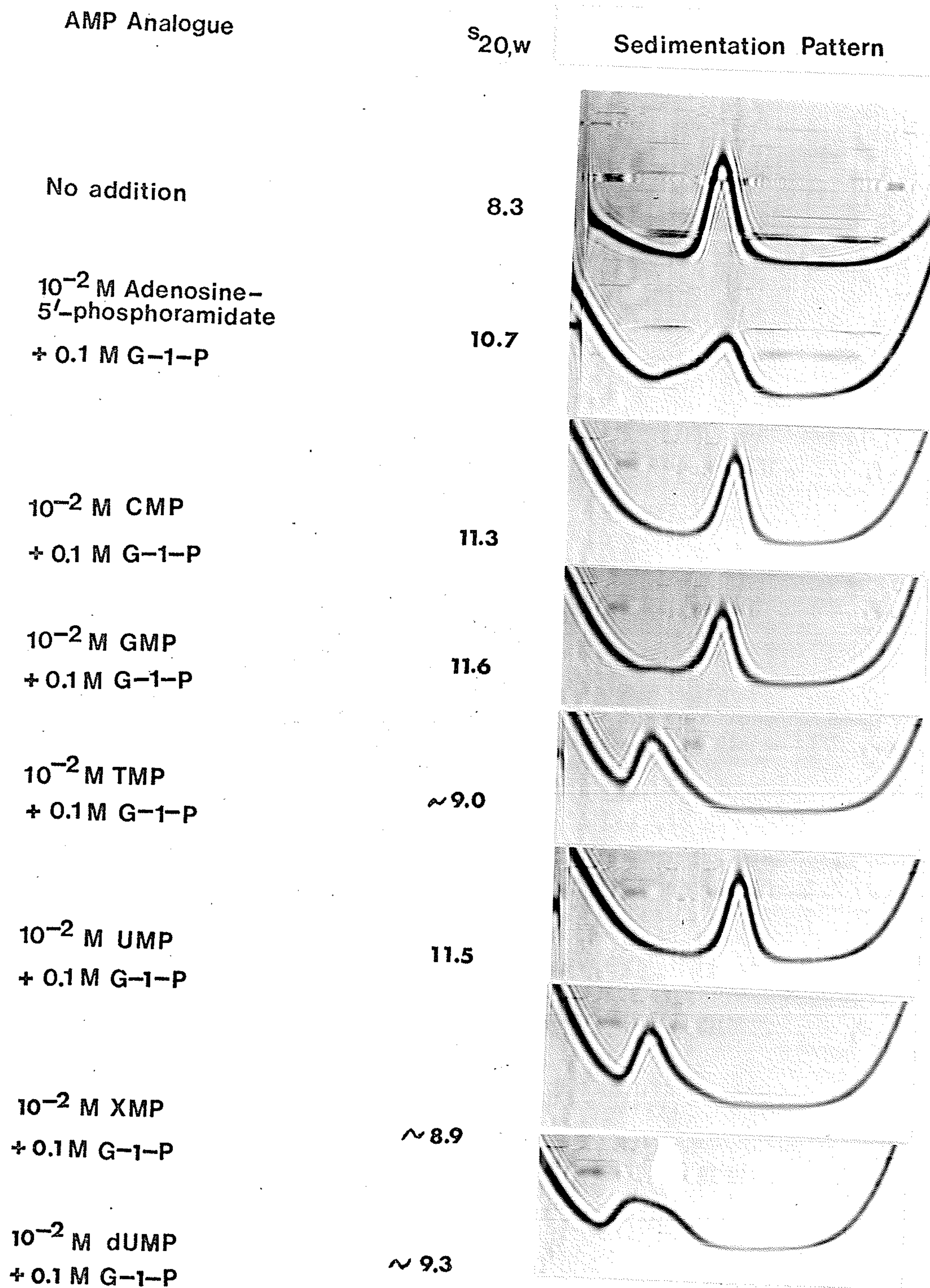
D



C

FIGURE 14

Effect of structural analogues of AMP on the association of phosphorylase *b*. Ultracentrifugation was at 20° in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8. Protein concentration varied from 4.5 to 6.0 mg/ml. Time of sedimentation was 32 minutes at 59,780 rpm. Direction of sedimentation was from left to right.


AMP Analogue
$s_{20,w}$
Sedimentation Pattern
No addition
8.3
10^{-2} M Adenosine-5′-phosphoramidate
+ 0.1 M G-1-P
10.7
10^{-2} M CMP
+ 0.1 M G-1-P
11.3
10^{-2} M GMP
+ 0.1 M G-1-P
11.6
10^{-2} M TMP
+ 0.1 M G-1-P
~9.0
10^{-2} M UMP
+ 0.1 M G-1-P
11.5
10^{-2} M XMP
+ 0.1 M G-1-P
~8.9
10^{-2} M dUMP
+ 0.1 M G-1-P
~9.3

caused a change in the kinetic properties of the enzyme, the specificity of the nucleotide effect on enzyme association was independent of the presence of the fluoride anion. In the presence of 0.3 M NaF, no association of phosphorylase b could be achieved in the presence of either IMP or glucose-1-P, but considerable association occurred in the presence of both the activator and the substrate (Figure 15B, lower). In the latter case, all of the enzyme existed essentially as the fast sedimenting component. Similar results were observed for the AMP induced association of phosphorylase b in the presence of polyamines (16). Thus, the extent of enzyme association was enhanced by both NaF and polyamines.

13. Effect of modifiers on stability of phosphorylase b

In addition to its effect on the sedimentation pattern of phosphorylase b, AMP also protected the enzyme against harmful treatments. Similar results were obtained in the presence of IMP. At a low temperature (0°) at pH 6.0, phosphorylase b can be slowly inactivated (80). This inactivation is completely blocked by AMP. Table VIII shows that a similar protection could be provided by 0.01 M IMP. Glucose-1-P (0.1 M) also protected the enzyme against cold inactivation, although it was slightly less effective than either nucleotide. The resolution of pyridoxal phosphate from phosphorylase b by imidazole and citrate (109) could also be prevented by AMP. Table VIII illustrated that IMP or glucose-1-P also had a protective effect. These results, therefore, suggest that structural alterations can occur in the presence of either glucose-1-P or IMP.

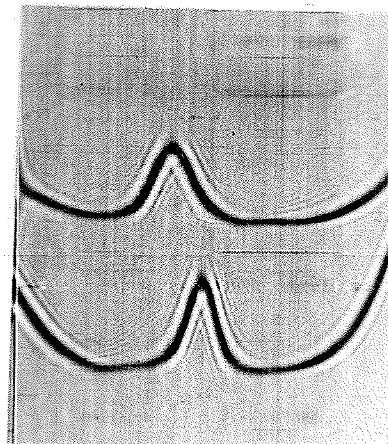
Conditions	$s_{20,w}$	**Sedimentation Pattern**
0.3 M NaF	**8.4**	
0.3 M NaF **0.1 M G-1-P**	**8.1**	
0.3 M NaF **10^{-2} M IMP**	**8.5**	
0.3 M NaF **10^{-2} M IMP** **0.1 M G-1-P**	**12.6**	

FIGURE 15

Effect of sodium fluoride on the IMP induced association of phosphorylase b. Ultracentrifugation at 20° of phorphorylase b (4.5 mg/ml) in 0.03 M cysteine-0.04 M glycerophosphate, pH·6.8. All pictures were taken 32 minutes after attainment of maximum speed (59,780 rpm). Direction of sedimentation was from left to right.

TABLE VIII

EFFECTS OF MODIFIERS ON STABILITY OF PHOSPHORYLASE *b*

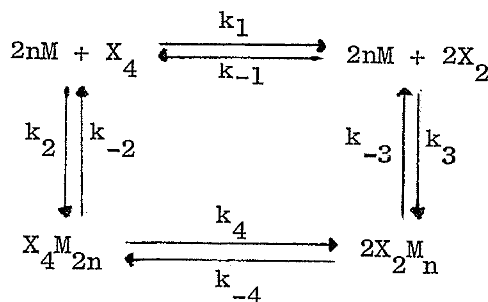
	Original activity				
	Cont.	AMP 10^{-3} M	IMP 10^{-2} M	G-1-P 0.1 M	10^{-2} M IMP + 0.1 M glucose-1-P
	%	%	%	%	%
Resolution[a]	44	95	79	100	78
Cold Inactivation[b]	32	100	100	82	100

[a] 0°, 20 minutes in pH 6.5, 0.4 M imidazole-citrate and 0.1 M cysteine.

[b] 0°, 3 hours in pH 6.0, 0.04 M glycerophosphate-0.03 M cysteine + 0.1 M NaCl.

B. Studies on the Activation of Phosphorylase a by Various Modifiers

As previously described in the literature review, the work of Graves and coworkers (7, 8, 18) has shown that incubation of glycogen phosphorylase a with AMP, glucose, or certain polysaccharides results in a time-dependent activation of this enzyme. Recent studies have established the following facts. (a) Free phosphorylase a exists in equilibrium between a more active dimer and a less active tetramer (8, 110) and (b) activation by modifiers can be correlated with an enhanced dissociation of tetramer into dimer (7, 8, 111). Therefore the activation of phosphorylase a may be represented by the following reaction scheme



SCHEME I

where the modifier M binds preferentially to the more active enzyme species, dimer X_2, where $k_3/k_{-3} > k_2/k_{-2}$. In addition, modifiers may also have a direct effect on the dissociation of the tetrameric species X_4. Since the enzyme dissociation proceeds at a slow rate (18, 111), an enhanced initial activation of phosphorylase a by these modifiers could be detected if binding of modifiers to the tetramer facilitates dissociation, i.e. $k_4 > k_1$. In the present study, the effect of glucose or AMP on both initial rate and final extent of phosphorylase a activation was examined in detail.

1. Activation of phosphorylase a by preincubation with glucose

As previously stated, prior incubation of phosphorylase a with glucose results in a time-dependent activation of the enzyme (7). The effect of preincubation of phosphorylase a with different concentrations of glucose is illustrated in Figure 16A. The initial rate of activation was strongly dependent upon glucose concentration. An increase in glucose concentration from 0.005 M to 0.2 M resulted in a 40-fold enhancement of the activation rate. Since activation can be correlated with an enhanced dissociation of the tetrameric species of the enzyme into a dimer (7), these data were in agreement with the postulate that glucose can directly enhance the conversion of the less active tetramer to a more active dimer (see Scheme I). In Figure 16B the initial rate of phosphorylase a activation is plotted against glucose concentration. Since the experimental conditions were such that the free enzyme existed essentially in the tetrameric form (8), the enhanced rate of phosphorylase a activation should be proportional to the concentration of enzyme-modifier complex, X_4M_{2n}. Figure 16B, therefore, represents the saturation curve of the glycogen phosphorylase a tetramer with glucose. This curve was sigmoidal suggesting that the binding of glucose to the enzyme exhibited homotropic interactions. To analyse the relationship between the initial rate of activation and the glucose concentration for this sigmoidal curve, a modified form of Hill's empirical equation (26, 27) was applied

$$\log [a/(A - a)] = n \log S - \log K \qquad \text{V}$$

where a is either the initial rate (a_i) or extent (a_{ex}) of activation and A the maximum rate (A_i) or extent (A_{ex}) of activation. When the

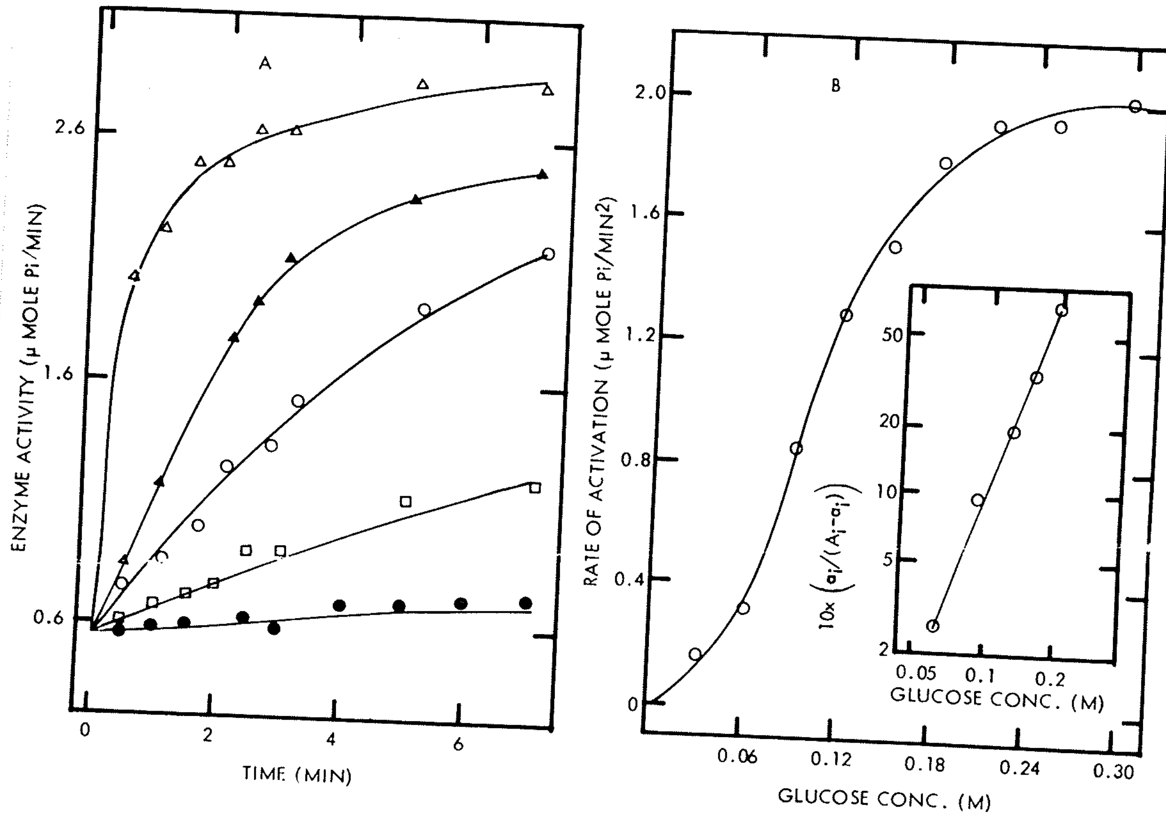


FIGURE 16

Effect of glucose concentration on the initial rate of phosphorylase a activation. A, phosphorylase a (0.45 mg per ml) was incubated in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8, at 20° (●), or in buffer with glucose concentrations of: (□) 0.005 M; (○) 0.05 M; (▲) 0.07 M; and (△) 0.2 M. At various intervals, aliquots were removed for activity measurement (30 second assay) in the presence of 0.04 M glucose-1-P, 1% glycogen, 0.025 M AMP, and 0.15 M glucose. B, phosphorylase a (0.45 mg per ml) was incubated in buffer containing various concentrations of glucose for 20 seconds prior to activity measurement. The buffer and assay conditions were as in A. Inset: the initial rate of enzyme activation, a_i, is defined as the difference in activity between enzyme preincubated in the presence and absence of glucose. Data are arranged in the form of a Hill plot.

above equation, arranged in the form of a Hill plot (26), was applied to the data in Figure 16B, a straight line with a slope of 2.9 was obtained (Figure 16B, inset).

The dependence of the final extent of phosphorylase a activation upon glucose concentration is depicted in Figure 17. At low concentrations, glucose was more effective in enhancing the extent than the rate of enzyme activation (Figure 16B versus Figure 17). For instance, at 0.02 M glucose where 90% of the maximum extent of activation was achieved, the initial rate of phosphorylase a activation was only 3.5% of its maximum. These results suggest that the extent of phosphorylase a activation by glucose may be described by simplification of reaction Scheme I into the following equation*.

$$X_4 + 2nG \rightleftharpoons 2X_2G_n \qquad \text{VI}$$

This equation indicates that the extent of activation by glucose (G) is proportional to the concentration of the X_2G_n dimeric complex. The sigmoidal curve in Figure 17, therefore, suggests that homotropic interactions occur in the binding of glucose to the dimeric form of phosphorylase a. The Hill coefficient for this interaction was 1.5 (see inset of Figure 17). This value was approximately half of the Hill coefficient for the interaction of glucose with phosphorylase a tetramer (Figure 16B).

2. Activation of phosphorylase a by preincubation with AMP

Wang and Graves (8) have observed that preincubation of phosphorylase a with AMP prior to enzymic assay results in a time-dependent

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* The specific activity of phosphorylase a at 20° is essentially independent of enzyme concentration from 0.1 to 0.6 mg per ml (101). Therefore the formation of free phosphorylase dimer may be considered negligible over 70% of the activation.

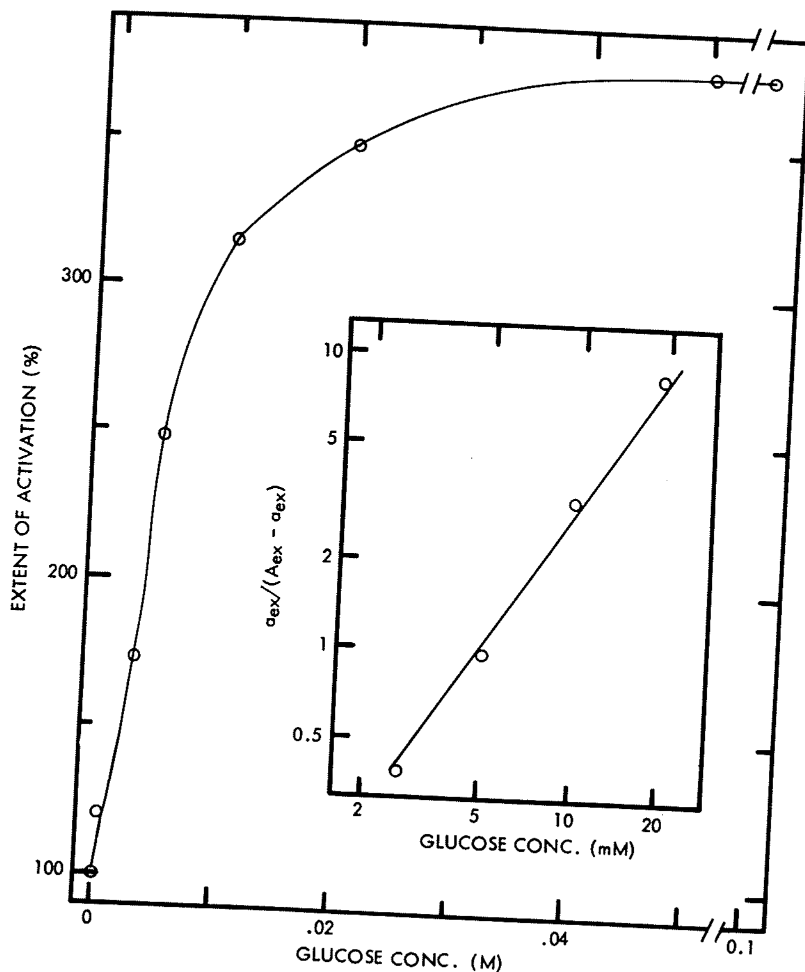


FIGURE 17

Effect of glucose concentration on the extent of phosphorylase $\underline{a}$ activation. Phosphorylase $\underline{a}$ (0.5 mg per ml) was preincubated at 20° for 90 minutes in buffer containing various concentrations of glucose. Aliquots were then removed for activity measurements (30 second assay) in 0.032 M glucose-1-P, 1% glycogen, 10^{-3} M AMP, and 0.1 M glucose. Inset: data are arranged in the form of a Hill plot. The extent of activation, a_{ex}, is expressed as the percentage of enzyme activity incubated in buffer alone.

activation and an enhanced dissociation of the enzyme. The data illustrated in Figure 18 show that the initial rate of AMP activation is also dependent upon the modifier concentration. At three minutes, activation with 2×10^{-5} M AMP was approximately 1.5 times greater than that observed with 10^{-6} M AMP. However, due to the slight extent of this activation, the initial rate could not be accurately measured. This difficulty in making accurate measurements of the initial rate of activation was evident at low concentrations of the nucleotide, as can be observed from the scattered points in Figure 18.

3. Effect of glycogen on phosphorylase *a* activation by AMP

Phosphorylase *a* also exhibits a time-dependent activation upon preincubation with glycogen (18). However, the mechanism of glycogen activation appears to be different from that of AMP or glucose. Metzger, Helmreich and Glaser have postulated that phosphorylase *a* activation by glycogen results only from the preferential binding of the polysaccharide to the dimeric form of the enzyme (111). Their postulate was based on observations that (a) the initial rate of glycogen activation of the enzyme is independent of the polysaccharide concentration (111), and (b) glycogen effectively blocks the association of dimeric phosphorylase *a* into a tetramer (8). Thus, it is possible that the dimeric species of phosphorylase *a* formed during AMP activation may be stabilized in the presence of low glycogen concentrations. Figure 19 shows the dependence of activation rate upon AMP concentration in the presence of 0.1% glycogen. At this constant, low level of glycogen, the initial rate of AMP activation was dependent only upon the nucleotide concentration. A change in AMP concentration from 10^{-6} M to 20×10^{-6} M

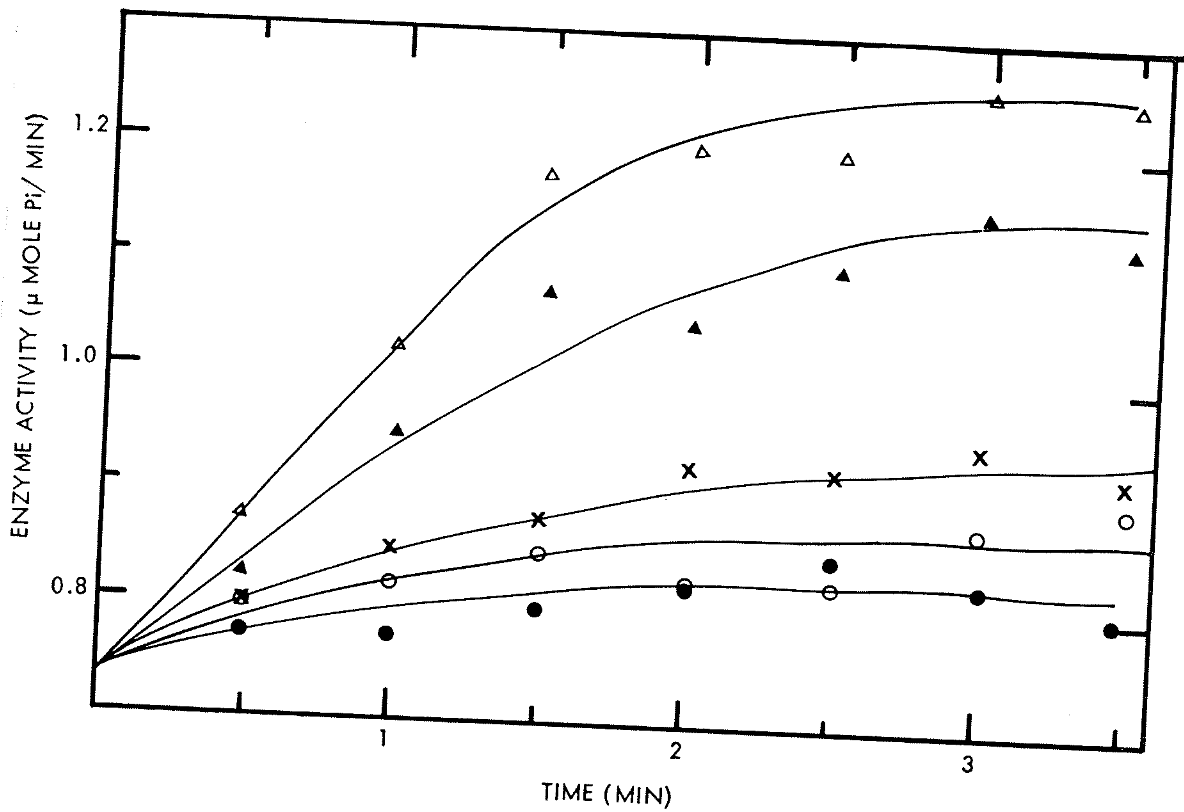


FIGURE 18

Effect of AMP concentration on the initial rate of phosphorylase a activation. Phosphorylase a (0.45 mg per ml) was incubated at 20° in buffer (●), or in buffer with AMP concentrations of: 10^{-6} M (○), 2×10^{-6} M (X), 6×10^{-6} M (▲), and 2×10^{-5} M (△). At various intervals, aliquots were removed for activity measurements in 0.044 M glucose-1-P, 1% glycogen, and 2×10^{-3} M AMP (30 second assay).

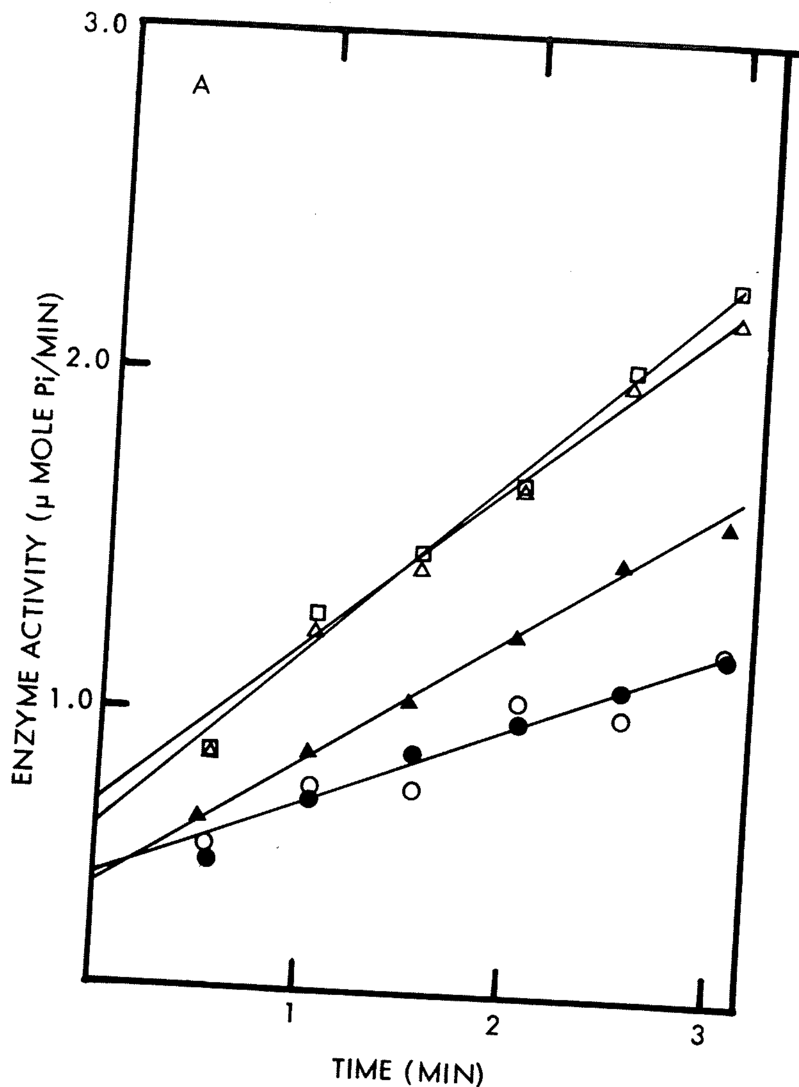


FIGURE 19

Dependence of activation rate on AMP concentration in the presence of glycogen. Phosphorylase a (0.43 mg per ml) was incubated at 20° in buffer containing 0.1% glycogen (●), or in buffer with 0.1% glycogen and AMP at concentrations of: 10^{-6} M (○), 4 x 10^{-6} M (▲), 7 x 10^{-6} M (△), and 20 x 10^{-6} M (□). At various intervals, aliquots were removed for activity measurements as in Figure 18.

resulted in a 2.5 fold increase in the rate of enzyme activation. At all AMP concentrations tested, the activity increment was linear with incubation time for at least 3 minutes.

In Figure 20, the extent and initial rate of phosphorylase a activation in the presence of 0.1% glycogen are plotted against AMP concentration. The nucleotide concentration required to achieve the half maximum extent of activation was 3.9 μM. This value may be compared with the nucleotide concentration required to obtain half maximum initial rate of activation of phosphorylase a, 4.4 μM. In contrast to the activation of the enzyme by glucose (see Figure 16 and 17), these values were only slightly different.

4. Reversal of glucose activation by AMP

Investigations have shown that although glucose and AMP can activate and dissociate phosphorylase a, the activation and dissociation of the enzyme by glucose can be reversed by AMP (7). Figure 21 illustrates that the activation of phosphorylase a by glucose was independent of the order of the nucleotide addition. While addition of AMP to the glucose activated enzyme resulted in a decrease in catalytic activity, the same final activity could be achieved by the initial addition of both glucose and AMP to the enzyme. This activation was, therefore, independent of the order of ligand addition. Thus, the enzymic activity of the ligand preincubated sample was determined only by the equilibrium conditions between the enzyme and the ligands. It is well known that during initial velocity measurements of the enzyme catalyzed reaction a true equilibrium between the enzyme and ligands are rarely obtained (112).

The data in Figure 22 show the dependence of catalytic activity

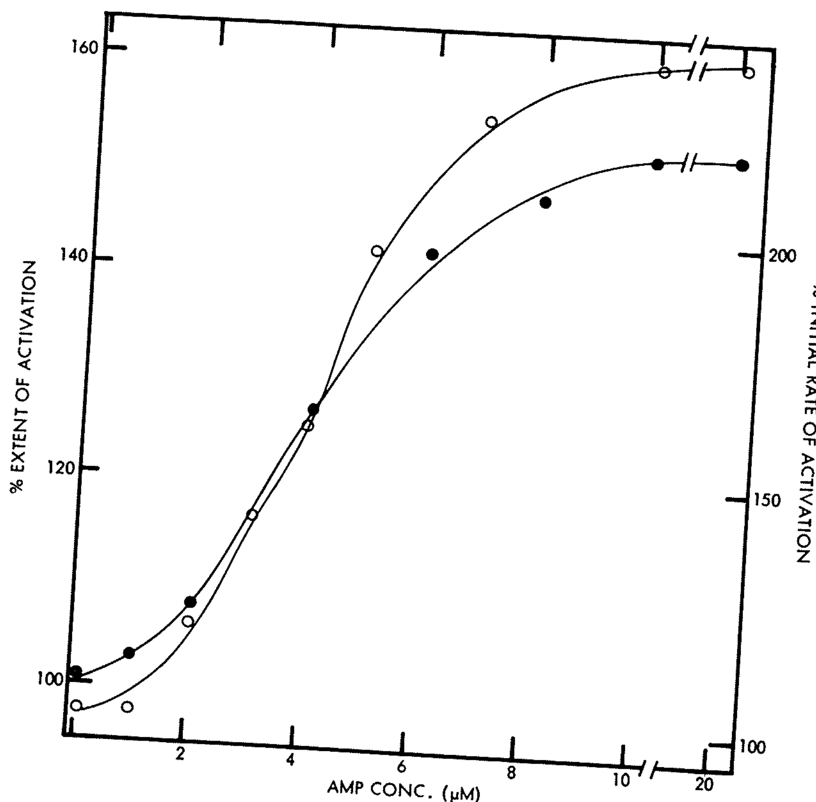


FIGURE 20

Dependence of extent and initial rate of phosphorylase a activation upon AMP concentration. Extent of activation (●): Phosphorylase a (0.45 mg per ml) was incubated in buffer containing various concentrations of AMP at 20° for 90 minutes. Aliquots were then removed for activity measurements as in Figure 18. Activity of the enzyme incubated with buffer alone was arbitrarily taken as 100%. Initial rate of activation (○): the rate of phosphorylase a (0.43 mg per ml) activation was determined as in Figure 18 in the presence of 0.1% glycogen and various concentrations of AMP. The rate of activation by glycogen alone was taken arbitrarily as 100%.

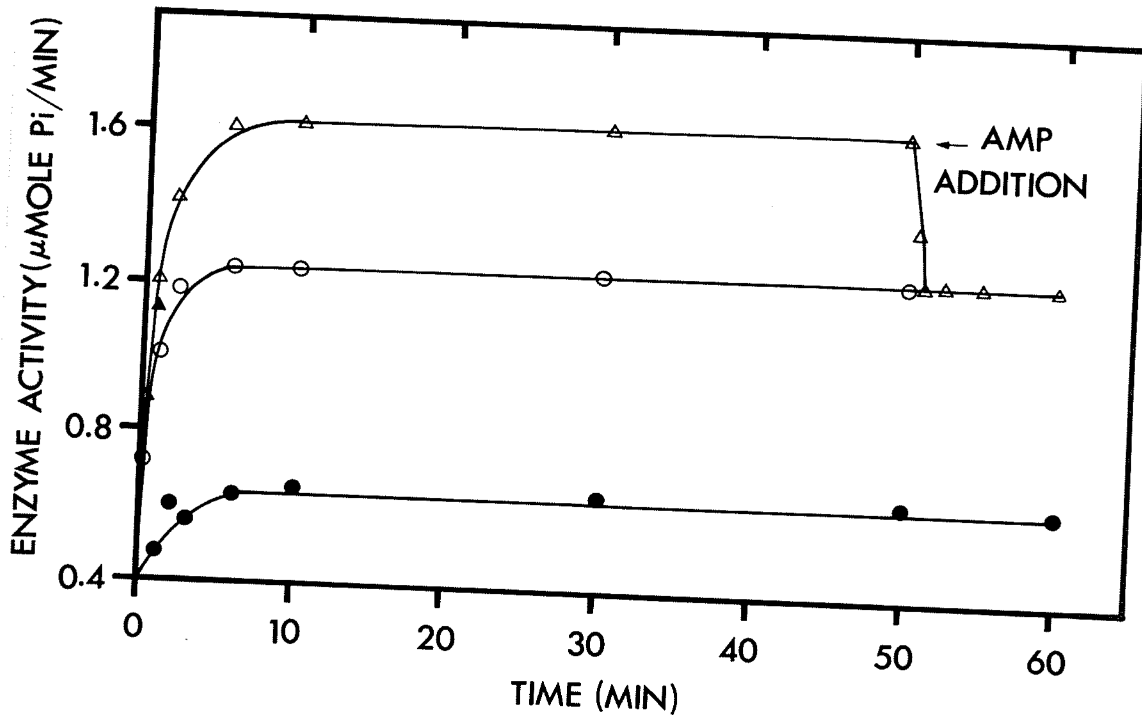


FIGURE 21

Effect of the order of ligand addition on the initial rate of phosphorylase a activation. Phosphorylase a (0.43 mg per ml) was incubated in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8, at 20° (●), or in buffer containing (0.05 M) glucose (○) or 10^{-3} M AMP (△). The arrow indicates the time of addition of AMP to a final concentration of 10^{-3} M to the incubation tube. At various intervals, aliquots were removed for activity measurements (30 second assay) in 0.032 M glucose-1-P, 1% glycogen, 2 x 10^{-3} M AMP, and 0.1 M glucose.

upon varying glucose concentrations at several fixed levels of AMP. Increasing amounts of AMP, at low glucose concentrations, partially reversed glucose activation. While the maximum activation of the enzyme by glucose was independent of AMP, the higher the concentration of the nucleotide the greater the glucose concentration required to achieve maximum activation. These data indicated that the effect of AMP upon glucose activation had kinetic characteristics similar to competitive interaction. Thus AMP, in addition to activating phosphorylase a, decreased the affinity of the enzyme toward glucose. Since AMP and glucose are not structural analogues, it seems unlikely that they could compete for the same binding site on the enzyme. The fact that the homotropic cooperative effect of glucose becomes pronounced with increasing AMP concentrations (Figure 22) suggested that the interaction of these two modifiers was allosteric in nature. As depicted in Figure 23, the Hill coefficient for the interaction of glucose with phosphorylase a increased from an n value of 1.4 to 2.1 as the nucleotide concentration was changed from 0 to 5×10^{-5} M. The limiting value of 2 for the Hill coefficient agrees with the view that there are 2 glucose binding sites per phosphorylase a dimer.

5. Reversal of AMP activation by glucose

In Figure 24, the reciprocal effects of glucose upon AMP activation of phosphorylase a are represented. In the absence of glucose or in the presence of a low glucose concentration (0.002 M), the enzyme activity increased as the AMP concentration was raised. At glucose concentrations of 0.005 M to 0.05 M an increase in AMP concentration led to an inhibition of the enzyme. Since no further change in enzyme activity

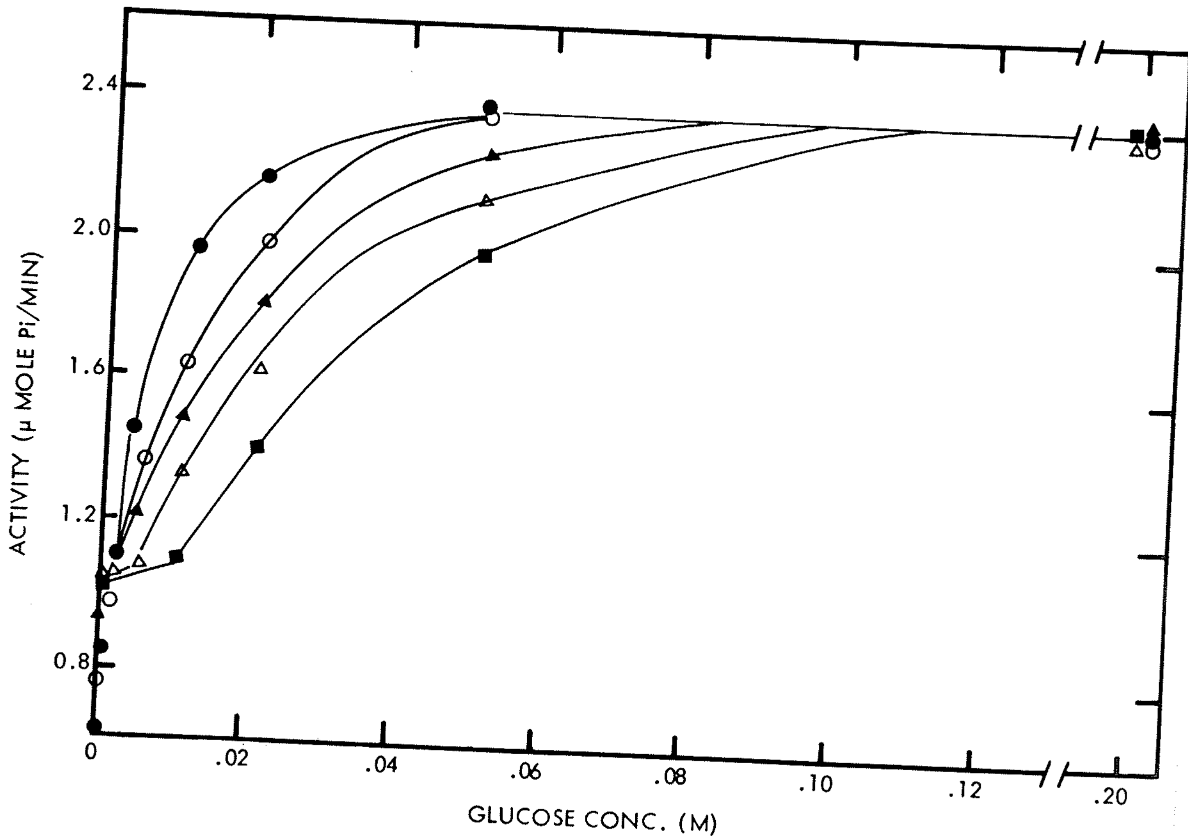


FIGURE 22

Effect of AMP on the activation of phosphorylase a by glucose. Phosphorylase a (0.5 mg per ml) was preincubated in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8, containing various concentrations of glucose at fixed AMP concentrations of: (●) 0; (○) 2.5 x 10^{-6} M; (▲) 5 x 10^{-6} M; (△) 10 x 10^{-6} M; and (■) 50 x 10^{-6} M. Aliquots were removed at 90 minutes and activity was assayed for 1 minute at 20° in substrate containing 0.032 M glucose-1-P, 1% glycogen, 1 x 10^{-3} M AMP, and 0.1 M glucose.

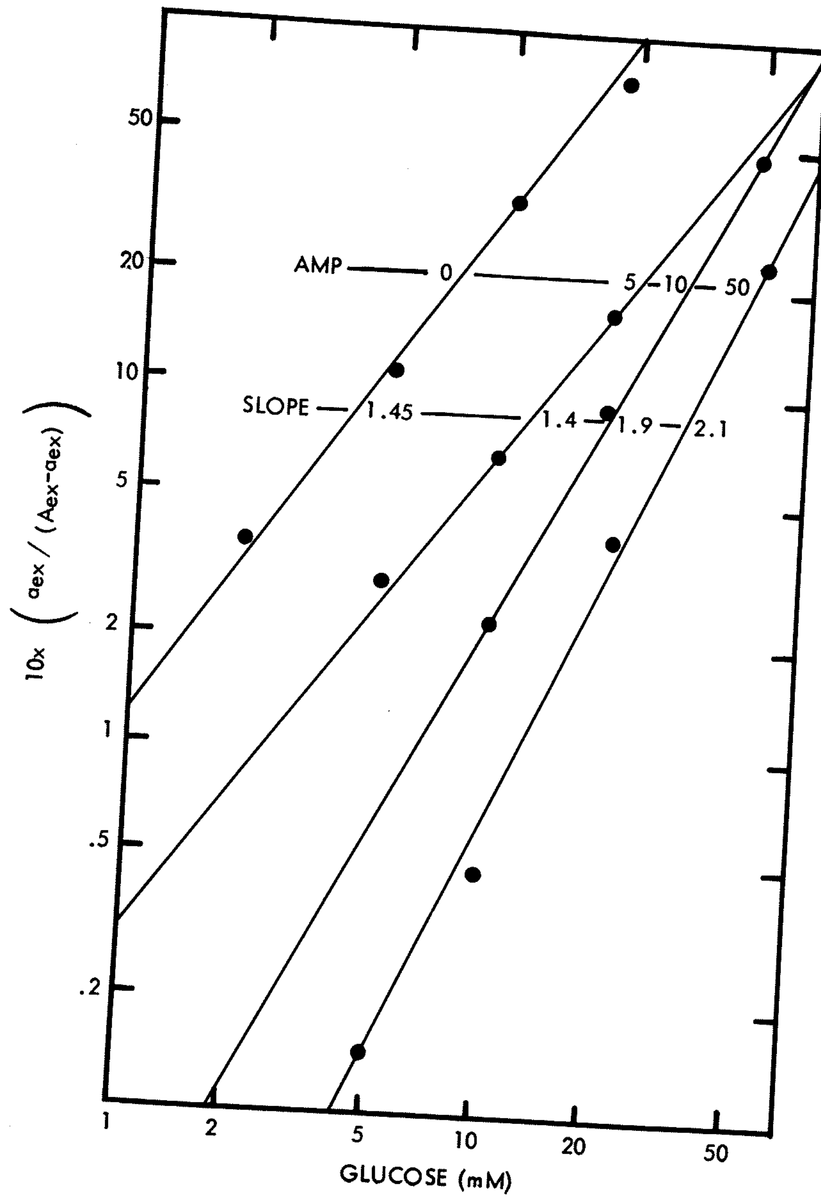


FIGURE 23

Calculated reaction order of the data from Figure 22.

could be observed at all levels of glucose when the AMP concentration was raised from 4 x 10^{-5} M to 10^{-4} M, it therefore appeared that an AMP concentration of 4 x 10^{-5} M was sufficient to saturate the enzyme. The activity of phosphorylase a at saturating concentrations of AMP was dependent upon glucose concentration. Therefore, depending upon the glucose concentration, AMP could either activate or inhibit phosphorylase a. At low glucose concentrations, where phosphorylase a exists mainly as the free enzyme, the activation effect of AMP on the enzyme was expressed. When a significant amount of the enzyme existed as the enzyme-glucose complex (at high glucose concentrations), AMP caused enhanced dissociation of this complex. This effect resulted in an inhibition of the enzyme since the enzyme-glucose complex (see Figure 17) was more active than the enzyme-AMP complex (see Figure 20). Thus, inhibition of the enzyme by AMP at higher glucose concentrations can be visualized as being due to a reversal of glucose activation. This reversal is incomplete since the final activity achieved by saturating concentrations of AMP was highly dependent upon the glucose level (Figure 24). This activity at 0.05 M glucose was approximately 70% greater than the activity in the presence of 0.005 M glucose. At 0.2 M glucose, neither inhibition nor activation of phosphorylase a could be detected, even when the AMP concentration was increased to 10^{-4} M. These results further substantiate the view that the two effectors do not compete for the same binding site on phosphorylase a. Furthermore, these results also suggest that the activity of the enzyme when saturated with both AMP and glucose is similar to the activity of the glucose saturated enzyme.

6. Reversal of glucose dissociation of the enzyme by AMP

To verify that AMP can not reverse phosphorylase a activation

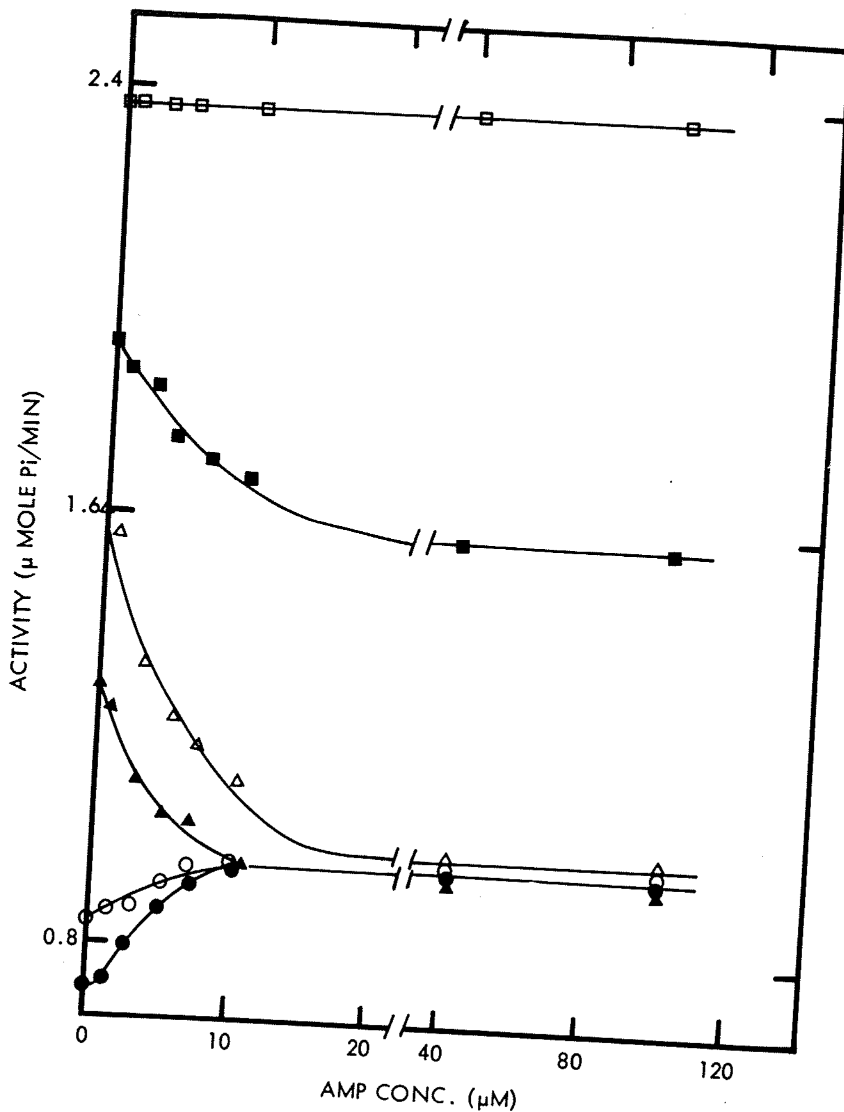


FIGURE 24

Effect of glucose on activation of phosphorylase a by AMP. Phosphorylase a (0.5 mg per ml) was preincubated in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8, containing various concentrations of glucose: (●) 0; (○) 2 x 10^{-3} M; (▲) 5 x 10^{-3} M; (△) 10 x 10^{-3} M; (■) 50 x 10^{-3} M; and (□) 200 x 10^{-3} M. Aliquots were removed at 90 minutes and activity assayed as in Figure 22.

at high glucose concentrations, ultracentrifugal studies on the interaction of the two modifiers with the enzyme were performed (Figure 25). While the dissociation by 0.05 M glucose of phosphorylase a to a dimer ($s_{20,w}$ = 8.9 S) was nearly reversed by saturating concentration of AMP (7), this reversal was incomplete at higher glucose concentrations. With 10^{-3} M AMP, the percentage of the tetrameric form of the enzyme (4 mg per ml) at 0.08 M, 0.15 M and 0.2 M glucose was estimated as 90%, 50% and 20%, respectively. As is to be expected from mass action effects, the extent of reversal was dependent upon protein concentration. No reversal of the enzyme dissociation by AMP could be observed with 0.20 glucose if the enzyme concentration was 1 mg per ml. Therefore the incomplete reversal of glucose dissociation by AMP was compatible with the failure of this nucleotide to completely reverse glucose activation (see Figure 24).

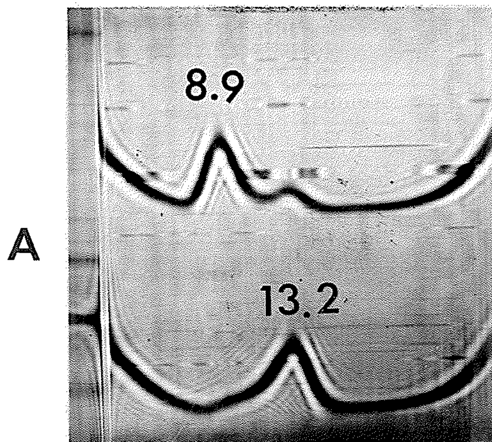
7. Effect of glucose and AMP on tryptic inactivation of phosphorylase a

To further investigate the effect of modifiers on the structure of the enzyme, tryptic inactivation of phosphorylase a has been examined after preincubation of the enzyme with glucose and AMP. Investigators have observed that while AMP completely prevents the inactivation of phosphorylase a by trypsin (81), glucose significantly enhances the initial rate of this inactivation (110). These experiments have been repeated in our laboratory. In addition, the combined effect of glucose and AMP on the tryptic inactivation of phosphorylase a has also been examined. Although at a high concentration of glucose (0.2 M) the dissociation of phosphorylase a was reversed only to a small extent by 10^{-3} M AMP (Figure 25C), the glucose enhanced tryptic inactivation could be completely reversed

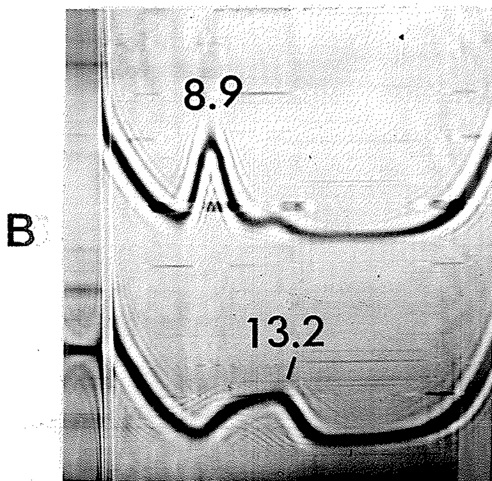
FIGURE 25

Effect of AMP on the dissociation of phosphorylase *a* by glucose. Ultracentrifugation at 20° of phosphorylase *a* (4 mg per ml) in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8. Upper curves with glucose concentrations of: (A) 0.08 M; (B) 0.15 M; (C) 0.2 M. Lower curves as in upper with 10^{-3} M AMP.

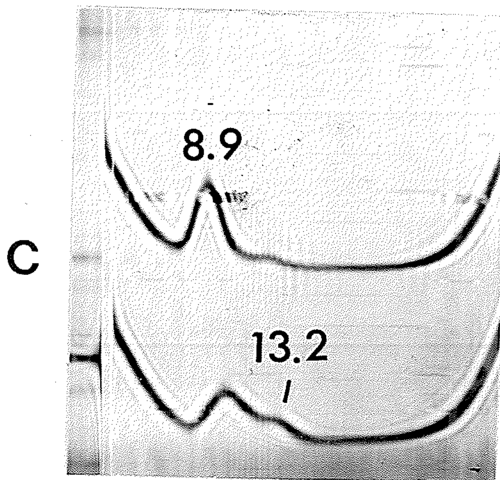
A



B



C



by this nucleotide (Figure 26). Figure 26 shows that AMP completely protected the inactivation of phosphorylase a by trypsin (81), whereas glucose significantly enhanced the initial rate of inactivation (110). Indeed, the initial rate of inactivation by trypsin was much slower with phosphorylase a preincubated in the presence of both glucose and AMP than with the control. These results indicate that there is no correlation between the modifier-induced dissociation and the degree of tryptic digestion. Presumably the alterations in both dissociation and tryptic digestion reflect conformational changes in the subunits. The data in Figure 26 may be explained if the dimeric forms of phosphorylase a in the presence of glucose and in the presence of both glucose and AMP assume different conformational states. The possibility is remote that AMP exhibits its protection through binding at the region of phosphorylase a which is highly susceptible to tryptic digestion. It is known that the enzyme form produced from the tryptic digestion of phosphorylase a, phosphorylase b', is still able to bind this nucleotide (81).

8. Effect of glucose and AMP on HMB inhibition of phosphorylase a

Madsen and coworkers (68, 72, 73) have extensively studied the interaction of phosphorylase a with HMB. In the present study, it was observed that glucose and AMP, in addition to their effect on the tryptic digestion of phosphorylase a, could also influence the inhibition of the enzyme by HMB. Figure 27A shows that 0.2 M glucose significantly retarded the rate of phosphorylase a inhibition by HMB. Although AMP could slightly protect the enzyme against HMB inactivation, it can partially reverse the protective effect of glucose. In the presence of 0.2 M glucose, an enhanced rate of HMB inhibition of phosphorylase a could be consistently

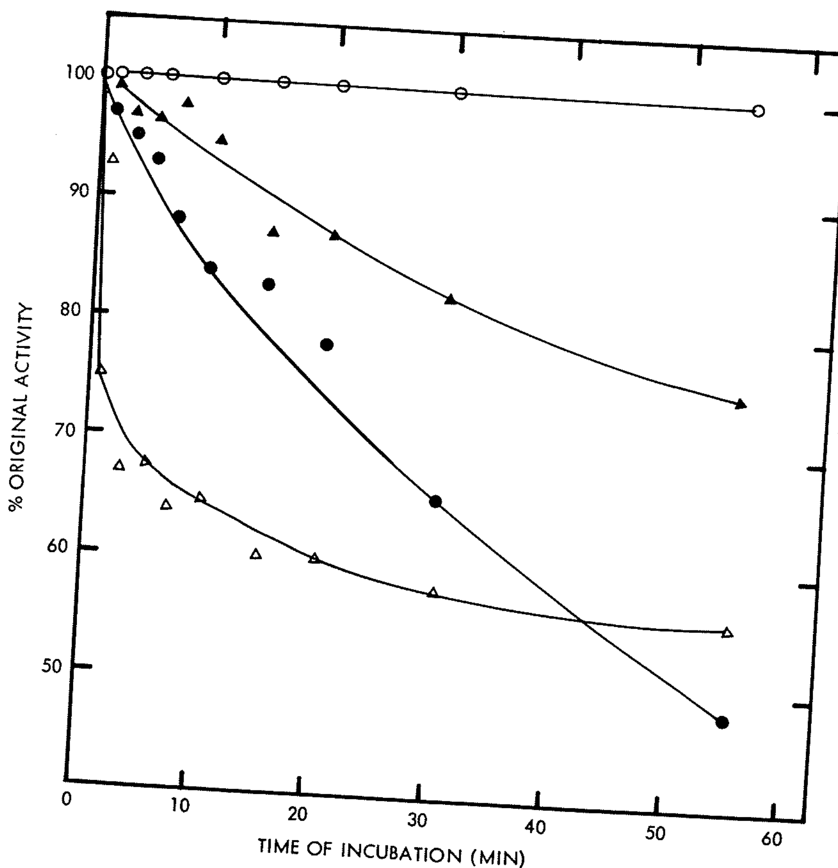


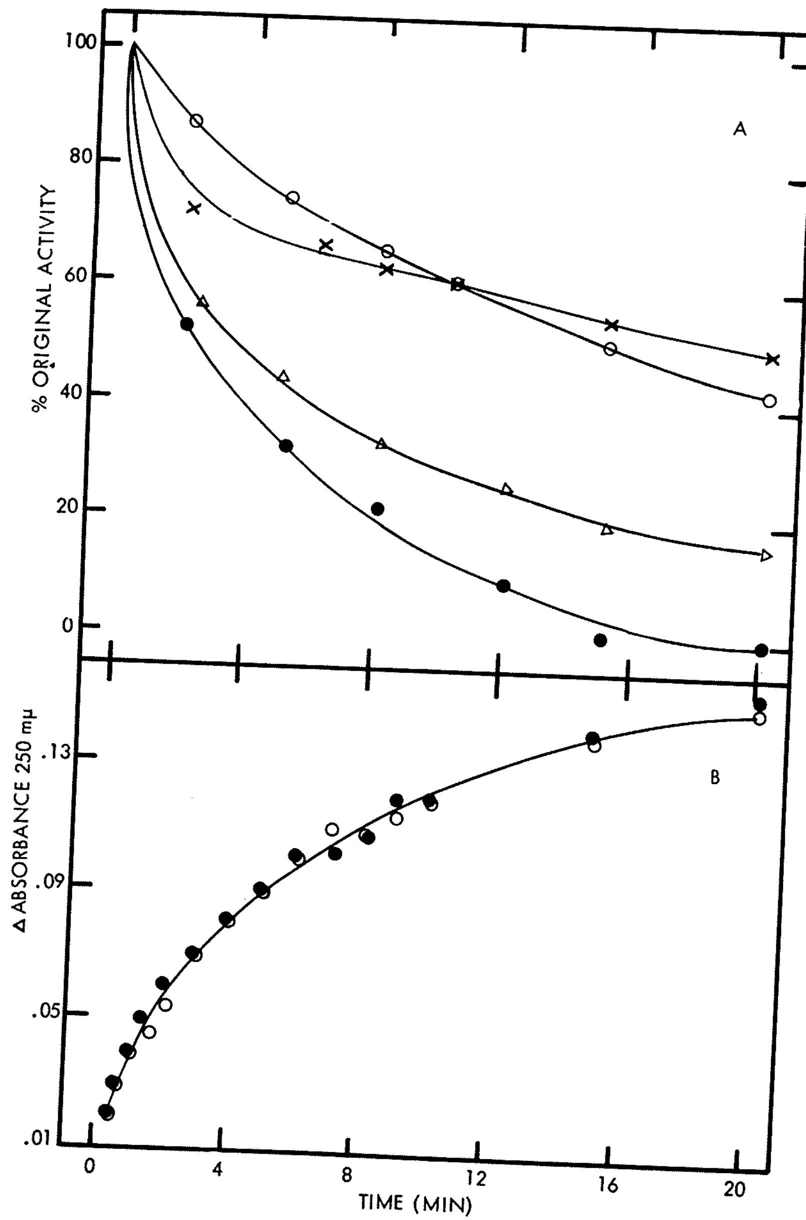
FIGURE 26

Effect of AMP and glucose on the tryptic digestion of phosphorylase a. Prior to the addition of trypsin (20 µg per ml), phosphorylase a (2.4 mg per ml) was preincubated for 60 minutes at 20° in 0.03 M cysteine-0.04 M glycerophosphate, pH 6.8 (●), containing (○) 10^{-3} M AMP, (△) 0.2 M glucose, and (▲) 0.2 M glucose with 10^{-3} M AMP. At various intervals after trypsin addition, aliquots were removed and diluted in buffer containing trypsin inhibitor (60 µg per ml). Enzyme activity was measured for 5 minutes at 30° in substrate containing 0.032 M glucose-1-P, 1% glycogen, and 10^{-3} M AMP.

observed with 10^{-3} M AMP. Thus, it is unlikely that AMP protection was due to a direct interaction of the nucleotide with the sulfhydryl groups. Since maximum activation of phosphorylase a was attained with 0.2 M glucose in the presence of 10^{-3} M AMP, the reversal of glucose protection of phosphorylase a against HMB inhibition could not be due to the dissociation of glucose from the enzyme by the nucleotide. It is, therefore, also unlikely that glucose protected phosphorylase a through a direct interaction with the sulfhydryl groups. Furthermore, Figure 27B shows that the rate of interaction of HMB with the sulfhydryl groups in phosphorylase a was not affected by glucose. In their investigations, Madsen et al (68, 72, 73) have provided strong evidence to suggest that the inactivation of phosphorylase by HMB resulted from the conformational change of the enzyme following modification of the sulfhydryl groups. These results further support the view that AMP or glucose causes a change in the intramolecular interaction of phosphorylase a. This change in enzyme structure does not directly result from dissociation of the enzyme. Figure 28A shows that glucose could also protect phosphorylase a at 30° when the enzyme concentration was lowered from 2 mg per ml to 0.1 mg per ml. It has been suggested that the enzyme exists mainly in the dimeric form under these conditions (8). Furthermore, phosphorylase b, the nonphosphorylated dimeric form of phosphorylase, was also protected by glucose against HMB inhibition (Figure 28B). These data, therefore, further support the postulate that the change in dissociation of phosphorylase a caused by binding of modifiers is accompanied with conformational changes of the enzyme.

FIGURE 27

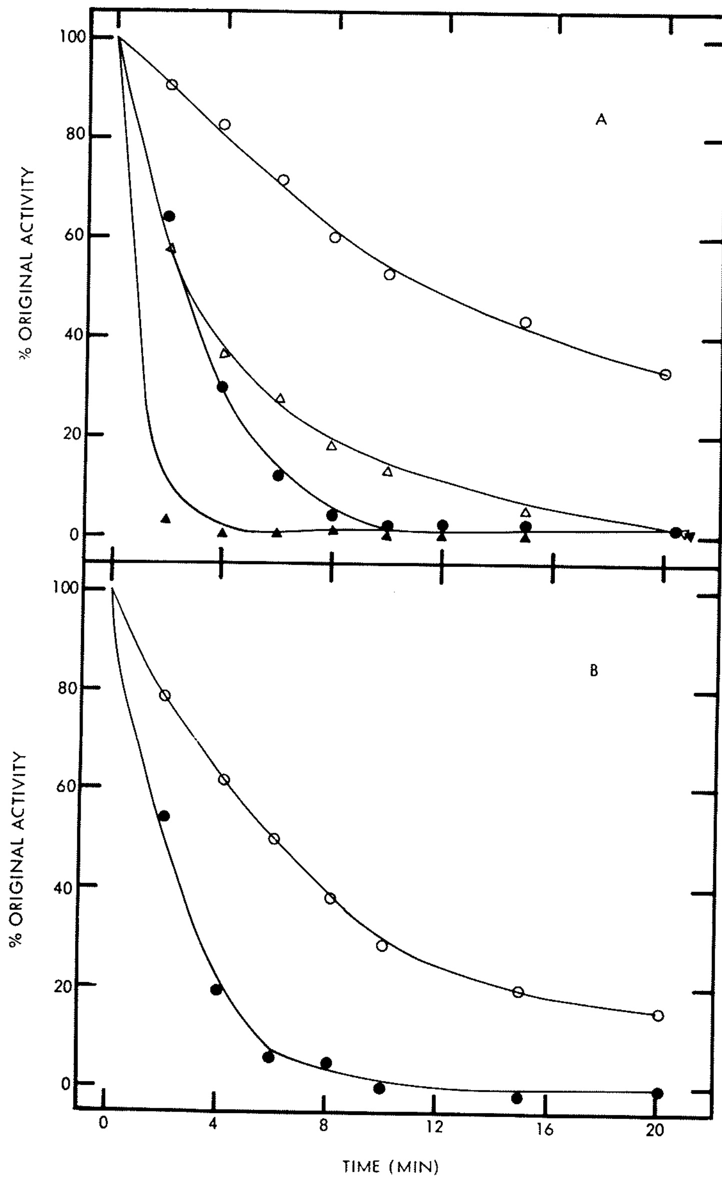
Effect of glucose and AMP on the interaction of phosphorylase a with HMB. A, phosphorylase a (2 mg per ml) was preincubated for 50 minutes at 20° in 0.04 M glycerophosphate-0.001 M EDTA buffer, pH 7.0, (●), or in buffer containing 10^{-3} M AMP (△), 0.2 M glucose (○), or 10^{-3} M AMP and 0.2 M glucose (X). HMB (7×10^{-4} M) was then added to the samples. Aliquots were removed at various intervals for activity measurement in 0.032 M glucose-1-P, 1% glycogen, and 10^{-3} M AMP (1 minute assay). B, phosphorylase a (0.95 mg per ml) was incubated for 50 minutes with the same buffer as in A (●), or buffer containing 0.2 M glucose (○) prior to the addition of 2×10^{-4} M HMB. The absorbance at 250 mμ was determined after HMB addition.



A
% ORIGINAL ACTIVITY
100
80
60
40
20
0
B
Δ ABSORBANCE 250 mμ
.13
.09
.05
.01
0
4
8
12
16
20
TIME (MIN)

FIGURE 28

Glucose protection of the dimeric forms of phosphorylase against HMB inactivation. A, phosphorylase a (2 mg per ml) was incubated at 30° for 50 minutes in buffer (●), or in buffer with 0.1 M glucose (○) prior to addition of 7 x 10^{-4} M HMB. Enzyme activity was determined at various intervals as in Figure 27A. ▲ and △ are similar to ● and ○ except that the enzyme concentration used was (0.1 mg per ml). B, phosphorylase b (1.7 mg per ml) was incubated in buffer (●), or buffer with 0.1 M glucose (○) for 50 minutes prior to the addition of HMB (7 x 10^{-4} M). Activity measurement at various intervals was as in Figure 27A.


100
80
60
40
20
0
% ORIGINAL ACTIVITY
A
B
0
4
8
12
16
20
TIME (MIN)

V. DISCUSSION

V. DISCUSSION

For simplicity, this discussion is divided into two main sections: (A) the allosteric activation of phosphorylase b by nucleotides, and (B) the allosteric activation of phosphorylase a by AMP and glucose.

A. The Allosteric Activation of Phosphorylase b by Nucleotides

Until recently, AMP was thought to be highly specific for phosphorylase b activation since only one other nucleotide, IMP, caused slight activation of this enzyme (15). The data in Table I indicated that other nucleotides, in fact, could activate phosphorylase b, this activation being dependent upon the concentration of glucose-1-P. These activators, as well as many of the non-activating compounds listed in Table I, were competitive inhibitors of AMP activation (see Figures 2 and 3) indicating that they shared a common binding site on the enzyme with AMP. The inhibition constants for these compounds are listed in Table II.

1. Effect of structural modifications of AMP on phosphorylase b activity

The kinetic data obtained from nucleotide activation studies (Tables, I, II, and III) permitted an analyses of the functional groups in the AMP molecule required for binding to and activation of phosphorylase b. Although it is assumed that enzyme regulation is achieved through the binding of effectors at specific regulatory sites on the enzyme surface and the subsequent allosteric transitions of the enzyme (6, 28, 113), the

exact chemical nature of the interaction between allosteric enzymes and effector molecules is not clearly understood. During the course of this investigation two laboratories have presented evidence concerning the identity of the functional groups on AMP which participate in the activation of phosphorylase b (97, 98). These researchers used assay systems different from those employed in the present work. Mott and Bieber (97) utilized a system which does not yield zero or first order kinetics (4). In the presence of a low concentration of glucose-1-P (0.016 M), studies of nucleotide activation indicated a high degree of specificity for AMP which was markedly altered in the presence of salmine. Among 20 structural analogues tested, Mott and Bieber (97) observed that only the previously inactive compounds did not activate phosphorylase b in the presence of salmine. In a more detailed study employing a coupled assay system (86), Okazaki et al (98) observed the effect of 38 structural analogues of AMP on phosphorylase b activity. Among the compounds tested, twenty-one were activators and the mode of their activation was analysed kinetically. From these studies, Okazaki et al (98) determined the functional groups of AMP important for the binding to and the activation of the enzyme. Since the results of this study were presented earlier in the review of the literature, they will not be described here. Similar conclusions as well as additional deductions were obtained in the present study. Therefore, wherever possible, these results will be compared with the findings of Okazaki et al (98).

Among the analogues tested, AMP had the highest affinity for phosphorylase b, the binding capacities of the other compounds being less than one-tenth that of AMP. Adenine, adenosine, ribose or ribose-5-P, all components of the AMP molecule, failed to activate phosphorylase b. Thus

it appears that the presence of all three components of AMP--namely, adenine, ribose, and phosphate--appear to be important in the activation of the enzyme. Similar conclusions were deduced by Okazaki et al (98). The fact that ribose or ribose-5-P did not cause any significant inhibition of the enzyme whereas adenine and adenosine did, suggests that the specificity of the AMP binding site is directed towards the adenine ring. Each component of the AMP molecule was examined to separately determine it's effect on both activation and binding.

a. Modification in the 5'-phosphate moiety. -- As previously reported by Okazaki et al (98), among the analogues tested in this group only adenosine-5'-phosphoramidate activated phosphorylase b (Table I). Analogues of AMP in which the phosphate was attached to the ribose at either the 2' or 3' positions or present in a cyclic 2'3' or 3'5' ring failed to activate the enzyme. Likewise, deletion (adenosine) or substitution (adenosine-5'-nicotinate) of the 5'-phosphate moiety rendered AMP inactive. These results indicate that the presence of the monophosphate group is an absolute requirement for activation and that it must be attached to the ribose at the 5' position. In the present study, it was observed that diadenosine-5'-pyrophosphate failed to activate the enzyme. Due to the bulky nature of this compound, enzyme action may be blocked by a misalignment of the key catalytic groups. This suggests that the nucleotide binding site has a strict stereospecific requirement for the 5'-monophosphate moiety attached to adenosine.

b. Modification in the adenine ring. -- All compounds in this group have been reported to activate phosphorylase b (98). In this work these nucleoside-5'-monophosphates have, with the exception of XMP, A_{max}

values of the same order of magnitude at 100 mM glucose-1-P (see Table I). It was difficult to analyse the effects on phosphorylase b of the analogues in this group since there was no apparent correlation between the A_{max}, $K_{a'}$, and $K_{i'}$, values for each compound. IMP and GMP are analogues of AMP in which the amino group at position 6 of the adenine ring was replaced by an hydroxyl group. These two nucleotides exhibited 30 times higher $K_{a'}$ values and 14 to 22 times higher $K_{i'}$ values than AMP, suggesting that the C-6 amino group of AMP is important for binding to and possibly activation of phosphorylase b. The reason for the low kinetic parameters of XMP was not apparent. XMP and GMP, in addition to hydroxyl groups at C-6 of the purine ring, have either an hydroxyl or an amino group at position 2. Since these nucleotides had lower A_{max} values and binding capacities than IMP, which had an hydroxyl group only at position 6, it appeared that the addition of a group at C-2 influenced both binding and activation, possibly either by steric hindrance or indirect binding to the enzyme.

The pyrimidine-nucleotides CMP, UMP, and TMP generally have higher activation and inhibition constants than do the purine nucleotides. The $K_{a'}$ values for the pyrimidine nucleotides increased over that for AMP by at least 60 fold, twice the values than those for the purine nucleotides. This difference in binding capacities can be attributed to the absence of the imidazole ring, which may possibly be implicated in the binding of AMP to phosphorylase b.

c. Modifications in the ribose and base moieties. -- Among the analogues tested in this group only 2'-dAMP and TMP activated phosphorylase b (98). Other 2'-deoxyribonucleotides failed to activate the enzyme, whereas their corresponding ribonucleotides gave varying degrees of

activation. Since 2'-dAMP had higher $K_{a'}$ and $K_{i'}$ values than AMP, suggested that the 2'-hydroxyl group on the ribose moiety participates in the binding to and the activation of the enzyme. Because it was not available, the effects of 3'-dAMP could not be tested. However, Okazaki et al (98) have shown that this compound had the same A_{max} and $K_{a'}$ values as AMP. The fact that cyclic-2'3'-AMP had a $K_{i'}$ value two fold higher than cyclic-3'5'-AMP, as described previously, supports the view that a free hydroxyl group at C-2' of the ribose is involved in the binding of AMP to phosphorylase b.

In summary, the monophosphate group at position 5' of the ribose moiety is absolute for activation. The 2'-hydroxyl group on the ribose participates in both activation and binding while the amino group at position 6 and possibly the imidazole moiety of the purine ring contribute to the binding of AMP to the enzyme.

2. Probable mode of AMP interaction with the regulatory site on the enzyme

The limited use of analogues with only minor modifications in the AMP molecule did not permit a detailed study of the nature of the chemical interaction of the nucleotides with the enzyme. At the pH used in these studies, pH 6.8, one can only postulate the probable ionic state of the functional groups. The 5'-monophosphate residue is a strong dibasic acid with pK_a values of $\sim$1.0 and 6.0 for the dissociation of the two free hydroxyl groups. At pH 6.8 the phosphate should exist in the ionized state. If one of it's hydroxyl groups was replaced by an amino group, as in adenosine-5'-phosphoramidate, or was in the cyclic configuration the binding capacity of the 5'-monophosphate was decreased by over 40 fold. It, therefore, appears that one of the two hydroxyl groups may be either involved in hydrogen or electrostatic binding to the enzyme. The mode of binding of the 2'-hydroxyl group on the ribose moiety, at pH 6.8, may be

through a hydrogen bond to the enzyme molecule. The 6-amino group of AMP, with a pK_a value of 3.7, should not be in the ionized state under the assay conditions (pH 6.8). Therefore, it is probable that a hydrogen bond is also responsible for the interaction between the amino group and the enzyme rather than an electrostatic interaction. Although the experimental evidence was not conclusive, it appears that the heterocyclic ring of AMP must have some interaction with the protein, possibly through a π-electron system of the purine ring with the enzyme regulatory site. This may be similar to the base stacking forces that stabilize the helical structures of polynucleotides (114).

In analysing these data, it appeared that at least two combinations of these essential groups were necessary for tight binding to the enzyme. The differences in the K_i, values between the nucleosides and their corresponding nucleotides indicated that loss of the 5'-monophosphate moiety resulted in decreased binding capacity. Substitution of the C-6 amino group by an hydroxyl group, as in IMP or GMP, or removal of the 2'-hydroxyl or imidazole groups also decreased the affinity of activators for the enzyme. However, if two or more essential groups were modified, that is both the C-6 amino, 2'-hydroxyl and/or 5'-monophosphate groups, there was virtually no binding. This became evident when CMP and UMP were compared. CMP , a pyrimidine nucleotide, having an amino group at position C-4, had a K_i value near that of the purine nucleotide, IMP. However, UMP, which had both the C-4 amino and imidazole groups missing had an extremely low binding capacity for the enzyme. Therefore, for maximum binding and activation, cooperation must exist among all functional groups on AMP.

3. Effect of ionic species and limited tryptic digestion on nucleotide activation

Although in the present study phosphorylase b and b' showed no differences in their nucleotide specificity, these enzyme forms did show differential responses to several ionic species. These ions may be classified into two groups, namely, anions and polycations. Since it has been shown that among various anions the anion of NaF has the most pronounced effect on phosphorylase b (82, 89), the effects of this anion on the nucleotide activation of phosphorylase b and b' were examined. Similarly, protamine and spermine were chosen to represent the polycations. Sealock and Graves (82) have attributed the activation of phosphorylase b by NaF to the "salting out" of a specific region of the enzyme surrounding the serine residue so that it mimics the ionic environment of the phosphoseryl residue in phosphorylase a. More recently, Graves and coworkers (88) have reported that NaF did not enhance AMP activation of phosphorylase b', thus providing support for their original postulate. However, it has been shown in this thesis that the activation by nucleotides other than AMP could be enhanced by NaF. Therefore, it appears that the NaF effect is not uniquely related to the presence of the hexapeptide. The fact that Graves et al (88) observed that the apparent Michaelis constants of phosphorylase b' for substrates and activators were independent of the concentration of glycogen, P_i, and AMP does suggest the possibility that the hexapeptide removed by trypsin could play a role in the allosteric properties of the enzyme.

The effect of polycations on the activity of phosphorylase b appears to be related to the direct binding of these ionic species to the enzyme (16). The fact that activation of phosphorylase b' by AMP or other nucleotides was not enhanced by protamine suggests that the specific hexapeptide is essential for the polycationic effect. Since it has been shown

that there are a large number of positively charged amino acid residues in the vicinity of the serine residue in the hexapeptide (79, 81), direct interaction of polycations with this region seems unlikely. However, it may be postulated that this site may interact electrostatically with a highly negative region of the enzyme (16). Polyamines could bind to this polyanionic site causing a displacement of the polycationic region of phosphorylase _b_. This structural alteration could lead to a conformation having enhanced catalytic activity in the presence of nucleotides. On removal of the hexapeptide region, phosphorylase _b'_ was not activated by polyamines suggesting that the enzyme has become desensitized (16).

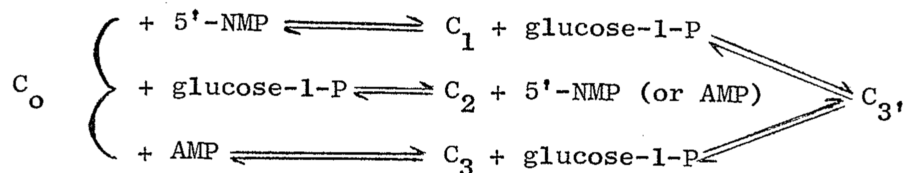
4. Comparison of the kinetic data with the allosteric model proposed by Monod, Wyman, and Changeux

Since AMP shared a common binding site on the enzyme with other nucleoside-5'-monophosphates, it was surprising that the kinetic behavior of nucleotide activation was different from that of AMP activation. Analyses of the kinetic results of AMP activation of phosphorylase _b_ according to the predictions of the theoretical model proposed by Monod, Wyman, and Changeux, (28) has led to confusion in the literature. The evidence reported by Buc (13, 14), and Helmreich and Cori (86) suggested that each of the substrates glucose-1-P, P_i, and glycogen reciprocally decreased the K_m values for AMP with no change in the V_{max}, thus classifying phosphorylase _b_ as an enzyme of Class K (28). The data reported in this thesis indicated that both the V_{max} and the K_m for glucose-1-P are dependent upon AMP concentrations. Similar evidence has recently been reported by Madsen (11, 12), and by Sealock and Graves (82). Thus, under our experimental conditions, the kinetic results of AMP activation indicated that phosphorylase _b_ belongs to a mixed V and K class enzyme

system. When attempts were made in the present study to apply the model of Monod et al (28) to the kinetics of IMP activation discrepancies became apparent. Since IMP appeared to affect the V_{max} but not the affinity towards glucose-1-P, phosphorylase b should be classified as an allosteric enzyme of class V (28). Similarly, other nucleoside-5'-monophosphates also increased the V_{max} of the enzyme without affecting the K_m of glucose-1-P further supporting the classification of phosphorylase b as a V class enzyme system. For an enzyme system of this class, according to the "concerted transition" theory of Monod et al (28), the substrate should not show homotropic cooperative interactions. Nevertheless, strong homotropic interactions of glucose-1-P with phosphorylase b were observed in the presence of IMP and in the presence of most of the other nucleoside-5'-monophosphates. Atkinson, Hathaway, and Smith (25) have reported similar kinetic evidence for yeast NAD-specific isocitrate dehydrogenase (25) and yeast phosphofructokinase (32) in which the intersite interactions remained strong in the presence of activators. The fact that classification of an enzyme according to its kinetic behavior is dependent upon the activator used is again not accountable by the model of Monod, Wyman, and Changeux (28). Therefore, the prediction of this model that effectors should change the cooperativity of the substrate saturation curve does not appear to be necessary for phosphorylase b.

To explain the kinetic results obtained in the present study, it may be assumed that the allosteric transition involved in nucleotide activation of phosphorylase b consists of two stages: (a) that of enhancing enzyme affinity toward glucose-1-P, and (b) that of enhancing catalytic efficiency (V_{max}). Thus, binding of the various nucleoside-5'-monophosphate analogues of AMP to the enzyme induces one allosteric transition stage,

enhancing the catalytic efficiency of phosphorylase b. On the other hand, AMP can induce both stages of allosteric transition so that both V_{max} and the enzyme affinity for glucose-1-P are increased. The fact that glucose-1-P exhibited homotropic interactions suggests that the binding of one glucose-1-P molecule to the enzyme facilitates binding of the second glucose-1-P. Therefore, this substrate can also induce the stage of allosteric transition involved in the enhancement of enzyme affinity. However, glucose-1-P does not affect the catalytic efficiency of phosphorylase b, since no activity could be shown in the absence of nucleotides. The observation that the homotropic interactions of glucose-1-P with phosphorylase b are independent of the concentration of most of the nucleoside-5'-monophosphates, but can be nearly eliminated by increasing AMP concentration, is in agreement with the above explanation. The various allosteric transitions in phosphorylase b that are induced upon binding with nucleotides and glucose-1-P can be represented in Scheme II



SCHEME II

where 5'-NMP is any nucleoside-5'-monophosphate activator other than AMP. It is postulated that the enzyme in its free state exists in an unique conformation, C_o, which can undergo conformational alterations upon binding of the various metabolites or their combinations. The enzyme forms existing in these various conformations show distinct allosteric properties. The conformation stabilized by 5'-NMP, C_1, has potential catalytic activity, but exhibits poor affinity for glucose-1-P, whereas C_3, the AMP-stabilized conformation, is both catalytically active and the form favored by glucose-1-P. In contrast to the other two forms of the enzyme, the

glucose-1-P-induced conformation, C_2, has a higher affinity for the substrate but is inactive. Although conformation C_3, is postulated for the enzyme when both substrate and a nucleotide are bound, it is possible that this conformation of the enzyme is essentially the same as conformation C_3.

An important feature of this postulated scheme (Scheme II) is that various effectors can each induce a distinct enzyme conformation. This postulation can be experimentally tested. Although the establishment of an absolute conformation for an enzyme requires rigorous physical techniques, it is possible to detect change in protein conformation by an examination of the alteration in certain structural properties of the enzyme. Recently Gerhart and Schachman (115) have used changes in reactivity of sulfhydryl groups and the sedimentation constants of aspartate transcarbamylase as a criterion for following the conformational alterations in this enzyme. In the present study, sedimentation velocity experiments showed that, in contrast to AMP, neither 5'-NMP nor glucose-1-P could induce the association of dimeric phosphorylase b into a tetrameric species. This result, therefore, supports the view that C_3, the conformational state stablized by AMP, is different from either C_1 or C_2, conformations stabilized by 5'-NMP and glucose-1-P, respectively. However, the fact that IMP and glucose-1-P protected the enzyme against cold inactivation as well as imidazole-citrate resolution of pyridoxal phosphate suggests that these effectors can cause changes in enzyme conformation. Furthermore, when both glucose-1-P and IMP or other nucleoside-5'-monophosphates were present together enzyme association occurred, suggests that the C_3, conformation is distinct from either the C_1 or C_2 form.

Koshland (45-48, 116), in his "induced fit" theory, was the first

to suggest the possibility that effectors could induce conformational alterations in the structure of the enzyme. This theory was later extended by Atkinson and coworkers (25) into a model explaining allosteric transitions of regulatory enzymes. An important feature of Koshland's hypothesis was the assumption that more than one group at the catalytic and regulatory sites would have to be involved in enzyme action and, therefore, the precise alignment of these groups with the effector would be necessary for enzyme action. This assumption received strong support in the present study. The substitution or elimination of functional groups on the AMP molecule caused dramatic changes in enzyme activity. For example, the substitution of a 6-hydroxyl for the 6-amino group on the adenine ring of AMP introduced a group of essentially the same size but different in chemical properties. The resultant compound, IMP, had entirely different kinetic properties than AMP. Similarly, when the functional amino and imidazole groups were both altered, as in UMP, a very low binding capacity for the enzyme was exhibited. These lines of evidence suggest that for the activator to induce a conformational change in the enzyme leading to total catalytic activity there must be participation of all functional groups on the effector molecule as well as interaction between such groups and active groups at the regulatory site of the enzyme. Cooperativity between all such groups would induce a maximum change in the enzyme conformation. Such an alteration could lead to a thermodynamically stable conformation which has the appropiate alignment of residues at the active site for binding of the substrate, glucose-1-P. Since AMP has the highest affinity and catalytic efficiency for phosphorylase _b_, the conformation induced by this nucleotide, C_3, is assumed to be that which has under gone the greater alteration in conformation. This was supported by the ability of AMP to induce the association of phosphorylase _b_ in the absence of glucose-1-P. An inter-

esting anomaly exists between the properties of dAMP and AMP. The kinetic observation that dAMP, like AMP, could nearly eliminate the homotropic interactions of glucose-1-P with phosphorylase b suggests that this deoxynucleotide could also induce conformation C_3 in Scheme II. Therefore, one would expect that dAMP would behave similarly to AMP in inducing the association of the enzyme in the absence of glucose-1-P. However, this was not the case, which seems to indicate that the 2' hydroxyl group on the ribose moiety may play a role in the AMP induced association of phosphorylase b. This received further support from the fact that dAMP had a 10-fold lower binding and activation capacity than had AMP. In addition, although dIMP was not tested, other deoxynucleotides, in contrast to their respective nucleotides, were not able to activate phosphorylase b and had very low binding capacities. These data add further support to the postulate that the 2'hydroxyl group on the ribose moiety is one of the functional groups of AMP dictating a particular conformational state. Thus the assumption of Koshland (116) that interaction between all functional groups on the enzyme and the effector molecule is necessary to induce the proper conformation essential for complete enzyme action is upheld. It is possible that each nucleotide, depending on its interaction strength with the enzyme, could induce its own specific conformation having varying degrees of affinity for glucose-1-P intermediate between conformations C_1 and C_3. This could account for the variations in the effects of the nucleotides on the interaction strength of glucose-1-P. However, since all AMP analogues have little effect on the concentration of the substrate at half-V_{max} and are similar in their associative properties, they have, for reasons of simplification, been depicted as stabilizing conformation C_1.

The postulation embodied in Scheme II, that allosteric transitions in the nucleotide activation of glycogen phosphorylase _b_ are biphasic, can also be applied to the kinetic data obtained in the presence of NaF. There were two changes in the kinetic properties of phosphorylase _b_ that occurred in the presence of the anion of NaF. First, the homotropic interactions of substrate as well as activators were no longer detected. Second, the reciprocal effects of nucleotide and glucose-1-P on each other's enzyme affinity were eliminated. Both anionic effects can be readily explained by the two-stage allosteric transition hypothesis if it is assumed that the allosteric transition involved in the enhancement of enzyme affinity toward glucose-1-P is "desensitized" by NaF.

B. The Allosteric Activation of Phosphorylase a by AMP and Glucose

1. Relationship between enzyme dissociation and allosteric transition

Glycogen phosphorylase a exists in equilibrium between a tetrameric and a dimeric species (7, 8, 18, 110, 111). Many investigators have concluded that the dimeric form of the enzyme is more active than the tetramer, since various effectors which are known to activate the enzyme upon preincubation can enhance formation of the dimeric species (7, 8, 18, 95, 110, 111). In the present study, the correlation between enhanced dimerization and activation of phosphorylase a is again demonstrated. The differences in the extent of enzyme activation by AMP and glucose could be related to the differences between these modifiers in promoting dimer formation. Furthermore, a correlation existed between the AMP reversal of both the activation and the dissociation caused by glucose since these two effects exhibited the same dependence upon glucose concentration. It is, therefore, reasonable to assume that activation of phosphorylase a arises from dissociation of the tetrameric form of the enzyme into the dimeric species.

In 1943, Cori, Cori, and Green (4) observed that glucose is a competitive inhibitor of glycogen phosphorylase a with respect to glucose-1-P. This inhibition can be reversed by AMP. In the presence of glucose the plot of initial velocity versus glucose-1-P is sigmoidal. Thus, glucose inhibition of phosphorylase a has the kinetic characteristics of an allosteric interaction. At high enzyme concentrations phosphorylase a exists mainly as a tetramer, which upon preincubation with glucose results in the dissociation of the enzyme to an active dimer (7). This activation is similar to glucose inhibition in that it also exhibits allosteric kinetics

and can be reversed by glucose-1-P or AMP. The observation that glucose activation of phosphorylase a can be correlated with enzyme dissociation (7), therefore, lends support to the postulation that allosteric transitions are mediated through the structural alterations of enzyme molecules.

Frieden and Colman (117) have concluded that for glutamate dehydrogenase the cooperative interactions of effectors result from association of the enzyme. However, since phosphorylase a exists mainly as a dimeric species under the usual assay conditions (7, 8, 18, 110, 111), the possibility that the allosteric interactions in phosphorylase a observed in the present study arose from enzyme association is remote. Furthermore, phosphorylase b is also inhibited allosterically by glucose without involving the process of dissociation (4). The fact that allosteric transition is rapid whereas the dissociation is slow also argues against the equivalence of these two processes. It is, however, generally believed that the change in dissociation of a polymeric protein in the presence of low molecular weight effectors reflects a change in the subunit conformation (118). The fact that the plots of initial rate of activation versus effector concentrations were both sigmoidal and modifier-concentration dependent suggests that a change in the intramolecular interactions within the tetramer precedes dimer formation. Although this change in enzyme conformation manifests itself through an enhanced dissociation of the enzyme, it is not essential for the enzyme to exist as a tetramer. Since the plot of the extent of activation versus glucose concentration also exhibited homotropic interactions, it appears that the conformational change which occurs in the dimer decreases the tendency for its association into the tetramer. The observation that AMP and glucose

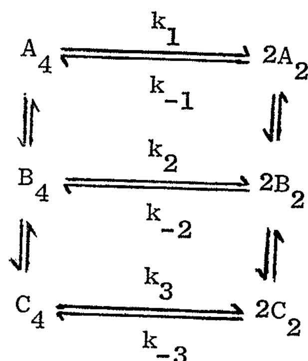
influenced the rate of enzyme inactivation by HMB and trypsin also suggests that these modifiers can cause structural alterations of the enzyme. The possibility that the change in enzyme stability arises from a change in the tetramer-dimer equilibrium can be ruled out since, as mentioned previously in the "Results", there was no correlation between the effect of modifiers on enzyme dissociation and the rate of inactivation.

In summary, experimental evidence presented in this thesis strongly suggested that glucose and AMP can each cause conformational changes in phosphorylase _a_. Depending upon the conditions and criteria applied, these conformation changes can be manifested in several ways. At high protein concentrations, where the free enzyme exists predominately in the tetrameric form, the rate or the extent of enzyme dissociation is enhanced by the effectors. On the other hand, under the usual assay conditions where the enzyme is essentially in the dimeric form, allosteric inhibition by glucose or activation by AMP is observed.

2. Comparison of data with the allosteric model proposed by Monod, Wyman and Changeux

Several investigators (12-14, 95, 119) have shown that the kinetic and modifier saturation properties of glycogen phosphorylase agree to a large extent with the predictions of the allosteric model proposed by Monod _et al_ (28). Therefore, the results of phosphorylase activation by various modifiers in the present work have been compared with this model. According to this model, the homotropic interaction observed with glucose indicated that this effector did not bind significantly to the predominant form of free phosphorylase _a_. Although the plot of initial rate versus AMP concentration for phosphorylase _a_ was also

sigmoidal, this plot did not represent a modifier saturation curve due to the high affinity of AMP for phosphorylase a. Under the experimental conditions employed in the present study, the concentration of AMP bound to phosphorylase a cannot be considered negligible relative to total nucleotide concentration. By direct measurements of the binding of AMP to phosphorylase a, Helmreich et al (95) have concluded that there is no homotropic interaction in the binding of AMP to this enzyme. Since it is justifiable to state that the change in phosphorylase a dissociation is a reflection of conformational changes of the enzyme, the enhanced dissociation of the phosphorylase a tetramer suggests that AMP also favors an enzyme conformation that differs from the predominating form of the free enzyme. Since AMP and glucose interact antagonistically with the enzyme, the model of Monod et al (28) further suggests that the conformational states favored by these two modifiers are different. Thus three conformational states have to be postulated for phosphorylase a to account for the observed effect of glucose and AMP on the activity of the enzyme. The equilibrium condition existing between tetrameric and dimeric phosphorylase a in the free enzyme is represented in Scheme III



SCHEME III

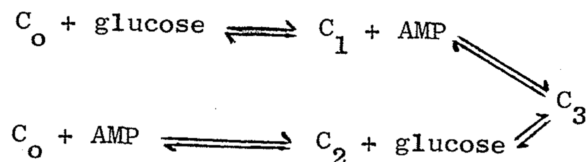
where A, B, and C represent the monomeric units existing in the three

conformational states. If free phosphorylase a exists mainly in the B form, and the favored conformations for glucose and AMP are the A and C forms, respectively, the dependence of initial rate of enzyme activation upon glucose and AMP concentration would indicate that both k_1 and k_3 are greater than k_2. Since glucose showed a pronounced homotropic interaction, the model predicts that at a saturating concentration of glucose, phosphorylase a exists predominately in the A form. When certain conditions prevailed in which the free enzyme or enzyme-AMP complex existed mainly as a tetramer, saturating glucose caused complete dissociation of the tetrameric enzyme. This observation supports the view that the predominant enzyme conformation at high concentrations of glucose differs from that in the free enzyme and in the presence of saturating AMP. Due to the slight extent of phosphorylase a activation by AMP, two alternative possibilities have to be considered. (a) Conformation C is only slightly favored over Conformation B by AMP, or (b) the predominant enzyme form at saturating AMP being Conformation C, the dissociation constant for C_4, k_3/k_{-3}, is only slightly higher than that for B_4, k_2/k_{-2}. The fact that AMP completely protected phosphorylase a against tryptic inactivation suggests that the predominant form of phosphorylase a in the presence of AMP differs from that in the free enzyme. Thus, this observation supports the second alternative.

If phosphorylase a exists predominately either in the A, or the C form in the presence of saturating glucose or AMP, respectively, the model of Monod et al predicts that the enzyme when saturated with both modifiers would exist as a mixture of these two enzyme forms (28). However, the observation that phosphorylase a at saturating concentrations of both modifiers existed mainly as a dimeric species similar to the A form,

but which resembled the C form in showing considerable resistance toward tryptic inactivation was incompatible with this prediction. Instead, these results indicated that the enzyme at high concentrations of both modifiers assumes a conformation which is different from any of the postulated conformations, A, B, or C. Thus, an additional conformation has to be postulated for this enzyme. A summary of the conformational states that have to be postulated for the allosteric transition of phosphorylase a are given in Table IX. Although the results of Helmreich et al (95) on the binding of AMP to phosphorylase a are adequately rationalized in terms of a two-conformation model, the findings in the present study require that for only two effectors used to activate the enzyme, four conformational states must be proposed and thus are not accomodated by the model of Monod et al (28).

One striking feature emerging from this analysis is that, in addition to the predominant conformational state present in the free enzyme, in the presence of each modifier and each combination of modifiers tested a different conformational state must be postulated. This would suggest that the basic assumption of the model of Monod et al concerning the pre-existence of equilibrated conformations is too restrictive to account for the allosteric transitions in glycogen phosphorylase a. However, the present results may be satisfactorily explained by a direct application of the "induced-fit" theory of Koshland (48) as shown in Scheme IV



SCHEME IV

TABLE IX

POSTULATED CONFORMATIONAL STATES OF PHOSPHORYLASE *a*

Modifier	Conformation
None	C_0
Glucose	C_I
AMP	C_{II}
Glucose + AMP	C_{III}

where the enzyme in its free state assumes a distinct conformation C_o which can be directly altered upon the binding of the effectors or their combinations. Upon complexing with glucose or AMP, the free enzyme undergoes conformational alterations into C_1 or C_2, respectively. In addition, the combination of both effectors will induce a different conformational state, C_3. This scheme is similar in many respects to that described previously for the allosteric transitions of phosphorylase b, both schemes resembling the model proposed by Atkinson, Hathaway, and Smith (25) described in the literature review.

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