Aspects of glycogen phosphorylase catalysis and regulation

Ronald Joseph Uhing

Iowa State University

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Aspects of glycogen phosphorylase catalysis and regulation

by

Ronald Joseph Uhing

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
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For the Graduate College

Iowa State University
Ames, Iowa
1980
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DEDICATION

To my parents, for a lifetime of support and encouragement.
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AMP</td>
<td>Adenosine 5'-monophosphate</td>
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<tr>
<td>AMPS</td>
<td>Adenosine 5'-thiophosphate</td>
<td></td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-monophosphate</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
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<tr>
<td>glucose-1-P</td>
<td>glucose-1-phosphate</td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>Inosine 5'-monophosphate</td>
<td></td>
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<tr>
<td>MES</td>
<td>2(N-Morpholino)ethane sulfonic acid</td>
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<tr>
<td>Pi</td>
<td>Orthophosphate</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>UDPG</td>
<td>Uridine 5'-diphosphoglucose</td>
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HISTORICAL BACKGROUND

Glycogen phosphorylase (α-1,4-glucan : orthophosphate glycosyl transferase, E.2.4.1.1) catalyzes the initial step in the degradation of glycogen:

\[(\alpha-1,4\text{-glucoside})_n + P_i \rightleftharpoons (\alpha-1,4\text{-glucoside})_{n-1} + \text{glucose}-1\text{-P}.\]

Although the equilibrium constant for this reaction is 0.3 at pH 6.8 (1), the enzyme acts in the direction of glycogen degradation in the cell due to the high ratio of inorganic phosphate to glucose-1-P (2). In muscle, the breakdown of glycogen is coupled to the energy needs of contraction, whereas in liver it is linked primarily to blood glucose homeostasis.

The reaction sequence outlined above is an important control step in carbohydrate metabolism and glycogen phosphorylase has been shown to be part of a complex regulatory system involving several enzymes and the levels of substrates, divalent cations, nucleotides, and hormones. In addition, skeletal muscle phosphorylase has been suggested to be a reservoir for pyridoxal phosphate (3), which is an indispensable part of the enzyme (4), so that glycogen metabolism may also be affected by the nutritional intake of this vitamin.

In 1943, Cori and Green reported that rabbit skeletal muscle glycogen phosphorylase exists in two forms, phosphorylase a and phosphorylase b (5). Subsequent studies have elucidated an interconversion mechanism involving the following phosphorylation-dephosphorylation sequence:
Phosphorylase a from skeletal muscle is a tetrameric enzyme composed of identical subunits (6). From sequence studies a monomeric molecular weight of 97,412 daltons has been determined (7). The phosphorylation site on the enzyme is a seryl residue located fourteen amino acids from the amino terminus (8). The a form of the enzyme exhibits activity in the absence of an allosteric effector, although the activity is stimulated about 25% by AMP (9). The b form of the enzyme is a dimer (10), and exhibits an absolute requirement for a nucleotide activator (9).

Although in the absence of any effectors phosphorylase a exists as a tetramer and phosphorylase b as a dimer, there exists a significant dimer-tetramer equilibrium for both forms. This equilibrium is dependent upon various factors including ionic strength, pH, temperature, and the presence of substrates and allosteric effectors (11). Huang and Graves have shown that the dimeric form of phosphorylase a is several fold more active than the tetramer (12). Under normal
physiological conditions both phosphorylases a and b exist as dimers. Phosphorylases a from several sources, e.g. lobster (13) and rabbit liver (14), exist only as dimers.

Both the phosphorylation and dephosphorylation reactions are rigidly controlled. Phosphorylase kinases from both skeletal muscle and liver have been shown to be activated via phosphorylation by cAMP-dependent protein kinase (15,16). Physiologically, this phosphorylation is a result of hormonal stimulation. Phosphorylase kinase can also be activated through an autophosphorylation mechanism initiated by the presence of calcium ions (17). This may be an important regulatory mechanism in skeletal muscle where nervous stimulation results in the release of calcium into the cytoplasm from the sarcoplasmic reticulum.

The controls on phosphorylase phosphatase activity have not been resolved to the same extent as those on the kinase. The substrate specificity of the phosphatase is apparently broader than that of phosphorylase kinase (18). Thus, one set of controls appears to be substrate competition for the enzyme. Hers and colleagues have suggested that this is important in the simultaneous inactivation of phosphorylase and activation of glycogen synthase in vivo (19), and Witters and Avruch have also found evidence for this from studies utilizing perfused liver (20).

Phosphatase activity is also regulated by the presence of various heat stable protein inhibitors (21-24). The presence of type I heat stable inhibitor is increased by epinephrine (25), suggesting that
both the phosphorylation and dephosphorylation of phosphorylase may be regulated by cAMP-dependent protein kinase. It has been suggested that the activity of phosphoprotein phosphatase is also regulated by glucocorticoids and insulin (26), thus providing further hormonal control.

The metabolic interconversion of phosphorylase can also be regulated by substrate directed effects. The phosphorylation by phosphorylase kinase is inhibited by glucose-6-P and activated by glycogen. Both of these effects are mediated primarily by binding of the effectors to phosphorylase (27). The action of phosphoprotein phosphatase on phosphorylase is inhibited by AMP, and activated by caffeine, glucose, glycogen, and glucose-6-P. All these effects occur through binding to the phosphorylase molecule (28-31).

Glycogen phosphorylase, which was originally purified from skeletal muscle, was shown to require AMP for activity (32). This was probably the earliest recognized example of allostery for an enzyme, and has been thought to have physiological implications, both because AMP binds at physiologically relevant concentrations and because of the inhibition of AMP binding by intracellular concentrations of ATP and glucose-6-P (11). More recent evidence suggests that increases in phosphorylase activity during exercise is not due to changes in ATP and glucose-6-P since the concentrations of these metabolites are not decreased under conditions where glycogen degradation is stimulated (33).

Although AMP is the most proficient activator various other nucleotides (e.g. GMP and IMP) are able to activate phosphorylase to a significant extent (11). Allosteric activation by AMP results in a
lower $K_m$ for the substrate, glucose-1-P, and an increase in the enzyme's maximal velocity. Binding of the nucleotide is a cooperative process showing both a homotropic interaction for nucleotide binding and a heterotropic cooperativity between nucleotide and substrate. The degree of cooperativity depends on a variety of factors, including buffer conditions, ionic strength, the nucleotide used as activator, and the presence of inhibitors.

The locus of activator binding has been identified by x-ray crystallographic studies to be near the amino terminus of the molecule (34). This site has been found to be indiscriminate in the binding of phosphoryl ligands. The crystalline enzyme readily binds AMP, ADP, ATP, IMP, glucose-1-P, glucose-6-P, UDPG, fructose-1-P, and P$_i$ (35). The importance of the phosphoryl portion of nucleotides in the activation process has been previously delineated from chemical studies (36). The recognition of phosphoryl groups appears to be through the basic pair, Arg 308, Arg 309 (35). Recognition of the aromatic base has also been emphasized, most notably by the ability of 8-(m-(-fluorosulfonyl-benzamido))benzylthioadenine to activate through covalent modification, yet this analog does not contain a phosphoryl group, nor any anionic substituent (37,38). The use of this analog in the sequence determination of phosphorylase has located tyrosine 155 as the site of modification (7). Tyrosine 75 has also been localized in this region (39), and has been implicated in AMP binding since binding is abolished when this residue is modified by ferrate ion. Spectral studies have also implicated an interaction of the nucleotide activator with an aromatic residue (40,41).
Not all phosphorylase isozymes exhibit similar activation by nucleotides. Plant phosphorylases such as those isolated from potato (42) and maize (43) are not stimulated by AMP, nor is the enzyme isolated from yeast (44). Various mammalian isozymes, such as those from corpus luteum (45), kidney (46), leukocyte (47), and liver (14), exhibit only slight activation in the presence of nucleotides. Liver phosphorylase \( b \) can be further activated with the inclusion of salts (48) or in the presence of high concentrations of glucose-1-P (49). Heart isozyme I exhibits a much greater inhibition of AMP binding by the competitive inhibitor, glucose-6-P, than does the enzyme from skeletal muscle (50). These studies suggest a metabolic adaptation of the nucleotide activation process.

Although AMP has long been implicated as the physiologically important activator of skeletal muscle glycogen phosphorylase, recent experimentation by Rahim et al. (33,51), has suggested that IMP may be the actual regulator. Using phosphorylase kinase deficient mice it was found that, under stress conditions where the rate of glycogen degradation was increased, IMP concentration was elevated whereas that of AMP decreased slightly (51). These authors further found that the increased activity of phosphorylase under stress could not be accounted for by decreases in the concentrations of the inhibitors ATP and glucose-6-P, since the concentration of ATP stayed the same while that of glucose-6-P increased.

Early calorimetric studies suggested the presence of a second nucleotide binding site on phosphorylase (52). Recently, the location of
this site on phosphorylase \(a\) has been provided by crystallographic studies and its kinetic characteristics determined (53). Nucleoside binding at this site is competitive with glucose-1-P, which binds 10 Å away. This site is approximately 30 Å distant from the nucleotide activator binding site. Of the compounds capable of inhibiting phosphorylase by binding to this inhibitor site, the more hydrophobic compounds such as adenosine, caffeine, and riboflavin are more effective than the more hydrophilic nucleotides. Soman and Philip have shown that the ability of various aromatic compounds to inhibit phosphorylase is directly related to their hydrophobicity (54). The inhibition of skeletal muscle by purines or methylated oxypurines has been known since 1955 (55), although, until recently, it was thought that their action was indicated through the activator site. The nature of this inhibitor site has been shown by x-ray crystallographic studies to be a hydrophobic slot between the side chains of Phe 285 and Tyr 612 (56).

Binding at the two nucleotide binding sites on phosphorylase stabilizes different conformers of the enzyme. In the terminology of the Monod model (57), the T (tight) conformer is inactive and is stabilized by caffeine or glucose. The R (relaxed) conformer is active and is stabilized by AMP and substrates. The x-ray crystallographic studies on phosphorylase have been undertaken on the inactive conformer, using glucose in the crystallization of phosphorylase \(a\) and IMP for phosphorylase \(b\) (58,59). The conformational changes which take place during the T + R transition have been inferred by inducing the R form
with glucose-1-P in crystals which have been lightly crosslinked with glutaraldehyde (60). In the presence of glucose-1-P, the crystals crack but gradually reanneal. Significant structural changes are seen in residues 1-75, 103-124, 240-287, and 383-397. Although all these residues are in the amino terminal half of the molecule, they link all the major ligand binding sites. In the presence of UDPG (which also stabilizes the R conformer), Phe 285 moves about 5 Å, thus eliminating binding capability at the nucleoside inhibitor site (35). The competitive inhibition seen by nucleoside binding at this site is thus explained by stabilization of an inactive enzyme conformer rather than a steric competition for binding. The same transitional changes result in helix 49-75 moving inward by about 5 Å, thus possibly facilitating AMP stabilization of the R conformer by providing an interaction with Tyr 75 (35).

The relative amounts of the two conformers depend on a variety of conditions other than those mentioned above. These include ionic strength, phosphorylation state, temperature, and the buffer used. The allosteric constant, $L(L = T/R)$, varies from 6100 at 29° C to 0.05 at 4° C (61). Phosphorylase $a$, in the absence of ligands, exhibits a ratio between 3 and 13 at 23° C (62). Thus, the activity in the absence of a nucleotide activator for the $a$ form is probably a result of the stabilization of the active conformer through phosphorylation of the enzyme.

Various authors have postulated additional conformational states in an attempt to explain apparent incongruities in their results with the
Monod model. Kastenschmidt et al. (61) have suggested an additional state, R', to explain their results at low temperatures or low concentrations of AMP. They have suggested that this state is the dimeric form which associates to tetramers at low temperatures or high protein concentrations. Wang and Black (63) have also postulated an additional state in order to explain the effects of glucose and AMP on phosphorylase a activation and dissociation.

Although additional states may be necessary to explain various properties of phosphorylase, an alternative explanation may be that the incongruities arise due to alterations in the binding characteristics under certain conditions. Most of the major ligands for phosphorylase have been found to bind to more than one site on the enzyme (35) and their affinities towards the various binding sites may be dependent upon the experimental conditions, so that one molecule may stabilize different conformers under the proper conditions.

The kinetic mechanism for phosphorylase has been suggested to be a rapid equilibrium random Bi-Bi based on studies utilizing phosphorylases a (64,65) and b (66) from rabbit skeletal muscle, rabbit liver phosphorylase (67), and E. coli maltodextrin phosphorylase (68). The catalytic mechanism thus probably differs from that of sucrose phosphorylase which catalyzes a similar reaction. The kinetics for this enzyme are Ping-Pong and the reaction has been shown to proceed through a covalent glucosyl-enzyme intermediate (69-71). 1,5-Gluconolactone has been found to be a potent inhibitor of glycogen phosphorylase and this
has been interpreted to mean that the transition state of the reaction is an oxonium ion in a half-chair conformation (72,73).

X-ray crystallographic data, in conjunction with the sequence data, have provided information on the residues present in the active site region of phosphorylase (35,56,74). Two problems remain in using this information to provide a detailed description of the catalytic reaction. Refinement of the crystal data to the precision necessary in order to provide exact orientation of the side chains has not been presented, and all the crystal data to date have been on the inactive T conformer; thus, the structure of the catalytic site for the active molecule still remains to be elucidated. The location of various residues in this region however does allow speculation on possible involvements in catalysis. From data for crystals obtained in the presence of glucose, Asn 133 is over the roof of the cavity, while Gly 135 and Leu 136 are closer above the glucose, and Leu 139 is toward the C-6 of glucose (35,56). These residues are part of the "glycine loop" (74) which extends from the nucleoside inhibitor site to the active site. This chain has been found to change orientations during the T → R transition (60). Asn 483 and Val 454 are also near the C-6 of glucose, while Gly 674, Ser 673, and Ala 672 are located in the region of the 0-3 and 0-6. In the area around the C-1 carbon and the potential phosphate binding region are found Asn 283, Asp 284, Arg 568, Tyr 572, and Glu 671 (33,56).

Pyridoxal phosphate has been shown to be an essential component of glycogen phosphorylase since its discovery in the enzyme in 1957 (75).
Recently, Parrish et al. (76) showed that the pyridoxal reconstituted enzyme was active in the presence of noncovalently bound phosphate or various phosphate analogs. These authors further showed that pyrophosphate was bound stoichiometrically, one mole per subunit, to the pyridoxal enzyme and competitively inhibited both the binding of the noncovalent phosphate and of the substrate, glucose-1-P, thus suggesting the presence of the pyridoxal phosphate at the catalytic site. This was confirmed soon afterward from crystallographic studies when the phosphoryl portion of pyridoxal phosphate was found to be within 7 Å of the substrate phosphate (77).

Removal of the pyridoxal phosphate results in an apoenzyme devoid of catalytic activity (78). Reconstitution of the apoenzyme with pyridoxal phosphate derivatives modified in positions 2, 3, and 6 have eliminated their possible involvement in the catalytic reaction (79-82). Reduction of the azomethine bond linking the pyridoxal phosphate to Lys 679 results in an enzyme which still retains essentially all of its activity (83). Thus the role of the coenzyme in phosphorylase must be different from that of the more classical B₆ enzymes. A drastic change in the basicity of the pyridinium nitrogen, through the introduction of fluorine in position 6, still results in an active enzyme species, thus suggesting that it is also not directly involved in catalysis (82). All recent attention has focused on the phosphoryl portion of the coenzyme and its potential involvement in catalysis. Various studies have indicated the necessity of a tetrahedral dianion at the 5 position (76,83-88). All other derivatives result in a total loss of enzymatic
activity. Helmreich and coworkers have shown, through $^{31}$P NMR studies, that a shift of the phosphorus resonance occurs upon activation of the enzyme (86,89). They have interpreted this as a deprotonation of the phosphoryl portion of the coenzyme and have suggested that this group donates a proton during the catalytic reaction. This appears to be inconsistent with the results of Parrish et al. (76) which showed that fluorophosphate was a good activator of the pyridoxal enzyme and yet this analog has a $pK_2$ one and a half pH units below that of the phosphate of pyridoxal phosphate and thus would not be expected to be as readily involved in a protonation-deprotonation step under the same conditions as pyridoxal phosphate.

Recently, Yan et al. (90) have shown that the dianionic form of the coenzyme is also necessary in order for various effectors at the active site and the nucleoside inhibitor site to be able to alter the rate of dephosphorylation of phosphorylase $a$. The state of the coenzyme did not appear to be important in order for effects on the dephosphorylation to be propagated through the nucleotide activator site.

Various authors have estimated that, in skeletal muscle, between 60% and 96% of the total vitamin B$_6$ in the tissue is associated with glycogen phosphorylase (3,91). Phosphorylase constitutes as much as 5% of the soluble proteins in skeletal muscle (92), and muscle is approximately 40% of the total body mass. Therefore it has been suggested that phosphorylase constitutes a major reservoir for vitamin B$_6$ in the body (3). Various studies have substantiated this idea. The specific activity of glycogen phosphorylase has been found to
substantially decrease in B₆ deficient animals (93,94). Black et al. (91) have recently reported that skeletal muscle phosphorylase activity and total B₆ content increased steadily, in nearly a constant ratio, in rats fed a high level of pyridoxine over a six week period. Under the same conditions, there was only a slight increase in two other B₆ containing enzymes, alanine and aspartate aminotransferases. These results suggest that glycogen phosphorylase meets criteria necessary for it being a reservoir for the vitamin. Black et al. (95) have further shown that another condition, starvation, is necessary for the initiation of phosphorylase depletion.
PART I. THE EFFECTS OF ORGANIC SOLVENTS ON THE CATALYTIC AND COENZYME PROPERTIES OF GLYCOGEN PHOSPHORYLASE
INTRODUCTION

The activity of glycogen phosphorylase is controlled by an equilibrium between inactive and active conformations. The equilibrium is shifted towards an active form by substrates, phosphorylation, and the allosteric activator AMP (60-62). Early studies suggested that a second nucleotide binding site exists on phosphorylase (52) and x-ray crystallographic studies have shown that this site is located in a crevice of the protein some 30 Å away from the AMP binding site (53). AMP does not bind effectively to this site but IMP, nucleosides, and purine analogs, e.g. caffeine and theophylline, do. Recent studies show that binding of these compounds competitively inhibits the binding of the substrate, glucose-1-P (53). This nucleoside binding site is distinct from that for glucose-1-P but in close proximity. Binding at this site stabilizes the inactive T conformation of the enzyme.

Various techniques have been utilized in order to unravel the conformational changes involved in the T → R transition, most recently through x-ray crystallographic (60) and $^{31}$P NMR (86,96) studies, in order to gain insight into the catalytic mechanism and its controls.

Organic solvents are found to also effect the allosteric equilibrium of glycogen phosphorylase by shifting the equilibrium towards an active conformation. This report characterizes the properties of various phosphorylase isozymes in the presence of an organic solvent, 1,2-dimethoxyethane, in order to further investigate the role of the allosteric equilibrium in the expression of catalytic activity.
The cofactor of glycogen phosphorylase, pyridoxal phosphate has been determined to be an indispensable component of the enzyme since its removal results in the loss of catalytic activity (78). Both structural (97-99) and catalytic (86,89) roles for the coenzyme have been suggested. The organic solvent 1,2-dimethoxyethane is also found to affect both the resolution and reconstitution roles of the coenzyme with phosphorylase b and apophosphorylase b, respectively. Its use in the resolution of rabbit skeletal muscle phosphorylase a is described in this report. Also described are further studies on the structural role of the coenzyme, especially that of the active site region of the enzyme.
EXPERIMENTAL PROCEDURES

Preparation of Enzymes

Rabbit skeletal muscle glycogen phosphorylase was prepared according to the method of Fischer and Krebs (100) and recrystallized at least three times before use. Residual AMP was removed by treatment with Norite A. Rabbit liver glycogen phosphorylase was prepared as described by Appleman et al. (14). Muscle phosphorylase a and liver phosphorylase a were prepared from the respective phosphorylase b forms by phosphorylation with rabbit skeletal muscle phosphorylase kinase (101). Apophosphorylase was prepared according to Shaltiel et al. (102) and passed over a Sephadex G-25 column which had been equilibrated with 0.2 M imidazole and 0.05 M L-cysteine at pH 6.0. Apophosphorylase a was prepared from phosphorylase a by a modification of the method of Shaltiel et al. (102). The major changes were the inclusion of 10% (v/v) 1,2-dimethoxyethane in the resolution buffer and a higher incubation temperature (22°-23° C). Complete details of the procedure are presented under RESULTS. Phosphorylase a reconstituted with pyridoxal phosphate analogs was prepared by incubating apophosphorylase a with a 5-fold excess of pyridoxal phosphate or a 25-fold excess of pyridoxal phosphate analogs in 40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol at pH 6.8. After incubation for 30 minutes at 30° C, the reconstituted enzyme was precipitated by the addition of an equal volume of a neutralized saturated ammonium sulfate solution. The reconstituted enzyme was dialyzed extensively against 40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol at pH 6.8 and recrystallized before use.
Preparation of $[^{14}\text{C}]$Glycogen

$[^{14}\text{C}]$Glycogen was prepared in a 1 ml volume containing 50 mM $[^{1-14}\text{C}]$glucose-1-P (specific activity: 1 μCi/μmole), 3% glycogen, 25 mM β-glycerophosphate, 19 mM 2-mercaptoethanol, and 50 μg of phosphorylase $\alpha$ at pH 6.8. The reaction mixture was incubated for 19 hours at 30°C and then cooled on ice. Protein was precipitated by the addition of 0.1 ml of 100% (w/v) trichloroacetic acid. The resulting suspension was centrifuged and the pellet was washed with 0.4 ml of cold 10% trichloroacetic acid. The supernatant fractions were combined and the $[^{14}\text{C}]$glycogen was precipitated by the addition of absolute ethanol to a final concentration of 67%. The insoluble $[^{14}\text{C}]$glycogen was centrifuged and the resulting precipitate was redissolved in 1 ml of $\text{H}_2\text{O}$. The ethanol precipitation was repeated three more times and the resulting $[^{14}\text{C}]$glycogen pellet was dissolved in 0.75 ml of $\text{H}_2\text{O}$. The supernatant fraction of the final precipitation step contained less than 0.1% of the radioactivity of the insoluble fraction.

Enzymatic Assays

Phosphorylase activity in the direction of glycogen synthesis was measured either by the liberation of inorganic phosphate from glucose-1-P as described by Illingworth and Cori (103) or by the incorporation of $[^{U-14}\text{C}]$glucose into glycogen from $[^{U-14}\text{C}]$glucose-1-P utilizing the filter paper assay of Thomas et al. (104). When the radioactive assay was used, absolute ethanol was substituted for acetone in the final wash. Unless otherwise specified, the assay system
contained 1% glycogen, 16 mM glucose-1-P, 20 mM β-glycerophosphate, and 15 mM 2-mercaptoethanol at pH 6.8 and 30°C. 1 mM AMP was also included for the measurement of phosphorylase b activity. Activity is expressed as IU/mg, where 1 IU is 1 μmole of inorganic phosphate liberated or 1 μmole of [U-14C]glucose incorporated, per minute.

In the direction of glycogen degradation utilizing [14C]glycogen, the reaction conditions were as described in the figure legend in a total volume of 0.04 ml. The reaction was terminated by the addition of 0.2 ml of cold 70% (v/v) ethanol. 0.02 Ml of cold nonradioactive 10% glycogen was added and the suspension centrifuged. 0.1 Ml of the resulting supernatant was removed for the determination of radioactive glucose-1-P liberated.

31P NMR Measurements

31P NMR measurements were performed in collaboration with Dr. Paul Schmidt at the Oklahoma Medical Research Foundation, Oklahoma City, OK. 31P Fourier-transformed NMR spectra were obtained at 109.4 MHz utilizing a 63.4 kilogauss Bruker superconducting magnet, a homebuilt detection transmitter/receiver, and a Nicolet 1180 computer and software. D2O was used for field/frequency lock. The sample tube was replaced with one containing phosphoric acid for chemical shift referencing. The appropriate experimental conditions are included in the figure legends.
Other Methods

The extent of resolution of phosphorylase a or b was tested by preincubating the apoenzyme, at 5 mg/ml, in the presence and absence of a 5-fold excess of pyridoxal phosphate at 30°C for 15 minutes. The resulting phosphorylase activity was measured by the method of Illingworth and Cori (103). The apoenzyme used in these experiments was better than 99% resolved and could be reconstituted to a specific activity comparable to the enzyme from which it was prepared.

The extent of reconstitution with pyridoxal phosphate analogs was similarly determined by preincubating in the presence or absence of a 5-fold excess of pyridoxal phosphate. Reconstitution for all enzyme forms utilized in these experiments was found to be 100%.

Phosphorylase concentrations were measured spectrophotometrically, using an extinction coefficient, $\epsilon_{1%}^{10 \text{ mm}}$ at 280 nm of 13.2 (105). The monomeric molecular weight of phosphorylase was taken to be 97,400 daltons (7).

Sedimentation velocity experiments were performed with a Beckman Spinco Model E ultracentrifuge. The movement of protein boundaries was followed by Schlieren optics and the Schlieren patterns analyzed with a microcomparator. $S_{20,w}$ values were calculated using a linear regression program of an HP-25 calculator. Viscosity measurements were performed using a Hewlett Packard Auto Viscometer Model #5901B. pK values were determined by the protocol of Albery and Massey (106).
Values for the $K_a$ for substrates, the $K_a$ for activators, and the Hill coefficients ($n_H$) for these effectors were determined either graphically or, more commonly, through the use of a linear regression program of an HP-25 calculator utilizing the equation for a Hill plot (107). The maximal velocities utilized in these plots were extrapolated graphically from plots of $(\text{initial velocity})^{-1}$ versus $(\text{effector concentration})^{-1}$. Hill plots were used because of the significant curvature of the double reciprocal plots.

Materials

AMP, glucose-1-P, L-cysteine, pyridoxal, pyridoxal phosphate, caffeine, and β-glycerophosphate were obtained from Sigma Chemical Company and used without further purification. Grade 1 imidazole from Sigma was used after three times recrystallization from reagent grade acetone. Shellfish glycogen from Sigma was further purified according to the method of Anderson and Graves (38). 2-Mercaptoethanol was obtained from Aldrich and $[\text{U-}^{14}\text{C}]$ glucose-1-P from Amersham. 1,2-Dimethoxyethane was obtained from Eastman Kodak. Phosphorous acid from Fisher Scientific Company was dried over phosphorus pentoxide, neutralized with sodium hydroxide, and used as sodium phosphite.
RESULTS

Inhibition of the AMP-induced activation of rabbit skeletal muscle phosphorylase b by various hydrophobic compounds has been described by Anderson and Graves (38). Soman and Philip (54) have found a direct correlation between the ability of various aromatic compounds to inhibit phosphorylase and their hydrophobicity. Recently, the binding of various hydrophobic compounds such as caffeine and adenosine to a nucleoside inhibitor site on phosphorylase a has been described (53). The apparent hydrophobic nature of these binding sites has prompted the use of organic solvents to further examine the nucleotide control of phosphorylase.

A representative list of the organic solvents examined is presented in Table I. It was found that most of the solvents tested, which were miscible with water, resulted in an activation of the AMP-induced phosphorylase b activity, while the cyclic compounds tested, tetrahydrofuran and dioxane, inhibited the enzyme. The most pronounced activation was found to be with methyl ethers of glycols. There appeared to be no direct correlation between the extent of activation of phosphorylase and the dielectric constant of the medium (e.g. glycerol, which has a lower dielectric constant than water, inhibits while dimethyl sulfoxide, which at the concentration used does not significantly alter the dielectric constant, activates). However, most of the solvents which activate have lower dielectric constants than water. The inhibition seen by some of the compounds may be due to binding at one or more of
Table I. Activation of phosphorylase b by organic solvents

<table>
<thead>
<tr>
<th>Solvent (10% v/v)</th>
<th>Activity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.00</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.22</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.10</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.87</td>
</tr>
<tr>
<td>Ethyleneglycol</td>
<td>0.93</td>
</tr>
<tr>
<td>Propyleneglycol</td>
<td>1.12</td>
</tr>
<tr>
<td>2-methoxyethanol</td>
<td>1.28</td>
</tr>
<tr>
<td>1,2-dimethoxyethane</td>
<td>1.34</td>
</tr>
<tr>
<td>1,1-dimethoxyethane</td>
<td>1.30</td>
</tr>
<tr>
<td>Dimethoxymethane</td>
<td>1.24</td>
</tr>
<tr>
<td>Trimethyloorthoformate</td>
<td>1.24</td>
</tr>
<tr>
<td>2-butoxyethanol</td>
<td>0.00</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.24</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>1.24</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>0.16</td>
</tr>
<tr>
<td>Dioxane</td>
<td>0.44</td>
</tr>
</tbody>
</table>

\(13\mu g/ml\) of phosphorylase b was assayed in the presence of 16 mM glucose-1-P, 1% glycogen, 1 mM AMP, 10 mM \(\beta\)-glycerophosphate, 7.5 mM 2-mercaptoethanol and 10% (v/v) of the indicated solvents at pH 6.8 and 30°C. The measurements were performed in duplicate and the data are presented as the average. The activity ratios for the two determinations varied less than 0.04.
the many sugar binding sites on phosphorylase since these compounds were used at relatively high concentrations and the inhibitory compounds contained hydroxyl groups or were cyclic compounds with ring oxygens.

Further studies were initiated with the most potent activator, 1,2-dimethoxyethane, because preliminary results indicated that it was also effective in reducing inhibition by hydrophobic compounds. The effect of 10% (v/v) 1,2-dimethoxyethane on various kinetic parameters is summarized in Table II. Inclusion of the solvent increases the maximal velocity of phosphorylase \( b \) obtained in the presence of 1 mM AMP by 19%. The \( K_m \)'s for the substrates, glucose-1-P and glycogen, were decreased slightly with the inclusion of 1,2-dimethoxyethane. The presence of this organic solvent also affects the cooperative interactions. The slight homotropic cooperativity usually seen for glucose-1-P binding in the presence of saturating AMP is eliminated when the solvent is included.

The most dramatic effect of 1,2-dimethoxyethane has been found to be on the nucleotide binding parameters of phosphorylase. Recently, Madsen and coworkers have shown that, in addition to a nucleotide activator site, there is a nucleoside inhibitor site where binding is competitive with the substrate, glucose-1-P (53,60). Using phosphorylase \( a \), these authors have suggested an interaction between nucleoside binding sites from a plot of \( \log \left( \frac{v_i}{v_0} - v_1 \right) \) versus the log of caffeine concentration, where \( v_i \) is the velocity at constant substrate concentration in the presence of caffeine and \( v_0 \) is the velocity in the absence of the inhibitor. This plot yields a slope of -1.4. Figure 1A
Table II. The effect of 1,2-dimethoxyethane on the kinetic parameters of phosphorylase b

<table>
<thead>
<tr>
<th>Kinetic Parameter</th>
<th>In the absence of 1,2-dimethoxyethane</th>
<th>In the presence of 10% 1,2-dimethoxyethane (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ glucose-1-P&lt;sup&gt;a&lt;/sup&gt; (mM)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>$n_H$ glucose-1-P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (IU/mg)</td>
<td>62</td>
<td>74</td>
</tr>
<tr>
<td>$K_m$ glycogen&lt;sup&gt;c&lt;/sup&gt; (%)</td>
<td>0.019</td>
<td>0.013</td>
</tr>
<tr>
<td>$K_a$ AMP&lt;sup&gt;d&lt;/sup&gt; (μM)</td>
<td>79</td>
<td>8</td>
</tr>
<tr>
<td>$n_H$ AMP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>$K_i$ caffeine&lt;sup&gt;e&lt;/sup&gt; (mM)</td>
<td>0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>$n_H$ glucose-1-P at $K_i$&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured in the presence of 1 mM AMP, 1% glycogen, 20 mM β-glycerophosphate, 10 mM 2-mercaptoethanol, 10 μg/ml enzyme and glucose-1-P concentrations between 1 and 15 mM at pH 6.8 and 30°C.

<sup>b</sup> Extrapolated from plots of (velocity)$^{-1}$ vs. (glucose-1-P)$^{-1}$ from a.

<sup>c</sup> Measured in the presence of 1 mM AMP, 16 mM glucose-1-P, 15 mM β-glycerophosphate, 11 mM 2-mercaptoethanol, 13 μg/ml enzyme and glycogen concentrations between 0.0025% and 0.0625% at pH 6.8 and 30°C.

<sup>d</sup> Measured in the presence of 1% glycogen, 16 mM glucose-1-P, 15 mM β-glycerophosphate, 11 mM 2-mercaptoethanol, 13 μg/ml enzyme and AMP concentrations between 10 and 100 μM in the absence of 1,2-dimethoxyethane or between 0.25 and 12.5 μM in the presence of 1,2-dimethoxyethane at pH 6.8 and 30°C.

<sup>e</sup> Measured in the presence of 1% glycogen, 1 mM AMP, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 9 μg/ml enzyme, glucose-1-P concentrations between 2 and 10 mM, and caffeine concentrations between 0 and 0.5 mM in the absence of 1,2-dimethoxyethane and between 0 and 7 mM in the presence of 1,2-dimethoxyethane at pH 6.8 and 30°C.
Figure 1. Caffeine inhibition of glycogen phosphorylase.

Enzymatic activity was measured as described by Illingworth and Cori (103) in the presence of 1% glycogen, 1 mM AMP, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 4 μg/ml of enzyme, glucose-1-P concentrations between 2 and 10 mM, and caffeine concentrations of 0 (○), 0.1 mM (●), 0.2 mM (□), 0.3 mM (▲), and 0.5 mM (△), at pH 6.8 and 30° C.

A. The experimental data are presented in the form of a double reciprocal plot. $V_{\text{m}}$ is determined from the y-intercept.

A. (inset) The same data are presented in the form of a Hill plot for the determination of $K_{\text{app}}$.

B. Determination of the $K_i$ for caffeine inhibition.
shows the effect of caffeine on the kinetics of phosphorylase b with respect to glucose-1-P in the absence of added solvent. A large increase in cooperativity is seen with increasing caffeine. This could be due to either cooperative inhibitor binding or increased cooperativity of substrate binding in the presence of the inhibitor. A Hill plot of the same data shows that the Hill coefficient ($n_H$) for glucose-1-P binding increases from 1.1 in the absence of caffeine to 1.7 at 0.5 mM caffeine. A replot of $K_{app}/V_m$ versus the concentration of caffeine (Fig. 1B) is linear. A $K_i$ of 0.3 mM for caffeine is determined from the x-intercept.

Binding at the nucleoside inhibitor site facilitates the R $\rightarrow$ T transition by stabilizing the T conformer (35,53). The lack of apparent cooperativity seen for binding to this conformer was further investigated using glucose which binds to the active site region of phosphorylase and also stabilizes the inactive T conformer (35,62). The data obtained for this inhibitor are similar to those obtained for caffeine (Fig. 2). A double reciprocal plot shows increasing cooperativity at increasing glucose concentrations. This is again attributable to substrate binding since the Hill coefficient for glucose-1-P increases from 1.1 in the absence of the inhibitor to 1.9 at 15 mM glucose. Again a replot of $K_{app}/V_m$ versus inhibitor concentration is linear and a $K_i$ of 6 mM is determined. The Hill coefficient for glucose-1-P at the $K_i$ value for both inhibitors is the same, 1.4.
Figure 2. Glucose inhibition of glycogen phosphorylase b.

Enzymatic activity was measured as described by Illingworth and Cori (103) in the presence of 1% glycogen, 1 mM AMP, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 4 µg/ml of enzyme, glucose-1-P concentrations between 2 and 10 mM, and glucose concentrations of 0 (○), 3 mM (●), 6 mM (□), 9 mM (■), 12 mM (△), and 15 mM (▲) at pH 6.8 and 30°C.

A. The experimental data are presented in the form of a double reciprocal plot for the determination of $V_m$.

A. (inset) The same data are presented in the form of a Hill plot for the determination of $K_{app}$.

B. Determination of the $K_i$ for glucose inhibition.
The effect of the inclusion of 10% (v/v) 1,2-dimethoxyethane on the binding parameters at the two nucleotide sites is summarized in Table II. The solvent exerts an opposite effect on the two sites. In the presence of the solvent, the $K_a$ for AMP is lowered 10-fold. The $K_a$ for phosphorylase $b$ in the presence of 1,2-dimethoxyethane is similar to that reported for phosphorylase $a$ (62). The solvent also eliminates the apparent cooperativity seen for AMP binding. The presence of 1,2-dimethoxyethane causes a 6-fold increase in the $K_i$ for caffeine binding at the nucleoside inhibitor site. The solvent again exhibits an effect on the cooperative interactions of phosphorylase. The apparent cooperativity for glucose-1-P binding in the presence of the inhibitor is reduced. The Hill coefficient at the $K_i$ value decreases from 1.4 in the absence of organic solvent to 1.1 in the presence of 10% (v/v) 1,2-dimethoxyethane. The data presented here are consistent with the organic solvent being able to alter the allosteric equilibrium since AMP binds with greater affinity to the R conformer (62) while caffeine appears to bind exclusively to the T conformer (35,53,96).

In order to further investigate the effect of 1,2-dimethoxyethane on the allosteric equilibrium, its effect on the different forms of phosphorylase was investigated since phosphorylase $a$ has a lower allosteric constant, i.e. a larger relative amount of the active R conformer (61,62). As is shown in Figure 3, the stimulation of phosphorylase activity by the organic solvent is highly dependent upon the enzyme's activity state. Phosphorylase $b$ in the presence of saturating AMP and phosphorylase $a$ in the absence of a nucleotide
Figure 3. 1,2-Dimethoxyethane activation of glycogen phosphorylase.

Enzymatic activity was measured as described under EXPERIMENTAL PROCEDURES in the presence of the indicated amounts of 1,2-dimethoxyethane. The assay mixture also contained:

A. 13 µg/ml of rabbit skeletal muscle phosphorylase b in the presence of no nucleotide (□), 1 mM AMP (○), or 1 mM IMP (△);

B. 13 µg/ml of rabbit skeletal muscle phosphorylase a in the presence of no nucleotide (□) or 1 mM AMP (○);

C. 15 µg/ml of rabbit liver phosphorylase b in the presence of no nucleotide (□), 5 mM AMP (○), or 5 mM IMP (△).
The graphs show the activity of enzymes as a function of the percentage of (v/v) 1,2-Dimethoxyethane. Each graph plots IU/mg against the percentage of 1,2-Dimethoxyethane. The graphs are labeled A, B, and C.
activator exhibit comparable maximal stimulations by the solvent of 35%.
Phosphorylase \(a\) in the presence of 1 mM AMP has a maximal stimulation of
only 9% in the presence of 1,2-dimethoxyethane. The effect of the
solvent on the maximal velocities and the \(K_m\)'s for glucose-1-P for the
various enzyme forms is presented in Table III. The differences seen
in the values for phosphorylase \(b\) between Tables II and III are due to
the different enzyme preparations used as well as the different
ranges of glucose-1-P concentrations utilized. At 10% (v/v)
1,2-dimethoxyethane phosphorylase \(b\) exhibits the largest effect of
the solvent on both substrate binding and the maximal velocity.
Phosphorylase \(a\) in the absence of AMP has a 13% stimulation of the
maximal velocity as compared to 14% for phosphorylase \(b\) in the presence
of 1 mM AMP. However, phosphorylase \(a\) exhibits a smaller effect on
substrate binding. Phosphorylase \(a\) in the presence of AMP shows a
decrease in the maximal velocity in the presence of 10% (v/v)
1,2-dimethoxyethane. This enzyme form also shows no effect of the
solvent on glucose-1-P binding.

The results on the parameters for nucleotide binding obtained with
1,2-dimethoxyethane suggested that this organic solvent could serve
as a useful probe to examine the different nature of these sites and
possible controls of phosphorylase activity by differential binding at
either an activator or inhibitor site on the same molecule. The
different effects of 1,2-dimethoxyethane on the two nucleotide binding
sites can be seen by using a single nucleotide, IMP (Fig. 3A).
The IMP-induced activity increases from 1.9 IU/mg in the absence of
Table III. The effect of 1,2-dimethoxyethane on kinetic constants for the different forms of rabbit muscle glycogen phosphorylase

<table>
<thead>
<tr>
<th>% 1,2-dimethoxyethane (v/v)</th>
<th>Kₘ glucose-1-Pᵃ (mM)</th>
<th>Vₘₐₓᵇ (IU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Phosphorylase b (-AMP)</td>
<td>-50</td>
<td>6.4</td>
</tr>
<tr>
<td>Phosphorylase b (+AMP)</td>
<td>5.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Phosphorylase a (-AMP)</td>
<td>3.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Phosphorylase a (+AMP)</td>
<td>2.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

ᵃMeasured in the presence of 1% glycogen, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 4 µg/ml of enzyme, and glucose-1-P concentrations between 2 and 10 mM at pH 6.8 and 30°C whether in the presence or absence of 1 mM AMP as indicated for phosphorylase b (+AMP), phosphorylase a (-AMP), and phosphorylase a (+AMP). For phosphorylase b (-AMP), the Kₘ was measured in the presence of 1% glycogen, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 67 µg/ml of enzyme, and glucose-1-P concentrations between 10 and 160 mM at pH 6.8 and 30°C in the absence of 1,2-dimethoxyethane. In the presence of 1,2-dimethoxyethane the same enzyme form was assayed in the presence of 1% glycogen, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 20 µg/ml of enzyme, and glucose-1-P concentrations between 1 and 15 mM at pH 6.8 and 30°C.

ᵇExtrapolated from plots of (velocity)^-1 versus (glucose-1-P)^-1 from a.
organic solvent to 46.1 IU/mg at 12.5% (v/v) 1,2-dimethoxyethane. IMP has about equal affinities for the nucleotide binding sites, 
\[ K_i = K_a = 2 \text{ mM} \] in the absence of added solvent (108), so that decreased binding at the inhibitor site and increased binding at the activator site is seen as the allosteric equilibrium is shifted with increasing concentrations of 1,2-dimethoxyethane. At the highest concentrations of 1,2-dimethoxyethane, IMP activation of phosphorylase \( b \) is only slightly below that of AMP. These results are consistent with those of Black and Wang (109) which showed that, in the presence of high glucose-1-P, IMP activation was comparable to that of AMP.

Figure 3C depicts the activity of rabbit liver phosphorylase \( b \) in the presence of no nucleotide, 5 mM IMP, and 5 mM AMP with increasing concentrations of 1,2-dimethoxyethane. The activation patterns for the two nucleotides exhibit identical profiles. The profiles exhibit similar patterns at 1 mM nucleotide. The maximum activation by liver phosphorylase \( b \) by 1,2-dimethoxyethane in the presence of AMP or IMP is similar to the activity of liver phosphorylase \( a \) in the absence of nucleotide. Liver phosphorylase \( a \) activity is not stimulated by 10% (v/v) 1,2-dimethoxyethane.

Phosphorylase \( b \) activity is no longer as dependent on a nucleotide activator when the activity is measured in the presence of an organic solvent (Table III, Figs. 3A and 3C). For phosphorylase \( b \) activity measured in the absence of activators (AMP or 1,2-dimethoxyethane), the recrystallized enzyme was Norite A treated five times. No change in the \( A_{260}/A_{280} \) ratio was observed for the last two treatments. The
enzyme used in the experiment in the absence of any activators had a specific activity of 40 IU/mg in the presence of AMP. The extent to which activation can be achieved in the absence of AMP using 1,2-dimethoxyethane is shown in Figure 4. AMP activation has been reported to be characterized primarily by a decrease in the $K_m$ for substrates (110). Activation by 1,2-dimethoxyethane in the absence of nucleotides, is characterized by a change in the maximal velocity between 10% and 20% (v/v) 1,2-dimethoxyethane, with only a slight change in the affinity for the substrate. Dreyfus et al. (111) have also recently investigated the action of organic solvents on phosphorylase b. These authors reported that at lower concentrations of organic solvent there was an increase in substrate binding, the $K_m$ for glucose-1-P being lowered from 11 to 2.3 mM as the t-butanol concentration was raised from 5 to 10%. The $K_m$ for glucose-1-P would be expected to be changing at lower concentrations of 1,2-dimethoxyethane also since the substrate affinity of phosphorylase b in the absence of activators is much higher than that seen in the presence of 10% (v/v) 1,2-dimethoxyethane. The maximal velocity obtained at the highest solvent concentration used is 46 IU/mg or 74% of that obtained in the presence of saturating AMP. No activity measurements were performed at concentrations of 1,2-dimethoxyethane higher than 25% because at these concentrations glycogen was precipitated. Another property of nucleotide activators which was also found to be characteristic of organic solvent activation was the reduction of homotropic cooperativity for the substrate. The Hill coefficient decreases from 1.6 to 1.0 as the concentration of
Figure 4. 1,2-Dimethoxyethane activation of phosphorylase b in the absence of nucleotides.

Activity was measured according to Illingworth and Cori (103) in the presence of 1% glycogen, 20 μg/ml of phosphorylase b, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 10% (○), 15% (□), or 20% (△) (v/v) 1,2-dimethoxyethane, and the indicated amounts of glucose-1-P at pH 6.8 and 30° C.
1,2-dimethoxyethane increases from 10% to 20%. A plot of the reciprocal of the maximal velocity versus the reciprocal of the 1,2-dimethoxyethane concentration exhibits significant curvature similar to the cooperativity seen for AMP activation. Since, as mentioned above, the higher concentrations of 1,2-dimethoxyethane could not be used, neither the $K_a$ for solvent activation nor the maximal velocity obtainable for saturating solvent effect could be determined.

Dreyfus et al. (111) have reported that t-butanol affects the aggregation state of phosphorylase b in the presence of AMP. The effect of organic solvents on the dissociation-association properties of phosphorylase has been further examined in order to investigate whether the effects of these solvents on the activity properties could be explained by an alteration of this equilibrium. Phosphorylase exists as either a dimer or tetramer depending on the state of phosphorylation and the presence of various effectors of the enzyme. Under the buffer conditions utilized in most of these experiments, phosphorylase b sediments as a dimer and phosphorylase a as a tetramer both in the presence and absence of 1,2-dimethoxyethane (Figure 5A and 5B). In the presence of AMP, phosphorylase b sediments primarily as a tetramer at 10° C although a significant amount of dimer is still present (Figure 5C). With the inclusion of the solvent this profile is shifted totally towards the tetramer in agreement with the results of Dreyfus et al. (111). The slight difference in the rates of sedimentation for the dimer and tetramer in the presence of the solvent can be explained by a change in the viscosity of the solution due to
Figure 5. The effect of 1,2-dimethoxyethane on the aggregation properties of glycogen phosphorylase.

Sedimentation velocity experiments were performed as described under EXPERIMENTAL PROCEDURES at 52000 rpm.

A. The sample contained 4.0 mg/ml of phosphorylase \( b \) in 36 mM \( \beta \)-glycerophosphate, 27 mM 2-mercaptoethanol at pH 6.8 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 10° C. \( S_{20,w} \) values are 8.3s (top) and 8.4s (bottom).

B. The sample contained 3.9 mg/ml of phosphorylase \( a \) in 36 mM \( \beta \)-glycerophosphate, 27 mM 2-mercaptoethanol at pH 6.8 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 21° C. \( S_{20,w} \) values are 12.2s (top) and 12.8s (bottom).

C. The sample contained 3.7 mg/ml of phosphorylase \( b \) in 1 mM AMP, 36 mM \( \beta \)-glycerophosphate, 27 mM 2-mercaptoethanol at pH 6.8 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 10° C. \( S_{20,w} \) values are 12.6s (top) and 13.1s (bottom).
the presence of the solvent. Inclusion of 10% (v/v) 1,2-dimethoxyethane results in a 36.8% increase in the viscosity of the solution at 10° C.

Using phosphorylase \( a \), the effect of 10% (v/v) 1,2-dimethoxyethane on the specific activities of both the dimeric and tetrameric forms of this enzyme was examined as well as the effect of the solvent on the dimer-tetramer equilibrium under the normal assay conditions. Huang and Graves (12) have shown that both the dissociation constant for this equilibrium and the specific activities for the two forms can be determined from activity measurements. Figure 6A shows the specific activity of phosphorylase \( a \) as a function of protein concentration. As can be seen, the curve differs slightly when 1,2-dimethoxyethane is included in the assay. Figures 6B and 6C show extrapolations to obtain the specific activities of the dimer and tetramer, respectively. The plot for Figure 6B is from the equation (12):

\[
\frac{1}{\bar{\phi} - \phi_T} = \frac{1}{K_d(\phi_D - \phi_T)^2} \times \frac{E_0(\bar{\phi} - \phi_T)}{\phi_D - \phi_T}
\]

where \( \bar{\phi} \) = measured specific activity, \( \phi_T = \lim_{E_0 \to 0} \phi \), \( E_0 \) = total enzyme concentration, and \( \phi_D \) = specific activity of the dimer. Figure 6B shows that there is a slight difference for the specific activity of the dimer due to the presence of the solvent. This increase of the specific activity of the phosphorylase \( a \) dimer amounts to about 6% under these conditions. The curvature from the squared term of the above equation is most evident in the absence of added solvent, thus providing difficulty in obtaining accurate values for \( \phi_D \) and \( K_d \). The differences due to the
Figure 6. The effect of 1,2-dimethoxyethane on the specific activity of phosphorylase.

A. Activity was assayed as described under EXPERIMENTAL PROCEDURES in the presence of 1% glycogen, 18 mM \(\beta\)-glycerophosphate, 0.9 mM EDTA, 0.5% KCl and the indicated amounts of phosphorylase \(a\) at pH 6.8 and 24° C in the absence (\(\circ\)) or presence (\(\bullet\)) of 10% (v/v) 1,2-dimethoxyethane. For enzyme concentrations below 0.5 mg/ml, 0.5 mg/ml bovine serum albumin was also included in the assay.

B. The data from A are plotted as (\(\phi - \phi_T\))\(^{-1}\) versus \([E_0(\phi - \phi_T)]\) in order to obtain \(\phi_D\) and \(K_D\).

C. The data from A are plotted as \(\bar{\phi}\) versus \(E_0\)\(^{-1}\) in order to obtain \(\phi_T\).
presence of 1,2-dimethoxyethane thus may be actually less than estimated here. The results suggest that there is little change in the
dissociation constant for the dimer-tetramer equilibrium, the $K_d$ being
increased from $7.8 \times 10^{-3}$ to $9.1 \times 10^{-3}$ g/l due to the inclusion of
10\% (v/v) 1,2-dimethoxyethane. The tetramer, which under the conditions
used here has about 10\% of the activity of the dimer in the absence of
1,2-dimethoxyethane, exhibits a much larger stimulation due to the
presence of the solvent. As can be determined from Figure 6C, this
stimulation is approximately 60\%. Since 1,2-dimethoxyethane affects
the specific activities of both the dimeric and tetrameric forms of
phosphorylase $a$ while doing little to alter the dissociation constant,
it seems unlikely that the changes seem in the activity properties
of the enzyme could be explained by large changes in the enzyme's
aggregation state.

Further evidence that the organic solvent activates by shifting the
allostERIC equilibrium is presented in Figure 7. The model of Monod
et al. (57) predicts that for allosteric inhibitors which bind to the
R form, activation should be seen at low concentrations of these
inhibitors. ATP, an allosteric inhibitor of phosphorylase (11),
inhibits the AMP activation of phosphorylase $b$, probably via direct
competition for the AMP site. In the presence of 10\% (v/v)
1,2-dimethoxyethane, ATP serves to activate the enzyme. Both experiments
were performed at the $K_a$ for AMP under the respective conditions
(Table II). The apparent lack of inhibition for the 1,2-dimethoxyethane
Figure 7. The effect of 1,2-dimethoxyethane on ATP inhibition of phosphorylase b activity.

Enzymatic activity was measured as described by Illingworth and Cori (103) in the presence of 1% glycogen, 16 mM glucose-1-P, 15 mM β-glycerophosphate, 11 mM 2-mercaptoethanol at pH 6.8 and 30° C in the presence of 80 µM AMP (○) or 8 µM AMP and 10% (v/v) 1,2-dimethoxyethane (●) at the indicated concentrations of ATP.
induced activity further suggests the presence of an "a-like" molecule since phosphorylase a is not effectively inhibited by ATP (11).

The effect of 1,2-dimethoxyethane on the pH profile of glycogen phosphorylase b was undertaken in order to further investigate the mechanism by which the enzyme is allosterically activated by the solvent. As can be determined from Figure 8A there is little effect of the solvent on the alkaline limb of the pH profile. Kasvinsky and Meyer (112) have suggested that this ionization is that of a histidyl group based on the observed pK_a and heat of ionization. This assumption is also consistent with the fact that the pK_2 is not perturbed by a possible environmental change induced by the presence of the organic solvent.

The acidic limb of the pH profile exhibits a pronounced dependency on the environment. The pK_1 is shifted due either to the presence of the solvent itself or to a conformational change induced by the solvent. The change in the pH profile for the AMP-activated phosphorylase b in the presence of 1,2-dimethoxyethane cannot be explained by a change in the pK_a of the substrate due to the presence of the solvent, since in the absence of AMP, the 1,2-dimethoxyethane-induced profile appears to have a similar pK_1 to nonactivated phosphorylase b (Fig. 8B). The differences in the profiles are also not due to a perturbation of the buffer pH by the solvent, since as is seen in Figure 8C the same shift in the acidic limb is seen in Tris-Imidazole buffer. As was mentioned above, the ionization of imidazole should not perturbed by the presence of organic solvents.

Apparently, the changes seen here are due a perturbation of the
Figure 8. The effect of pH on the stimulation of phosphorylase b activity by 1,2-dimethoxyethane.

Enzymatic activity was measured according to Illingworth and Cori (103) in the presence of 1% glycogen and 50 mM glucose-1-P at the indicated pH values and at 30° C. The presence of other substances were as indicated. Both substrates and buffers were adjusted to the indicated pH values.

A. 16 µg/ml of phosphorylase b was assayed as described above in the presence of 50 mM Tris-maleate, 10 mM AMP for the pH values of 5.6 to 6.2, 3 mM AMP for the pH values of 6.4 to 8.0, in the absence (■) or presence (●) of 10% (v/v) 1,2-dimethoxyethane.

B. 49 µg/ml of phosphorylase b in the presence of 10% (v/v) 1,2-dimethoxyethane (▲), or 3 mg/ml phosphorylase b in the absence of 1,2-dimethoxyethane (○), was assayed as described above in the presence of 50 mM Tris-maleate.

C. Assay conditions were as described in A with the substitution of 50 mM Tris-50mM imidazole for the Tris-maleate buffer.
ionization of some residue on the enzyme which is important for the catalytic reaction rather than the binding of substrates of activators, since the concentrations of all effectors used here should be saturating according to the results reported by Kalvinsky and Meyer (112) and Hollo et al. (113).

As has been alluded to earlier, the presence of the organic solvent affects the binding of effectors to the active site. The binding of the substrates, glucose-1-P and glycogen, are slightly improved in the presence of AMP, while the binding of the inhibitor, glucose, is significantly reduced, the $K_d$ being increased from 6 mM to 21 mM with the inclusion of 10% (v/v) 1,2-dimethoxyethane. Since as was mentioned earlier, glucose binds to and stabilizes the inactive T conformer (35,62), the decreased affinity under conditions where the R conformer is further stabilized is not surprising.

Figure 9A shows the effect of 1,2-dimethoxyethane on the inhibition by 1,5-gluconolactone. This compound has been suggested to be a transition analog for the phosphorylase reaction (72,73). The increased inhibition by this compound in the presence of the solvent cannot be explained by a change in the rate of its hydrolysis since, as is seen in Figure 9B, the inclusion of 1,2-dimethoxyethane actually accelerates this process. The increased inhibition by this compound is suggestive of a more stabilized transition state under conditions where the maximal velocity is also increased.

Bunton and coworkers (114,115) have provided evidence the acid hydrolysis of glucose-1-P proceeds through an $S_N^1$ mechanism via an
Figure 9. The effect of 1,2-dimethoxyethane on the inhibition of phosphorylase b activity by 1,5-gluconolactone.

A. Assay conditions were as described under EXPERIMENTAL PROCEDURES in the presence of 13 µg/ml phosphorylase b, no (○) or 10% (●) (v/v) 1,2-dimethoxyethane and the indicated amounts of 1,5-gluconolactone.

B. The effect of 1,2-dimethoxyethane on the hydrolysis of 1,5-gluconolactone. 3 mM gluconolactone was incubated in the presence of 40 mM β-glycerophosphate and no (○) or 10% (●) (v/v) 1,2-dimethoxyethane at pH 6.8. At the indicated times, aliquots were removed and hydrolysis determined as in Tu et al. (72).
[1,5-gluconolactone] (mM)

% Inhibition

A

[1,5-gluconolactone] (mM)

A₅₄₀

B

Time (min.)
oxonium intermediate through the pH range 2 to 4, thus providing an analogy with the phosphorylase reaction. Below this range, there is an increase in rate which is not correlatable with the increase in hydrogen ion concentration (115). Tu et al. (72) have shown an isotope effect, $k_H/k_D$, of 1.13 for the hydrolysis of glucose-1-P in 1.1 N HCl. This is also suggestive of an $S_N^1$ mechanism. Figure 10 depicts the rate of hydrolysis of glucose-1-P in 1.2 N HCl as a function of chloride concentration. The rate exhibits a near linear dependency on the salt concentration as well as a further stimulation by the organic solvent, 1,2-dimethoxyethane. Bunton and coworkers have previously reported a salt effect on the hydrolysis of glucose-1-P (115) and from their work or electrolyte effects an acid-catalyzed ester hydrolyses (116) have suggested that the carbonium intermediate is stabilized by an increase in water activity as well as by specific salt interactions. Although the effect of salts has been shown previously, the data in Figure 10 are presented because the conditions used are similar to those showing the kinetic isotope effect (72) and also to present the effect of a lower dielectric on this process.

Shaltiel et al. (102) found that pyridoxal phosphate could be resolved from phosphorylase $b$ under mild conditions using a deforming buffer, imidazolum citrate, and a stereospecific aldehyde trapping agent, L-cysteine. Incubation of the enzyme with imidazolum citrate resulted in the formation of monomers and a rapid rate of exchange with exogenous $[^{32}\text{P}]$pyridoxal phosphate. These authors further reported that phosphorylase $a$ could not be resolved under these conditions. Other,
Figure 10. The effect of ionic strength and dielectric constant on the acid hydrolysis of glucose-1-P.

200 mM glucose-1-P was incubated in 1.2 N HCl with the concentration of chloride anion adjusted to the indicated levels by addition of KCl. The reaction was carried out in the absence (○) or presence (●) of 10% (v/v) 1,2-dimethoxyethane (dielectric constant = 13) at 30° C. At intervals between 5 and 45 minutes, aliquots were removed and phosphate determined in a manner analogous to that utilized for the phosphorylase reaction (103).
more drastic, conditions have been utilized in the resolution of the cofactor from a form of the enzyme (79). However, these conditions result in an apophosphorylase a which can only be reconstituted to about half of the original activity.

It was found that inclusion of 10% (v/v) 1,2-dimethoxyethane in the resolution medium accelerates the rate of resolution of phosphorylase b. The faster rate can be attributed to the increased accessibility of the pyridoxal phosphate (Figs. 11A and 11B). In the presence of 0.4 M imidazolium citrate, but in the absence of L-cysteine, there is a gradual appearance of the 415 nm band of the bound pyridoxal phosphate. This band has been attributed to the exposed protonated imine (117). The presence of 10% (v/v) 1,2-dimethoxyethane results in a large acceleration in the rate of appearance of this band. The process observed is not due to resolution of the cofactor since it was found that no loss of enzymatic activity occurs under these conditions.

Shaltiel et al. (102) suggested on the basis of exchange experiments that the effect of the deforming buffer was to expose the coenzyme. However, no data have been presented previously which correlate this hypothesis with spectral evidence.

Figure 12 demonstrates that the presence of 1,2-dimethoxyethane also results in an acceleration in the reverse reaction. The change in the reconstitution rate may be due to a change in the aggregation state of the enzyme since apophosphorylase b exhibits an increased predominance of the lower molecular weight forms in the presence of the solvent [Fig. 12 (inset)].
Figure 11. The effect of the resolution conditions on the spectral properties of pyridoxal phosphate bound to phosphorylase b.

9 mg/ml of phosphorylase b was incubated in the presence of 0.4 M imidazolium citrate, 42 mM β-glycerophosphate, 9 mM 2-mercaptoethanol at pH 6.0 in the absence (A) or presence (B) of 10% (v/v) 1,2-dimethoxyethane. At the times indicated by the numbers on the curves, spectra were recorded on a Cary 15 recording spectrophotometer.
Figure 12. The effect of 1,2-dimethoxyethane on the rate of reconstitution of phosphorylase b with pyridoxal phosphate.

5 mg/ml of apophosphorylase b was incubated in the presence of 50 μM pyridoxal phosphate, 30 mM β-glycerophosphate, 23 mM 2-mercaptoethanol at pH 6.8 and 19° C in the absence (○) or presence (●) of 10% (v/v) 1,2-dimethoxyethane. At the indicated times aliquots were removed and assayed as described under EXPERIMENTAL PROCEDURES.

(inset) The effect of 1,2-dimethoxyethane on the aggregation state of apophosphorylase b.

Sedimentation velocity experiments were performed as described under EXPERIMENTAL PROCEDURES at 52000 rpm. The sample contained 2.5 mg/ml of apophosphorylase b in 36 mM β-glycerophosphate, 27 mM 2-mercaptoethanol at pH 6.8 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 20° C. S20,w values are 8.2s and 18.0s (top) and 8.5s (bottom).
Since inclusion of the solvent resulted in an increased rate of resolution of the $b$ form of phosphorylase, the possibility of its use in the resolution of phosphorylase $a$ was investigated. From Figure 13 it can be seen that no resolution of phosphorylase $a$ is seen at 30° C under the resolution conditions described by Shaltiel et al. (102). Inclusion of 10% (v/v) 1,2-dimethoxyethane results in a rapid loss of activity which has been found to be due to resolution of the coenzyme since incubation of pyridoxal phosphate, upon removal of the resolution buffer, results in a return of the enzymatic activity. Shaltiel et al. (102) have suggested that formation of monomers may be a prerequisite for resolution of the coenzyme based upon the finding that phosphorylase $b$ formed monomers in the presence of the deforming buffer. As is seen in Figure 13 (inset), the results obtained with phosphorylase $a$ are also in agreement with this suggestion. In the presence of the deforming buffer alone, phosphorylase $a$ sediments predominantly as the dimer and is not capable of being resolved. When the deforming buffer contains 10% (v/v) 1,2-dimethoxyethane, conditions under which the enzyme would be resolved if L-cysteine were included, the enzyme sediments as a monomer.

Good preparations of apophosphorylase $a$ can be prepared directly from phosphorylase $a$ by modification of the procedure of Shaltiel et al. (102). Phosphorylase $a$ at 5 mg/ml is incubated for approximately 12 hours at room temperature (22°-23° C) in the presence of 0.4 M imidazolium citrate - 0.1 M L-cysteine - 10% (v/v) 1,2-dimethoxyethane at pH 6.0. The solvent is added to the deforming buffer immediately
Figure 13. The effect of 1,2-dimethoxyethane on the resolution of phosphorylase a.

1.3 mg/ml of phosphorylase a was incubated in the presence of 0.4 M imidazolium-citrate, 0.1 M L-cysteine at pH 6.0 and 30° C in the absence (O) or presence (©) of 10% (v/v) 1,2-dimethoxyethane. At the indicated times aliquots were removed and assayed as described under EXPERIMENTAL PROCEDURES.

(inset) The effect of the resolution buffer on the aggregation state of phosphorylase a.

Sedimentation velocity experimentation was performed as described under EXPERIMENTAL PROCEDURES at 52000 rpm. The sample contained 3.5 mg/ml phosphorylase a and 0.4 M imidazolium-citrate at pH 6.0 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 23° C. S_{20,w} values are 9.5s (top) and 4.2s (bottom).
before use in order to minimize the precipitation of salts. After the incubation period, an equal volume of neutralized saturated ammonium sulfate is added and the suspension centrifuged. The precipitate is dissolved in 0.2 M imidazolium citrate - 0.05 M L-cysteine at pH 6 and desalted over Sephadex G-25 which had been preequilibrated with the same buffer. The protein-containing fractions are combined, made 50% saturated ammonium sulfate, and the precipitate is dissolved in 0.04 M β-glycerophosphate - 0.03 M 2-mercaptoethanol at pH 6.8 and dialyzed against the same buffer. The protein crystallizes when dialyzed in the cold. The resulting apoenzyme preparation is greater than 99% resolved based on activity and can be reconstituted, at 5 mg/ml phosphorylase with a 5-fold molar excess of pyridoxal phosphate, to the same specific activity as the phosphorylase a from which it is prepared. The reconstituted enzyme has the same properties as native phosphorylase a as determined by sedimentation characteristics, enzymatic activities, and as a substrate for a phosphoprotein phosphatase (90). The yield of apophosphorylase a is routinely greater than 75%. Higher incubation temperatures during the resolution result in lower yields, even at shorter incubation times. Higher incubation temperatures also result in a lower specific activity for the reconstituted enzyme. Lower incubation temperatures greatly decrease the rate of resolutions.

Feldmann and Hull (86) have reported a line width of 55-60 Hz for the $^{31}$P NMR signal of the pyridoxal phosphate bound to phosphorylases a and b. The line width of 20 Hz for the serine phosphate of
apophosphorylase a (Fig. 14A) suggests that this is much less rigidly fixed. The signal position corresponds to the dianion when compared to a peptide serine phosphate (Fig. 15). Hoerl et al. (118), using thio-phosphorylated phosphorylase a, reported a line width and peak position for the thiophosphorylated serine corresponding to that of a dianion fixed in a salt linkage. The flexibility of the serine phosphate in the apoenzyme compared to the native is consistent with results on the trypsin sensitivity of this portion of the molecule (119) and the increased dephosphorylation rate for the apoenzyme (90). The accessibility of the serine phosphate is further emphasized in Figure 15. The serine phosphate of the apoenzyme exhibits a pH titration profile similar to that of the tetradecapeptide corresponding to the region around this residue for the pH range 6.0–7.5. Spectra could not be obtained at lower pH values due to denaturation of the protein.

For pyridoxal reconstituted phosphorylase a the $^{31}$P NMR signal exhibits considerable line broadening when compared to the apoenzyme (Fig. 14B). Although this spectrum constitutes less than one half the number of scans as that of the apoenzyme, it is quite evident that even at an equivalent number of scans the peak would be broadened. This result suggests a more structured amino terminal region for the pyridoxal enzyme. A more structured conformation for this enzyme derivative has been previously suggested on the basis of hybrid enzyme (97) and spectral (98,99) studies.

The structural role for the coenzyme was further investigated using activity measurements. The unorganized structure for the amino terminal
Figure 14. The effect of the coenzyme on the structure of phosphorylase a as determined by $^{31}$P NMR spectra of the serine phosphate.

A. Apophosphorylase a at 30 mg/ml in 50 mM Tris, 30 mM 2-mercaptoethanol, and 0.3 M NaCl at pH 7.5. The spectrum represents 24,552 scans with an acquisition time of 0.20 sec and a pulse width of 24 μsec. The spectrum was obtained at 24° C.

B. Pyridoxal reconstituted phosphorylase a at 38 mg/ml in 50 mM Tris, 30 mM 2-mercaptoethanol, and 0.4 M NaCl at pH 7.5. The spectrum represents 10,000 scans with an acquisition time of 0.20 sec and a pulse width of 24 μsec. The spectrum was obtained at 24° C.
Figure 15. pH Titration of the phosphoserine of a tetradecapeptide and apophosphorylase a using $^{31}$P NMR.

$^{31}$P NMR spectra of apophosphorylase a (○) were obtained using the same conditions as those described in Figure 14. The pH was adjusted with acetic acid. The points represent the peak position obtained from spectra for 24,552 scans at pH 7.5 and 10,000 scans at the lower pH values. $^{31}$P NMR spectra for the tetradecapeptide (NH$_2$-SerAspGlnGluLysArgGlnIleSer(PO$_4$)ValArgGlyLeu-COOH) (○) were obtained in the presence of 0.24 mM peptide and 50 mM imidazole at 24° C. The initial pH was 4.2 and the pH was adjusted to other pH values by the addition of 2 M Tris base. The points represent the peak positions obtained for 1,000 scans with an acquisition time of 0.20 sec. and a pulse width of 24 μsec.
region of the apoenzyme suggests that the absence of activity for this form may be due to a low affinity for substrates. Figure 16A presents the activity as a function of phosphate concentration for native phosphorylase $a$ and a 400-fold excess of an apophosphorylase $a$ preparation which was greater than 99.95% resolved. Activity was measured in the direction of glycogen degradation since Parrish et al. (76) have shown that the presence of noncovalent phosphate or phosphate analog is necessary for the expression of activity for pyridoxal-reconstituted phosphorylase. The results obtained here exhibit similar profiles for the apo and native enzymes, suggesting that the low activity of the apoenzyme is probably due to residual native phosphorylase $a$. No significant difference is seen in the profiles even at phosphate concentrations as high as 0.05 M.

Honikel and Madsen (120) have reported that the iodide quenching of pyridoxal phosphate fluorescence is eliminated for phosphorylase $b$ in the presence of AMP and glucose-1-P together, thus suggesting that the coenzyme is inaccessible in the presence of substrate. This is further suggested from the results of Helmreich and Klein (89). These authors reported that, for E. coli maltodextrin phosphorylase, the ability of the phosphoryl portion of the coenzyme to be titrated is lost in the presence of the active site inhibitor, glucose. These results suggest that the ability of glucose-1-P to bind to the pyridoxal enzyme in the absence of a phosphate analog should be expressed by its ability to inhibit the binding of the analog. Data for this are presented in Figure 16B. This result shows that at two concentrations of
Figure 16. The effect of the coenzyme structure on properties of the active site of phosphorylase a.

A. Determination of the activity properties of apophosphorylase a. The assay mixture contained 1% [14C]glycogen, 10 mM β-glycerophosphate, 7.5 mM 2-mercaptoethanol, 1 mg/ml apophosphorylase a (○) or 2.5 µg/ml phosphorylase a (●), and the indicated amounts of P_i at pH 6.8 and 30° C. The reaction mixture was incubated for 10 minutes and then terminated as described under EXPERIMENTAL PROCEDURES. The activity is expressed as counts per minute of reaction.

B. The effect of the substrate on the K_m for phosphite and pyridoxal reconstituted phosphorylase a. Activity was assayed as described by Illingworth and Cori (103) in the presence of 1% glycogen, 10 mM β-glycerophosphate, 7.5 mM 2-mercaptoethanol, 0.2 mg/ml pyridoxal phosphorylase a, 16 mM (●) or 37.5 mM (○) glucose-1-P, and the indicated amounts of sodium phosphite at pH 6.8 and 30° C.
glucose-1-P, 16 mM and 37.5 mM, the \( K_m \) for phosphite is unchanged while the maximal velocity is substantially increased. This suggests that glucose-1-P may be incapable of binding to the active site in the absence of the phosphoryl portion of the coenzyme.
DISCUSSION

Rabbit skeletal muscle glycogen phosphorylase b, as initially purified, was found to require AMP for activity yet this nucleotide was not utilized during the catalytic process (32), thus presenting an early recognition of the allosteric control of this enzyme. Further allosteric controls on phosphorylase activity have subsequently been recognized. These include the phosphorylation of the enzyme to the a form and the presence of a second nucleotide site where binding is inhibitory. In 1965, Monod et al. (57) provided a model for enzymatic allosteric transitions assuming an inactive T state and an active R state. The existence of these two conformations have been confirmed for the phosphorylase molecule by various methods, most notably through recent x-ray crystallographic studies which have delineated the sections of the molecule which undergo major conformational changes during the T → R transition (60).

Kastenschmidt et al. (61) have reported an allosteric constant, L (L = T/R), for phosphorylase b in the absence of ligands of 6100 at 29° C. This equilibrium constant corresponds to a ΔG for the T → R transition of 5.2 kcal mol⁻¹. Helmreich et al. (62) have found that for phosphorylase a this same allosteric constant is between 3 and 13 at 23° C. This corresponds to a ΔG of 0.6 to 1.5 kcal mol⁻¹, thus phosphorylation results in a stabilization of the allosteric equilibrium by 3.7 to 4.6 kcal mol⁻¹. Recent crystallographic studies have shown that the serine phosphate of phosphorylase a interacts with Arg 69
near the subunit interface (35). This interaction probably accounts for the stabilization of the allosteric equilibrium upon phosphorylation of the enzyme.

Besides the ability of AMP and phosphorylation of the enzyme to shift the allosteric equilibrium in favor of the R state, recent x-ray crystallographic and kinetic evidence have shown that a second nucleotide site exists on phosphorylase where binding is competitive with the substrate, glucose-1-P, which binds 10 Å away (53). Binding at this site stabilizes the T conformer. Of the compounds capable of inhibiting phosphorylase by binding to this site, the more hydrophobic compounds such as adenosine, caffeine, and riboflavin are more effective than the more hydrophilic nucleotides.

Madsen and coworkers have suggested from kinetic studies using phosphorylase a, that there is an interaction between the nucleoside inhibitor sites on a dimer (53). Using phosphorylase b, the data presented here show that this apparent cooperativity is attributable to glucose-1-P binding, and replots exhibit linearity suggesting no significant interaction between inhibitor sites under these conditions. The kinetic plots of Madsen and coworkers are incapable of providing a distinction between cooperativity of inhibitor binding and increased cooperativity of substrate binding induced by the inhibitor (53). The effect of the inhibitor on glucose-1-P binding is that predicted by the model of Monod et al. (57). Since caffeine and glucose stabilize the T conformer, the cooperativity of glucose-1-P would be expected to increase since the degree of cooperativity is dependent upon the
allosteric constant. The linearity of the replots for these inhibitors may be due to the relative amounts of T and R conformers and need not imply that there is no interaction between subunits for the T conformer.

Kasvinsky et al. (121) have shown a synergistic relationship between glucose binding and binding at the nucleoside inhibitor site. These authors calculated that the inhibition constant for caffeine and phosphorylase a decreased from 0.2 mM in the absence of glucose to 0.06 mM in the presence of saturating glucose. This is probably due to the increased predominance of the T state in the presence of glucose. The effect of 1,2-dimethoxyethane on the binding parameters of caffeine and glucose is consistent with the ability of this solvent to facilitate the T \rightarrow R conversion. The larger effect of 10% (v/v) 1,2-dimethoxyethane on caffeine binding may be due to a direct solvent effect. The inclusion of an organic solvent increases the hydrophobicity of the medium, so that the affinity of a hydrophobic compound for an apolar site on the enzyme would be expected to decrease. The ability of organic solvents to "solvate" hydrophobic substrates or inhibitors from their binding site has been previously described for α-chymotrypsin (122).

Nucleotide activation of rabbit liver phosphorylase exhibits properties markedly different from the muscle isozyme. The liver enzyme is only slightly active in the presence of AMP. Further activation requires the presence of high salt concentrations (48,49,123) or high concentrations of glucose-1-P (123). These conditions are similar to those found necessary for best activation of skeletal muscle
phosphorylase \textit{b} in the presence of IMP (109). The presence of the nucleoside inhibitor site on the liver isozyme has been reported by Kasvinsky \textit{et al.} (121), although no information is available on the relative affinity of nucleotides for the two sites.

The results presented here on nucleotide activation of liver phosphorylase \textit{b} in the presence of 1,2-dimethoxyethane suggest that, in contrast to the situation observed with skeletal muscle phosphorylase, AMP may have an affinity for the nucleoside inhibitor site which is comparable to or greater than its affinity for the activator site. Only after AMP inhibition at the inhibitor site is reduced by the solvent can activation by the nucleotide be seen. This suggestion is consistent with the report that the low activity with liver phosphorylase \textit{b} is due to a high $K_m$ for glucose-1-P (123).

These results also suggest that 1,2-dimethoxyethane may be useful in determining total phosphorylase levels in liver extracts. The current assay utilizing high levels of fluoride or sulfate (49) is inadequate since these anions inhibit liver phosphorylase \textit{a} activity while giving only partial activation of phosphorylase \textit{b}. In contrast, 10% (v/v) 1,2-dimethoxyethane does not affect liver phosphorylase \textit{a} activity and activates liver phosphorylase \textit{b} in the presence of AMP or IMP to a level comparable to that of the \textit{a} form. These results suggest that 1,2-dimethoxyethane may be useful in more accurately determining activities for the different forms of liver phosphorylase utilizing the low glucose-1-P assay described under "EXPERIMENTAL PROCEDURES".
The usefulness of this assay has recently been verified by Chrisman and Exton for phosphatase inactivation of liver phosphorylase $a$ (16). While phosphorylase $a$ activity decreased 85%, there was essentially no change in total phosphorylase activity when it was assayed as suggested here. These authors also utilized the low $K_a$ for AMP and skeletal muscle phosphorylase $b$ in the presence of 10% (v/v) 1,2-dimethoxyethane in order to detect low levels of AMP (16). They reported that as little as 1 μM AMP caused a detectable increase in activity under these conditions.

The effect of 1,2-dimethoxyethane on the different forms of skeletal muscle phosphorylase presents further evidence that this solvent activates by shifting the allosteric equilibrium. Phosphorylase $a$ in the presence of AMP already appears to be nearly fully activated. Phosphorylase $a$ in the absence of AMP can be activated by the solvent to the same extent as that obtained with AMP. AMP has been reported to lower the $K_m$ for glucose-1-P and phosphorylase $a$ (64) and this same effect is seen with 1,2-dimethoxyethane.

The data presented here suggest that the activation properties of the solvent cannot be explained by a difference in the aggregation state of the enzyme. Inclusion of the solvent facilitates the AMP-induced tetramerization of phosphorylase $b$. This is again probably due to the ability of the solvent to stabilize the $R$ form since glucose-1-P which facilitates this transition in the presence of AMP (61), also increases the amount of tetramer in the presence of the nucleotide (109). Helmreich and coworkers have invoked an additional conformational state
for the tetramer in order to explain its allosteric properties (61). The lower activity of the tetramer and the difference in the solvent stimulation for this form are also consistent with this suggestion.

The results obtained with 1,2-dimethoxyethane in the absence of nucleotides show that, in the presence of substrates, the allosteric equilibrium can be shifted to a large extent to the active form by this solvent alone. The activity obtained cannot be explained by contaminating AMP in the enzyme preparation or substrates since further increases in the solvent concentration result in an increased maximal velocity with little change in the $K_m$ for glucose-1-P. AMP activation is characterized by a change in the $K_m$ for substrates at limiting concentrations of this nucleotide (110). Further evidence that organic solvent activation is not attributable to endogenous nucleotides has been presented by Dreyfus et al. (124). These authors reported that butanedione modification of an essential arginine residue eliminated the ability of phosphorylase $b$ to be activated by AMP while there was no affect on $t$-butanol activation. Still further evidence for differences in solvent activation and that of AMP is provided by the differences in the acidic limb of the pH profiles.

The effects of pH on glycogen phosphorylase activity obtained here suggest that $pK_1$ exhibits a wide variance dependent upon the activity state of the enzyme. Kasvinsky and Meyer have reported that phosphorylase $a$ exhibits a $pK_1$ at least one-half pH lower than that of phosphorylase $b$ in the presence of AMP (112). The results obtained here show that 1,2-dimethoxyethane activation of phosphorylase $b$ in the presence of
AMP correlates with a substantial lowering of the $pK_1$ again suggesting the presence of a phosphorylase $a$-type molecules under these conditions. This change in the pH profile can be attributed to the catalytic reaction since the concentrations of effectors used here should be saturating according to the results of Kasvinsky and Meyer (112) utilizing $P_i$ and the consideration of the lower $pK_2$ for glucose-1-P which was utilized in these experiments. Phosphorylase $b$ in the absence of activators and in the presence of 10% (v/v) 1,2-dimethoxyethane as the activator exhibits a $pK_1$ which is approximately 0.2 units higher than that of AMP-activated phosphorylase $b$.

Various authors have invoked an ionization of the phosphoryl portion of pyridoxal phosphate to explain the acidic limb of the pH profile (112,125). Although protonation of a carboxyl group could also be suggested to account for this ionization, it should be noted that Feldmann and Hull using $^{31}P$ NMR and phosphorylase $b$ found that the presence of AMP and a substrate analog resulted in a deprotonation of the phosphoryl portion of pyridoxal phosphate (86). These authors further presented evidence that the resonance for phosphorylase $b$ in the absence of effectors was insensitive to pH between 5.8 and 8.5. Addition of the activator AMPS and the substrate analog arsenate together, or arsenate alone resulted in a titration of the resonance below pH 6.4. These results, together with the x-ray crystallographic results which locate the coenzyme at the active site (74,77), makes it attractive to suggest that the acidic ionization is due to the
protonation of the phosphoryl portion of the coenzyme and that the formation of the dianion is an important step in the activation process.

Using water-soluble carbodiimide modification, Ariki and Fukui have presented evidence for the presence of an essential residue with a pK between 5.0 and 5.5 (126). These authors further showed that inactivation was stimulated considerably by the addition of glucose-1-P or P$_i$. The modification was inhibited by α-glucosyl fluoride, which exhibits inhibition kinetics similar to those of glucose (127). These results suggest that this residue is modified when the enzyme is present in the R conformation. These authors presented evidence that addition of glycine ethyl ester in the presence of the carbodiimide resulted in its covalent attachment to the enzyme. Since glucosyl amine affected the modification in a manner similar to that of glycine ethyl ester while 2-amino-2-deoxy glucose did not, these authors suggested that the modified residue was located near the C1 carbon of the sugar. These results suggest the presence of a phosphoryl or a carboxyl residue, with a pK similar to that seen in the activity pH profiles, near the point of catalysis.

The results obtained on ionic strength activation of the acid hydrolysis of glucose-1-P suggests the importance of ions on this process while evidence supports an S$_N^1$ mechanism under these conditions (72,114). From their work on ester hydrolysis, Bunton et al. (116) have presented evidence that this stimulation is attributable to the anion and that the rate enhancement is due to stabilization of the developing carbonium, both via an increase in the activity coefficient of the medium and
through specific interactions. Further, these authors found that the catalytic order of strong acids and salts suggested that the lower the charge density of the anion the more it stabilizes transition states with carbonium ion character (116).

The effect of low charge density reagents is clearly seen in micellar catalysis (128). The ability of the cationic surfactant, hexadecyltrimethylammonium bromide, to catalyze unimolecular decarboxylations and dephosphorylations is greatest when a high charge density anion in the initial state is converted with a low charge density transition state. The anion in the transition state is stabilized by the low charge density cationic, trimethylammonium, head of the surfactant much more readily than the initial high charge density anion.

The stimulation by anions and the further stimulation by a lower dielectric suggests that a similar situation may be important in the action of glycogen phosphorylase. The most likely candidates for such a function in phosphorylase would be the dianionic phosphoryl group of pyridoxal or a carboxylate anion. The increased inhibition by the transition state analog, 1,5-gluconolactone, in the presence of 1,2-dimethoxyethane suggests that the transition state is stabilized under conditions where the maximal velocity is also increased. This oxonium intermediate can be considered to be a low charge density cation due to the delocalization of charge between the Cl carbon and the ring oxygen. As mentioned previously, Feldmann and Hull (86) have shown that activation results in a deprotonation of the phosphoryl group of
pyridoxal phosphate. The results presented here also suggest the
importance of the activity state of the enzyme on the $pK_a$ of the pH
profile. By analogy with the results on the acid hydrolysis of
glucose-1-P, the activation process may be such as to allow the dianion
of pyridoxal phosphate to stabilize the developing oxonium transition
state. It should be noted however, that x-ray crystallographic studies
on the T form of the enzyme also show the presence of two carboxyl
groups near the catalytic site (35). The theoretical studies of
Warshel and Levitt on lysozyme (129) suggest that electrostatic
stabilization of the carbonium ion may be considerable. These authors
have suggested that the presence of Asp 52 lowers the energy of the
carbonium ion relative to the ground state by 9 kcal mol$^{-1}$.

An involvement of the phosphoryl dianion as suggested here would
allow for nucleophilic assistance by this group as well as electrostatic
stabilization of the developing transition state. Such a mechanism
would be considered to have both $S_N^1$ and $S_N^2$ characteristics, thus
offering a possible explanation for the absence of a secondary isotope
effect reported by Firsov et al. (130) under conditions where no
covalent intermediate is found.

Helmreich and coworkers have proposed a role for the phosphoryl
portion of the coenzyme in the protonation of the bridge oxygen during
the catalytic reaction (86,89). However, this suggestion conflicts with
the results of Parrish et al. (76) which showed that fluorophosphate
was similar to phosphite in its ability to activate pyridoxal
reconstituted phosphorylase $b$. The difference in the ionization of
these two analogs ($pK^2 = 4.8$ for fluorophosphate as compared to $pK^2 = 6.2$ for phosphite), suggests that fluorophosphate should not be as proficient in a protonation step during the reaction at the pH utilized in their experiments.

The results reported here show that good preparations of apophosphorylase $a$ can be made by using 1,2-dimethoxyethane in the resolution medium. The effect of this solvent on the resolution does not appear to be related to its effect on the allosteric equilibrium since both AMP and caffeine have been reported to inhibit the resolution of phosphorylase $b$ (131). Good preparations of apophosphorylase $a$ allow the effects of different pyridoxal phosphate analogs on the properties of phosphorylase $a$ to be determined. Yan et al. (90) have recently reported that the structure of the coenzyme is important for the regulation by glucose and caffeine of the dephosphorylation by phosphatase, and their regulations of the dimer-tetramer equilibrium. These authors have presented evidence for the importance of the dianionic form of the phosphoryl portion of the coenzyme in this regulation. The importance of the dianionic form in the expression of activity of the $b$ form of the enzyme has been previously suggested by Parrish et al. (76).

The results presented here suggest that removal of the coenzyme results in flexibility for the serine phosphate when the linewidth for this residue is compared to that reported for the phosphoryl group of the coenzyme (86). Although no direct correlation can be made with the serine phosphate of the native enzyme due to the fact that the
$^{31}P$ NMR resonances of the coenzyme and the serine phosphate coincide, various evidence suggests that the resonance seen for the apoenzyme has a narrower linewidth than that of the native enzyme. As is seen in Figure 13B, addition of the pyridoxal portion of the coenzyme results in considerable linebroadening. Also, $^{31}P$ NMR spectra obtained an apophosphorylase reconstituted with the phosphonethyl analog of pyridoxal phosphate exhibit a broader linewidth for the serine phosphate than that exhibited here for the apoenzyme (R. J. Uthing, S. C. B. Yan, D. J. Graves, Department of Biochemistry and Biophysics, Iowa State University and P. Schmidt, Oklahoma City Medical Research Foundation, unpublished observations). The accessibility of the serine phosphate may account for the increased rate of dephosphorylation seen for the apoenzyme (90).

The results obtained on the activity of the apoenzyme in the presence of increasing concentrations of inorganic phosphate suggest that the small amount of activity is due either to residual phosphorylase in the preparation or to an apoenzyme with a similar $K_m$ for substrates but which has a maximal velocity which is at least a thousand-fold lower than that seen for the native enzyme. No evidence can be seen for an enzyme form with a higher $K_m$ for the substrate. Parrish et al. (76) have reported that pyridoxal reconstituted phosphorylase $b$ has a four-fold higher $K_m$ for glucose-1-P. Shimomura and Fukui have found that removal of the coenzyme from the α-glucan phosphorylase of potato tubers resulted in the loss of its ability to bind polysaccharide (132). For these reasons it is suggested that the activity profile presented here represents residual native phosphorylase $a$ activity rather than
any intrinsic activity of the apoenzyme. Furthermore, the results obtained with pyridoxal reconstituted phosphorylase \( a \) suggest that the presence of the phosphoryl portion of the coenzyme is necessary for the binding of substrates.

Shimomura and Fukui have reported that pyridoxal phosphorylase \( b \) has a structure similar to the native enzyme as determined by circular dichroism and ultraviolet absorption studies (98,99). Hybrid enzyme studies (97) have shown that the pyridoxal enzyme is capable of inducing activity in the immobilized native monomer, whereas the apoenzyme is not. The results presented here on the \( ^{31}P \text{ NMR} \) of the serine phosphate also suggest that much of the native structure is retained in the pyridoxal enzyme, although conformational differences in the active site region must still be present since the data here suggests that the phosphoryl portion of the coenzyme is necessary for the binding of the substrate, glucose-1-P.

The suggestion that a single group on the enzyme may be responsible for both the catalytic reaction and the binding of substrates is supported from other studies. Kasvinsky and Meyer have suggested, from a plot of \( pK_m \) versus pH, that the ionization of a residue with a \( pK \) of 5.1 is important in the binding of substrates by phosphorylase \( a \) (112). These authors have suggested that a group of the same \( pK \) is also important for the maximal velocity profile, from a plot of \( \frac{V_{\text{max}}}{K_m} \) versus pH.
CONCLUSION

Organic solvents are found to affect the allosteric equilibrium of glycogen phosphorylase. This is evidenced by changes in the activity properties of the enzyme. Inhibitors which stabilize the inactive T conformation of the enzyme, exhibit decreased affinities in the presence of the solvent, 1,2-dimethoxyethane. The $K_1$ caffeine is increased 6-fold in the presence of the solvent. Substrates and activators which bind to the active R conformation of the enzyme exhibit increased affinities in the presence of the solvent. This is most evident for the activator, AMP, which exhibits a 10-fold reduction for its $K_a$ in the presence of 1,2-dimethoxyethane. The $K_m$'s for the substrates, glucose-1-P and glycogen, is also reduced. 1,2-Dimethoxyethane is also able to activate the enzyme in the absence of a nucleotide activator to a considerable extent.

The allosteric activation of glycogen phosphorylase results in a change of the $pK_1$ for the activity profile. The formation of the dianionic form of the phosphoryl group of the coenzyme, pyridoxal phosphate, may account for this change. By analogy to the effects of anions and a change in dielectric on the acid hydrolysis of glucose-1-P, it is suggested that the dianion of the coenzyme stabilizes the developing positive charge on the oxonium intermediate.

The use of 1,2-dimethoxyethane allows for resolution of pyridoxal phosphate from glycogen phosphorylase a. Results from $^{31}P$ NMR and kinetic studies suggest that the coenzyme also has a structural role...
and that substrates may not bind in the absence of the phosphoryl portion of the coenzyme.
ACKNOWLEDGEMENTS

Thanks are extended to Dr. Al Janski for performing some of the early experiments which led to this research as well as many stimulating discussions during the early part of this work. I am also indebted to Steve Lentz for providing assistance in part of the experimentation.
PART II. THE EFFECT OF DIETARY PYRIDOXINE ON GLYCOGEN METABOLISM IN THE MOUSE
INTRODUCTION

In 1954, Beaton and Goodwin reported that carbohydrate metabolism was altered in the vitamin B\textsubscript{6} deficient rat (133). These alterations were evidenced by a reduction in blood glucose and a lowering of liver glycogen levels. Changes in blood glucose occurred within five days of vitamin depletion. In 1957, pyridoxal phosphate was found to be a prosthetic group of the glycogen degrading enzyme, glycogen phosphorylase (75). Subsequent studies have led to the suggestion that this enzyme functions as a reservoir for the vitamin (3). Glycogen phosphorylase activity in skeletal muscle is significantly reduced during pyridoxine deficiency (93,94).

Although this enzyme is involved in a complicated regulatory scheme, the effects of its reduction during the vitamin deficiency on the other regulated enzymes concerned with glycogen metabolism have not been investigated to any extent. Furthermore, the effect of pyridoxine deficiency on glycogen metabolism in the liver has been largely ignored. This organ serves as the primary pool for carbohydrate storage in the body. It also appears to serve a major role in the metabolism of the various forms of vitamin B\textsubscript{6} (134). Pyridoxine deficiency results in the preferential depletion of the vitamin in the cytosolic fraction of this organ (135), although the effect on specific metabolic processes is not understood.

A role for vitamin B\textsubscript{6} in diabetes mellitus has been suggested since treatment of various forms of the disease (e.g., gestational diabetes) with the vitamin improved the glucose tolerance curve but did not affect
plasma insulin levels (136). Adams et. al. (137) have suggested that the improved glucose tolerance is due to the increased formation of a tryptophan metabolite, quinolinic acid, which is an inhibitor of hepatic phosphoenolpyruvate carboxykinase.

The present study was initiated in order to investigate the effects of pyridoxine deficiency on carbohydrate metabolism in the mouse. The activity levels of several enzymes concerned with glycogen metabolism in both skeletal muscle and liver were determined. These included the enzymes responsible for its synthesis and degradation, glycogen synthase and glycogen phosphorylase, as well as the enzymes responsible for the interconversion between the various forms of these enzymes. The activity levels of these enzymes were determined in both the normal and diabetic state in order to investigate the effect of the pyridoxine nutritional state on this condition.
EXPERIMENTAL PROCEDURES

Animals

Three week old male mice from Biolab Corporation, St. Paul, Minnesota, were fed either a pyridoxine deficient diet (ICN Biochemicals, Cleveland, Ohio) or the same diet which had been supplemented with 3 ppm pyridoxine. The animals were allowed continuous access to water and food. The mice were maintained on the diet for seven weeks before being sacrificed.

After five weeks on the diet, a portion of the group of mice had diabetes induced by intraperitoneal injections of streptozotocin (130 mg per kg of body weight). The diabetic condition was ascertained by elevated serum sugar levels.

Preparation of Tissue

Mice were sacrificed between 8 and 10 a.m. and portions of the hind leg skeletal muscle and of the liver were rapidly excised and homogenized in the cold in either 40 mM \( \beta \)-glycerophosphate, 30 mM 2-mercaptoethanol, 1 mM EDTA, and 100 mM sodium fluoride at pH 6.8 (buffer A), or 50 mM imidazole, 30 mM 2-mercaptoethanol, and 3 mM EDTA at pH 7.5 (buffer B). The remaining tissue was frozen in dry iceacetone for later determination of glycogen content. Homogenates were centrifuged with a Beckman microfuge for 5 minutes at 4°C and the supernatant used for enzyme assays.
Enzymatic Assays

Skeletal muscle glycogen phosphorylase was assayed utilizing the filter paper assay of Thomas et al. (104). The assay system contained 1% glycogen, 16 mM glucose-1-P, 16 mM β-glycerophosphate, 12 mM 2-mercaptoethanol, and 10 mM sodium fluoride at pH 6.8 and 30°C in the absence (phosphorylase a) or presence (total phosphorylase) of 1 mM AMP.

Liver glycogen phosphorylase was assayed according to the conditions of Stalmans and Hers (49) using the filter paper assay of Thomas et al. (104).

Skeletal muscle glycogen synthase activity was assayed in the presence of 10 mM UDPG, 1% glycogen, 50 mM Tris, 5 mM EDTA, 30 mM sodium fluoride, and either 14 mM sodium sulfate (synthase I) or 14 mM glucose-6-P (synthase D + I) at pH 7.8 and 30°C utilizing the filter paper assay of Thomas et al. (104).

Liver glycogen synthase activity was determined in a similar manner as the skeletal muscle isozyme with the exception that the concentration of glucose-6-P was 28 mM for the determination of glycogen synthase D + I.

Phosphorylase kinase activity was assayed as described by Brostrom et al. (138) in the presence of 0.2 mM calcium. Activity was determined by the incorporation of \([^{32}\text{P}]\)phosphate into skeletal muscle glycogen phosphorylase b from \([\gamma-^{32}\text{P}])\)ATP.

Phosphorylase phosphatase activity was assayed as previously described (139) using \([^{32}\text{P}]\)phosphate release from \([^{32}\text{P}]\)phosphorylase a.

Aspartate aminotransferase activity was assayed as described by Black et al. (91) at 23°C using 150 units of malate dehydrogenase.
The activity of cAMP-dependent protein kinase was determined as described by Reimann et al. (140) (Method B). The assay system contained 22 mM MES, 1.8 mM theophylline, 22 mM sodium fluoride, 11 mM magnesium acetate, 3.6 mg/ml casein, and 0.11 mM [γ-32P]ATP in the presence or absence of 11 μM cAMP at pH 6.9 and 30°C.

The enzymatic reactions were initiated by the addition of the homogenation supernatant in buffer B for phosphorylase phosphatase and aspartate aminotransferase, and by the homogenation supernatant in buffer A for the remainder of the enzymes. All activity measurements were performed in duplicate and were linear with time. Activities are expressed in terms of milligrams of protein in the supernatant.

Other Methods

Protein was determined in the homogenation supernatants using the Bradford assay (141). Blood glucose was determined using glucose oxidase reagent ("Glucostat", Worthington Biochemical Corporation). Glycogen concentrations were determined by the phenol-sulfuric acid method (142).

Statistical evaluation of the data was performed using t-test analysis. Differences with p values <0.10 are presented. Smaller differences are considered to be not significant (n.s.). Values for p₁ are from comparisons with the nondiabetic mice maintained on the diet containing 3 ppm pyridoxine. Values for p₂ are from comparisons of the deficient diabetic mice with the deficient nondiabetic mice. Data are presented as the mean ± the standard deviation.
Materials

AMP, ATP, UDPG, glucose-1-P, β-glycerophosphate, Tris, MES, and theophylline were obtained from Sigma Chemical Company and used without further purification. Grade 1 imidazole from Sigma was used after three times recrystallization from reagent grade acetone. Shellfish glycogen from Sigma was further purified according to the method of Anderson and Graves (38). 2-Mercaptoethanol was obtained from Aldrich. [U-\(^{14}\)C] glucose-1-P and [U-\(^{14}\)C]UDPG were obtained from Amersham. [γ-\(^{32}\)P]ATP was prepared by the method of Glynn and Chappell (143).

Rabbit skeletal muscle glycogen phosphorylase \(b\) was prepared according to the method of Fischer and Krebs (100). Skeletal muscle glycogen phosphorylase \(a\) was prepared by rabbit skeletal muscle phosphorylase kinase (101) using [γ-\(^{32}\)P]ATP. Malate dehydrogenase was obtained from Sigma.
RESULTS

Figure 1 depicts the growth rate of mice maintained on a diet containing no or 3 ppm pyridoxine. Mice maintained in the absence of the vitamin exhibited a slower growth rate as well as an earlier growth plateau reached. Increasing the vitamin content to 30 ppm resulted in a slight enhancement of the growth rate over that seen at 3 ppm pyridoxine. McHenry (144) has reported that the lower body weight for B₆ deficient rats is due to a decrease in body fat while the amount of protein in the deficient animal is similar to that of rats maintained on a diet which included the vitamin.

The data included in this report are for mice which had been maintained for seven weeks on diets containing no or 3 ppm pyridoxine. After five weeks on the diet approximately one-half of the mice in each group was made diabetic by injection of streptozotocin. The deficient diabetic mice appeared to be healthier than their nondiabetic counterparts as evidenced by increased activity and appetite and a lower mortality rate.

The effect of the diet and the diabetic condition on the levels of blood glucose and storage glycogen is summarized in Table I. N₃ and D₃ refer to the normal and diabetic mice maintained on the diet containing 3 ppm pyridoxine. N₀ and D₀ refer similarly to the mice maintained on the diet lacking the vitamin. The values in parentheses refer to the number of mice in the determination. B₆ deficiency results in a significant (p₁ < 0.001) reduction in the blood glucose level as has been previously reported by Beaton and Goodwin (133). This reduction
Figure 1. The effect of dietary pyridoxine on the growth rate of mice.

Mice were maintained on diets in the absence of pyridoxine (○) or in the presence of 3 ppm pyridoxine (●). At the indicated times the average weight was determined. The values in parentheses indicate the number of mice in each group.
Table I. Blood glucose and storage glycogen levels

<table>
<thead>
<tr>
<th></th>
<th>N_3</th>
<th>D_3</th>
<th>P_1</th>
<th>N_0</th>
<th>P_1</th>
<th>D_0</th>
<th>P_1</th>
<th>P_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose</td>
<td>160 ±20</td>
<td>410 ±120</td>
<td>&lt;0.001</td>
<td>90 ±10</td>
<td>&lt;0.001</td>
<td>370 ±70</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(mg %)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(7)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver glycogen</td>
<td>29 ±12</td>
<td>14 ± 11</td>
<td>&lt;0.05</td>
<td>40 ±37</td>
<td>n.s.</td>
<td>25 ±24</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>(µg/mg of tissue)</td>
<td>(10)</td>
<td>(7)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle glycogen</td>
<td>1.59± 0.45</td>
<td>1.80± 0.31</td>
<td>n.s.</td>
<td>2.24± 0.78</td>
<td>&lt;0.05</td>
<td>1.84± 0.91</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>(µg/mg of tissue)</td>
<td></td>
<td></td>
<td></td>
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</table>
may be a result of an alteration in the hormonal control in the deficient animal since induction of the diabetic state results in an elevation of the blood glucose to levels similar to those seen for diabetic mice maintained on the B₆ supplemented diet. The lower blood sugar levels do not appear to be due to a reduction in storage glycogen in the deficient animal since, as is seen in Table I, these levels are similar to those of the animals which have been supplied with the vitamin.

The liver glycogen levels exhibit a much larger variance for the deficient animal when compared to the B₆ supplemented animal. Whereas the levels were maintained within a narrow range for the supplemented animals in both the normal and diabetic state, those of the deficient mice varied between 1 and 120 µg per mg of tissue for the normal mice and between 2 and 65 µg per mg of tissue for the diabetic mice. In contrast to those of liver, muscle glycogen levels do not appear to be as significantly affected by the experimental conditions. Lyon and Porter (94) have previously reported that skeletal muscle glycogen levels do not change in B₆ deficiency.

The effect of B₆ deficiency on the activities of the glycogen metabolizing enzymes in skeletal muscle is summarized in Table II. The deficient condition results in a significant reduction in total phosphorylase levels as has been previously reported (93-95). Also similar to that reported previously (93), this reduction of total phosphorylase activity occurs while normal levels of the active form, phosphorylase a, are maintained.
Table II. Glycogen metabolizing enzymes (muscle)

<table>
<thead>
<tr>
<th></th>
<th>N₃</th>
<th>D₃</th>
<th>P₁</th>
<th>N₀</th>
<th>P₁</th>
<th>D₀</th>
<th>P₁</th>
<th>P₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase a</td>
<td>0.54±0.19</td>
<td>0.49±0.14</td>
<td>n.s.</td>
<td>0.68±0.18</td>
<td>n.s.</td>
<td>0.55±0.18</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>(μmole/min • mg)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phosphorylase</td>
<td>2.24±0.33</td>
<td>2.12±0.24</td>
<td>n.s.</td>
<td>0.99±0.24</td>
<td>&lt;0.001</td>
<td>0.89±0.12</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>(μmole/min • mg)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorylase a/total</td>
<td>0.24±0.08</td>
<td>0.24±0.09</td>
<td>n.s.</td>
<td>0.69±0.15</td>
<td>&lt;0.001</td>
<td>0.61±0.16</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthase I</td>
<td>25.4±3.4</td>
<td>24.4±4.0</td>
<td>n.s.</td>
<td>12.3±5.6</td>
<td>&lt;0.001</td>
<td>9.6±5.2</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>(nmole/min • mg)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthase D + I</td>
<td>48.5±7.8</td>
<td>54.1±7.2</td>
<td>n.s.</td>
<td>59.6±22.9</td>
<td>n.s.</td>
<td>44.0±4.3</td>
<td>n.s.</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(nmole/min • mg)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthase I/(D+I)</td>
<td>0.54±0.11</td>
<td>0.46±0.08</td>
<td>&lt;0.1</td>
<td>0.21±0.06</td>
<td>&lt;0.001</td>
<td>0.22±0.11</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
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<td>(11)</td>
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</tr>
</tbody>
</table>
Although the levels of total glycogen synthase in muscle are similar in the B\textsubscript{6} supplemented and B\textsubscript{6} deficient mice, there is a significant (p\textsubscript{l} < 0.001) reduction of the active I form of the enzyme in the deficient state. This condition apparently does not lead to mobilization of glycogen stores since, as is shown in Table I, the glycogen levels in B\textsubscript{6} deficient mice are at least as large as those of the animals which had been supplemented with the vitamin.

The data summarized in Table III show that although dramatic effects are seen for the activity ratios of glycogen phosphorylase and glycogen synthase (Table II), corresponding large significant increases in the activity ratios of phosphorylase kinase and cAMP-dependent protein kinase are not witnessed. The pH 6.8 and pH 8.2 activities of phosphorylase kinase decrease in both the normal and diabetic B\textsubscript{6} deficient animals. Although there appear to be slight changes in the cAMP-dependent protein kinase activity under the different conditions, corresponding changes are not seen for phosphorylase kinase. Also seen in Table III is a slight increase in phosphorylase phosphatase activity for B\textsubscript{6} deficient mice in both the normal and diabetic state.

The data presented in Table IV show that in the deficient state liver glycogen phosphorylase exhibits a more pronounced reduction in activity than that seen for the muscle isozyme (Table II). In contrast to the situation exhibited by the muscle enzyme, this reduction is similar for phosphorylase a and total phosphorylase. The activity ratio for phosphorylase in the deficient state is similar to that obtained for the B\textsubscript{6} supplemented animals. Phosphorylase in liver accounts for
Table III. Interconverting enzymes (muscle)

<table>
<thead>
<tr>
<th></th>
<th>( N_3 )</th>
<th>( D_3 )</th>
<th>( P_1 )</th>
<th>( N_0 )</th>
<th>( P_1 )</th>
<th>( D_0 )</th>
<th>( P_1 )</th>
<th>( P_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase kinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.8 (nmole/min • mg)</td>
<td>1.86±0.32</td>
<td>1.65±0.35</td>
<td>n.s.</td>
<td>1.40±0.24</td>
<td>&lt;0.01</td>
<td>1.23±0.49</td>
<td>&lt;0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
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<tr>
<td>Phosphorylase kinase</td>
<td>6.93±1.75</td>
<td>6.46±0.94</td>
<td>n.s.</td>
<td>4.65±1.21</td>
<td>&lt;0.01</td>
<td>4.17±1.17</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>pH 8.2 (nmole/min • mg)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>0.27±0.05</td>
<td>0.26±0.07</td>
<td>n.s.</td>
<td>0.31±0.06</td>
<td>n.s.</td>
<td>0.29±0.09</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>pH 6.8/pH 8.2</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorylase phosphatase</td>
<td>2.17±0.63</td>
<td>2.32±0.41</td>
<td>n.s.</td>
<td>2.70±0.52</td>
<td>&lt;0.10</td>
<td>2.96±0.57</td>
<td>&lt;0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>(nmole/min • mg)</td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein kinase -cAMP (pmole/min • mg)</td>
<td>17.7 ± 7.9</td>
<td>25.7 ± 13.4</td>
<td>n.s.</td>
<td>31.1 ± 17.8</td>
<td>&lt;0.05</td>
<td>9.3 ± 4.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(7)</td>
<td></td>
<td>(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein kinase +cAMP (pmole/min • mg)</td>
<td>28.6 ± 7.7</td>
<td>38.4 ± 11.0</td>
<td>&lt;0.05</td>
<td>36.3 ± 13.5</td>
<td>n.s.</td>
<td>19.0 ± 7.9</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>(10)</td>
<td>(8)</td>
<td></td>
<td>(8)</td>
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<td>(11)</td>
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<tr>
<td>Protein kinase -cAMP/+cAMP</td>
<td>0.60±0.16</td>
<td>0.65±0.28</td>
<td>n.s.</td>
<td>0.78±0.22</td>
<td>&lt;0.10</td>
<td>0.64±0.31</td>
<td>n.s.</td>
<td>n.s.</td>
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<td></td>
<td>(10)</td>
<td>(8)</td>
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<td>(7)</td>
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approximately 10% of the pyridoxal phosphate in the organ compared to approximately 90% in skeletal muscle (135). The depletion of liver phosphorylase activity found here is much greater than that reported by Lyon and Porter (94) and suggests that the B₆ nutritional effect on the liver isozyme is significant. The assay system employed by the above authors was the same used for the measurement of skeletal muscle glycogen phosphorylase activity and has been found to be inadequate for the determination of the activity of the liver isozyme (49,123).

The total activity of liver glycogen synthase is similar for mice maintained on the B₆ deficient and B₆ supplemented diets. The percentage of the active I form of the enzyme exhibits a significant (p₁ < 0.05) increase in the deficient animal. This significance is lost upon induction of the diabetic state. The results presented here suggest significant differences between muscle and liver in the deficient state which cannot be explained by the total content of the glycogen metabolizing enzymes. In skeletal muscle the relative level of the active form of phosphorylase is increased in the deficient state with a corresponding reduction in the level of glycogen synthase I. Similar changes are not observed in liver. In fact, while the percentage of phosphorylase a remains constant, there is a slight increase in the percentage of glycogen synthase I in the deficient state.

As has been mentioned previously, the liver glycogen levels exhibit a large variability in the B₆ deficient state. As is seen in Table IV, similar phenomena are observed for both the levels of glycogen synthase and glycogen phosphorylase. The variability of glycogen and its
<table>
<thead>
<tr>
<th></th>
<th>N3</th>
<th>D3</th>
<th>P1</th>
<th>N0</th>
<th>P1</th>
<th>D3</th>
<th>P1</th>
<th>P2</th>
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<tbody>
<tr>
<td>Phosphorylase α</td>
<td>Nmole/min • mg&lt;br&gt;236 ±30 (10)&lt;br&gt;285 ±36 (8)&lt;br&gt;&lt;0.01 49 ±34 (8)&lt;br&gt;&lt;0.001 49 ±34 (11)&lt;br&gt;&lt;0.001 n.s.&lt;br&gt;&lt;0.001 n.s.&lt;br&gt;&lt;0.001 n.s.&lt;br&gt;&lt;0.001 n.s.</td>
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<tr>
<td>Total phosphorylase</td>
<td>Nmole/min • mg&lt;br&gt;205 ±31 (10)&lt;br&gt;239 ±32 (8)&lt;br&gt;&lt;0.05 42 ±28 (8)&lt;br&gt;&lt;0.001 42 ±26 (11)&lt;br&gt;&lt;0.001 n.s.&lt;br&gt;&lt;0.001 n.s.&lt;br&gt;&lt;0.001 n.s.&lt;br&gt;&lt;0.001 n.s.</td>
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<td>Phosphorylase α/total</td>
<td>α/total&lt;br&gt;1.15 ± 0.05 (10)&lt;br&gt;1.20 ± 0.07 (8)&lt;br&gt;n.s. 1.18 ± 0.05 (8)&lt;br&gt;n.s. 1.15 ± 0.11 (11)&lt;br&gt;n.s. n.s. n.s.</td>
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<td>Synthase I</td>
<td>Nmole/min • mg&lt;br&gt;2.0 ± 1.1 (10)&lt;br&gt;1.5 ± 0.6 (8)&lt;br&gt;n.s. 3.0 ± 1.9 (8)&lt;br&gt;n.s. 2.1 ± 1.7 (11)&lt;br&gt;n.s. n.s. n.s.</td>
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<tr>
<td>Synthase D + I</td>
<td>Nmole/min • mg&lt;br&gt;36.2 ± 7.2 (10)&lt;br&gt;41.3 ± 3.6 (8)&lt;br&gt;&lt;0.10 33.2 ±21.0 (8)&lt;br&gt;n.s. 33.6 ±25.2 (11)&lt;br&gt;n.s. n.s. n.s.</td>
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<tr>
<td>Synthase I/(D+I)</td>
<td>I/(D+I)&lt;br&gt;0.05± 0.02 (10)&lt;br&gt;0.04± 0.01 (8)&lt;br&gt;n.s. 0.10± 0.06 (8)&lt;br&gt;&lt;0.05 0.07± 0.03 (11)&lt;br&gt;n.s. n.s. n.s.</td>
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metabolizing enzymes appear to be interrelated since, as is shown in Figures 2 and 3, the levels of these enzymes exhibit linear relationships with the amount of glycogen present for the individual mice. Linear regression analysis yields similar slopes for the two lines, 0.69 for glycogen synthase. This suggests that the variability of glycogen content is not due to relative differences in the levels of these enzymes. Although total enzyme activity levels are plotted here, similar profiles are obtained when the data are presented as the activities of the active form versus glycogen concentrations.

The data presented in Table V suggest that the levels of the interconverting enzymes in liver are increased in the vitamin deficiency. Although no significant difference is seen for the activity ratio of phosphorylase kinase, there is a substantial increase in the activity ratio of cAMP-dependent protein kinase. There is also an increase in the activity of phosphorylase phosphatase. A similar larger relative increase in glycogen synthase phosphatase activity as compared to cAMP-dependent protein kinase activity could explain why there is an increase in the relative amount of glycogen synthase I in the deficient state. In the deficient diabetic state, there is a still further increase in the activity of the protein kinase. Correspondingly, the relative amount of glycogen synthase I is no longer significantly different from the B₆ supplemented animal.

The data on another B₆ containing enzyme, aspartate aminotransferase (Table VI), suggest that the decreases observed for glycogen phosphorylase under these experimental conditions are not specific.
Figure 2. The interrelationship between total glycogen synthase activity and liver glycogen content in the B6 deficient mouse.

The data are presented for the determinations from each mouse. The data are for both the deficient nondiabetic (•) and the deficient diabetic (○) mice.
Total Synthase Activity \( \frac{\text{nmole}}{\text{min} \cdot \text{mg}} \)
Figure 3. The interrelationship between total glycogen phosphorylase activity and liver glycogen content in the B₆ deficient mouse.

The data are presented for the determinations from each mouse. The data are for both the deficient nondiabetic (●) and the deficient diabetic (○) mouse.
Total Phosphorylase Activity \((\frac{\text{n mole}}{\text{min} \cdot \text{mg}})\)
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>N_1</th>
<th>D_3</th>
<th>P_1</th>
<th>N_0</th>
<th>P_1</th>
<th>D_0</th>
<th>P_1</th>
<th>P_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase kinase pH 6.8 (nmole/min • mg)</td>
<td>0.14± 0.04</td>
<td>0.13± 0.02 n.s.</td>
<td>0.20± 0.06 &lt;0.05</td>
<td>0.19± 0.08 &lt;0.10 n.s.</td>
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<tr>
<td>Phosphorylase kinase pH 8.2 (nmole/min • mg)</td>
<td>0.15± 0.05</td>
<td>0.13± 0.02 n.s.</td>
<td>0.21± 0.06 &lt;0.10</td>
<td>0.20± 0.09 n.s. n.s.</td>
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<tr>
<td>Phosphorylase phosphatase pH 6.8/pH 8.2 (nmole/min • mg)</td>
<td>0.25± 0.13</td>
<td>0.48± 0.33 &lt;0.05</td>
<td>0.59± 0.14 &lt;0.001</td>
<td>0.65± 0.21 &lt;0.001 n.s.</td>
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<tr>
<td>Protein kinase -cAMP (pmole/min • mg)</td>
<td>38 ±35</td>
<td>30 ±19 n.s.</td>
<td>98 ±56 &lt;0.05</td>
<td>105 ±32 &lt;0.001 n.s.</td>
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<tr>
<td>Protein kinase +cAMP (pmole/min • mg)</td>
<td>63 ±38</td>
<td>50 ±12 n.s.</td>
<td>130 ±43 &lt;0.01</td>
<td>106 ±33 &lt;0.05 n.s.</td>
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<tr>
<td>Protein kinase -cAMP/+cAMP</td>
<td>0.45± 0.27</td>
<td>0.57± 0.27 n.s.</td>
<td>0.82± 0.13 &lt;0.01</td>
<td>1.00± 0.13 &lt;0.001 &lt;0.01</td>
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</table>
Table VI. Aspartate aminotransferase activity

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<tr>
<th></th>
<th>N&lt;sub&gt;3&lt;/sub&gt;</th>
<th>D&lt;sub&gt;3&lt;/sub&gt;</th>
<th>P&lt;sub&gt;1&lt;/sub&gt;</th>
<th>N&lt;sub&gt;0&lt;/sub&gt;</th>
<th>P&lt;sub&gt;1&lt;/sub&gt;</th>
<th>D&lt;sub&gt;0&lt;/sub&gt;</th>
<th>P&lt;sub&gt;1&lt;/sub&gt;</th>
<th>P&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle aspartate aminotransferase (ΔA&lt;sub&gt;340&lt;/sub&gt;/min mg)</td>
<td>0.28±0.08</td>
<td>0.32±0.08</td>
<td>n.s.</td>
<td>0.14±0.05</td>
<td>&lt;0.001</td>
<td>0.14±0.04</td>
<td>&lt;0.001</td>
<td>n.s.</td>
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<td></td>
<td>(10)</td>
<td>(8)</td>
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<td>(8)</td>
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<td>(11)</td>
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</tr>
<tr>
<td>Liver aspartate aminotransferase (ΔA&lt;sub&gt;340&lt;/sub&gt;/min mg)</td>
<td>0.16±0.06</td>
<td>0.22±0.06</td>
<td>&lt;0.05</td>
<td>0.07±0.07</td>
<td>&lt;0.01</td>
<td>0.05±0.04</td>
<td>&lt;0.001</td>
<td>n.s.</td>
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<tr>
<td></td>
<td>(10)</td>
<td>(8)</td>
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<td>(8)</td>
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<td>(11)</td>
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</table>
The relative decreases seen for skeletal muscle and liver aspartate aminotransferases are similar to those observed for glycogen phosphorylase. Various aminotransferases are important in the maintenance of carbohydrate levels by providing gluconeogenic precursors from amino acids. It is thus conceivable that alterations of carbohydrate metabolism under various conditions could be due to changes in the activities of these enzymes.
DISCUSSION

In 1964, Krebs and Fischer (3) proposed that skeletal muscle glycogen phosphorylase serves as a reservoir for vitamin \( B_6 \) based on the substantial amount of the body's content of this vitamin which is contained in the enzyme. Since then, various studies have been reported which seem to support this hypothesis. Phosphorylase activity per gram of muscle or per milligram of protein is significantly reduced in \( B_6 \) deficiency and this reduction is reversed by resupplementation with the vitamin (93-95). Black et al. (91) have reported that rats fed high levels of pyridoxine exhibited increases in skeletal muscle glycogen phosphorylase activity in a constant ratio with the increased \( B_6 \) content of the tissue. Smaller increases, which plateaued within two weeks, were observed for two other \( B_6 \) containing enzymes, alanine and aspartate aminotransferases. A recent study (95) has shown that a further condition is necessary for the depletion of skeletal muscle glycogen phosphorylase. This condition is the starvation which results from the anorexia associated with \( B_6 \) deficiency. These authors further reported that starvation also resulted in reductions of skeletal muscle phosphorylase for rats which had been supplemented with \( B_6 \). Smaller changes were observed for aspartate aminotransferase while no significant reduction was observed for alanine aminotransferase.

Black et al. (95) have reported that aspartate aminotransferase decreases to the same extent as that observed for glycogen phosphorylase during \( B_6 \) deficiency. A similar observation was made here for skeletal muscle. This effect appears to be specific for \( B_6 \) containing enzymes,
since other enzymes concerned with glycogen metabolism do not exhibit corresponding decreases in activity.

The results on liver show that glycogen phosphorylase in this organ exhibits a larger relative decrease in activity during the deficient state when compared to the same enzyme in skeletal muscle. Again there was a corresponding decrease in the activity of another B₆ containing enzyme, aspartate aminotransferase. Muscle glycogen phosphorylase was approximately 55% lower than the control after seven weeks on the deficient diet, whereas liver phosphorylase activity was approximately 80% below the control. Other enzymes associated with glycogen metabolism in the liver are not depleted in the deficiency state.

Skeletal muscle glycogen phosphorylase meets criteria for being a reservoir in that its concentration increases when the vitamin is present in surplus amounts, a large proportion of the body's content of vitamin B₆ is contained in this single enzyme, and deficiency leads to release of the vitamin from this enzyme (3,91,93-95). The results in this report show, however, that deficiency also leads to a decrease in the activity of liver glycogen phosphorylase and both liver and skeletal muscle aspartate aminotransferases. The relative decrease in activity for these enzymes is at least as great as that seen for skeletal muscle glycogen phosphorylase. The effect of these losses on the maintenance of the vitamin pool would not be expected to be as great as the effect of skeletal muscle glycogen phosphorylase depletion because of the smaller relative amounts of these enzymes.
The results obtained here support the studies by McHenry and co-workers (133, 144) suggesting that B₆ deficiency results in alterations of carbohydrate metabolism. These alterations are manifested by a reduction in blood sugar and a large variability in the liver glycogen content. The reduced blood sugar levels do not appear to be a result of a starvation condition since all the deficient animals had lower blood sugar levels independent of whether liver glycogen levels were below normal or well above it. Furthermore, induction of the diabetic state in these animals resulted in the elevation of blood sugar to levels similar to those of mice which had been supplemented with the vitamin. The change in blood sugar levels in the deficient state may be due to changes in the amounts of circulating hormones. If such changes do exist they are not manifested by changes in the activity ratios of either liver glycogen phosphorylase or liver glycogen synthase. Furthermore, Lyon and Porter (94) have reported that B₆ deficiency does not produce changes in glucose-6-phosphatase activity in liver.

The variability of liver glycogen concentrations does not appear to be due to alterations in the activities of glycogen phosphorylase or glycogen synthase. The activity ratios of these enzymes do not exhibit changes correlating with the concentrations of glycogen. The activity levels of the interconverting enzymes do not decrease during the deficiency state, instead they all exhibit significant increases. The levels of these enzymes do not appear to correlate with the levels of liver glycogen synthase activities with glycogen concentration under conditions where the levels of other enzymes involved with glycogen
metabolism do not exhibit corresponding variabilities, suggesting that this phenomenon may be due to the greater affinity of these metabolizing enzymes for the polysaccaride.

A similar condition does not appear to exist in skeletal muscle. There appears to be no corresponding changes between the levels of the glycogen metabolizing enzymes and the levels of glycogen for the individual animals. Furthermore, it has been reported that during surplus B₆ supplementation, where skeletal muscle glycogen phosphorylase increases more than five-fold, no corresponding increase for tissue glycogen is observed (145).

Bosron et al. (135) have reported that B₆ deficiency results in preferential depletion of the cytosolic fraction of pyridoxal phosphate in the liver. Furthermore, these authors reported that glycogen phosphorylase accounts for 10% of the pyridoxal phosphate in this tissue and aspartate aminotransferase accounts for an additional 4 to 7%. The results presented here show that these enzymes exhibit large decreases in activity in the deficient state. These enzymes could account for a significant portion of the loss of the cytosolic pool of the vitamin during deficiency.

Illingworth et al. (93) have suggested that the lack of a change in phosphorylase a activity in skeletal muscle in the deficient state, where total phosphorylase activity was significantly reduced, may be a mechanism to maintain normal glycogen metabolism. If this were the cause, one would not expect to find a concomitant decrease in the percent I of glycogen synthase. This observation suggests that the
actual cause is probably more complex. The increase in phosphorylase a and decrease in synthase I may represent a change in the metabolic state of the tissue. The phosphorylation states of these enzymes are dependent not only on the relative activities of the kinases and phosphatases but also on the levels of various metabolites. The activity states of these enzymes would suggest that skeletal muscle would be more actively degrading glycogen, although glycogen levels in the B₆ deficient animals are not lower than normal.

Diabetes mellitus is evidenced by significant alterations in carbohydrate metabolism. A role for B₆ in this condition has been suggested since treatment of various forms of this disease (e.g., gestational diabetes) with the vitamin improved the glucose tolerance curve but did not affect plasma insulin levels (137). It was of interest, therefore, to investigate the effect of the diabetic condition on the enzyme levels of glycogen metabolism in the normal and B₆ deficient state. The results presented here show that neither of the B₆ containing enzymes examined in this study were significantly altered during the two week diabetic period. Also, the effects of the diabetic condition appeared to be similar in the normal and deficient mouse, even though phosphorylase levels were significantly reduced in the latter.

In summary, the effect of vitamin B₆ deficiency on enzymes involved in glycogen metabolism is evidenced primarily by a decrease in the levels of glycogen phosphorylase. The condition results in a change in the activity ratios of the glycogen metabolizing enzymes in skeletal muscle but not in liver. The levels of the interconverting enzymes exhibit
changes in both kinase and phosphatase activity. The results presented here support the concept of McHenry (144) that carbohydrate metabolism is altered as a result of vitamin B₆ deficiency.
B₆ deficiency results in alterations in glycogen metabolism in the mouse. These alterations are evidenced by a reduction in blood glucose and a large variability for the liver glycogen stores. Induction of the diabetic state in these animals results in an elevation of blood sugar levels to values similar to those seen for B₆ mice which had been supplemented with the vitamin.

B₆ deficiency results in preferential depletion of enzymes which contain the vitamin. Other enzymes associated with glycogen metabolism do not exhibit large decreases in activity. In fact, the interconverting enzymes in liver exhibit significant increases in activity in the deficient state. Important differences exist between skeletal muscle and liver in terms of the effect of B₆ deficiency on glycogen metabolism. Skeletal muscle exhibits an increase in the activity ratio of glycogen phosphorylase and a decrease in the activity ratio of glycogen synthase, suggesting an altered metabolic state. Liver does not exhibit similar changes, but shows a variance in the activities of these enzymes for the individual mice which correlates with the variability of glycogen content. The results also show that B₆ deficiency results in a larger decrease in activity for liver enzymes than for those of skeletal muscle.
Thanks are extended to the following people for performing assays during this experimentation: Drs. Sau Chi Betty Yan, Mikihiro Kobayashi, and Joyce Miller, as well as Bill Harris, Mike Hurst, Lee Graves, Chang Yem Chung, and Jesse Chan. Thanks are also extended to Terry Brooks for care of the animals.


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96. Withers, S. G., Sykes, B. D., Madsen, N. B. and Kasvinsky, P. J. (1979), Biochemistry 18, 5342.


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Thanks are also extended to the members of his research group for many stimulating discussions.