STUDIES ON THE MECHANISM OF ACTIVATION AND DEACTIVATION OF PHOSPHORYLASE KINASE FROM RABBIT SKELETAL MUSCLE

BY

TOOLSEE J. SINGH, B. Sc. (Hons.)

A Thesis

Presented to the

Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. Jerry H. Wang for his close supervision and enthusiasm during the course of this work. His comments on this manuscript during its preparation are highly appreciated.

I am grateful to Drs. K. Dakshinamurti and R. L. Khandelwal for reading this manuscript before it was typed in its final form and for making valuable suggestions. I am also thankful to Dr. Khandelwal for helpful discussions concerning the dephosphorylation of activated phosphorylase kinase.

Finally, Miss B. Persaud is to be thanked for typing this manuscript.

ABSTRACT

In this study the activation of phosphorylase kinase by the protein kinase-catalyzed reaction and deactivation of different phosphorylated forms of the enzyme by a homogeneous preparation of a phosphoprotein phosphatase were investigated. The stimulation of the nonactivated kinase by organic solvents and fatty acids was also examined.

In the presence of 10 mM Mg^{2+}, phosphorylase kinase can be activated to very high levels by the cAMP-dependent protein kinase-catalyzed reaction. These levels are much higher than those observed previously when the reaction was studied at 1 to 2 mM Mg^{2+} (Cohen, P. (1973) Eur. J. Biochem. 34, 1-14; Hayakawa, T. Perkins, J. P., and Krebs, E. G. (1973) Biochemistry 12, 574-580). The activation of phosphorylase kinase seems to occur in two phases. At low Mg^{2+} (1 to 2 mM) only the first phase is manifested and involves the incorporation of 2 moles of phosphate, 1 mole into each of subunits A and B. This results in a 30 to 50 fold activation of the enzyme. At high Mg^{2+} (10 mM), 7 to 9 moles phosphate could be incorporated, the additional sites phosphorylated being on subunit A. The extent of activation achieved by the kinase is 2 to 3 times higher than that observed when the reaction is studied at low Mg^{2+}. With substrates of protein kinase other than phosphorylase kinase the same extent of phosphorylation is achieved at low and high Mg^{2+}. This suggests that Mg^{2+} may be altering the conformation of phosphorylase kinase in such a way as to render more phosphorylation sites accessible to protein kinase.

Phosphorylase kinase activation could be reversed by a phospho-

protein phosphatase of broad substrate specificity (Khandelwal, R. L., Vandenheede, J. R., and Krebs, E. G. (1976) J. Biol. Chem. 251, 4850-4858). This phosphatase incompletely dephosphorylates phosphorylase kinase. A clearly defined resistant phase is observed regardless of the initial phosphorylation state of phosphorylase kinase used as a substrate for the phosphatase. Mg^{2+} or Mn^{2+} ions do not stimulate the dephosphorylation of this resistant phase. Examination of the subunit dephosphorylation pattern revealed that the release of ^{32}P radioactivity from subunit B is very rapid compared to subunit A. The resistant phase seems to reflect the dephosphorylation mainly of subunit A. For phosphorylase kinase containing 0.5 to 2 moles of phosphate, subunit B dephosphorylation results in the almost complete reversal of the kinase to the nonactivated state. However, phosphorylase kinase containing 5 to 10 moles of phosphate lost only 50 to 60% of its activity when subunit B dephosphorylation was complete. The remaining 40 to 50% activity is lost when sites on subunit A are dephosphorylated. These sites on subunit A can be rapidly dephosphorylated by a phosphatase present in both liver and skeletal muscle homogenates.

The activity of nonactivated phosphorylase kinase can be stimulated by high concentrations (1 M) of various organic solvents at pH 6.8 In increasing order of effectiveness methanol, 1-propanol, methyl isobutyl ketone, ethyl formate, formamide, ethanol, dimethyl sulfoxide, 2-propanol, tetrahydrofuran and acetone stimulated the kinase activity from 3-fold (methanol) to 28-fold (acetone). At higher concentrations ethanol was the most effective solvent. At 1.72 M a 46-fold enhancement in kinase activity was achieved. If phosphorylase kinase is

first activated by phosphorylation only a small degree of stimulation of its activity by organic solvents is observed. The stimulation of the nonactivated kinase by organic solvents is largely, but not totally, reversible upon removal of the solvent. In the presence of organic solvents, phosphorylase kinase is not dissociated to lower M. W. species nor does it lose its dependency on Ca^{2+} ions for activity. The stimulation of casein phosphorylation and the autophosphorylation of the kinase by organic solvents suggests that the stimulatory effect of organic solvents is directly on the phosphorylase kinase molecule. Further, the unsaturated fatty acids, palmitoleic and oleic acids, can also enhance the activity of nonactivated phosphorylase kinase. A 6-fold stimulation could be achieved at low concentrations (0.8 mM) of these fatty acids. These studies suggest that phosphorylase kinase may exhibit high activity without undergoing covalent modification.

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ABBREVIATIONS

5' - AMP, adenosine 5' - monophosphate

ATP, adenosine triphosphate

cAMP, adenosine 3', 5' - monophosphate

cGMP, guanosine 3', 5' - monophosphate

DTNB, 5, 5' - dithiobis -(2-nitrobenzoic acid)

EDTA, ethylenediamineteraacetic acid

EGTA, ethylene glycol bis (β- aminoethyl ether) - N, N' - tetraacetic acid

Mes, 2-(N-morpholino) - ethanesulfonic acid

Monomeric Unit, the minimal M. W. of phosphorylase kinase, 318,000 (116)

M. W., molecular weight

P_i, inorganic phosphate

SDS, sodium dodecyl sulfate

Tes, N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid

TN-I, inhibitory subunit of troponin

TN-T, tropomyosin-binding subunit of troponin

I. INTRODUCTION

Phosphorylase kinase (ATP: phosphorylase phosphotransferace, EC 2.7.1.38) is of pivotal importance in the enzyme system responsible for the glycogenolysis in skeletal muscle (1, 2, 3,). The enzyme is composed of three types of subunits, A, B, C and has the molecular structure A_4 B_4 C_4 (4). It can exist in nonactivated and activated states. The nonactivated enzyme can be converted to the activated form by protein phosphorylation. Two mechanisms have been elucidated for the enzyme activation. One of these is catalyzed by the cAMP-dependent protein kinase and the other by phosphorylase kinase itself (5). These mechanisms have been studied in great detail (4, 6, 7, 8). Both modes of activation involve the phosphorylation of subunits A and B. The incorporation of 1 mole of phosphate into each of subunits A and B by the protein kinase-catalyzed reaction result in a 30 to 50-fold activation of phosphorylase kinase. Enzyme activation seems to correlate best with the phosphorylation of subunit B (4). The autoactivation of phosphorylase kinase results from the incorporation of 7 to 9 moles of phosphate per monomeric unit of the kinase with subunit A phosphorylation being greater than that of subunit B in the ratio 2.5:1 (7). Enzyme activation can reach as high as 200-fold (8) and seems to depend on the phosphorylation of both subunits A and B (7).

An evaluation of the activation of phosphorylase kinase resulting from the autocatalytic and protein kinase-catalyzed reactions revealed that non-identical sites may be phosphorylated on the kinase by the two different mechanisms (7). While trying to reproduce this and similar results it was observed that in the presence of high concentrations of Mg^{2+}, phosphorylase kinase can be activated by the protein kinase-catalyzed reaction to higher levels than previously observed (9). In

fact, total phosphate incorporation, the fold enzyme activation and the phosphorylation pattern of the subunits are very similar to that observed for activation by the autocatalytic mechanism. The activation of phosphorylase kinase catalyzed by protein kinase at high Mg^{2+} concentrations has been characterized and compared to the autoactivation of the enzyme. The possible mechanism of the Mg^{2+} effect is discussed.

Phosphorylase kinase activation resulting from phosphorylation of the enzyme can be reversed by phosphoprotein phosphatase(s). Preliminary studies (10, 11, 12) on the reversal of the kinase activation by protein dephosphorylation have been attempted. However, detailed analysis of the dephosphorylation of phosphorylase kinase phosphorylated to different levels and by different mechanisms have not been done. In the present study phosphorylase kinase labelled at approximately 1, 2 or 5 to 7 sites by the cAMP-dependent protein kinase and 9 to 10 sites by the autocatalytic reaction were used as substrates for a homogeneous preparation of phosphoprotein phosphatase (13). The loss of enzyme activity was correlated with the dephosphorylation of particular subunits. The influence of the initial phosphorylation state of the phosphorylase kinase molecule on enzyme deactivation will be considered. An evaluation will be made on the importance of the phosphorylation state of individual subunits in influencing the dephosphorylation of other subunits. Also the preference of the phosphatase for kinase phosphorylated by the protein kinase-catalyzed reaction or by autophosphorylation will be assessed.

The possibility of an efficient activation of phosphorylase kinase by a non-covalent mechanism was also investigated. Previously glycogen, heparin (14), Mg^{2+} (15, 16) and neutral salts (17) were shown to

stimulate nonactivated phosphorylase kinase by various small extents. In addition, Ca^{2+} ions have been shown to be essential for the kinase activity (5, 18, 19). Hence, it has been hypothesized that glycogenolysis can be coupled to muscular contraction by the stimulation of phosphorylase kinase by Ca^{2+} ions released from the sarcoplasmic reticulum. However, it has been demonstrated that phosphorylase kinase is not converted to the activated form during the electrical stimulation of muscle (20, 21, 22) and the nonactivated kinase shows relatively low activity even in the presence of saturating amounts of Ca^{2+}. Such low activity cannot account for the rapid conversion of phosphorylase b to the a form observed during neural stimulation of muscle. Hence the possibility was considered that activators of phosphorylase kinase may exist that allow the enzyme to exhibit high activity under certain conditions without the need to be activated by phosphorylation. During the course of this study it was observed that high concentrations of various organic solvents can stimulate phosphorylase kinase in a largely reversible manner. The fold stimulation of the kinase activity achieved is similar to that obtained when two sites on the enzyme are phosphorylated (4). In addition unsaturated fatty acids were found to stimulate the nonactivated kinase 6 to 7 fold at low concentrations. The significance of these findings is discussed.

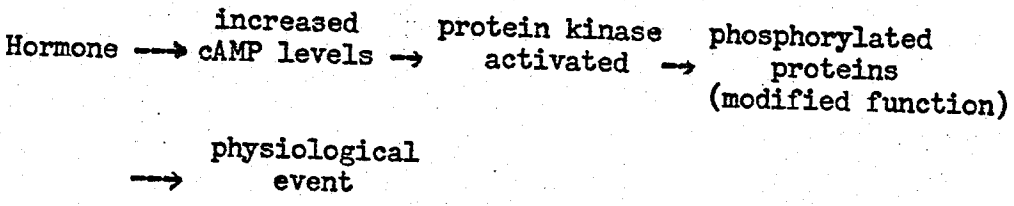
II. LITERATURE REVIEW

The literature review will be confined largely to an examination of the properties and detailed regulation of phosphorylase kinase. Hence, Section II. A is meant to be only a general introduction to the already large and rapidly expanding field of protein phosphorylation. More detailed accounts on the properties and regulation of protein kinases have been given elsewhere (3, 23, 24, 25, 26). Studies on some of the cellular processes controlled by phosphorylation-dephosphorylation reactions have also been documented. These include glycogen metabolism (2, 26, 27, 28,), lipid metabolism (29-34), protein biosynthesis (35-39), nervous function (40-44), membrane transport (45-49, 50-53) and smooth muscle contraction (54-56), among others.

A. Protein Phosphorylation and the Regulation of Cellular Function

Studies on glycogen phosphorylase revealed for the first time that protein phosphorylation is a potentially important mechanism for controlling cellular function. In the early stages of their studies on muscle phosphorylase, Cori and coworkers recognized that the enzyme exists in two enzymatically interconvertible forms called phosphorylase b and phosphorylase a, (57 - 58). Phosphorylase a is the physiologically active form of the enzyme (59). The conversion of phosphorylase b to the a form was shown to be catalyzed by a protein kinase (later called phosphorylase b kinase) and to involve phosphorylation of the enzyme (60) . The enzyme catalyzing the reverse reaction was called phosphorylase phosphatase (61).

Since the original discovery of cAMP by Sutherland and Rall (62) this nucleotide has been shown to affect numerous cellular processes (3, 63, 64). Many hormones can increase the levels of cAMP in target cells (64) and the nucleotide has been proposed to act as a "second messenger" (65) in these cells. The mechanism by which cAMP carries out its role as second messenger was solved by the discovery of a protein kinase (66) that could be activated by the nucleotide. The broad substrate specificity of the cAMP-dependent protein kinase (66) as well as its ubiquitous distribution (67) has led to the hypothesis that all actions of cAMP in higher organisms are mediated by protein kinase-catalyzed phosphorylation reactions (3, 67) . Scheme A outlines a postulated mechanism (3) to explain the mode of action of those hormones that cause an increase in the cellular level of cAMP.



Scheme A

Protein kinases have arbitrarily been divided into two classes on the basis of their response to cyclic nucleotides. The cyclic nucleotide-dependent protein kinases are active only in the presence of these nucleotides whereas the independent kinases are equally active either in the presence or absence of cyclic nucleotides. With the exception of a few cases, little is known about the regulation of the cyclic nucleotide independent kinases even though these have been purified and characterized

in many cases (68-76, 77) . The regulation of phosphorylase kinase is now understood in detail (see below). A cyclic nucleotide independent kinase that has been termed glycogen synthase kinase 2(71, 78, 79) was purified from skeletal muscle and shown to catalyze the phosphorylation and inactivation of glycogen synthase (79-81). A protein kinase that is converted from an inactive to an active form by phosphorylation and is involved in the control of protein synthesis in rabbit reticulocytes has been chacterized (37, 38, 75, 76, 82).

The cyclic nucleotide-dependent protein kinases can be subdivided on the basis of whether they are cAMP-dependent or cGMP-dependent. These kinases have a high affinity for cAMP and cGMP, respectively. Both classes of protein kinases show a wide distribution among mammalian tissues (67,83,84). In addition, these kinase activities have been detected in both the soluble and particulate fractions of cells (85-90). The soluble enzymes from a number of tissues have been extensively purified and characterized.

The cAMP-dependent enzyme can be subdivided into type I and type II protein kinases (91). The type I kinase from skeletal muscle (26, 92, 93) and the type II kinase from heart (23,94,95) have been purified to homogeneity. The activation of these protein kinases by cAMP was shown to involve the binding of the nucleotide to the holoenzyme and the subsequent dissociation of the latter into catalytic (C) and regulatory (R) subunits (96-98) as shown in Scheme B.

$$R_2 C_2 + 2\ cAMP \longrightarrow R_2 \cdot (cAMP)_2 + 2C$$

Scheme B

The type II protein kinase catalyzes the autophosphorylation of its R subunits. As a result the phosphorylated enzyme apparently binds cAMP with increased affinity and its dissociation into C and R subunits is facilitated (98, 99, 100). The type I enzyme is not phosphorylated (98). Both type I and II protein kinases are inhibited by a heat-stable protein inhibitor (101).

The cGMP-dependent protein kinase from several invertebrate (102-104, 105) and vertebrate (106, 107, 108-114) tissues has been characterized. The enzyme from lobster muscle (115) can apparently be dissociated, like the cAMP-dependent enzymes, into catalytic and regulatory subunits. However, a homogeneous preparation of cGMP-dependent protein kinase from bovine lung was shown to consist of two identical subunits each of which could bind cGMP and catalyze protein phosphorylation (111). Hence, the mammalian cGMP-dependent kinase does not seem to be composed of separate catalytic and regulatory subunits. A protein modulator that could stimulate the activity of the cGMP-dependent protein kinase has been described (116-118).

The change in cellular functions promoted by the protein kinase-catalyzed phosphorylation of certain proteins (Scheme A) can be reversed by the action of specific protein phosphatases on these phosphoproteins. Whether multifunctional phosphoprotein phosphatases exist, by analogy with the protein kinases, is still to be established beyond doubt. In a number of cases (13,119,120) phosphoprotein phosphatases have been purified to homogeneity and proved to be multifunctional. However, these phosphatases have M_r = 35,000 and may represent the catalytic subunit of a larger holoenzyme (121-123).

Many proteins have been shown to act as substrates for protein

kinases. Enzymes which are regulated by protein phosphorylation catalyzed by the cAMP-dependent protein kinase include phosphorylase kinase (see below), glycogen synthase (26), hormone-sensitive lipase (29), L-type pyruvate kinase (124), fructose 1, 6-biphosphatase (125), phenylalanine hydroxylase (126,127) and several others (32,34,128-137). Various non-enzymic proteins are also phosphorylated either by cAMP-dependent or independent kinases (28). Some of these have been demonstrated to occur in vivo and include the phosphorylation of histone and non-histone proteins (40,138-140), membrane proteins (28, 141-144) and cardiac troponin (145, 146). Relatively few proteins have been shown to be substrates for the cGMP-dependent protein kinase (147-149, 150, 151) and the role of this enzyme in cellular regulation remains largely obscure.

B. Properties of Phosphorylase Kinase

Phosphorylase kinase (ATP: phosphorylase phosphotransferace, EC 2.7.1.38) is a key enzyme in the glycogenolytic pathway of skeletal muscle and other tissues. The different sources from which the enzyme has been studied include brain (152), heart (153-157), liver (3,158-162), adipose tissue (163, 164), dogfish muscle (165) and yeast (166). However, the greatest amount of information has been accumulated on the enzyme from skeletal muscle. Hence, only studies dealing with the skeletal muscle enzyme will be reviewed here.

Phosphorylase kinase from rabbit skeletal muscle has been purified to homogeneity and characterised independently by two laboratories (4 , 167). This section discusses some of the more important properties of the phosphorylase kinase macromolecule.

1. Molecular Weight and Subunit Structure

Phosphorylase kinase is a very large molecule having an average M. W. of 1.28×10^6 (4) or 1.33×10^6 (167) as determined by sedimentation equilibrium studies. Electrophoretic analysis in the presence of sodium dodecyl sulfate revealed three types of subunits, designated α, β, γ (4) or A, B, C (167). The molecular weights of these subunits were determined to be 119,000—145,000, 108,000—128,000, and 41,000—45,000, respectively (4, 167). Cohen (4) found that the three types of subunits exist in equimolar quantities and therefore assigned a molecular formula of $A_4 B_4 C_4$ for the enzyme. Hayakawa et al. (167) proposed a formula of $A_4 B_4 C_8$.

2. Two Forms of Phosphorylase Kinase

Early studies on phosphorylase kinase revealed that this enzyme, like phosphorylase, is also capable of existing in two separate molecular forms: a phosphorylated and a nonphosphorylated form (14, 168). The nonphosphorylated form, also referred to as nonactivated phosphorylase kinase, is essentially inactive below pH 7 but shows significant activity at higher pH values. The phosphorylated or activated form of phosphorylase kinase is much more active than the nonactivated form below pH 7 and only slightly more active at high pH values (1, 168). An increase in the ratio of enzyme activity at pH 6.8 to activity at pH 8.2 has been used as an index of phosphorylase kinase activation which occurs *in vivo* as well as *in vitro* (14,20,169-173). Nonactivated phosphorylase kinase exhibits a pH 6.8/8.2 ratio of 0.02-0.05 (174) compared to 0.04 or higher for the activated form (4, 6, 18). Nonactivated phosphorylase kinase can be converted to the activated form by protein phosphorylation (see Section II. C).

3. Requirement for Calcium Ions

Phosphorylase kinase, either in the nonactivated or activated form, shows an obligatory requirement for low concentrations of calcium ions for its activity (5,18,19,175,176). The calcium-free nonactivated enzyme is thought to be totally inactive (5, 176). The purified enzyme contains both high and low-affinity calcium binding sites (5). Nonactivated phosphorylase kinase has apparent K_m values of 0.13 and 3 μM for Ca^{2+} at pH 8.2, while the activated enzyme has only one apparent K_m value of 0.22 μM at this pH.

4. Substrate Specificity

a) Phosphorylase b

Phosphorylase kinase catalyzes the conversion of phosphorylase b to phosphorylase a (60,177,178):

2 phosphorylase b + 4ATP ⟶ phosphorylase a + 4ADP

In this reaction the terminal phosphate group of ATP is transferred to a specific serine residue in phosphorylase (177, 179). Hence four moles of phosphate become incorporated per mole of phosphorylase a with the resultant full activation of the phosphorylase molecule. The above reaction is thought to be irreversible physiologically but phosphorylase b can be reformed through the action of a specific phosphorylase phosphatase (61, 180) . This latter enzyme hydrolyzes the phosphoester bonds in phosphorylase a.

b) Synthetic Peptides

Phosphorylase kinase can also catalyze the phosphorylation of various synthetic peptides (181, 182, 183). These peptides have different sequences but are all derived from the sequence, Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu. This latter peptide is

found in phosphorylase b itself. It contains the serine residue that becomes phosphorylated when either native phosphorylase b or the peptide is used as a substrate for phosphorylase kinase (184). However, native phosphorylase b is a better substrate than this tetradecapeptide (183). However, the latter permitted an examination of the specificity of phosphorylase kinase to be made (181). By manipulating such parameters as size and charge as well as substituting for different amino acid residues in the peptide, conclusions were made about which amino acids were essential or nonessential (181, 183). For instance, it was found that if residues surrounding the phosphorylatable serine (i.e. in the sequence Lys-Gln-Ile-Ser-Val-Arg) were substituted, a large decrease or total loss in phosphorylation capacity occurred (181). The tetradecapeptide and its derivatives have been shown to stimulate the autophosphorylation of phosphorylase kinase (8).

c) Phosphorylase Kinase

Phosphorylase kinase can catalyse its own phosphorylation and resultant activation (174, 185). This reaction will be dealt with in greater detail in Section II. C.

d) Other Proteins

In addition to using itself and phosphorylase b as substrates, phosphorylase kinase can catalyze the phosphorylation of a few other proteins.

(i) Casein

Delange et al. (174) found that phosphorylase kinase could use casein as a substrate. Although the activated enzyme catalyzed a two-fold higher rate of phosphorylation compared to the nonactivated enzyme, the overall incorporation of labelled phosphate into casein was

very low.

(ii) Histone

It was shown (2) that phosphorylase kinase is capable of phosphorylating several histone fractions. However, in the course of doing so, phosphorylase kinase itself becomes phosphorylated. This is the case because highly basic proteins such as histones can stimulate the autophosphorylation of phosphorylase kinase (2 , 8) . Disc gel analysis of histone phosphorylated by protein kinase and phosphorylase kinase, revealed that the pattern of phosphate incorporation into the different histone fractions was different for the two kinases (2).

(iii) Troponin

Phosphorylase kinase catalyzes the phosphorylation of troponin isolated from either skeletal or cardiac muscle. However, the rate of phosphorylation is low compared to that of phosphorylase b. Two of the three subunits of the troponin complex, TN-I and TN-T, serve as phosphate acceptors (186-190, 191). However, phosphorylase kinase seem to preferentially catalyze the phosphorylation of TN-T and TN-I in troponin isolated from skeletal and cardiac muscle, respectively (165, 190). The finding that freshly isolated troponin can contain up to 1 mole of covalently bound phosphate (190, 191) and that non-activated and activated phosphorylase kinase phosphorylate the TN-I subunit at the same rate (186), indicate that troponin phosphorylation can possibly be controlled by Ca^{2+} fluxes during muscular contraction. From the observation that in intact hearts the force of contraction is related to TN-I phosphorylation (145), it seems that troponin phosphorylation does indeed play an important physiological role.

(iv) Membranes

It has been reported that protein kinase-catalyzed phosphorylation of cardiac sarcoplasmic reticulum enhances the uptake of Ca^{2+} by this membrane. A membrane protein of M_r = 22,000 was shown to be phosphorylated (49,51,52,192). More recently, phosphorylase kinase has been shown to stimulate the uptake of Ca^{2+} by both cardiac and skeletal muscle sarcoplasmic reticulum (53) . This kinase catalyzed the phosphorylation of a membrane protein M_r = 95,000 thereby enhancing Ca^{2+} uptake by 48% in cardiac muscle and up to 60% by skeletal muscle sarcoplasmic reticulum. Similar findings of phosphorylase kinase-catalyzed phosphorylation and increased Ca^{2+} uptake have also been reported for cardiac sarcolemma (193).

5. Additional Properties

Phosphorylase kinase is a globular protein with a frictional ratio of 1.17 (167), an isoelectric point of 5.77 (167), an $A_{280}^{1\%}$ of 11.8 (167) or 12.4 (4), and a partial specific volume of 0.735 ml/gm (4).

The enzyme contains approximately 160 moles of cysteine per mole of protein. Of these, 20 residues react rapidly with DTNB with no loss of activity. The remaining cysteines react more slowly, and their titration inactivates the enzyme (167).

Phosphorylase kinase can be inhibited or stimulated under different conditions. Glucose and glucose-6-phosphate inhibit whereas glycogen and heparin could stimulate the nonactivated enzyme (14) . Low concentrations (0.1 M) of various neutral salts suppress the kinase activity; higher concentrations stimulate it (17) . The autophcsphorylation of phosphorylase kinase is inhibited by inorganic phosphate as

well as different phosphorylated metabolites (7) but this reaction is enhanced in the presence of substrates of the kinase (8). Free Mg^{2+} ions have also been demonstrated to stimulate phosphorylase kinase activity (194).

C. Activation of Phosphorylase kinase

Phosphorylase kinase isolated from rabbit skeletal muscle is normally in the nonactivated form (4, 167). In this form the enzyme is essentially inactive below pH 7, but can be converted to the activated form which shows high activity at this pH (1, 168). Two mechanisms can be used *in vitro* to activate the enzyme: phosphorylation and limited proteolysis.

1. Phosphorylation

Early studies on phosphorylase kinase showed that the enzyme becomes activated after incubation in the presence of ATP and Mg^{2+} (14) . Subsequent work proved that this activation was the result of phosphorylation of the phosphorylase kinase molecule (1, 174). In the course of these studies, it was observed that adenosine 3', 5'-monophosphate (cAMP) could cause a stimulation in the levels of both the phosphorylation and activation of phosphorylase kinase (1 , 14, 168, 174, 195). The extent of stimulation was much greater for the activation compared to the phosphorylation of the enzyme (174).

Since activation of phosphorylase kinase occurred in the absence of cAMP but was accelerated in the presence of this nucleotide, it was postulated that two separate mechanisms may be responsible for the activation of phosphorylase kinase (1, 174). This idea was further supported when it was found that no appreciable binding of cAMP to

phosphorylase kinase occurred during activation of this enzyme (174). In addition, the effect of cAMP was very marked in the presence of small amounts of crude muscle extracts (1), thus implying that a special factor (or enzyme) exists in the extracts that can interact with cAMP in some way and so cause an increased level in phosphorylase kinase activation.

In an elegant study Walsh et al. (185) proved conclusively that phosphorylase kinase can be activated by two separate mechanisms. One of these reactions is catalyzed by phosphorylase kinase itself (i.e. the reaction is autocatalytic), while the other is catalyzed by the cAMP-dependent protein kinase. In addition, more recent studies have demonstrated that phosphorylase kinase can be activated by a cGMP-dependent (150, 151), a protease-activated (196) and a modulator-dependent (77) protein kinase. These different mechanisms of activation of phosphorylase kinase will be considered in turn.

a) Catalyzed by the cAMP-dependent Protein Kinase

Walsh et al. (66) were the first to demonstrate that skeletal muscle extracts contain a kinase that could catalyze the activation of phosphorylase kinase. This enzyme has turned out to be the now familiar and well characterized cAMP-dependent protein kinase (see Section II. A). The activation reaction for phosphorylase kinase can be written:

$$\text{nonactivated phosphorylase kinase} \xrightarrow[\text{cAMP, ATP, } Mg^{2+}]{\text{protein kinase}} \text{activated phosphorylase kinase} \quad \text{(I)}$$

Since phosphorylase kinase is a Ca^{2+}-dependent enzyme (5) the above reaction can be studied in the absence of autoactivation by inclusion of the Ca^{2+} chelator, EGTA, in the reaction mixture. Further, the use of low ATP-Mg^{2+} in reaction (I) will keep activation by the autocatalytic

mechanism to a minimum (185). Phosphorylase kinase has a K_m for ATP of about 0.4 mM (14) compared to 0.01 mM (92) for protein kinase.

The protein kinase-catalyzed activation of phosphorylase kinase has been studied independently by two laboratories (4 , 6). Cohen (4) found that a total of 2 moles of phosphate were incorporated into the fully activated enzyme. Hayakawa et al. (6) found 0.8 to 1.5 moles. Correlation between enzyme activity and phosphorylation of individual subunits of phosphorylase kinase showed that the rapid increase in activity at pH 7 seem to correlate best with the phosphorylation of subunit B (4). Hayakawa et al. (6) found this correlation at pH 8.2; enzyme activity measured at pH 6.8 followed an independent course. Both laboratories found that there was an initial lag in subunit A phosphorylation, which only started after subunit B was already half-maximally phosphorylated. Whereas Cohen (4) found no additional increase in enzyme activity due to subunit A phosphorylation at pH 7.0, Hayakawa et al. observed that both enzyme activity and subunit A phosphorylation continued to increase at pH 6.8 long after subunit B phosphorylation seem to have ceased. A maximum of up to 1 mole of phosphate could be incorporated into subunit A under these circumstances compared to only about 0.45 mole for subunit B over a 60 min incubation period. Cohen (4) observed that subunits A and B each incorporated 1 mole of phosphate when the enzyme was maximally phosphorylated. Subunit C was reported not to be phosphorylated (4 , 6).

b) Catalyzed by Phosphorylase Kinase

Phosphorylase kinase activation by the autocatalytic mechanism was neglected until quite recently. Activation by this reaction was observed to be very slow and hence thought not to be important

physiologically (6, 185, 197). Even under favorable conditions, the extent of enzyme activation appears to be much lower than that catalyzed by protein kinase (4 , 6). However, two recent studies (7 , 8) suggest that autoactivation may indeed be important in regulating phosphorylase kinase activity.

In the presence of Ca^{2+}, ATP and Mg^{2+}, phosphorylase kinase can catalyze its own phosphorylation (6, 185):

$$\text{Nonactivated phosphorylase kinase} \xrightarrow[Ca^{2+},\ ATP,\ Mg^{2+}]{\text{phosphorylase kinase}} \text{Activated phosphorylase kinase} \qquad \text{(II)}$$

Since phosphorylase kinase is normally contaminated with trace amounts of protein kinase (185), a detailed study of reaction (II) can be undertaken only if all potential protein kinase activity is suppressed. This can be achieved by inclusion of the heat-stable protein inhibitor (101) in the autoactivation mixture. The inhibitor selectively binds to protein kinase and so blocks reaction (I) (198, 199).

The autophosphorylation of phosphorylase kinase has been reported to result in only a 3-fold increase in enzyme activity (6). Even after extended periods of incubation (up to 3 hr) incorporation of phosphate into the kinase molecule was low. Subunit B incorporated only about 0.2 moles phosphate compared to 0.75 moles incorporated by subunit A. Later work (7 , 8) suggests that the conditions used for the above study may have been non-optimal.

Wang et al. (7) found that the autocatalytic reaction can be severely inhibited by inorganic orthophosphate or organic phosphates such as β-glycerophosphate, fructose-1-phosphate, glucose-1-phosphate, glucose-6-phosphate, 2-phosphoglycerate and 2, 3-diphosphoglycerate. Since previous

studies (6, 185) were carried out using β-glycerophosphate, it would therefore be expected that both phosphate incorporation and enzyme activation would be low. However, autoactivation carried out in the presence of Mes instead of β-glycerophosphate buffer resulted in the incorporation of up to 7-9 phosphates per monomeric unit of phosphorylase kinase (7). Enzyme activation resulting from this phosphorylation was at least two-fold higher compared to the protein kinase-phosphorylated enzyme. These results seem to agree with those of another study (8) in which a high level of phosphate incorporation (up to 6 moles per monomeric unit) and enzyme activation were observed for the autocatalytic reaction. Analysis of the autoactivated kinase showed that all the phosphate incorporated were associated with subunits A and B. Subunits C was not phosphorylated. The ratio of the phosphate in subunits A and B was found to be 2.5:1 (7).

Under certain conditions the autocatalytic reaction can be stimulated. It was previously shown (174) that glycogen can increase the rate of autophosphorylation by 2-fold. Subsequent work proved that protein substrates of phosphorylase kinase can also stimulate. Both phosphorylase b and a tetradecapeptide corresponding to residues 5-18 at the amino terminus of glycogen phosphorylase (184) could stimulate autophosphorylation (8). This tetradecapeptide is a substrate of phosphorylase kinase (see Section II. B. 4. b) and in its presence phosphorylase kinase activity increases 13-fold compared to only 4-fold in its absence. At the same time the apparent rate of phosphorylation of subunits A and B was stimulated 5-fold (8). Subsequent studies demonstrated that high concentrations (0.9 M) of neutral salts can increase the rate of autophosphorylation up to 4-fold (17).

c) Catalyzed by other Protein Kinases

Besides the cAMP-dependent protein kinase and phosphorylase kinase itself, other protein kinases have been shown to activate phosphorylase kinase. A homogeneous preparation of cGMP-dependent protein kinase from bovine lung was shown to catalyze the phosphorylation (150) and activation (151) of phosphorylase kinase. Up to 5 moles of phosphate per monomeric unit were incorporated after 60 min at 30° (150). The degree of phosphorylation and activation of phosphorylase kinase that could be achieved by the cGMP-dependent protein kinase was the same as that effected by the cAMP-dependent protein kinase. However, higher concentrations of the cGMP-dependent enzyme were required (150, 151).

A protein kinase that is activated by a Ca^{2+}-dependent protease (200) was partially purified from rat brain (201). This enzyme, either in its inactive or active forms, does not bind cyclic nucleotides and is not inhibited by EGTA or the protein inhibitor of the cAMP-dependent protein kinase. In its active form the protein kinase could activate phosphorylase kinase (196). Up to 2 moles of phosphate per monomeric unit were incorporated, 1 mole into each of subunits A and B. Subunit B was phosphorylated at a faster rate than subunit A, an observation that is similar to the case when the cAMP-dependent protein kinase is used to phosphorylate phosphorylase kinase (4 , 6).

Yet another apparently different protein kinase has been recently reported to activate phosphorylase kinase (77) . This kinase is activated by the Ca^{2+}-dependent modulator (202-204), a protein known to be the activator of several other enzymes (205-210). The kinase and modulator protein when present together caused a marked stimulation of phosphorylase kinase activation. No activation was observed when the

protein kinase alone was added to the phosphorylation mixture (77). These studies were done with partially purified protein kinase preparations. This enzyme has now been obtained in homogeneous form and has been observed not to catalyze the activation of phosphorylase kinase (211). The activation of phosphorylase kinase observed in the earlier study (77) is probably due to the direct effect of the modulator protein on phosphorylase kinase.

2. Proteolysis

Treatment of phosphorylase kinase either with trypsin or a Ca^{2+}-dependent protease found in skeletal muscle results in activation of the enzyme (14, 212, 213). This activation was later shown to result from limited proteolysis of the phosphorylase kinase molecule (4, 6). When the time course of trypsin activation of phosphorylase kinase was correlated with the SDS-gel patterns at the different time intervals, it was found that subunit A was completely degraded in less than 2 min. At the same time the enzyme activity reached 80% of its maximal value. Further incubation resulted in the gradual disappearance of the B subunit with an additional small increase in enzyme activity. Subunit C was not degraded by trypsin (4, 6). After subunits A and B are completely degraded by trypsin, the smaller subfragments formed still show kinase activity indicating that the large molecular weight of the native enzyme is not essential for the conversion of phosphorylase b to the a form (214).

D. Reversal of Phosphorylase Kinase Activation

Several enzymes have been reported to exist in phosphorylated and dephosphorylated forms (see Section II. A). The phosphorylation of many

of these regulatory enzymes is catalyzed by the well studied multifunctional cAMP-dependent protein kinase. The reverse process of dephosphorylation is catalyzed by phosphoprotein phosphatases. In this section, progress in delineating the mechanism of dephosphorylation of phosphorylase kinase will be evaluated.

1. Early Studies

The reversal of phosphorylase kinase activation by a protein phosphatase was studied for the first time by Krebs and coworkers (11). They partially purified phosphorylase kinase phosphatase from rabbit skeletal muscle and showed that this enzyme could release $^{32}P_i$ from phosphorylase kinase previously labelled using [γ-^{32}P] ATP. This dephosphorylation led to an inactivation of the kinase which could again be activated in the presence of ATP after the phosphatase was inhibited by sodium fluoride. The phosphatase could also be inhibited by glycogen and showed a dependence on divalent cations to express its catalytic activity. The enzyme showed a wide tissue distribution, being higest in brain, skeletal muscle and lung. Phosphorylase kinase phosphatase was apparently different from phosphorylase phosphatase (215) since the latter enzyme did not use activated phosphorylase kinase as a substrate (11).

Further studies revealed that glycogen synthase D phosphatase (216) and phosphorylase kinase phosphatase are apparently the same enzyme (217). The partially purified phosphatase could inactivate and activate phosphorylated phosphorylase kinase and glycogen synthetase, respectively. These phosphorylated proteins were competitive substrates for the phosphatase. Further, the two activities were observed to copurify from skeletal muscle. The phosphatase could also dephosphorylate phosphorylated histone (216). Hence it was postulated that this enzyme

represents a multifunctional phosphoprotein phosphatase. It probably reverses phosphorylation reactions catalyzed by the cAMP-dependent protein kinase (217).

The phosphorylation state of the substrate, phosphorylase kinase, was postulated to be important in the regulation of phosphorylase kinase activity (10) . When phosphorylase kinase was phosphorylated by the cAMP-dependent protein kinase so that approximately 1 mole of phosphate was incorporated (subunit A=0.23 mole; subunit B = 0.75 mole) neither subunit A nor subunit B dephosphorylation proceeded in the absence of divalent cations. An increase in the phosphorylation state of phosphorylase kinase so that 2 moles of phosphate were incorporated (subunit A = 1.05 mole; subunit B = 1.05 mole) resulted in the dephosphorylation of the B-subunit in the absence of divalent cations. The kinase became inactivated in the process. The phosphatase studied in these experiments was reported to be a trace contaminant of the phosphorylase kinase preparation (10) . From the above experiments it was postulated that the rate of dephosphorylation of the B-subunit was determined by the phosphorylation state of the A-subunit, in the absence of divalent cations. This mechanism of controlling phosphorylase kinase phosphatase has been termed regulation by "second site phosphorylation" (10).

2. Distinct Phosphatases for Phosphorylated A and B Subunits

The early studies of Krebs and coworkers (11) evaluated the total phosphorylase kinase phosphatase activity in skeletal muscle extracts. Apparently this activity could be resolved into more than one species of phosphorylase kinase phosphatase. Recently two of these enzymes were isolated. One of the phosphatases is relatively specific for

the A-subunit, the other for the B-subunit of phosphorylase kinase (12, 218). Phosphorylase kinase that was predominantly phosphorylated in the A-subunit or the B-subunit was used as substrate for assaying the activity of phosphatase A and phosphatase B, respectively. Both of these phosphatases were partially purified from skeletal muscle. Phosphatase A has an apparent molecular weight of 170,000 and phosphatase B, 45,000-75,000. Phosphorylase kinase that was dephosphorylated by these phosphatases could be rephosphorylated by the cAMP-dependent protein kinase. This discounts the possibility that these phosphatases are really proteolytic enzymes that could release phosphopeptides from the phosphorylated kinase (12).

Additional studies revealed that phosphatase B which is relatively specific for phosphorylated B-subunits also dephosphorylates phosphorylase a and glycogen synthase D (219). It was therefore hypothesized, in agreement with an earlier report (217), that a single phosphoprotein phosphatase catalyzes the dephosphorylation reactions that inhibit glycogenolysis or stimulate glycogen synthesis. Phosphatase A is specific only for phosphorylated A-subunits and is postulated to serve a regulatory role by opposing the action of phosphatase B thereby prolongling the half-life of activated phosphorylase kinase. Regulation by "second site phosphorylation" (see above) is apparently relevant here (219).

3. Studies with Homogeneous Phosphoprotein Phosphatases

Phosphoprotein phosphatases have been partially purified from many sources (12, 216-217, 220-223) and proved to be of varying molecular weights (70,000-260,000). These phosphatases do not seem to have stringent substrate requirements. For instance, Nakai and Thomas (223) purified a phosphoprotein phosphatase 150-fold from bovine heart and showed that it could use as substrate the phosphorylated forms of

phosphorylase, glycogen synthase, phosphorylase kinase, histone and casein. From a previous study (222) the M. W. of this phosphatase was estimated to be 65,000-70,000. The ratios of the activities of the phosphatase for the different substrates remained constant during purification and hence it was concluded that the same enzyme could dephosphorylate several phosphoproteins (223).

In order to critically evaluate the studies done with partially purified enzymes, homogeneous preparations of phosphoprotein phosphatases were recently obtained from rabbit liver (13, 119) and skeletal muscle (119, 120). Unlike the partially purified enzymes, the homogeneous phosphoprotein phosphatases have M_r = 35,000. However, each of these enzymes could dephosphorylate several phosphoproteins, thereby agreeing with earlier findings. Khandelwal et al. (13) purified two phosphatases that seem to be representative of the homogeneous preparations. The enzymes, termed phosphoprotein phosphatase I and II, have M_r of 30,500 and 34,000, respectively. These enzymes could dephosphorylate phosphorylase *a*, glycogen synthase D, activated phosphorylase kinase, in addition to the phosphorylated forms of histone, casein and the inhibitory component of troponin (TN-I). Similar wide substrate specificity was also observed by the other workers (119). Since the dephosphorylation reactions could readily be reversed by rephosphorylation, it is unlikely that the phosphatases are contaminated with or are themselves proteases that release small phosphopeptides from the phosphorylated substrates (13) . It is not clear at present whether phosphatase I and II are isozymes or whether phosphatase I is derived from phosphatase II by proteolysis during purification.

The wide substrate specificity observed with the purified enzymes again raises the question of the existence of a multifunctional phosphoprotein phosphatase. Besides being able to dephosphorylate both phosphorylase a and glycogen synthase D, several other properties such as copurification, comigration on disc gel electrophoresis, competition between the two substrates for the enzyme, were used to show that the catalytic activity of phosphorylase a phosphatase and glycogen synthase D phosphatase are embodied in a single enzyme (224). This finding seem to agree with that of other workers (217, 219). However, the homogeneous phosphatases studied here are of lower molecular weight ($\sim$35,000) compared to that observed for the partially purified enzymes (M. W. 170,000-260,000) (12, 123) . It is possible that the low molecular weight enzymes may actually represent the catalytic subunit(s) of the much larger native enzyme (121-123).

E. Regulation of Phosphorylase Kinase

The activation of phosphorylase kinase by phosphorylation is now well established largely by studies conducted on the purified skeletal muscle enzyme (4 , 6 , 7 , 8 , 9 , 14). Various studies have been done to explain the in vivo regulation of this enzyme. Some of these studies are considered below.

1. Hormonal Regulation

Catecholamines can stimulate the activation of phosphorylase kinase in skeletal muscle (20-22,225-226). In one study (22) different concentrations of the catecholamine, isoproterenol, were used to stimulate glycogenolysis in muscle. Changes in cAMP levels and in phosphorylase kinase activity were measured. A 3-fold activation of phos-

phorylase kinase was observed when cAMP levels increased to 0.8 n moles/gm of muscle compared to basal levels (0.15 to 0.20 n moles/gm). Further increases in cAMP levels did not enhance the fold activation of the kinase. Another study (226) also showed a small degree of activation of phosphorylase kinase after the administration of epinephrine. There was a poor correlation between enzyme activation and phosphorylation of the enzyme. In fact, nonactivated phosphorylase kinase was shown to contain large amounts (0.709 ± 0.047 moles/100,000 gm protein) of covalently bound phosphate. Kinase activated as a result of epinephrine administration to animals showed only slightly higher values (0.840 ± 0.076 moles/100,000 gm protein). The low level of activation of phosphorylase kinase observed in these studies can possibly be explained by dephosphorylation of the activated enzyme during its purification. Even in the presence of sodium floride phosphorylase kinase phosphatase probably still has some activity.

Even though catecholamines increased cAMP levels in the above studies, the activation of phosphorylase kinase observed was not shown to have been catalyzed by the cAMP-dependent protein kinase. Since phosphorylase kinase can also be activated by self-phosphorylation (7 , 8 , 185) or by a Ca^{2+}-dependent protease found in muscle extracts (212, 213) it was important to establish that the activation is protein kinase-catalyzed. Cohen and coworkers (227, 228) demonstrated a 5-10 fold activation of phosphorylase kinase after the administration of epinephrine to rabbits. The sequence of amino acids of the phosphopeptides derived from the isolated activated phosphorylase kinase were shown to be the same when the enzyme was phosphorylated *in vivo* as when it was phosphorylated *in vitro* by the cAMP-dependent protein kinase (228).

2. Neural Regulation.

Both the non-activated and activated forms of phosphorylase kinase require low concentrations of Ca^{2+} for catalytic activity (5, 175, 212). The enzyme can be reversibly stimulated by Ca^{2+} at concentrations within the same range necessary for tension development in skeletal muscle (10^{-7} to 10^{-6}M). During electrical stimulation of muscle, it was observed that a very rapid conversion of phosphorylase b to the a form occurs. However no increase in the tissue levels of cAMP could be demonstrated nor was phosphorylase kinase significantly converted to the activated form (20, 21, 22) . These results indicate that activation of phosphorylase kinase is not a prerequisite for the conversion of phosphorylase b to the a form in skeletal muscle. As a result it has been hypothesized that upon electrical stimulation of muscle the Ca^{2+} released from the sarcoplasmic reticulum serves a dual role. It triggers muscle contraction (229) and stimulates phosphorylase kinase activity thereby linking muscular contraction and glycogenolysis (5, 18, 19) . However, nonactivated phosphorylase kinase has very low activity even in the presence of saturating amounts of Ca^{2+}. It has been estimated (4, 197) that all the nonactivated kinase in rabbit skeletal muscle will take about 15 - 30 sec to convert 50% of the phosphorylase b to the a form. This time is far greater than the 1 sec required when frog sartorius muscle is stimulated electrically (230). Hence, it is possible that activators of phosphorylase kinase may be important in regulating its activity. The observation that glycogen stimulates the enzyme activity is important in this respect (14) . Fatty acids, phospholipids and other membrane components may also be important in regulating the enzyme activity since it has been demonstrated that phosphorylase kinase is

partly particulate. This particulate kinase has a pH 6.8/8.2 ratio of about 0.5 (153, 231), typical of phosphorylase kinase which is already in the activated form.

3. Regulation in Glycogen Particles

A protein-glycogen complex can be isolated from rabbit muscle and has been proposed to be a structural and functional unit of the muscle fibre (232). The complex contains a substantial amount of the total cellular phosphorylase, phosphorylase kinase, phosphorylase phosphatase and glycogen synthase activities (176, 232).

The factors regulating the activation of phosphorylase were studied in the glycogen particles (176). Addition of ATP (1 mM), Mg^{2+} (5 mM) and Ca^{2+} (1 mM) resulted in the rapid conversion of all the phosphorylase b to the a form. After all the ATP was consumed the phosphorylase a was reconverted to the b-form by the phosphatase present. This cycle of activation-deactivation could be repeated by readdition of ATP and was termed "flash activation" of phosphorylase (176). The concentration of Ca^{2+} required to give half-maximum activation of phosphorylase kinase in this system was 2×10^{-6}M. Similar concentrations of Ca^{2+} are required to trigger muscle contraction (229, 233).

In the absence of cAMP, no change in phosphorylase kinase activity was observed during the "flash activation" of phosphorylase. Hence it was concluded that the kinase was not activated by phosphorylation and remained essentially in the nonactivated form (176). However, another report (197) indicated that phosphorylase kinase was activated 20% during one cycle of the "flash activation" of phosphorylase. The use of [γ-^{32}P] ATP confirmed that this activation of the kinase was the result of phosphorylation of this enzyme. The activation was reversed by phosphatase

activity present in the glycogen particles (197). Recent studies on purified phosphorylase kinase have shown that the enzyme could be activated to very high levels by autophosphorylation (7 , 8). Nevertheless, the criticism has been that autoactivation is too slow a process to play a significant role in controlling the enzyme activity. However, the 20% activation of the kinase mentioned above occurred in 1 min under the prevailing experimental conditions (197). These values can possibly be bettered in the presence of activators of the enzyme. Hence, autoactivation may indeed be a potentially important mechanism for regulating phosphorylase kinase activity in glycogen particles or *in vivo*.

III. EXPERIMENTAL PROCEDURES

A. Materials

1. Preparation of Enzymes

a) Phosphorylase b

Phosphorylase b was purified from commercial frozen rabbit muscle (Pel-Freeze Biologicals, Inc., Rogers, Arkansas) by the method of Fisher and Krebs (174, 234, 235). After the third crystallisation, the enzyme was lyophilized and the yellow powder stored in tightly capped vials at -70° (5). When ready to use one vial of crystals was retrieved from -70°, warmed up for 30 min and dissolved in a small amount (about 2 mls) of distilled water. This solution was centrifuged at 10,000 x g for 5 min to remove any denatured proteins and then left at 3° overnight to allow crystals to form. The crystals were collected, dissolved in 40 mM glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8 and treated with Norit A to remove 5'AMP (234). The concentration of the final AMP-free phosphorylase b was determined spectrophotometrically at 280 nm. An absorbancy index of 13.1 (4) for a 1% enzyme solution was used.

b) Phosphorylase Kinase

Nonactivated phosphorylase kinase from rabbit skeletal muscle was purified by the method of Krebs et al. (14) later modified by others (4 , 5, 167, 174) . The enzyme obtained after the Sepharose 4B step was in 50 mM glycerophosphate, 2 mM EDTA, 10% sucrose, pH 6.8. For a few experiments, an aliquot of the enzyme was dialyzed against 50 mM Mes, 2 mM EDTA, 10% sucrose, pH 6.8 for 18-20 hr. The kinase was stored frozen at -70° in small aliquots. The concentration of phosphorylase kinase solutions was calculated by monitoring the absorbance at 280 nm

and using 12.4 (4) as the absorbance for a 1% solution.

c) Cyclic AMP-dependent Protein Kinase

Partially purified cAMP-dependent protein kinase from rabbit skeletal muscle was prepared by the method of Reimann et al. (92) . The catalytic subunit of protein kinase from bovine skeletal muscle was purified to homogeneity (93) and was a generous gift of Drs. J. A. Beavo and E. G. Krebs of the University of California, Davis. For a few experiments, the catalytic subunit was partially purified from rabbit skeletal muscle essentially by the method of Kinzel and Kübler (236). The holoenzyme and catalytic subunit were stored at -70° and 3°, respectively. The catalytic subunit could also be stored at -20° in the presence of 50% glycerol.

d) Phosphoprotein Phosphatases

Phosphoprotein phosphatase I and II were purified to electrophoretic homogeneity from rabbit liver by the method of Khandelwal et al. (13) . These enzymes were generously provided by Dr. Khandelwal of the University of Manitoba.

Specific phosphatase activity for phosphorylated A-subunits of phosphorylase kinase was obtained by using crude material from two sources. Rabbit liver extract which was made in 20 mM Tris, 4 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 was used. The crude extract (in 4 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.0) from rabbit skeletal muscle was subjected to 60% ammonium sulfate fractionation. The pellet was recovered by centrifugation and dissolved in a minimal amount of 20 mM Tris, 2 mM EDTA, 50 mM 2-mercaptoethanol, pH 7.5 (buffer A). The dissolved pellet was extensively dialyzed against two changes of buffer A. This material was used as a source of phospha-

tase A.

2. Preparation of Other Proteins

a) Protein inhibitor of cAMP-dependent Protein Kinase

The heat-stable protein inhibitor of cAMP-dependent protein kinase was partially purified from rabbit skeletal muscle by the procedure of Walsh et al. (101). The Sephadex G-75 step was omitted. The powder obtained after lyophilization was dissolved in a minimal volume of 5 mM Tris-HCl, 1 mM EDTA, pH 7.5, clarified by centrifugation and stored in small aliquots at -70°. Protein concentration was determined by the method of Lowry et al. (237).

b) Protein Inhibitor of Phosphoprotein Phosphatase

A low molecular weight heat-stable inhibitor of phosphoprotein phosphatase was purified from rabbit liver by the method of Khandelwal and Zinman (238). The inhibitor was kindly supplied by Dr. Khandelwal of the University of Manitoba.

3. $[\gamma\text{-}^{32}P]$ ATP

In the early stages of these studies, $[\gamma\text{-}^{32}P]$ ATP was prepared from $^{32}P_i$ by a modification (239) of the method of Glynn and Chappel (240). These preparations normally had a specific activity of about 3×10^9 cpm per μmole. Later, the $[\gamma\text{-}^{32}P]$ ATP was purchased as such. Both $^{32}P_i$ and $[\gamma\text{-}^{32}P]$ ATP were from New England Nuclear Corporation.

4. Fatty Acids, Phospholipids and Detergents

Saturated fatty acids were purchased from Applied Science Laboratories, Inc., and the unsaturated fatty acids from Cal Biochem. Phosphatidyl choline was purchased from Serdary Research Laboratories. The mixed phospholipids (soy bean) was from Associated Concentrates.

Triton X-100 was from Sigma Chemical Company and Tween 20 from the Pierce Chemical Company.

Stock solutions of the fatty acids, phospholipids and detergents were made in absolute ethanol. Hence when these substances were added to the phosphorylase kinase assay mixture, there was a carryover of 0.34 M ethanol. The slight stimulation of the kinase by this concentration of ethanol (Fig. 15) was subtracted from that observed for each test substance.

5. Chemicals

Magnesium Acetate was purchased from Fisher Scientific Company. Mes, β-glycerophosphate and EGTA were from Sigma. Absolute ethanol was from Canadian Industrial Alcohols and Chemicals Ltd., Corbyville, Ontario. The other organic solvents tested for their ability to stimulate non-activated phosphorylase kinase (Table 3) were either from Fisher or the British Drug Houses Ltd.

All other chemicals were obtained from established suppliers and were reagent grade or better.

B. Methods

1. Determination of ^{32}P Incorporation into Protein Substrates

The phosphorylation of protein substrates in the phosphorylase kinase and protein kinase reactions was followed by removing aliquots of the reaction mixture at timed intervals and spotting these on filter squares as described previously (92, 241). After 5 sec the filter disks were dropped in cold 10% trichloroacetic acid (TCA) to quench the reaction. The filters were washed successively in 10% cold TCA (30 min), 5% TCA (20 min), 5% TCA (20 min) and 95% ethanol (10 min). The wire basket

used to hold the filter disks (241) was occasionally moved by a gentle up-down motion so as to change the positions of the filters and facilitate thorough removal of non-protein bound radioactivity. After the ethanol wash, the filters were dried for 5 min under a hair drier and the amount of radioactivity on each disc determined by scintillation counting. Samples from reaction blanks were treated exactly as above and the average counts from these filters subtracted from the others.

For the calculation of moles of phosphate incorporated into phosphorylase kinase, a minimal molecular weight of 318,000 (4) was used. This minimal molecular weight was referred to as a "monomeric unit" of the kinase (9).

2. Protein Kinase Activity

The cAMP-dependent protein kinase was assayed as previously described (241). The reaction mixture normally contained type II histone (Sigma), 1 mg/ml; [γ-^{32}P] ATP, 0.2 mM; magnesium acetate, 1 mM; EGTA, 0.1 mM; sodium acetate, 50 mM; diluted protein kinase. With the protein kinase holoenzyme, 10 μM cAMP was also added to the above ingredients. The reaction was started by the addition of protein kinase. Different dilutions of the enzyme were used. Samples were removed at different times for the determination of ^{32}P incorporation into the protein substrate. Reaction velocities were calculated from the linear part of the progress curves. One unit of protein kinase activity was defined as the amount of enzyme catalyzing the incorporation of 1 p mole of ^{32}P into histone per min at 30°.

3. Phosphorylase Kinase Activity

Phosphorylase kinase was assayed by following the incorporation of ^{32}P from [γ-^{32}P] ATP into phosphorylase b (5, 167). Unless

otherwise indicated, the reaction mixture normally contained phosphorylase b, 4 mg/ml; 2-mercaptoethanol, 15 mM; magnesium acetate, 10 mM; calcium chloride, 0.1 mM; [γ-^{32}P] ATP, 2.4 mM; buffer (25 mM Tris, 25 mM glycerophosphate, pH 6.8); diluted phosphorylase kinase. The kinase was diluted in 50 mM glycerophosphate (pH 6.8) containing 60 mM 2-mercaptoethanol immediately before use. The effect of the various organic solvents, fatty acids or phospholipids on the kinase activity was investigated by adding these substances directly to the above basic reaction mixture. All additions were made immediately before the reaction was initiated. Ethanol was used as a representative solvent that stimulates phosphorylase kinase activity mainly because of the relative ease of working with this solvent as well as the great stimulation obtained at higher concentrations (Fig. 15). At concentrations much greater than 1 M, many of the other solvents inhibit the kinase activity.

The reaction was usually initiated by the addition of phosphorylase kinase at 30°. After 5 min aliquots were removed for the determination of ^{32}P incorporation into phosphorylase b. One unit of kinase activity was defined as the amount of this enzyme that catalyzes the incorporation of 1 μmole of phosphate into phosphorylase b per min at 30°.

4. Activation of Phosphorylase Kinase

Phosphorylase kinase was phosphorylated by the cAMP-dependent protein kinase (catalytic subunit or partially pure holoenzyme) in a reaction mixture normally containing phosphorylase kinase, 0.30 to 0.40 mg/ml; 2-mercaptoethanol, 15 mM; EDTA, 0.5 to 1 mM; [γ-^{32}P] ATP, 0.28 mM; magnesium acetate, 1 mM or 10 mM; EGTA, 0.5 mM or 1 mM; sucrose, 2 to 5%; protein kinase; glycerophosphate buffer (pH 6.8), 50 mM. When the protein kinase holoenzyme was used, 10 μM cAMP was also included in the reaction

mixture. Activation of phosphorylase kinase by the autocatalytic reaction was carried out in a reaction mixture normally containing phosphorylase kinase, 0.30 to 0.40 mg/ml; 2-mercaptoethanol, 15 mM; EDTA, 0.5 to 1 mM; [γ-^{32}P] ATP, 2.4 mM; magnesium acetate, 10 mM; sucrose, 2-5%; calcium chloride, 0.1 mM; protein inhibitor, 5 μg/ml; Mes buffer (pH 6.8), 50 mM. At pH 8.2 the autocatalytic reaction was done in a buffer consisting of 20 mM Mes, 37.5 mM Tes. The activation reactions were initiated by [γ-^{32}P] ATP after allowing 2 min for temperature equilibration at 30°. Samples were removed at different times and assayed for phosphorylase kinase activity or phosphate incorporation.

5. Preparation of ^{32}P-labelled Phosphorylase Kinase

Phosphorylase kinase containing 2 phosphates per monomeric unit was prepared in a reaction mixture catalyzed by the catalytic subunit of protein kinase at 1 mM Mg^{2+} as described (Section III.B.4). Since the rate of phosphorylation of subunit B is much greater than subunit A (4 , 6 , 9) , phosphorylase kinase labelled predominantly in subunit B was prepared as above for the two site-labelled kinase but the reaction was stopped when 0.7-1.0 phosphate per monomeric unit was incorporated. For instance, phosphorylase kinase containing 0.86 phosphates per monomeric unit, showed 0.60 moles in subunit B and 0.26 moles in subunit A. Kinase containing greater than 2 phosphates per monomeric unit was prepared either by the protein kinase-catalyzed reaction at 10 mM Mg^{2+} or by the autocatalytic reaction (see Section III.B.4). About 5 to 7 moles of phosphate could reproducibly be incorporated by the former reaction. The slow rate at which the second phase phosphorylation occurs (9) as well as the 1 hr incubation normally used for these experiments may be the limiting factor for higher incorporation. Phosphorylation by

the autocatalytic reaction resulted in 9 to 11 phosphates being incorporated in the kinase after 1 hr at 30°.

The phosphorylation reactions were terminated by cooling down in iced water for 10 min. The labelled kinase was then extensively dialyzed against 50 mM Tris (pH 7.0) containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 3 to 5% sucrose. The dialyzed enzyme was stored at 3°. Phosphorylated phosphorylase kinase was made fresh every week.

6. Dephosphorylation of ^{32}P-labelled Phosphorylase Kinase

Phosphorylated phosphorylase kinase (0.30 mg/ml) was warmed for 3 min at 30°. The reaction was initiated by the addition of phosphatase. Samples were removed at different times and added to 50 mM glycerophosphate (pH 6.8) containing 15 mM 2-mercaptoethanol and 50 mM sodium fluoride to stop the reaction. Aliquots of the stopped reaction mixture were used for the determination of protein-bound radioactivity or phosphorylase kinase activity. Still other samples were used to run SDS-polyacryalmide gel electrophoresis for the determination of radioactivity into subunits A and B.

7. SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done at alkaline pH as described previously (167). Samples of phosphorylated phosphorylase kinase were boiled in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Approximately 10 μg of protein was applied to each gel. After electrophoresis, the gels were stained with Commassie Brilliant Blue and destained in a solution of 5% methanol, 7.5% acetic acid (242). For the determination of ^{32}P incorporation into the subunits of phosphorylase kinase, a procedure modified (243) from that of Basch (244) was used. The individual protein

bands were sliced manually (for a few experiments, the unstained gel was sliced into 1 mm slices by using a Gilson Aliquogel automatic gel fractionator) and dried overnight at 50°. Each gel slice was then digested with 0.6 ml NCS solubilizer in the presence of 0.1 ml of double-distilled water at 50° for 3½ hr. Radioactivity (^{32}P) was then determined in a Beckman LS 250 scintillation spectrophotometer after the addition of 0.02 ml glacial acetic acid followed by 12.5 ml of Omnifluor scintillation mixture.

8. Sedimentation Velocity Experiments

For these experiments, phosphorylase kinase was in 50 mM glycerophosphate (pH 6.8) containing 1 mM EDTA at a concentration of 2 mg/ml. Ethanol, when present, was at a concentration of 1.72 M. Runs were carried out in a Spinco Model E analytical ultracentrifuge with the rotor speed being 51,700 rpm and the temperature 20°. Pictures were taken at 7 min interval using a Schlieren Optical System.

IV. RESULTS

A. Protein Kinase-catalyzed Activation of Phosphorylase Kinase at Low and High Mg^{2+} Concentrations

1. Effect of Mg^{2+} Concentration on the Phosphorylation and Activation of Phosphorylase Kinase

Earlier studies (4) have indicated that the protein kinase-catalyzed activation of phosphorylase kinase results from the incorporation of 2 phosphates per monomeric unit of phosphorylase kinase (M.W. 318,000). In these early studies, the activations were carried out in the presence of EGTA and low concentrations of ATP and Mg^{2+} to minimize the autocatalytic reaction of phosphorylase kinase. In this study (9) it was found that the level of phosphorylation of phosphorylase kinase depends on the Mg^{2+} concentration in the protein kinase-catalyzed reaction. As is shown in Fig. 1A just over 2 moles of phosphate per 318,000 gm protein were incorporated into phosphorylase kinase when the reaction was carried out in the presence of 1 mM Mg^{2+}, thus confirming previous observations. However, when 10 mM Mg^{2+} was used in the reaction medium, more than 5 mol phosphate were incorporated per 318,000 gm phosphorylase kinase in 60 min. As is apparent in Fig. 1A, the phosphorylation reaction was not complete at 60 min. Experiments carried out for longer times or with higher concentrations of protein kinase resulted in the incorporation of up to 9 moles of phosphate per 318,000 gm phosphorylase kinase.

The additional phosphorylation of phosphorylase kinase during the protein kinase-catalyzed reaction at 10 mM Mg^{2+} is associated with a further

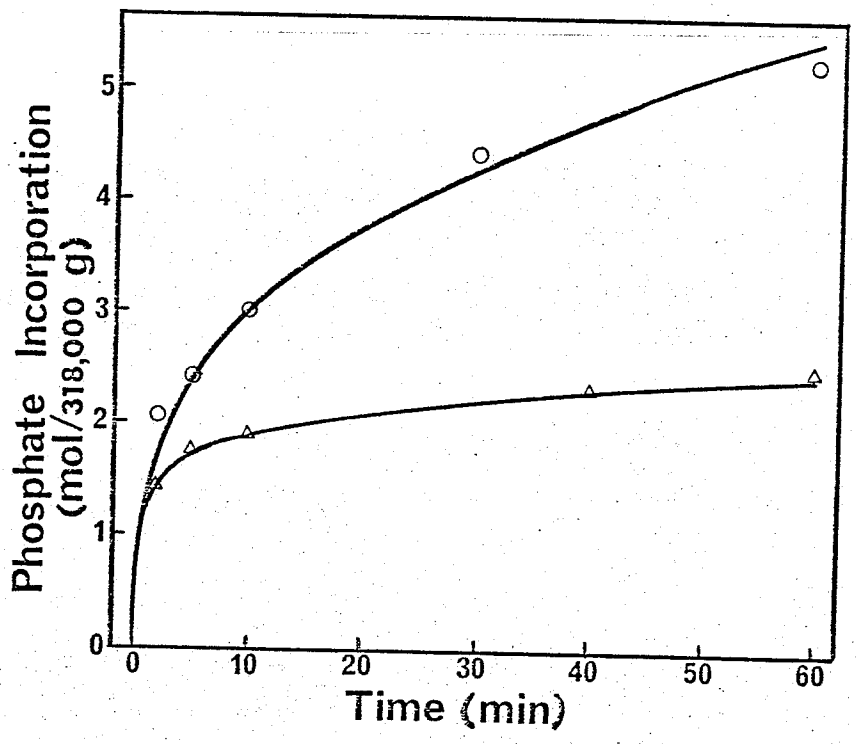


Fig. 1 (A). Phosphorylation of phosphorylase kinase at 1 mM and 10 mM Mg^{2+}. Phosphorylase kinase (0.73 mg/ml) was incubated with 0.28 mM ATP and either 1 mM (Δ) or 10 mM (o) Mg^{2+} in the presence of 25 mM NaF, 5% sucrose and partially purified protein kinase. Other details were the same as in "Methods". Aliquots were removed at different time intervals and assayed for phosphate incorporation into protein.

increase in enzyme activity over that observed at 1 mM Mg^{2+}. As is shown in Fig. 1B, phosphorylase kinase activated in the presence of 10 mM Mg^{2+} achieved an activity more than 2-fold higher than the enzyme activated at 1 mM Mg^{2+}. Cohen (4) has shown that the nonactivated phosphorylase kinase can be activated up to 50-fold during the protein kinase-catalyzed reaction. Conditions used in his study, however, resulted in the incorporation of 2 phosphates per monomeric unit of phosphorylase kinase. Using 10 mM Mg^{2+} in the reaction media, we have observed in this study up to 200-fold activation of phosphorylase kinase.

The activation and phosphorylation of phosphorylase kinase by the protein kinase-catalyzed reaction at 10 mM Mg^{2+} appear to consist of two phases. In the first phase, there is rapid enzyme activation and phosphorylation reaching, in a few minutes, a level similar to that achieved for the reaction done at 1 mM Mg^{2+}. In the second phase, there are further activation and phosphorylation at much reduced rates. The observation suggests that high concentrations of Mg^{2+} enhances the second phase of the reaction. The low levels of phosphorylase kinase activation and phosphorylation observed at 1 mM Mg^{2+} is not due to the loss of any of the reaction components, such as inactivation of protein kinase or hydrolysis of ATP. When phosphorylase kinase was first subjected to the protein kinase-catalyzed reaction at 1 mM Mg^{2+} for 20 min so that close to 2 mol phosphate per 318,000 gm of phosphorylase kinase has been incorporated, further phosphorylation of the enzyme could result by raising Mg^{2+} concentration in the reaction mixture to 10 mM (Fig. 2A). This further phosphorylation was accompanied by an additional activation of the enzyme (Fig. 2B).

The effect of Mg^{2+} on the protein kinase-catalyzed phosphory-

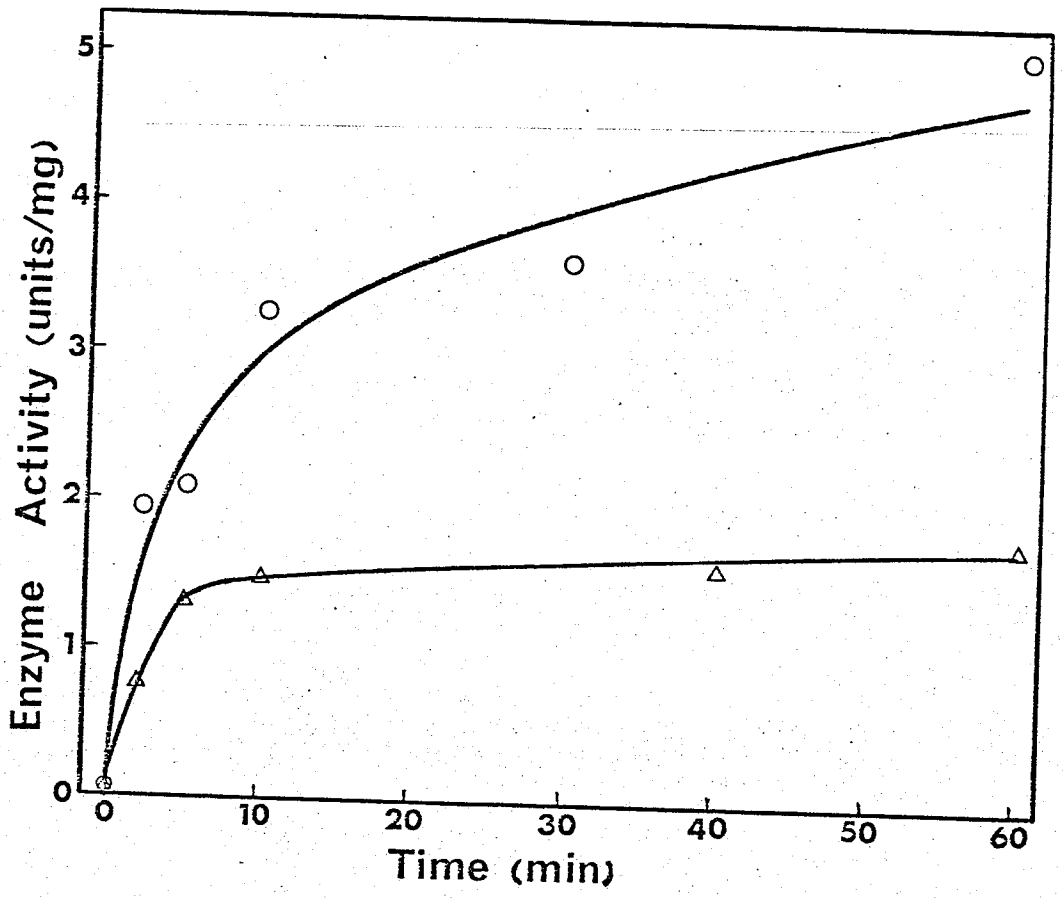


Fig. 1 (B). Activation of phosphorylase kinase at 1 mM (Δ) and 10 mM (O) Mg^{2+}. The reaction mixture was the same as that shown for Fig. 1 (A). At each time interval, an aliquot of the reaction mixture was removed and diluted in 12 mM EDTA to stop the reaction and assayed for enzyme activity.

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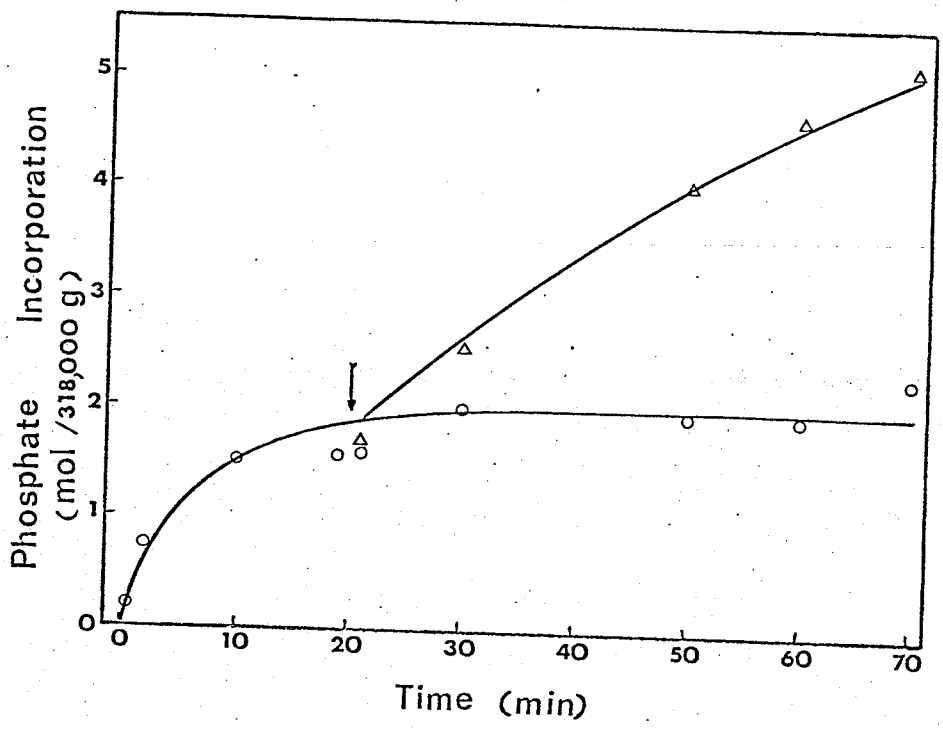

Fig. 2. (A). Change in Mg^{2+} concentration during the Phosphorylation of phosphorylase kinase. Phosphorylase kinase (0.30 mg/ml) was incubated with 0.28 mM ATP and 1 mM Mg^{2+} in the presence of the catalytic subunit of protein kinase. At the different times aliquots were removed and assayed for protein-bound phosphate. At 20 min (↓), a portion of the reaction mixture was withdrawn and mixed with 10 mM Mg^{2+} - 1 mM EGTA. Incubation was continued at 30°C. Aliquots were removed and assayed for phosphate incorporation.

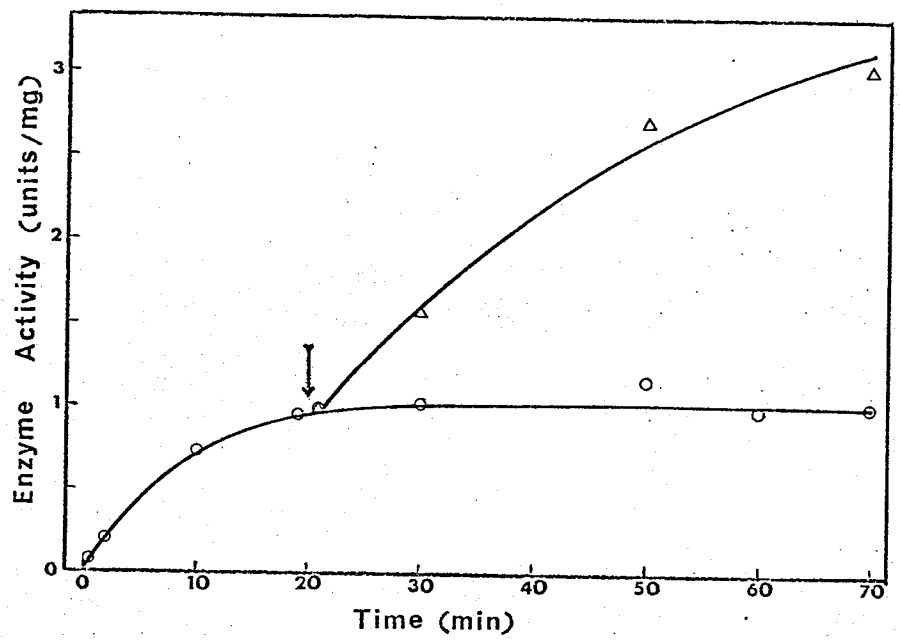

Fig. 2. (B). Change in Mg^{2+} concentration during the Activation of Phosphorylase Kinase. The reaction mixture was the same as that shown for Fig. 2 (A). At each time interval an aliquot of the reaction mixture was removed and diluted in 12 mM EDTA to stop the reaction and assayed for enzyme activity.

lation of phosphorylase kinase has been examined over a wide range of Mg^{2+} concentrations. An increase in the stimulation of the second phase phosphorylation of phosphorylase kinase was observed as the concentration of the cation was increased in the range of 2 to 8 mM. Further increase in Mg^{2+} concentration from 8 to 25 mM had little additional stimulation of the protein phosphorylation.

2. Evidence for a Protein Kinase-Catalyzed Reaction

The suggestion that the high levels of phosphorylation and activation of phosphorylase kinase at 10 mM Mg^{2+} is due to a protein kinase-catalyzed reaction is supported by several observations (Table 1). It is unlikely that the autocatalytic reaction of phosphorylase kinase contributed significantly to this phosphorylation since the reactions were routinely carried out in the presence of 1 mM EGTA. Furthermore, while glycerophosphate has been shown to drastically suppress the autophosphorylation of phosphorylase kinase (7), the enzyme phosphorylation and activation observed in this study were affected by this buffer only slightly (Fig. 3).

The possibility that phosphorylase kinase activation resulted from the action of a contaminating protease and that the high level of protein phosphorylation was secondary to the proteolytic modification of phosphorylase kinase has also been ruled out. The activation of phosphorylase kinase was found to depend on the simultaneous presence of both Mg^{2+} and ATP. Examination of phosphorylase kinase by SDS-gel electrophoresis indicated that the electrophoretic patterns of the enzyme both before and after its activation in 10 mM Mg^{2+} were identical. Previous studies (4 , 6) have shown that activation of phosphorylase kinase by limited tryptic digestion results in extensive degradation of

Table 1. Evidence in Support of a Protein Kinase-catalyzed Activation of Phosphorylase Kinase at 10 mM Mg^{2+}

Criteria	Activation of Phosphorylase Kinase Catalyzed		
	by Phos. Kinase[a]	by Protein Kinase[a]	at 10 mM Mg^{2+}
Inhibition by EGTA	+[b]	-[c]	-
Inhibition by protein inhibitor	-	+	+
Inhibition by phosphates	+	-	-
Depend on cAMP	-	+	+
Dependence on protein kinase	-	+	+
Presence of a lag	+	-	-

[a]The different factors that could be used to distinguish autoactivation from the protein kinase-catalyzed activation of phosphorylase kinase are from ref. 7, 185.

[b]+ = Yes

[c]- = No

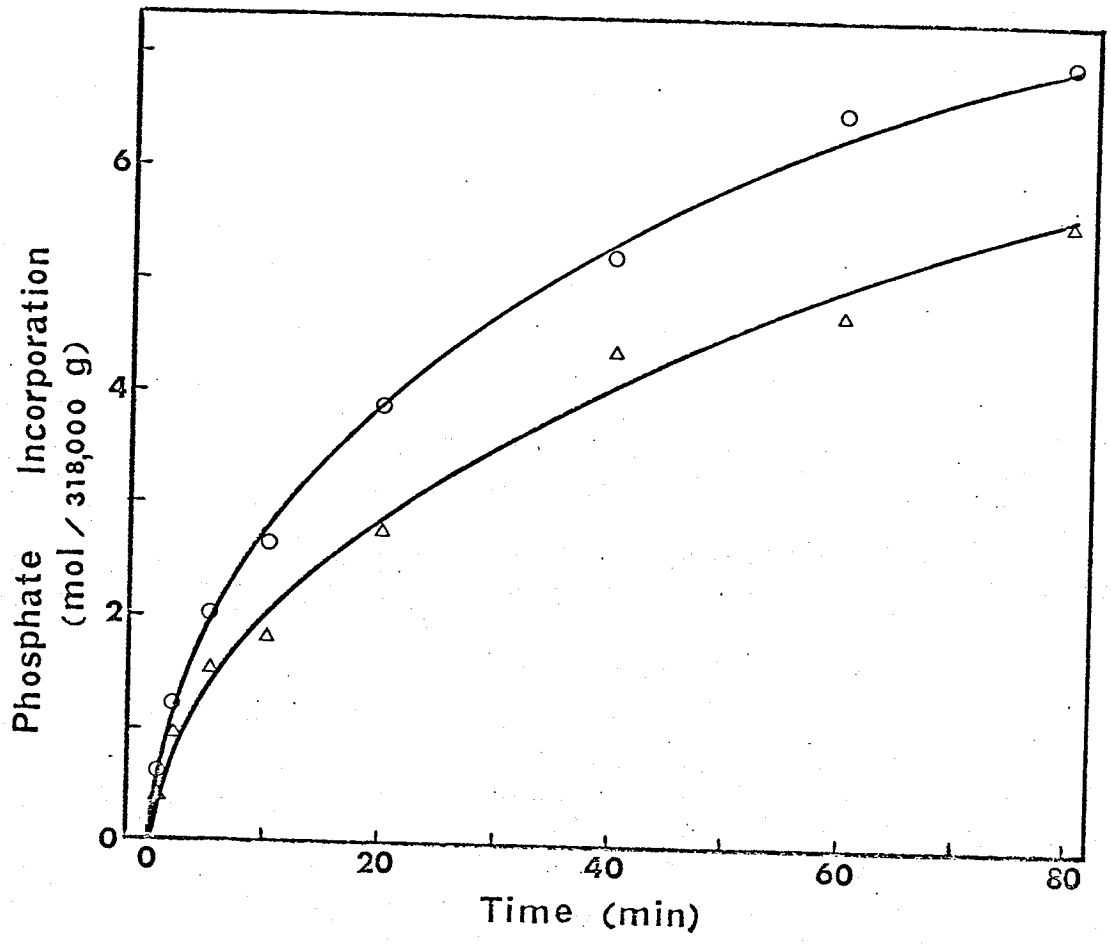

Fig. 3. Phosphorylation of phosphorylase kinase in glycerophosphate and MES buffers. Phosphorylase kinase (0.35 mg/ml) was incubated with 0.28 mM ATP, 10 mM Mg^{2+} and the catalytic subunit of protein kinase in either 50 mM glycerophosphate (Δ) or 50 mM MES (O) buffer, pH 6.8. Aliquots were removed at various times and assayed for protein-bound phosphate.

subunits A and B.

The enzyme activation and phosphorylation showed substantial dependence on the addition of exogenous protein kinase. When the protein kinase holoenzyme was used as the enzyme source, the reaction was significantly stimulated by cAMP. In contrast, if the purified catalytic subunit of the protein kinase was used, phosphorylase kinase could be activated and phosphorylated to high levels in the absence of cAMP. Since the purified catalytic subunit of protein kinase was found to be homogeneous by SDS-gel electrophoresis, the possibility that the reaction was catalyzed by another protein kinase is not too likely. In addition, Fig. 4 shows that the phosphorylation of phosphorylase kinase was markedly inhibited by the heat-stable protein inhibitor which has been shown to be specific for the cAMP-dependent protein kinase (101). Thus, from several lines of evidence, it may be concluded that the high levels of phosphorylation and activation of phosphorylase kinase at 10 mM Mg^{2+} are indeed due to a cAMP-dependent protein kinase-catalyzed reaction.

3. Patterns of Subunit Phosphorylation

At low concentrations of Mg^{2+}, subunits A and B of phosphorylase kinase became phosphorylated during the protein kinase-catalyzed reaction and approximately one phosphate was incorporated into each of these two subunits (4). When the protein kinase-catalyzed reaction was carried out in the presence of 10 mM Mg^{2+}, phosphorylation of phosphorylase kinase was also found to occur exclusively on subunits A and B. However, subunit A was phosphorylated to a much greater extent than subunit B, the ratio of phosphorylation of subunits A and B being 3.4 to 1. To further examine the pattern of subunit phosphorylation,

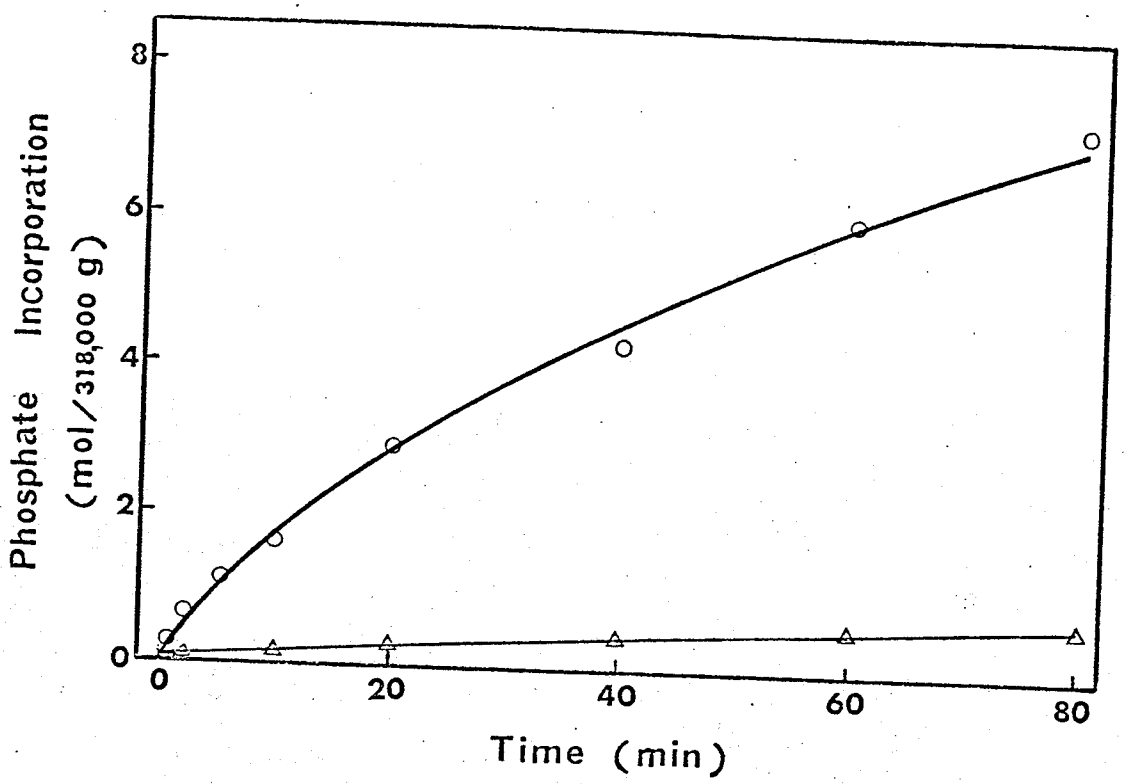

Fig. 4. Inhibition of phosphorylase kinase phosphorylation by the protein inhibitor of cAMP-dependent protein kinase. Phosphorylase kinase (0.38 mg/ml) was incubated with 0.28 mM ATP, 10 mM Mg^{2+} and the catalytic subunit of protein kinase either in the presence (Δ) or absence (0) of protein inhibitor. Aliquots were withdrawn at the various times and assayed for protein-bound phosphate.

samples of phosphorylase kinase in the protein kinase-catalyzed reaction were withdrawn at various times and phosphate in the various subunits was analyzed (see "Methods" for details). Results for such an experiment are shown in Fig. 5. As can be seen, the phosphorylation of subunits A and B followed different courses. Subunit B was phosphorylated at a rapid initial rate and reached a final level of about 1.5 moles of phosphate per subunit at 15 min. No further increase in phosphate incorporation seemed to occur over the next 85 min. The time course of the subunit A phosphorylation exhibited an initial lag of 2 to 3 min which was followed by a slow but continuous phosphorylation. At 100 min, there were 5.4 phosphates incorporated per subunit A. Previous studies (4) of the protein kinase-catalyzed phosphorylation at low concentrations of Mg^{2+} have indicated that subunit B is rapidly phosphorylated to a level of 1 phosphate per subunit; the phosphorylation of subunit A occurred at a slow rate after an initial 2 to 3 min lag and reached a final level of about 1 phosphate per subunit. Comparing the present results with the previous observations, it is suggested that the additional phosphorylation of phosphorylase kinase obtained at higher concentrations of Mg^{2+} results from the phosphorylation of subunit A.

As has been mentioned in a preceding section, the activation of phosphorylase kinase at high concentrations of Mg^{2+} appeared to consist of two phases: a rapid initial rise followed by a gradual further increase in the enzyme activity at pH 6.8. The correlation of the time courses of the enzyme activation and subunit phosphorylations is also depicted in Fig. 5. It is seen in this experiment that phosphorylase kinase was activated 50-fold in the first 5 min of incubation. This

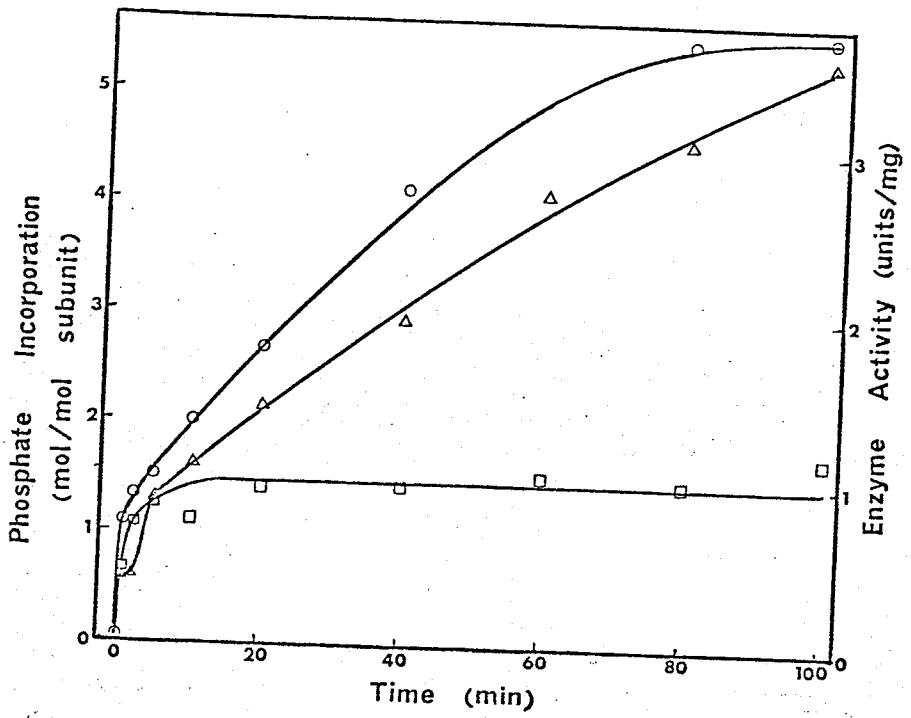

Fig. 5. Pattern of phosphorylation of the subunits of phosphorylase kinase. Phosphorylase kinase (0.30 mg/ml) was incubated with 0.28 mM ATP, 10 mM Mg^{2+} and the catalytic subunit of protein kinase. At the different time intervals aliquots were removed for the assay of enzyme activity (O) and phosphate incorporation into subunit A (Δ) and subunit B (□). See "Methods" for details.

rapid phase was followed by a slower further increase in enzyme activity. The final activation of the enzyme was reached after 90 min of incubation and the increase in enzyme activity achieved was 185-fold. The initial rapid enzyme activation seems to correlate with the rapid phosphorylation of subunit B whereas the slower activation phase appears to correlate with the phosphorylation of subunit A. Thus, in contrast to the previous suggestion (4) that the protein kinase-catalyzed activation of phosphorylase kinase results from the phosphorylation of subunit B, the present results suggest that phosphorylation of both subunits A and B contribute to activation of the enzyme. The possible involvement of subunit A phosphorylation in the activation of phosphorylase kinase has been suggested before (6).

4. Comparison between the Protein Kinase-Catalyzed Phosphorylation and Autophosphorylation

Recent studies on the autocatalytic mechanism of phosphorylase kinase activation (7 , 8) showed that the enzyme could be phosphorylated and activated to very high levels. In this respect autoactivation proves to be very similar to the activation catalyzed by protein kinase at high concentrations of Mg^{2+}. As shown in Fig. 6A, phosphate incorporation achieved by either mechanism seems to approach the same final level and these high levels of protein phosphorylation result in similar increases in enzyme activity. Furthermore, the pattern of subunit phosphorylation of phosphorylase kinase catalyzed by the protein kinase at 10 mM Mg^{2+} appears similar to that of the autocatalytic reaction (Table 2). These similarities raised the question as to whether the sites phosphorylated by the two different mechanisms are the same or different. A partial answer to this question was supplied

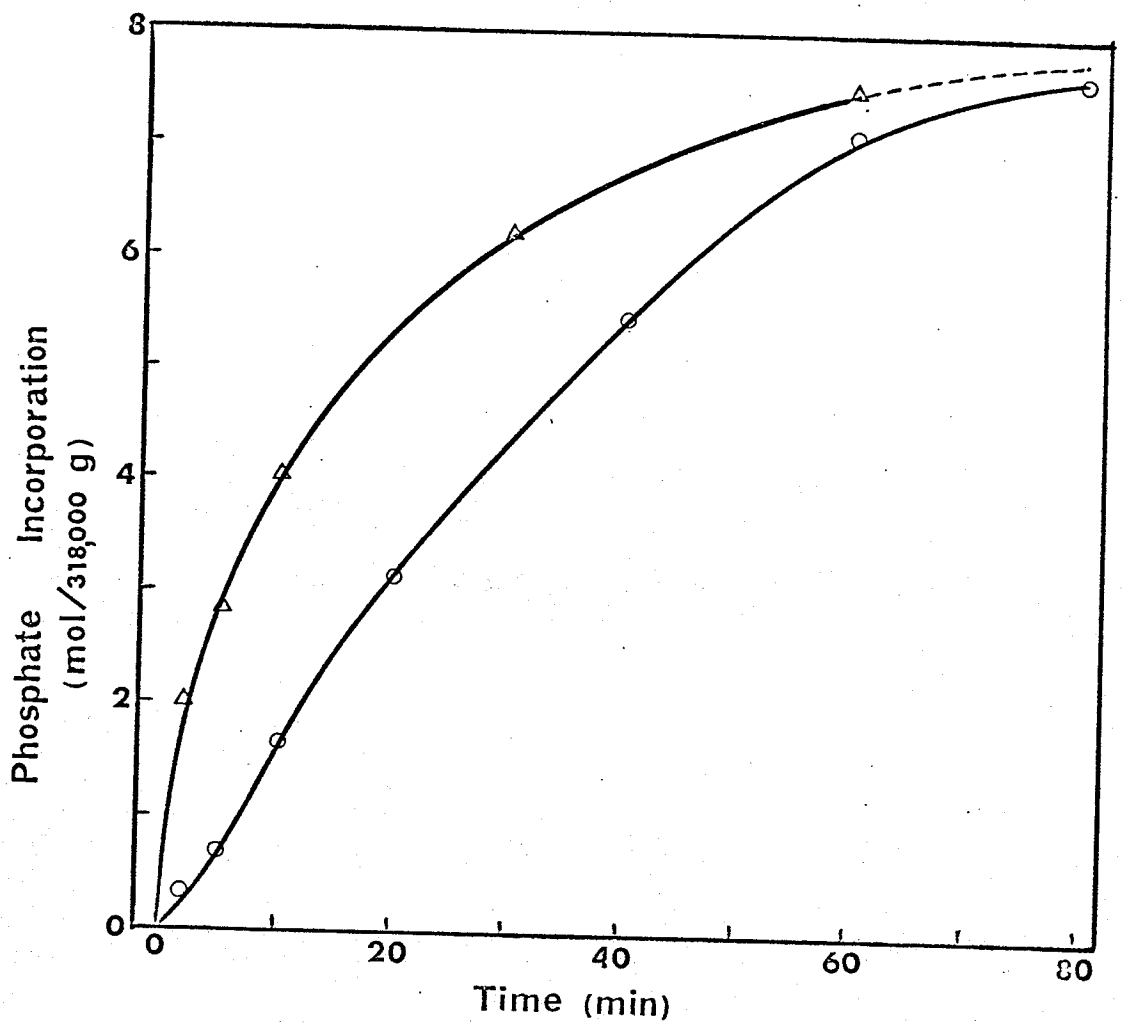

Fig. 6. (A). Comparison between autophosphorylation and the protein kinase-catalyzed phosphorylation of phosphorylase kinase. Phosphorylase kinase (0.36 mg/ml) was phosphorylated either by the autocatalytic reaction (O) or by protein kinase (Δ). Autophosphorylation was studied in a reaction mixture similar to that described in "Methods", with the exception that the reaction was done at pH 8.2 in 20 mM MES - 37.5 mM TES buffer. The protein kinase-catalyzed reaction was done in the presence of 0.28 mM ATP, 10 mM Mg^{2+} and partially pure protein kinase. Aliquots were removed at different times to determine protein-bound phosphate.

Table 2. Similarities between the Protein Kinase-catalyzed Activation and Autoactivation of Phosphorylase Kinase

Property	Activation of Phosphorylase Kinase Catalyzed by	
	Protein Kinase	Phos. Kinase
Phosphate incorporation (mol/318,000 gm)	7 - 9	7 - 9
Subunits phosphorylated	A, B	A, B [a, b]
A : B phosphorylation	3.6:1	2.5:1 [a]
Enzyme activation (fold)	44-185	60 - 200 [a, b]

[a, b] These data are from a, ref. 7 ; b, ref. 8.

by allowing phosphorylase kinase to be phosphorylated by the two different mechanisms simultaneously and separately. Fig. 6B shows that just over 6 moles of phosphate were incorporated ofter 80 min, and the phosphorylation curve shows the tendency to start levelling off, with possibly maximum phosphorylation reached after the incorporation of a further 2-3 phosphates. This result seems to agree with the hypothesis that common sites are phosphorylated by both mechanisms. The phosphorylation of separate sites will be more consistent with the incorporation of 15-18 phosphates. The implicit assumption is made in the above argument that phosphorylation by either mechanism can proceed independently of the other. Phosphorylation of phosphorylase kinase by the two different mechanisms acting separately also support the concept of a common set of sites. An experiment was done (data not shown) in which phosphorylase kinase was first maximally autophosphorylated and then used as a substrate for the protein kinase-catalyzed reaction at 10 mM Mg^{2+}. No further phosphorylation of phosphorylase kinase was observed, indicating that the autocatalytic mechanism phosphorylated the sites that would otherwise have been phosphorylated by protein kinase.

5. Possible Explanation for the Mg^{2+} Effect

Like many other kinases, the cAMP-dependent protein kinase uses the ATP-Mg^{2+} complex as a substrate. The preceding results suggest that the metallic ion has an additional role as an activator for the protein kinase-catalyzed phosphorylation of phosphorylase kinase. This effect of Mg^{2+} appears to be specific for phosphorylase kinase as the protein substrate, since when histone or casein was used as substrate for the protein kinase, phosphorylations at 1 and 10 mM Mg^{2+} were identical

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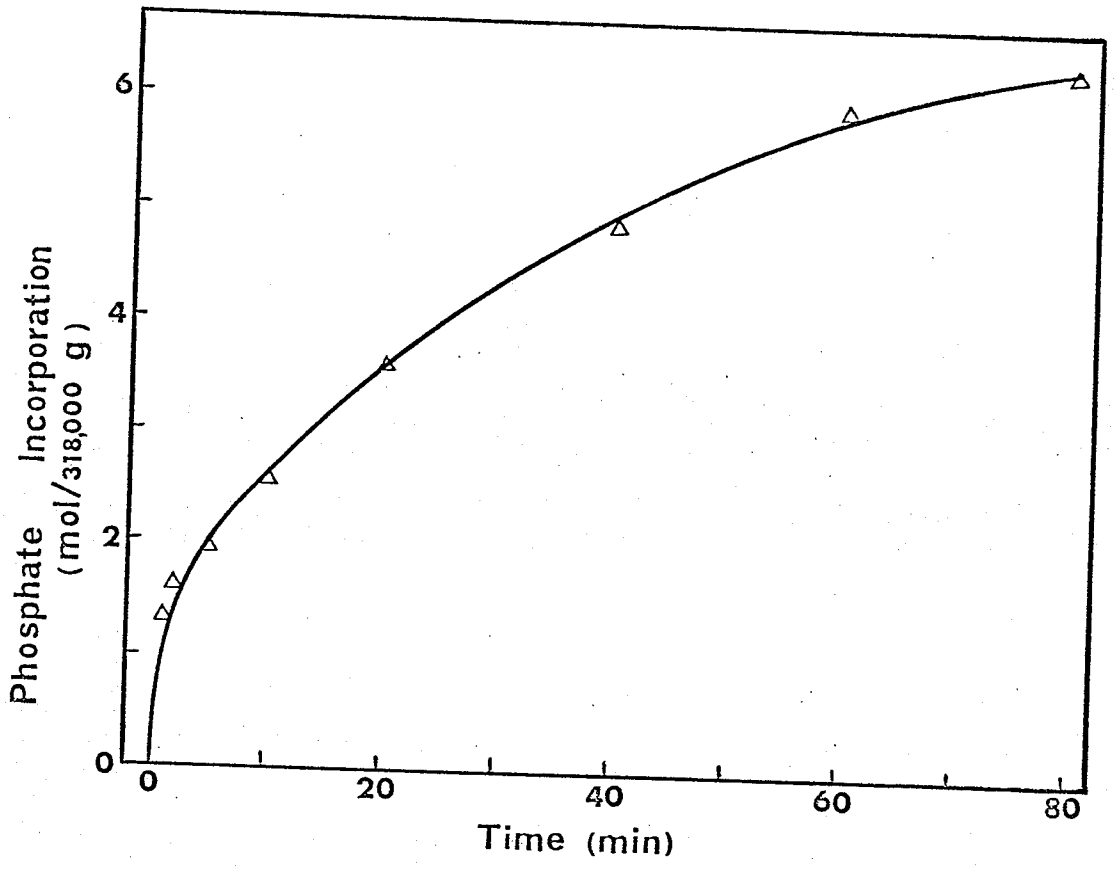

Fig. 6. (B). Phosphorylation of phosphorylase kinase by both the autocatalytic and protein kinase-catalyzed mechanisms. Phosphorylase kinase (0.36 mg/ml) was incubated in a reaction mixture containing 2.43 mM ATP, 11 mM Mg^{2+}, 0.10 mM Ca^{2+}, the catalytic subunit of protein kinase, 15 mM 2-mercaptoethanol, 0.75 mM EDTA, 3.8% sucrose, 52.5 mM MES buffer, pH 6.8. Aliquots were removed at the various times and assayed for protein-bound phosphate.

(Fig. 7). The results agree with the hypothesis that Mg^{2+} exerts its effect on phosphorylase kinase, presumably by changing the protein conformation to render more sites phosphorylatable. Since the additional sites were always phosphorylated slowly at 10 mM Mg^{2+}, the possibility that Mg^{2+} induces a slow conformational change in phosphorylase kinase has been considered. However, in experiments in which phosphorylase kinase was preincubated with and without high concentrations of Mg^{2+}, no difference was observed in the subsequent rates of phosphorylation.

Reimann et al. (92) have shown that an increase in Mg^{2+} concentration results in a decrease in the K_m for ATP by protein kinase when casein is used as the substrate. This effect of Mg^{2+} was not seen for the experiments in Fig. 7 since the ATP concentration used was saturating even at low concentrations of the metal ion.

6. Other Factors Affecting Phosphorylase Kinase Phosphorylation

The preceding results indicate that increases in Mg^{2+} concentration beyond 2 mM in the protein kinase-catalyzed reaction result in the phosphorylation of additional sites in phosphorylase kinase. Preliminary experiments have shown that phosphorylation of these additional sites may also be affected by other factors. At 10 mM Mg^{2+}, increasing ATP concentration from 0.3 to 1.4 mM resulted in an increase in the rate of phosphorylation of these sites.

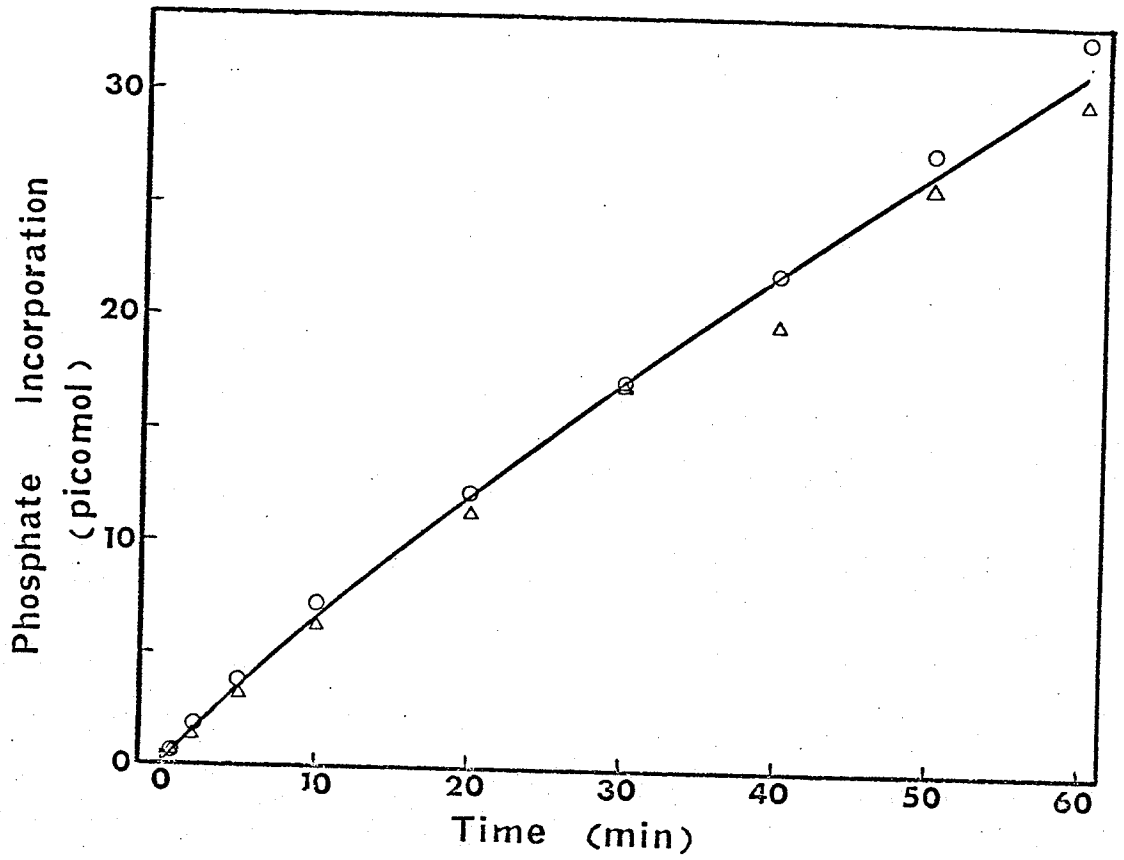

Fig. 7 (A). Phosphorylation of casein at 1 mM and 10 mM Mg^{2+}. Dephosphocasein (6 mg/ml) was incubated at 30°C with the catalytic subunit of protein kinase either in the presence of 1 mM Mg^{2+} - 0.1 mM EGTA (O) or 10 mM Mg^{2+} - 1 mM EGTA (Δ). Other ingredients present were, ATP, 0.28 mM; 2-mercaptoethanol, 15 mM; glycerophosphate buffer (pH 6.0), 50 mM. Aliquots were removed at various times and assayed for protein-bound phosphate.

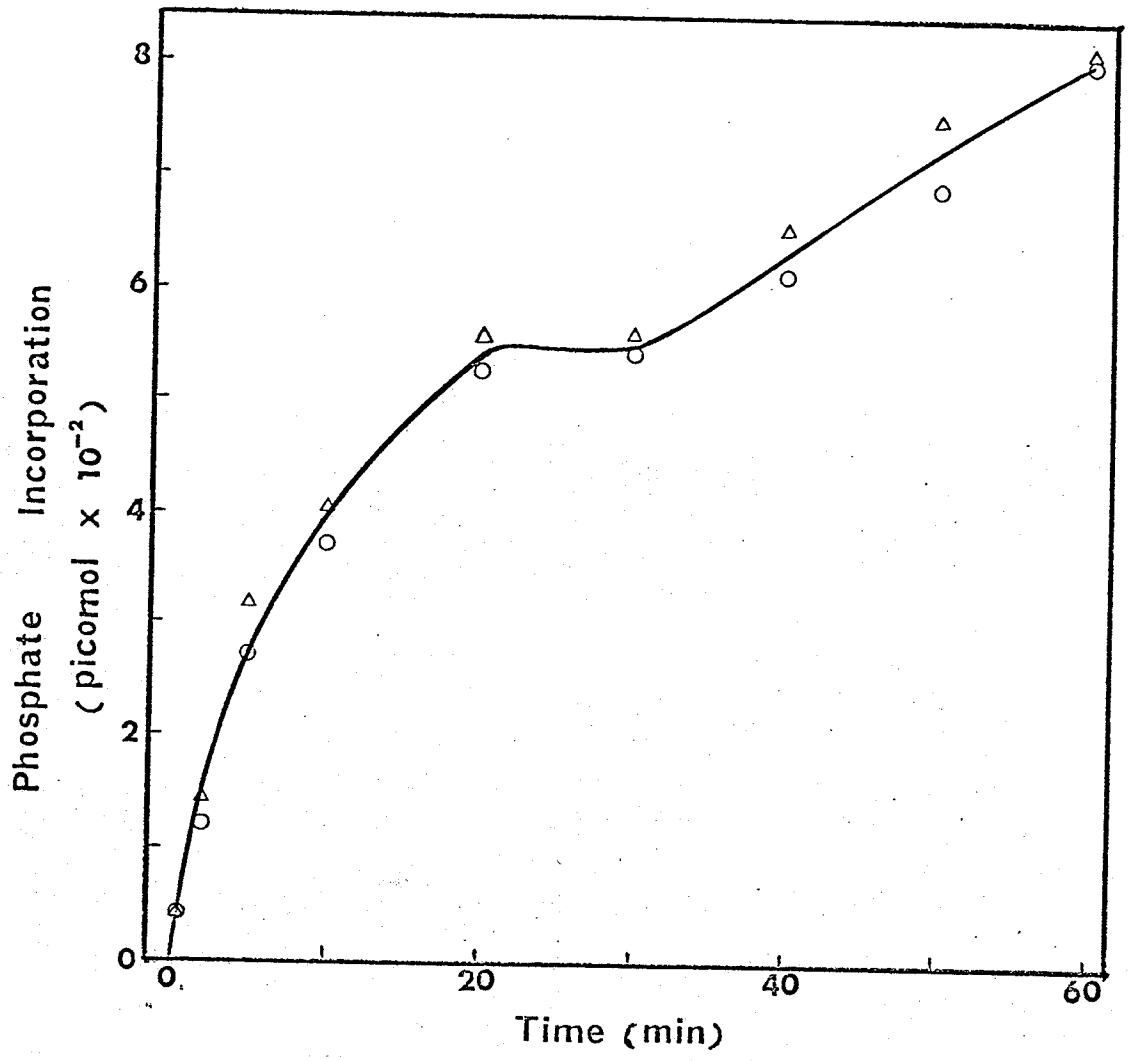

Fig. 7 (B). Phosphorylation of histone at 1 mM (O) and 10 mM (Δ) Mg^{2+}. Type II histone (1 mg/ml) was phosphorylated by the catalytic subunit of protein kinase under identical conditions as described in Fig. 7 (A).

B. Dephosphorylation and Inactivation of Different Phosphorylated Forms of Phosphorylase Kinase

1. Dephosphorylation of Phosphorylase Kinase by phosphoprotein phosphatase I and II

Khandelwal et al. (13) have reported that phosphoprotein phosphatase II can catalyze the dephosphorylation of activated phosphorylase kinase. Their observation has been repeated and extended in these studies. The different phosphorylated forms of phosphorylase kinase (see "Methods") were found to be protein substrates for both phosphoprotein phosphatase I and II. However in every case the dephosphorylation reaction was incomplete. For example, phosphorylase kinase labelled either at 2.4 sites (Fig. 8A) or 5.5 sites (Fig. 8B) was incompletely dephosphorylated by either phosphatase I or II. In both cases, the protein dephosphorylation shows an initial rapid phase follwed by a slow or resistant phase. Both phosphatase I and II seem to generate the same resistant phase since phosphatase I does not further dephosphorylate the resistant phase left after treatment with phosphatase II (data not shown). This suggests that the two phosphatases show the same specificity for the phosphorylated sites on the activated phosphorylase kinase.

The fact that the dephosphorylation of activated phosphorylase kinase does not proceed to completion (Fig. 8) may be due to one of several reasons. It is possible that the phosphatases may be very labile under the conditions of the study and hence become inactivated during the course of the reaction. However, the addition of excess phosphatase either at the start of the reaction or after the resistant phase has been produced did not cause the dephosphorylation reaction to proceed to completion. Metal ions are known to stimulate phosphatase activity (10, 11)

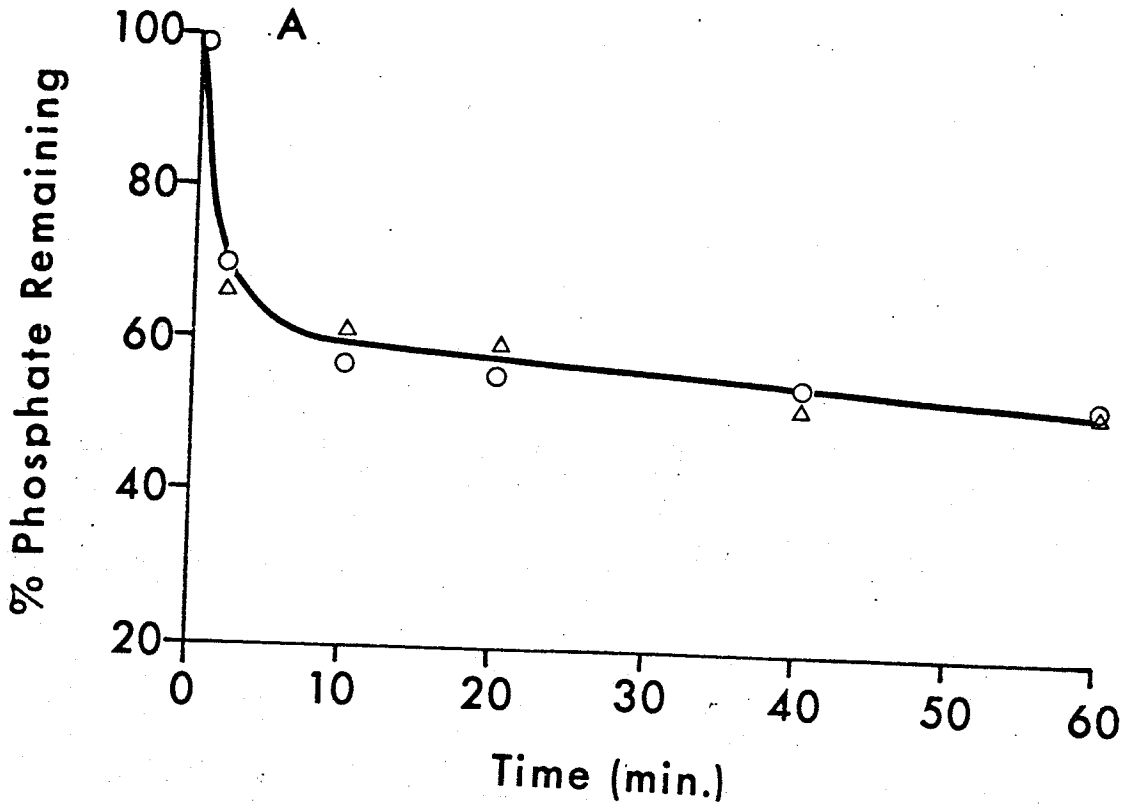

Fig. 8 (A). Dephosphorylation of phosphorylase kinase labelled at 2.4 sites by phosphoprotein phosphatase I and II. Phosphorylase kinase was phosphorylated by the protein kinase-catalyzed reaction and used as substrate for the phosphatases. Excess of either phosphatase I (o) or II (Δ) was used to initiate the reaction and the percent radioactivity remaining determined.

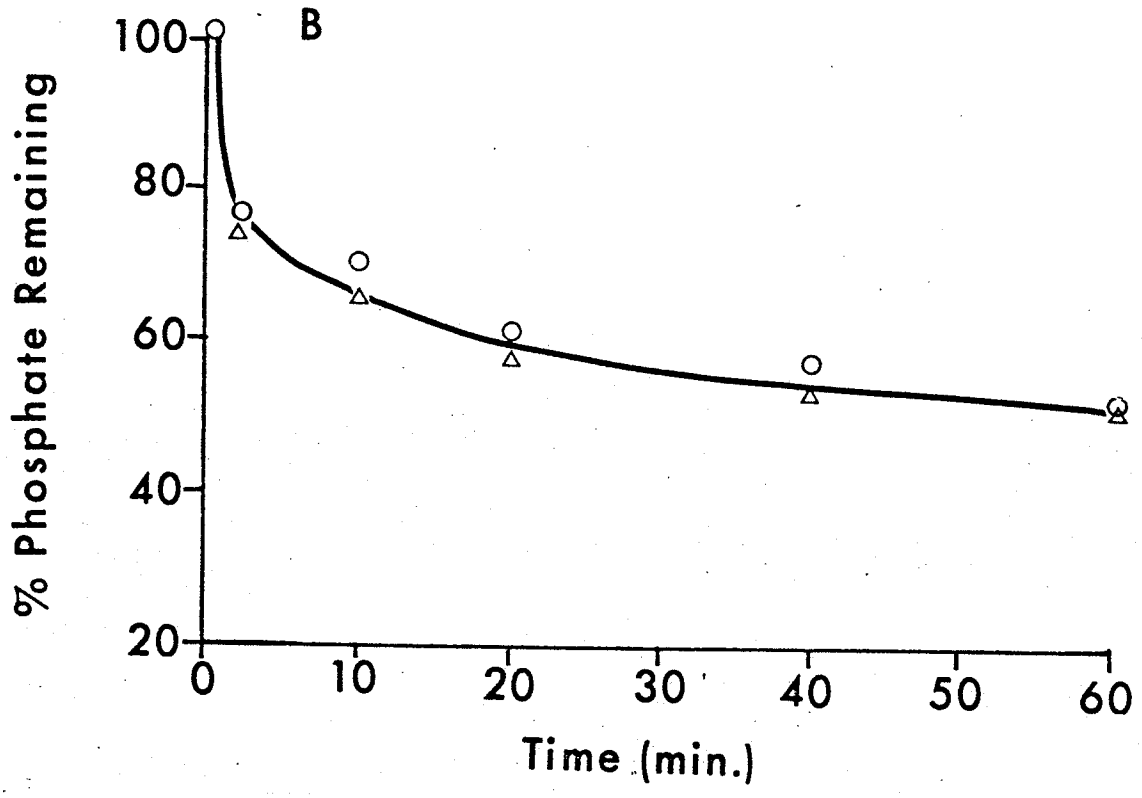

Fig. 8 (B). Dephosphorylation of phosphorylase kinase labelled at 5.5 sites by phosphoprotein phosphatase I (o) and II (Δ). Phosphorylase kinase used as substrate for the phosphatases was labelled by the protein kinase-catalyzed reaction. Other details are the same as for Fig. 8 (A).

but 1 mM Mn^{2+} or 10 mM Mg^{2+} did not enhance further the dephosphorylation of the resistant phase. Hence it was concluded that phosphatases I and II show specificity for only certain common sites on activated phosphorylase kinase.

The dephosphorylation of activated phosphorylase kinase by either phosphatase I or II leads to a deactivation of the enzyme. This deactivation is almost total for phosphorylase kinase initally labelled at 2 sites (Fig. 9A) or less than two sites (data not shown). These results are in agreement with those of a previous investigation (10). However, phosphorylase kinase which was labelled at 5 to 10 sites is only partially deactivated. Fig. 9B shows that 36% of the initial activity remains after treating phosphorylase kinase labelled autocatalytically at 9.2 sites for 1 hr with phosphatase. Similar results were also obtained when phosphorylase kinase labelled at greater than 5 sites by protein kinase (see "Methods") was used as a substrate for the phosphatase.

2. Pattern of Subunit Dephosphorylation of Phosphorylase Kinase

The time course of dephosphorylation of the different activated forms of phosphorylase kinase was examined in terms of their subunit structure. Fig. 10 shows the time course for the dephosphorylation of subunits A and B of phosphorylase kinase which was initially labelled at 2.4 sites by the cAMP-dependent protein kinase-catalyzed reaction. It can be seen that there is a rapid dephosphorylation of subunit B and a very slow dephosphorylation of subunit A. The very rapid and extensive dephosphorylation of subunit B leads to an equally rapid deactivation of the activated enzyme to less than 10% of its initial activity (see also Fig. 9A). Subunit A, it should be noted, loses about 20% of its original radioactivity over the 1 hr period of the reaction (Fig. 10). These

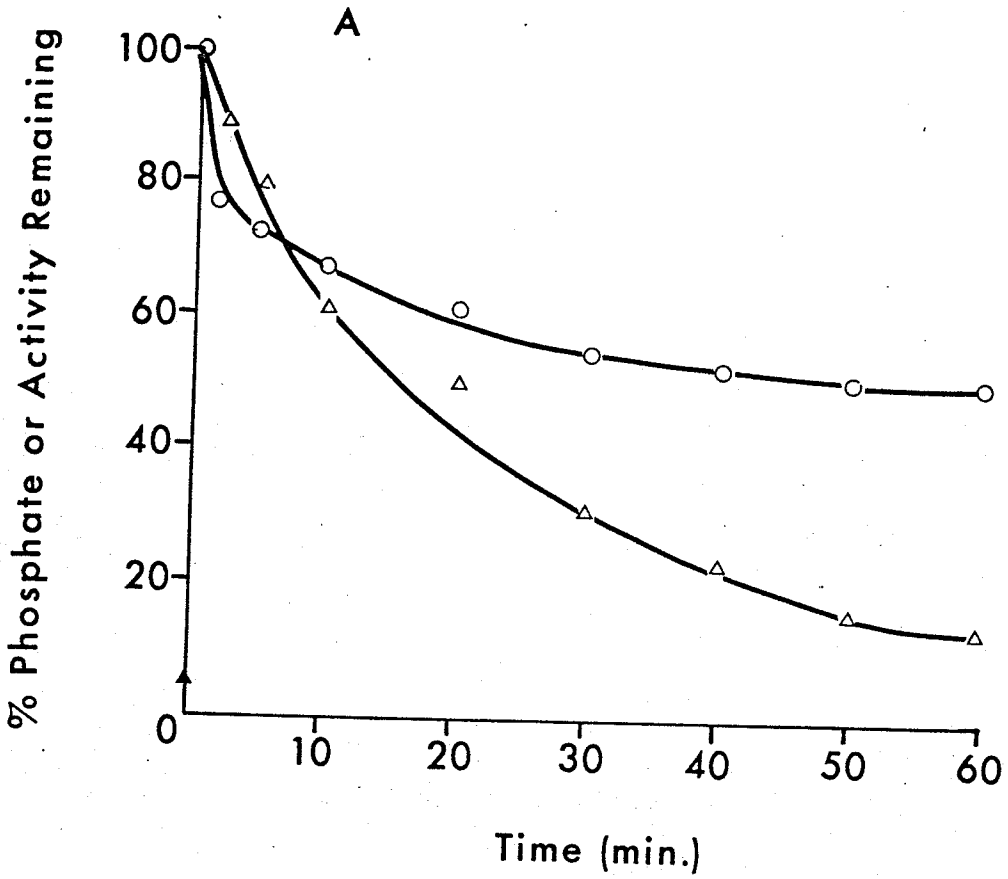

Fig. 9 (A). Correlation of the dephosphorylation of phosphorylase kinase labelled at 2.2 sites with enzyme deactivation. Phosphorylase kinase was labelled by the protein kinase-catalyzed reaction. The reaction was initiated by addition of phosphatase II. At different times aliquots of the reaction mixture were withdrawn and added to 50 mM glycerophosphate, pH 6.8 + 15 mM 2-mercaptoethanol + 50 mM NaF (kept on ice) to stop the reaction. Aliquots of the stopped reaction were used to determine enzyme activity (Δ) or protein-bound radioactivity (o). The activity of the nonactivated kinase is shown (▲).

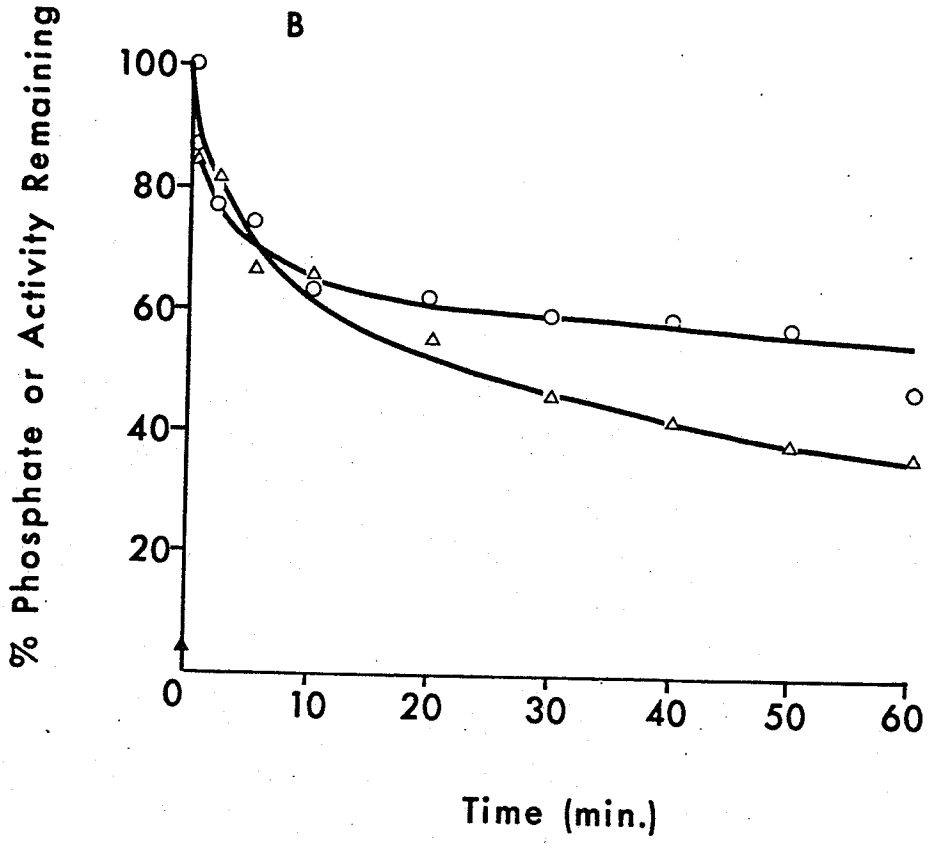

Fig. 9 (B). Correlation of the dephosphorylation of phosphorylase kinase labelled at 9.2 sites with enzyme deactivation. Phosphorylase kinase was labelled by the autocatalytic reaction. Other details are the same as for Fig. 9 (A). Enzyme activity (Δ) and protein-bound radioactivity (o) are shown. The activity of the nonactivated kinase is shown (▲).

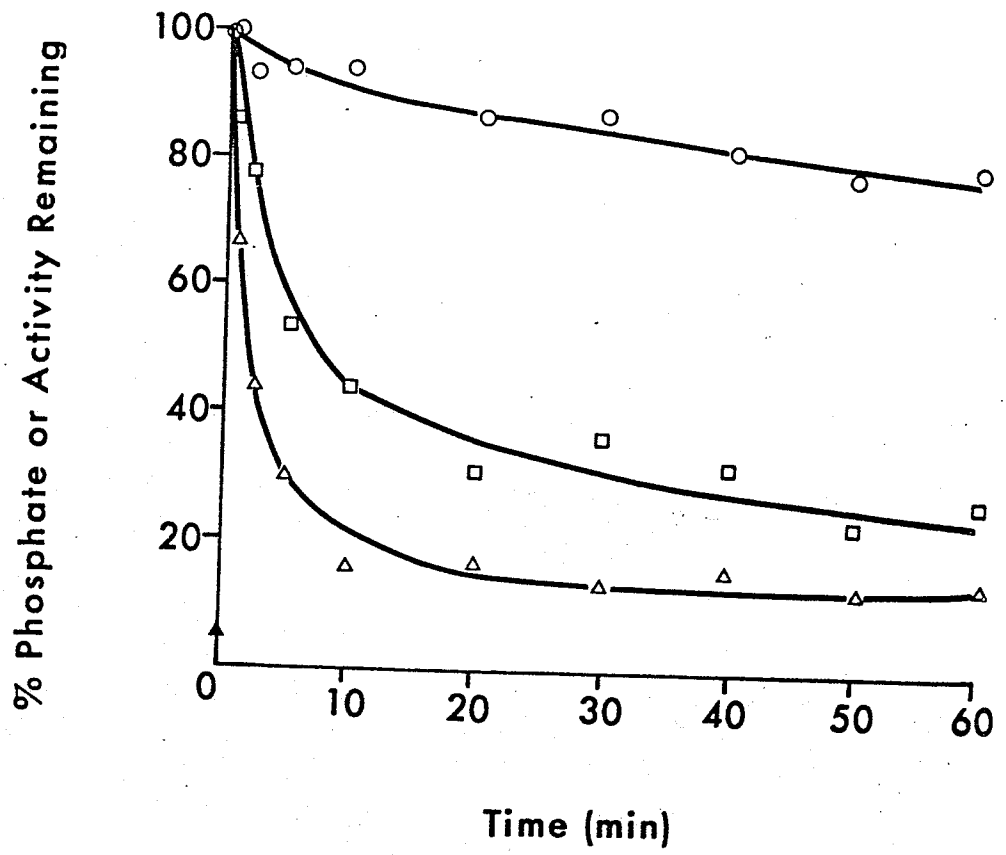

Fig. 10. Pattern of dephosphorylation of the subunits of phosphorylase kinase which was labelled at 2.4 sites by the protein kinase-catalysed reaction. The reaction was initiated by the addition of phosphatase II. Aliquots were removed at the different times and the reaction stopped as indicated in the legend to Fig. 9 (A). Enzyme activity (Δ) and the radioactivity in subunit A (O) and B (□) were determined as in "Methods". The activity of the non-activated kinase is shown (▲).

results are in general agreement with those obtained previously on the dephosphorylation of this form of activated phosphorylase kinase by phosphatase B (10, 12) suggesting that phosphatase I and II may be the same as or similar to phosphatase B.

It was previously postulated that the phosphorylation state of subunit A regulates the rate of dephosphorylation of subunit B and the deactivation of phosphorylase kinase (10) (see Section II.D.1). In the present studies, however, phosphorylase kinase containing less than 1 phosphate or up to 5 phosphates were found to be equally good substrates for the homogeneous phosphoprotein phosphatases. In one experiment phosphorylase kinase containing 0.73 (A = 0.20; B = 0.53), 2.4 (A = 1.4; B = 1.0) and 5.5 (A = 4.3; B = 1.2) moles of phosphate were used as substrates for phosphoprotein phosphatase II. In all cases subunit B was rapidly and extensively (release of 75% to 90% of radioactivity) dephosphorylated in 5 to 10 min. Hence increased phosphorylation of subunit A does not seem to enhance the rate of dephosphorylation of subunit B. This indicates that the homogeneous phosphoprotein phosphatases do not behave in the same way as the phosphatase (endogenous in the phosphorylated phosphorylase kinase preparations) utilized in the previous study (10).

Fig. 11 shows the dephosphorylation of the subunits of phosphorylase kinase which was labelled by the autocatalytic reaction and contains 9.2 moles of phosphate. Again there is a rapid dephosphorylation of subunit B and a much slower but significant dephosphorylation of subunit A. After exposure to the phosphatase for 1 hr, 44% of the phosphate on subunit A was released. Also shown in Fig. 11 is phosphorylase kinase activity. It should be noted that at least 50% of the original enzyme

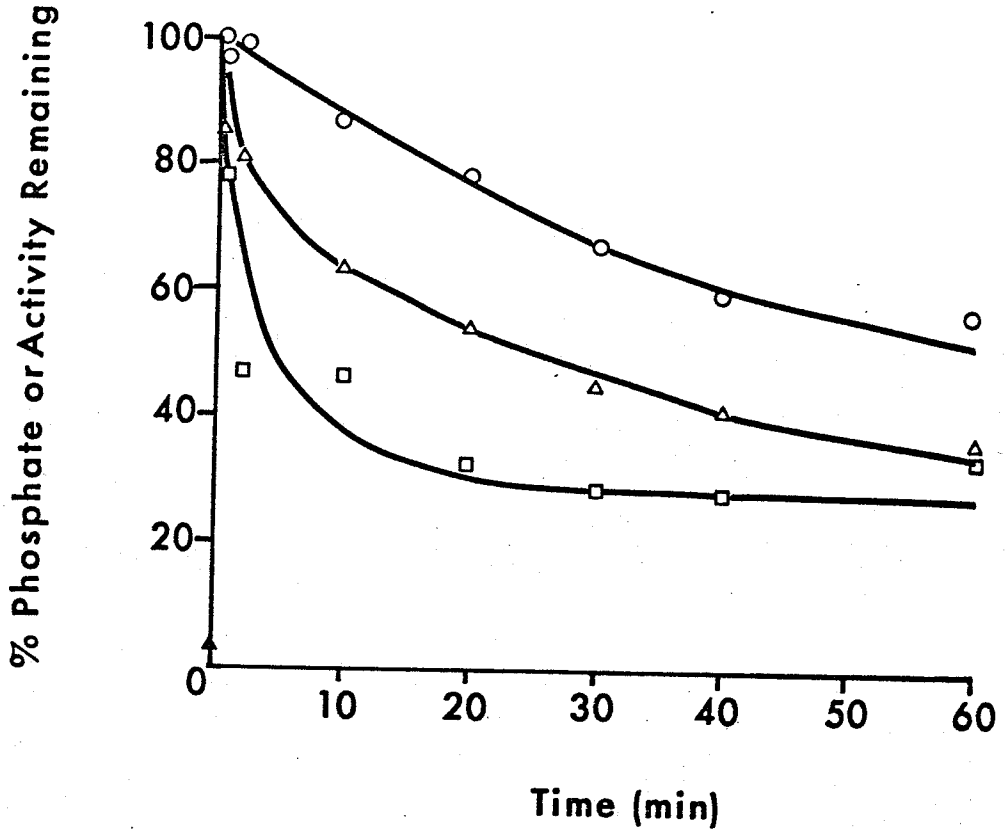

Fig. 11. Pattern of dephosphorylation of the subunits of phosphorylase kinase which was labelled at 9.2 sites by the autocatalytic reaction. The experimental details and the symbols used are the same as for Fig. 10. The activity of the nonactivated kinase is shown (▲).

activity remains after subunit B dephosphorylation is complete. A further 15% loss in activity also occurs and this seems to result from the dephosphorylation of sites on subunit A.

It is apparent from Figs. 10 and 11 that though most of the radioactivity on subunit B is rapidly released, about 25% is resistant to treatment with phosphatase. This finding raises the possibility of the existence of a susceptible set of sites (~75%) and a resistant set of sites (~25%) being present on subunit B. On the other hand, the resistant radioactivity may represent cross contamination of the B-band radioactivity by that from the A-band when the gels were sliced (see "Methods"). To check this latter possibility, the gels were sliced with an automatic gel slicer before and after treatment with phosphatase II for 1 hr. It can be seen from Fig. 12 that the A and B-band radioactivity are not completely resolved and hence cross contamination must have occurred between the bands. When phosphorylase kinase containing 1.7 moles of phosphate was treated with phosphatase for 1 hr, only a symmetrical radioactivity peak corresponding to subunit A remains (Fig. 12A). This result suggests that the resistant phase in subunit B seen in Figs. 9A & 10 is most likely due to contamination of band B radioactivity by that from band A. However, for the highly labelled phosphorylase kinase (Fig. 12B) a small amount of radioactivity remains in band B after treatment with phosphatase. Since cross contamination between bands A and B undoubtedly did occur, the exact percentage of the remaining phosphate in subunit B (Fig. 11 and Fig. 12B) is difficult to calculate accurately.

3. Existence of a Phosphatase Specific for Subunit A

It was concluded earlier that phosphoprotein phosphatase

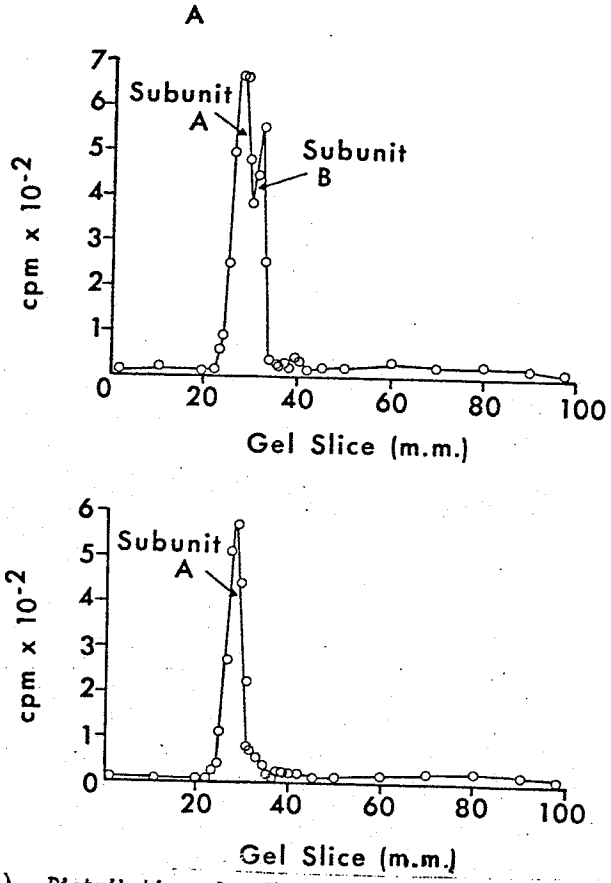

Fig. 12 (A). Distribution of radioactivity into the subunits of phosphorylase kinase labelled at 1.7 sites. Phosphorylase kinase was labelled by the protein kinase-catalyzed reaction. An aliquot of the labelled kinase was treated for 1 hr at 30° with excess phosphatase II. Samples of the phosphorylated and dephosphorylated enzyme were run on SDS-gels to separate the kinase into its subunits. These gels were then sliced into 1 mm segments on a Gilson Aliquogel automatic gel fractionator. The gel slices were treated and the radioactivity determined as described in "Methods". The top and bottom panels respectively represent the distribution of radioactivity before and after treatment with phosphatase.

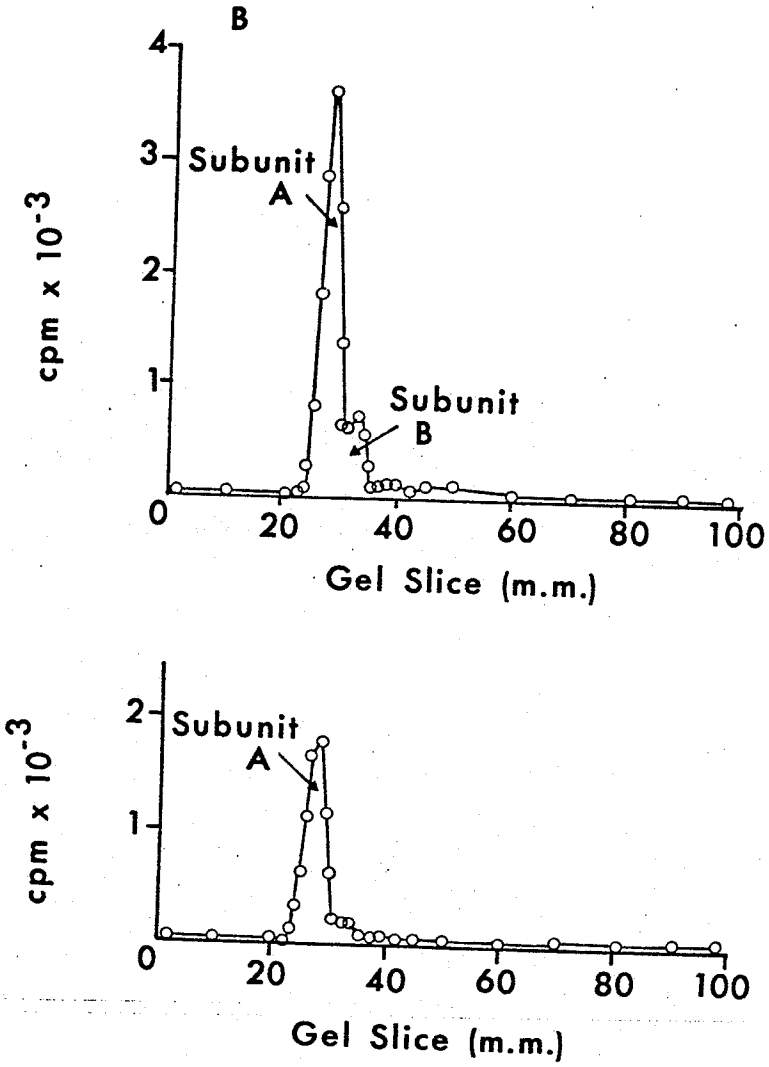

Fig. 12 (B). Distribution of radioactivity into the subunits of phosphorylase kinase labelled 5.2 sites. Phosphorylase kinase was labelled by the protein kinase-catalyzed reaction. An aliquot of the labelled kinase was treated for 1 hr at 30° with excess phosphatase II. Other details are the same as indicated for Fig. 12 (A).

I and II may be the same as or similar to phosphatase B discovered by Cohen and coworkers (12) . It could also be stated from the foregoing that the resistant phase seen in Fig. 8 is contributed mainly by phosphorylated subunit A. It was reported (245) that E. coli alkaline phosphatase can apparently cause selective dephosphorylation of subunit A. However, in the present study it was found that neither E. coli nor calf intestine alkaline phosphatase could further dephosphorylate the resistant phase produced by prior dephosphorylation of activated phosphorylase kinase by phosphatase I. However, phosphatase activity specific for phosphorylated subunit A was detected in skeletal muscle extract, in agreement with the previous report (12) . Such activity was also observed in liver extract as shown in Fig. 13. Catalytic amounts of phosphatase II were used to dephosphorylate subunit B mainly, thereby generating the resistant phase which is composed mostly of phosphorylated subunit A. Addition of an aliquot of either liver or enriched skeletal muscle extract in the presence of 10 mM Mg^{2+} caused a rapid loss of radioactivity from the resistant phase (Fig. 13).

4. Reversal of Phosphorylase Kinase Dephosphorylation

The ready interconversion of phosphorylase kinase between phosphorylated and dephosphorylated states has been demonstrated before (10, 219) . However, the phosphatase preparations used in these studies were only partially pure. Since the phosphoprotein phosphatases used in the present study have been purified to apparent homogeneity (13), it was important to establish that the deactivation of phosphorylase kinase catalyzed by these phosphatases can be readily reversed. Fig. 14 shows that the deactivation of phosphorylase kinase by phosphatase II could easily be reversed by inhibiting the phosphatase by a protein in-

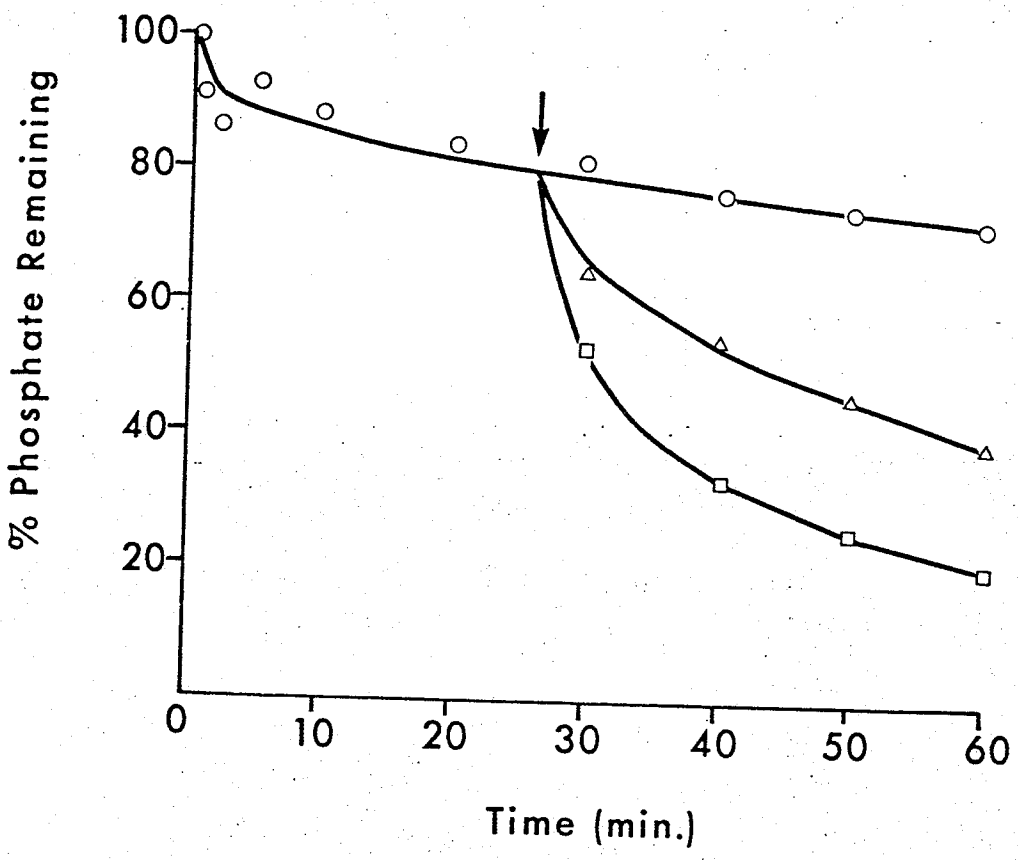

Fig. 13. Specific phosphatase activity directed against phosphorylated A-subunits. Phosphorylase kinase which was labelled at 5.2 sites by the protein kinase-catalyzed reaction was used in this experiment. The reaction was initiated with catalytic amounts of phosphatase II (O) so as to cause the dephosphorylation of the B-subunits in about 20 min but leaving the A-subunits largely in the phosphorylated form. At 26 min (↓) 123 μl aliquots of the initial reaction mixture (O) were removed and separately mixed with 10 μl of the crude rabbit liver extract (Δ) or 10 μl of the dissolved dialyzed pellet obtained by subjecting the crude extract from rabbit skeletal muscle to 60% Ammonium sulfate fractionation (□). Both of the latter dephosphorylations (Δ, □) were carried out in the presence of 10 mM Mg^{2+}.

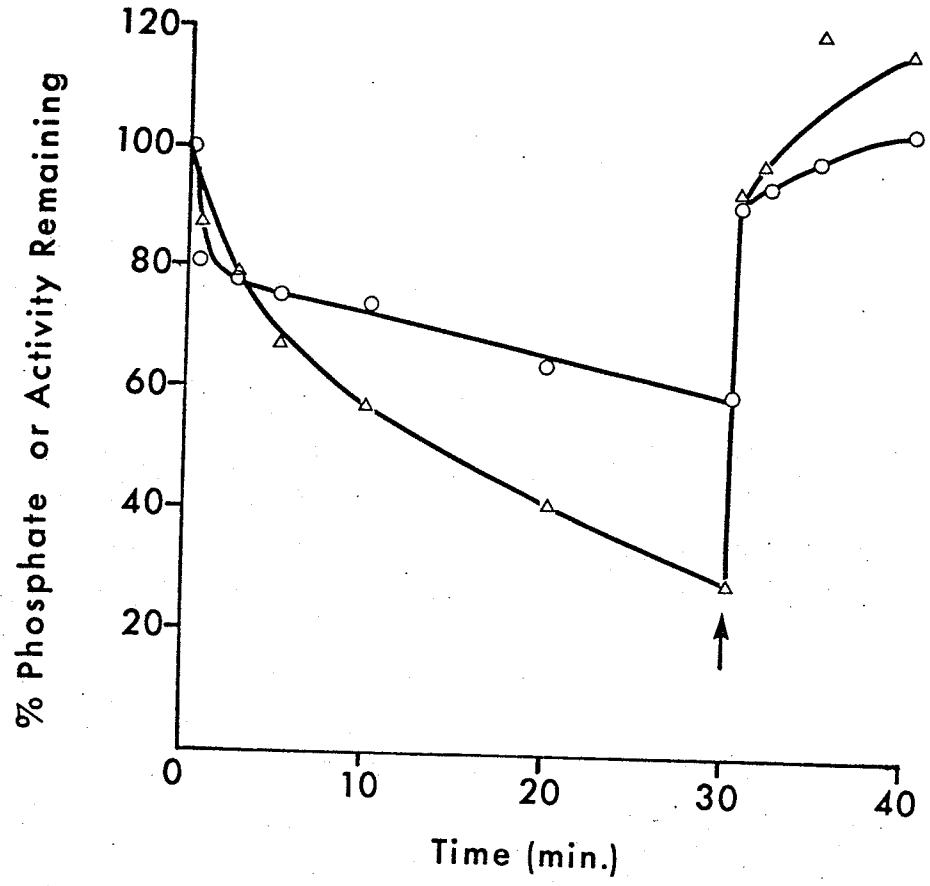

Fig. 14. Reversal of phosphorylase kinase dephosphorylation. Phosphorylase kinase labelled at 2.2 sites by the protein kinase-catalyzed reaction was used for this experiment. The reaction was started by addition of phosphatase II. Aliquots were removed at intervals for the determination of enzyme activity (Δ) or protein-bound radioactivity (0) as described in "Methods". At 30 min (↑) an aliquot of the reaction mixture was mixed with 0.22 mM ATP^{32}, 3 mM Mg^{2+}, 0.30 mM EGTA, catalytic subunit of protein kinase and excess phosphatase protein inhibitor. The incubation was continued at 30°. Aliquots were removed for the determination of enzyme activity or protein-bound radioactivity.

hibitor (238) and then treating phosphorylase kinase with the catalytic subunit of protein kinase in the presence of ATP-Mg^{2+}. It should be noted that the tendency of both phosphate incorporation and enzyme activation to exceed the starting values (0 min) is because Mg^{2+} was deliberately set at 3 mM so that the activation reaction catalyzed by protein kinase would be stimulated (9).

C. Activation of Phosphorylase Kinase by Organic Solvents

1. Stimulation of Nonactivated Phosphorylase Kinase by Organic Solvents

The conversion of phosphorylase b to phosphorylase a catalyzed by nonactivated phosphorylase kinase at pH 6.8 can be stimulated by high concentrations of various organic solvents (Table 3). At the 1 M level it can be seen that 2-propanol, dimethylsulfoxide and tetrahydrofuran give about an 18-fold stimulation. Acetone gave nearly a 28-fold stimulation and was the best solvent at the 1 M level for stimulating the activity of the nonactivated kinase. Ethanol at this concentration (1 M) gave only a 9-fold stimulation, but at higher concentrations (Fig. 15) much greater stimulation is achieved. At concentrations greater than 1.72 M, ethanol inhibits.

Besides the solvents shown in Table 3, others were also tested at the 1 M level. The higher alcohols: 1-butanol, 2-butanol, isoamyl alcohol besides being only slightly soluble in aqueous solution, also caused precipitation of the protein mixture. Dioxane was also tried but caused complete precipitation of the proteins. Unlike ethanol, ethanolamine and 2-chloroethanol gave no stimulation of phosphorylase kinase. Ethyl acetate stimulated the kinase 2-fold.

It is known that alkaline pH stimulates the activity of non-

TABLE 3. Stimulation of Nonactivated Phosphorylase Kinase by Different Organic Solvents[a].

Solvent	Stimulation (fold)
none	1.0
methanol	3.3
ethanol	8.8
1-propanol	3.3
2-propanol	18.6
acetone	27.5
methyl ethyl ketone	1.3
methyl isobutyl ketone	3.6
formamide	6.6
dimethyl sulfoxide	17.8
tetahydrofuran	18.6
ethyl formate	4.5
ethylene glycol	2.1
glycerol	1.1

[a]All organic solvents were tested at a concentration of 1 M. Each solvent was added directly to the basic reaction mixture as described in "Methods". The concentration of phosphorylase kinase used to catalyze the reaction was 0.52 μg/ml.

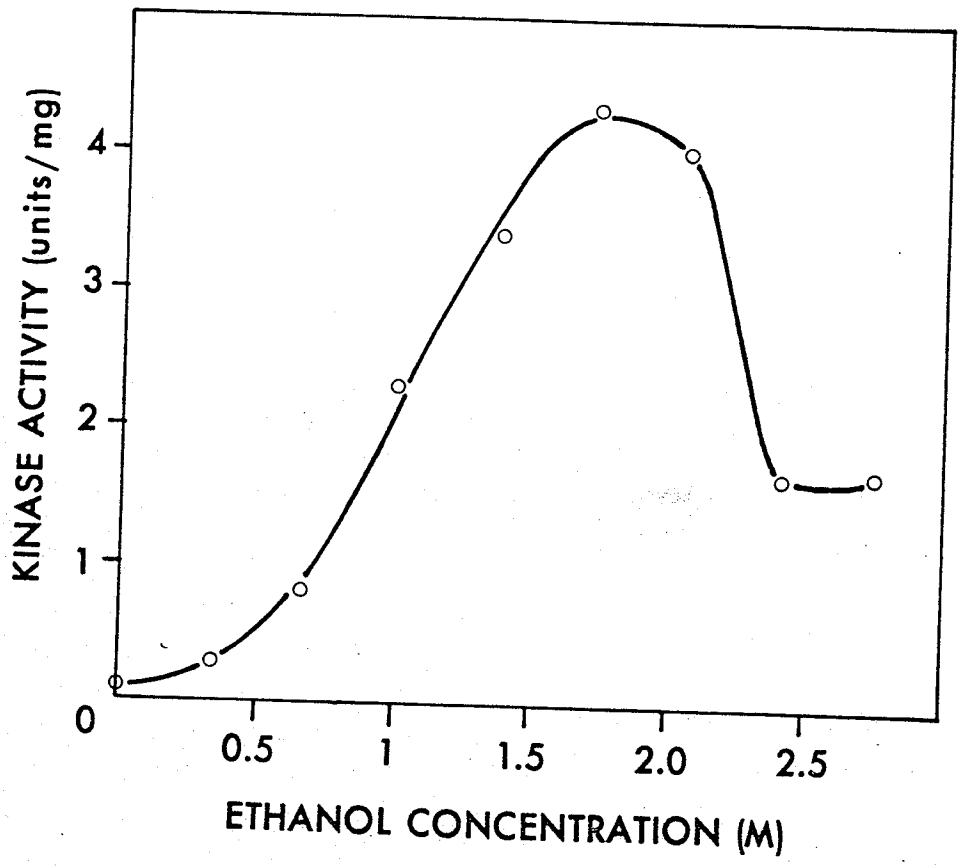

Fig. 15. Effect of various concentrations of ethanol on the activity of nonactivated phosphorylase kinase. Nonactivated phosphorylase kinase (1.04 μ g/ml) was assayed at 30° in a reaction mixture that contained 25 mM Tris-25 mM glycerophosphate, pH 6.8; phosphorylase b, 4 mg/ml; [γ-^{32}P] ATP, 2.28 mM; Mg^{2+}, 10 mM; Ca^{2+}, 0.1 mM; 2-mercaptoethanol, 6 mM; and different concentrations of ethanol as indicated. The reaction was initiated by kinase and after 5 min the incorporation of ^{32}P into protein was determined.

activated phosphorylase kinase (1). However it is unlikely that the high level of stimulation seen with some solvents (Table 3) could be explained by an increase in pH. When only the assay buffer and the appropriate solvent were mixed and the pH monitored, very little (0.1 to 0.2 pH units) or no change in the pH was observed.

If phosphorylase kinase is first activated by phosphorylation and then the effect of the organic solvent investigated, only a small stimulation of the kinase activity is observed (Fig. 16). In this experiment the activities of the activated and nonactivated kinase were assayed in the presence of 0.85 M ethanol. Increasing the concentration of ethanol up to 1.72 M did not cause any further stimulation of the activated kinase, unlike the case of nonactivated phosphorylase kinase (Fig. 15). The small degree of stimulation of the activated kinase compared to the nonactivated kinase suggests that the organic solvent stimulates the nonactivated enzyme by changing its conformation to one that is similar to that of the phosphorylated phosphorylase kinase.

Further evidence in support of this point may be obtained from an examination of the pH profile of phosphorylase kinase in the presence of organic solvents. Phosphorylase kinase in the activated and nonactivated state show characteristic pH dependence (1). Fig. 17A shows the change in pH dependence of the nonactivated kinase in the presence of 1.72 M ethanol. In the presence of the organic solvent, the pH activity profile of nonactivated phosphorylase kinase is almost identical to that of the activated kinase. In the absence of ethanol the enzyme showed a pH 6.8/8.2 activity ratio of 0.04 whereas the inclusion of 1.72 M ethanol in the assay changed this ratio to 0.64. As a comparison, the pH activity

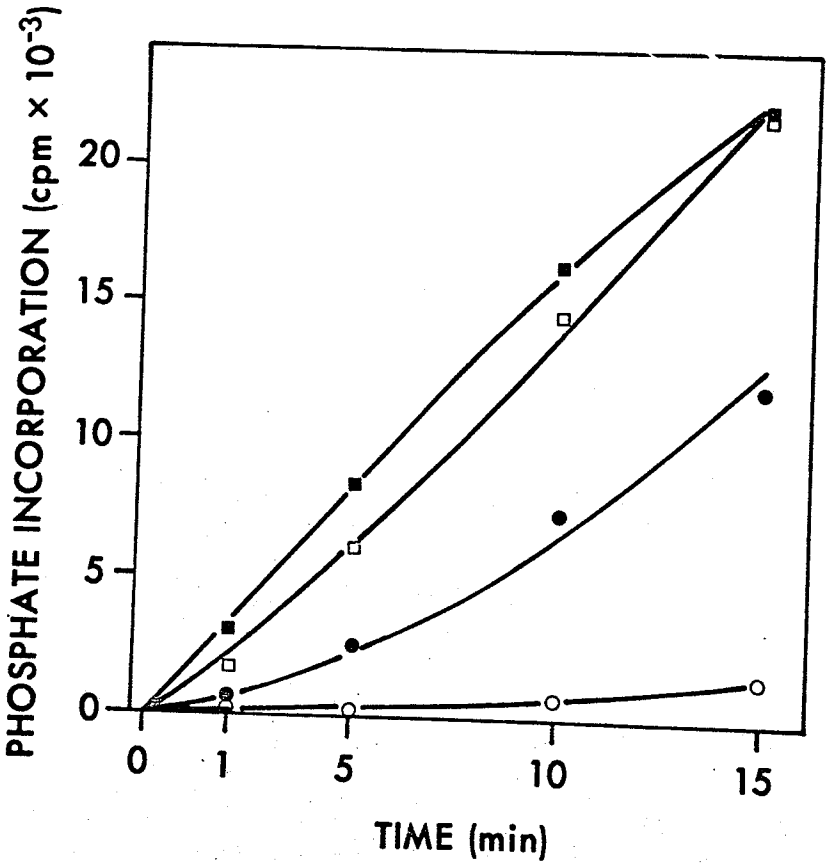

Fig. 16. Effect of ethanol on the activity of nonactivated and activated phosphorylase kinase. Nonactivated phosphorylase kinase (0.31 mg/ml) was activated by the catalytic subunit of protein kinase in a reaction mixture that contained 50 mM glycerophosphate buffer, pH 6.8; 2-mercaptoethanol, 14 mM; Mg^{2+}, 1 mM; [γ-^{32}P] ATP, 0.26 mM; EGTA, 0.5 mM; catalytic subunit of protein kinase. The reaction was initiated by the addition of protein kinase and stopped by cooling down in iced water after 30 min. During this time 2.1 moles of phosphate were incorporated into phosphorylase kinase. Kinase activity was assayed as described in "Methods". Where included, ethanol concentration was 0.85 M. (○) Nonactivated kinase (0.31 μg/ml); (●) Nonactivated kinase + ethanol; (□) Activated kinase (0.31 μg/ml); (■) Activated kinase + ethanol.

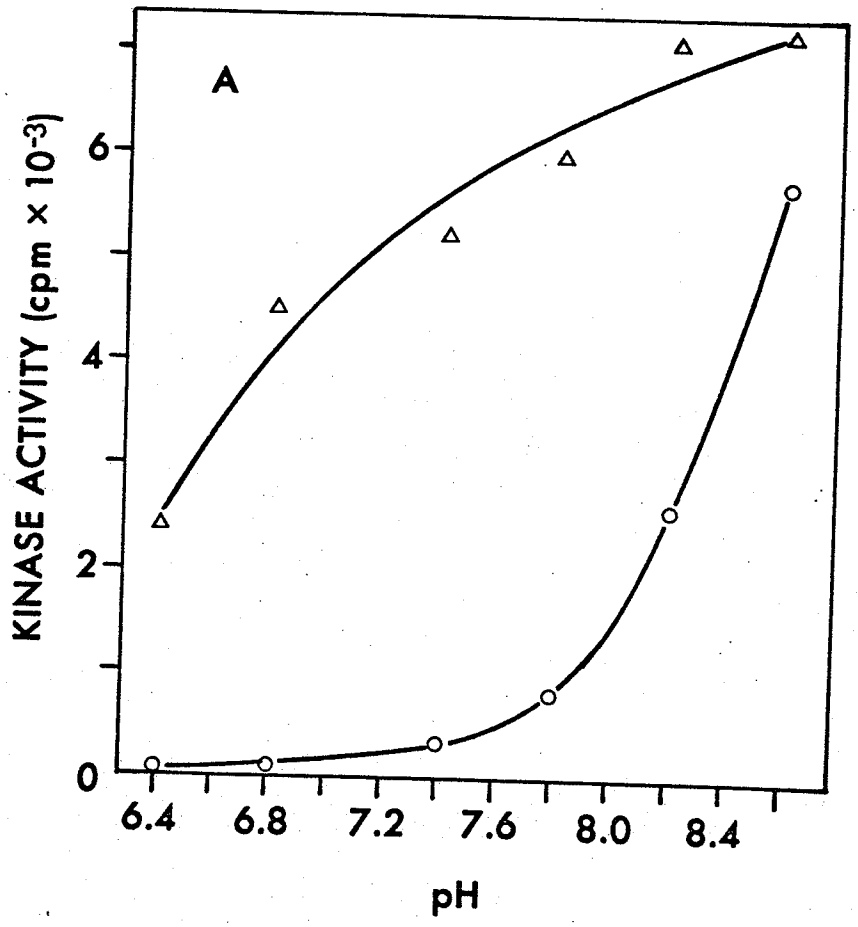

Fig. 17 (A). pH profile of nonactivated phosphorylase kinase in the presence and absence of ethanol. The reaction mixture contained 25 mM Tris-25 mM glycerophosphate adjusted to different pH's; phosphorylase b, 4 mg/ml; [γ-^{32}P] ATP, 2.28 mM; Mg^{2+}, 10 mM; Ca^{2+} 0.1 mM; 2-mercaptoethanol, 6 mM. Ethanol where included was 1.72 M. Nonactivated phosphorylase kinase at a concentration of 0.52 μg/ml was used to initiate the reaction at 30°. Aliquots were removed after 5 min for the determination of ^{32}P incorporated into protein. (○) Kinase alone; (Δ) Kinase + 1.72 M ethanol.

profiles of the phosphorylated kinase both in the absence and presence of 1.72 M ethanol are shown in Fig. 17B. The pH 6.8/8.2 activity ratios in the presence and absence of ethanol are 0.66 and 0.51, respectively. The pH activity profiles of nonactivated phosphorylase kinase in the presence of 1.72 M ethanol and the activated kinase (in the absence of ethanol) are very similar. This observation further supports the hypothesis that organic solvents permit the nonactivated kinase to assume a conformation similar to that of the activated kinase.

2. Stimulation by Organic Solvents also Observed with other Substrates

The possibility was considered that organic solvents such as ethanol may be affecting the substrate, phosphorylase, rather than the phosphorylase kinase molecule thereby increasing the rate of phosphorylation of phosphorylase. This possibility was investigated by using alternative substrates for the kinase. As shown in Fig. 18A the phosphorylation of casein can be stimulated by the inclusion of 0.85 M ethanol in the reaction mixture. This suggests that the stimulatory action of ethanol is directly on the phosphorylase kinase molecule rather than the substrate, phosphorylase. Further support for this concept comes from evaluating the effect of ethanol on the autophosphorylation of phosphorylase kinase. Fig. 18B shows that the autophosphorylation of the kinase is indeed stimulated by ethanol. Since phosphorylase kinase acts both as the enzyme and the substrate in this experiment, the direct effect of ethanol on the kinase is thereby established. While the initial rate of phosphorylation increases with the increase in ethanol concentration up to 1.02 M, the final extent of phosphorylation of phosphorylase kinase in 1.02 M ethanol is lower than that achieved in 0.34 M ethanol. The reason for this is that prolonged exposure of phosphorylase

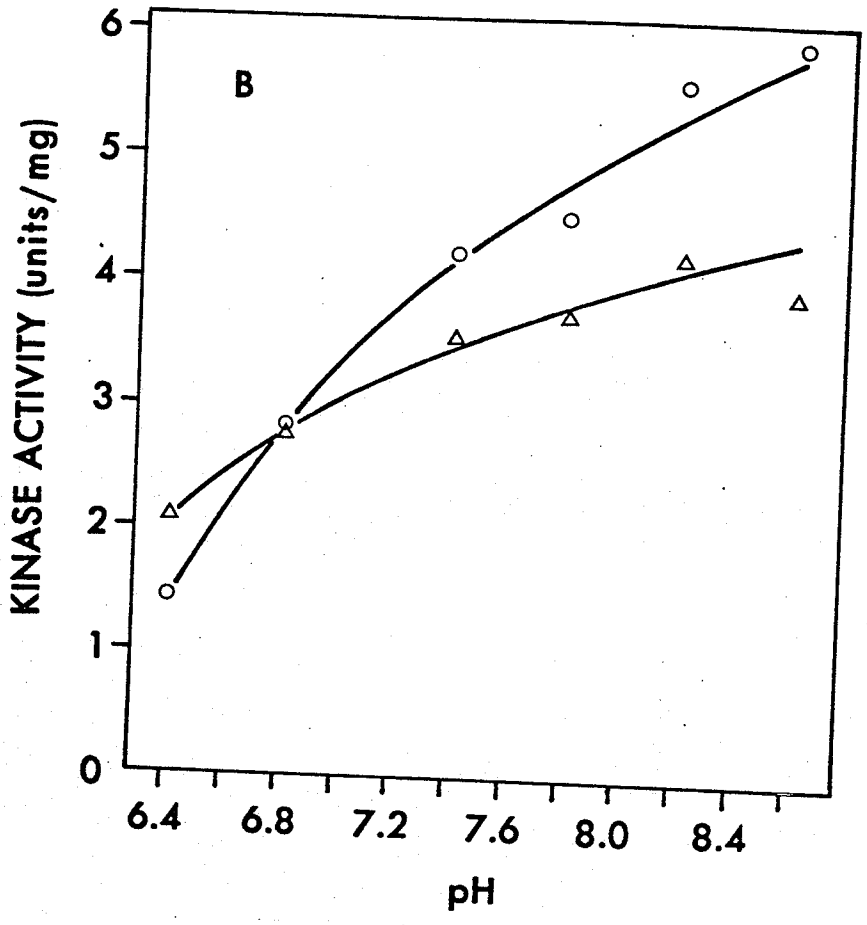

Fig. 17 (B). pH profile of activated phosphorylase kinase in the presence and absence of ethanol. Nonactivated phosphorylase kinase (0.31 mg/ml) was autoactivated in a reaction mixture that contained 50 mM Mes buffer, pH 6.8; Mg^{2+}, 10 mM; Ca^{2+}, 0.1 mM; [γ-^{32}P] ATP, 1.06 mM; 2-mercaptoethanol, 12 mM. The reaction was initiated by the addition of [γ-^{32}P] ATP and stopped by cooling down in iced water after 10 min at 30°C. Approximately 4 moles of phosphate were incorporated into the kinase. This activated kinase was assayed as in "Methods" at a concentration of 0.51 μg/ml at 30°. (O) Activated kinase alone; (Δ) Activated kinase + 1.72 M ethanol.

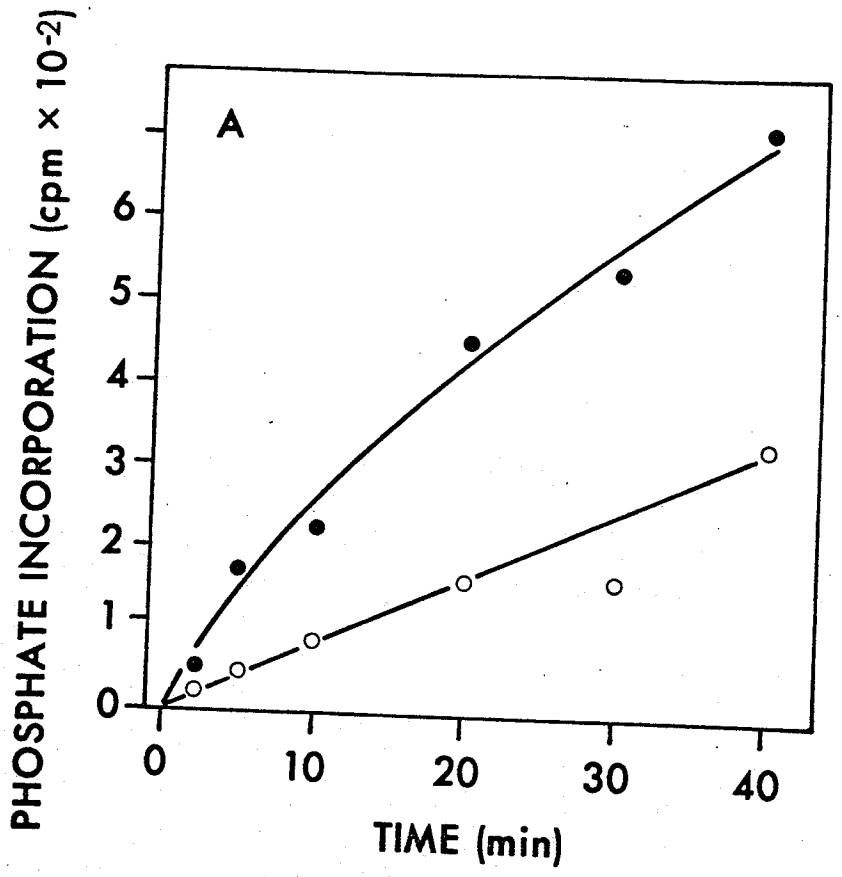

Fig. 18 (A). Phosphorylation of casein in the presence and absence of ethanol. Dephosphocasein (0.5 mg/ml) was phosphorylated in a reaction mixture that contained glycerophosphate buffer (pH 6.8), 50 mM; 2-mercaptoethanol, 13 mM; $[\gamma\text{-}^{32}P]$ ATP, 0.93 mM; Mg^{2+}, 10 mM; Ca^{2+}, 0.1 mM; phosphorylase kinase 10 μg/ml. The reaction was initiated by the addition of $[\gamma\text{-}^{32}P]$ ATP. Aliquots were removed at different times for the determination of ^{32}P incorporated into protein. (o) casein alone; (•) casein + 0.85 M ethanol.

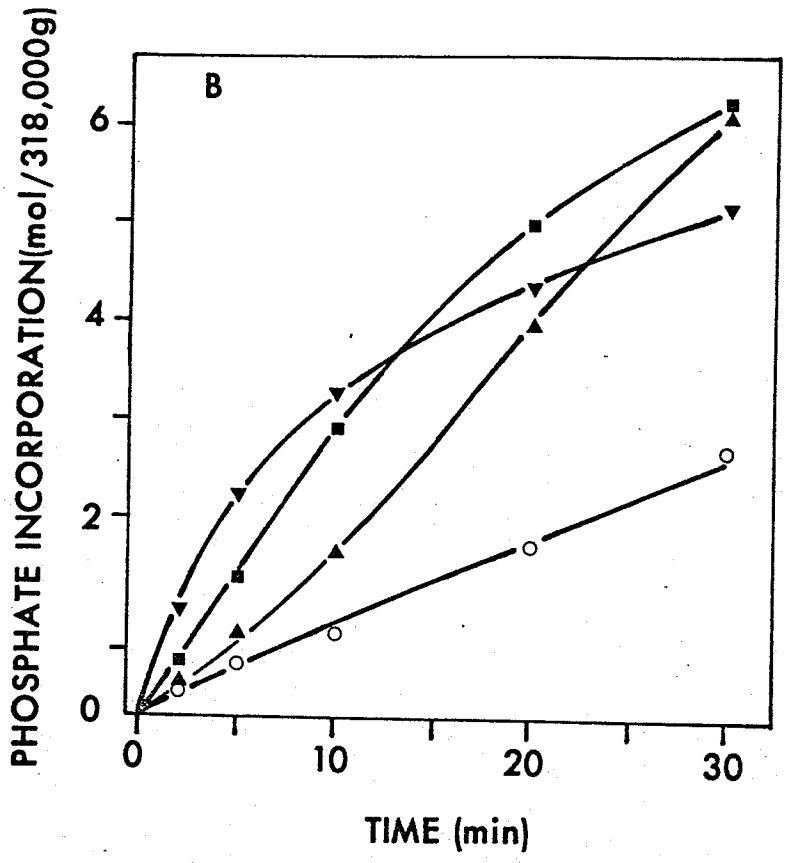

Fig. 18 (B). Autophosphorylation of phosphorylase kinase in the presence and absence of ethanol. Phosphorylase kinase (0.35 mg/ml) was autophosphorylated in a reaction mixture identical to that used for casein phosphorylation. Other details are the same as for Fig. 18 (A). (o) Autophosphorylation; (▲) autophosphorylation + 0.34 M ethanol; (■) autophosphorylation + 0.68 M ethanol; (▼) autophosphorylation + 1.02 M ethanol.

kinase to higher concentrations of ethanol results in its inactivation. In a separate experiment, it was shown that under the conditions described for Fig. 18B phosphorylase kinase lost nearly all of its activated activity after incubation at 30° for 30 min in 1.02 M ethanol.

3. Possible Mechanism of Activity Stimulation by Organic Solvents

To test whether the activation of phosphorylase kinase by organic solvents can be reversed by removal of the high concentration of the solvent, a sample of the kinase (0.5 mg/ml) was incubated with 0.85 M ethanol at 30° for 10 min. After a 1000-fold dilution the kinase activity was assayed both in the presence and absence of 0.85 M ethanol. The result showed that the preincubated enzyme behaved identical to a non-incubated sample, suggesting that the organic solvent effect depends on contact between the enzyme and the solvent during the enzyme assay. This particular point was further examined by the experiment shown in Fig. 19. Phosphorylase kinase was assayed in the presence and absence of 0.85 M ethanol (Fig. 19, Inset). After 5 min the ethanol was diluted to 0.17 M (curve B). Aliquots from the control received 0.17 M (curve A) or 0.85 M (curve C) ethanol. If the effect of this organic solvent was totally reversible, then it would be expected that the rate at which the activity increases in the presence of 0.17 M ethanol (curve A) would be the same as the rate for kinase also in the presence of 0.17 M ethanol but which was initially exposed for 5 min to 0.85 M ethanol (curve B). As can be seen from Fig. 19, the two curves (A and B) increase at different rates. However curve B shows a lower rate than curve C (0.85 M ethanol) indicating that the effect of the organic solvent is largely, but not totally, reversible. From the ability of ethanol to stimulate the autophosphorylation of phosphorylase kinase (Fig. 18B), it seems

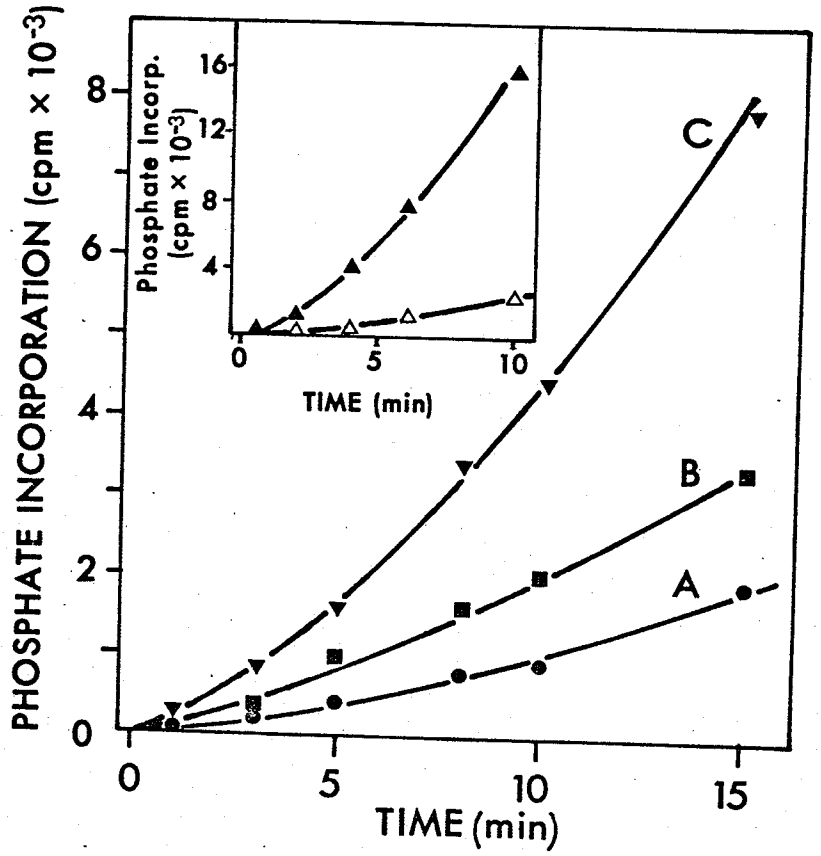

Fig. 19. Reversibility of ethanol stimulation of nonactivated phosphorylase kinase. Nonactivated phosphorylase kinase (1.51 μg/ml) was assayed in the presence (▲) and absence (△) of 0.85 M ethanol (Inset) as described in "Methods". After 5 min a 5-fold dilution was made to reduce the ethanol concentration to 0.17 M (curve B) and the kinase concentration to 0.30 μg/ml. All other ingredients were maintained at their initial concentrations and the incubation continued. Aliquots from the control (△) were similarly diluted but in addition received 0.17 M ethanol (curve A) or 0.85 M ethanol (curve C). Samples were removed at the different times for the determination of ^{32}P incorporated into protein.

possible that the difference in rates of curves A and B (Fig. 19) may be due to a slight autoactivation of phosphorylase kinase during the 5 min preincubation with 0.85 M ethanol (Fig. 19, Inset).

Another possibility to account for the stimulation of phosphorylase kinase activity by organic solvents is that the latter may be solubilising from the kinase endogenous lipids which may be acting as inhibitors of the enzyme activity. Hence if the putative lipids are separated out the kinase should show an increase in its pH 6.8/8.2 activity ratio, characteristic of phosphorylase kinase which is activated (1). This point was checked by preincubating nonactivated phosphorylase kinase with 1.72 M ethanol at 3° for 10 min, then passing the mixture through a small Sephadex G-25 column (1.1 x 20 cm) equilibrated with 50 mM glycerophosphate + 1 mM EDTA, pH 6.8 + 10% sucrose. The pH 6.8/8.2 ratio was the same (0.06) both before and after preincubation with ethanol. Hence, it is not likely that the stimulation of phosphorylase kinase by organic solvents is due to the solubilisation of inhibitory lipids.

The stimulatory effect of organic solvents on phosphorylase kinase could conceivably be explained by the dissociation of the enzyme to a more active state. However, analytical sedimentation velocity ultracentrifugation revealed no dissociation of the native enzyme in the presence of 1.72 M ethanol.

Phosphorylase kinase, whether in a nonactivated or activated form, is known to require Ca^{2+} for activity (5,18,19,175,176). It was therefore interesting to find out whether the organic solvent-stimulated kinase also showed this dependency. As can be seen in Fig. 20, in the absence of any added Ca^{2+} but in the presence of 1 mM EGTA, the kinase has no

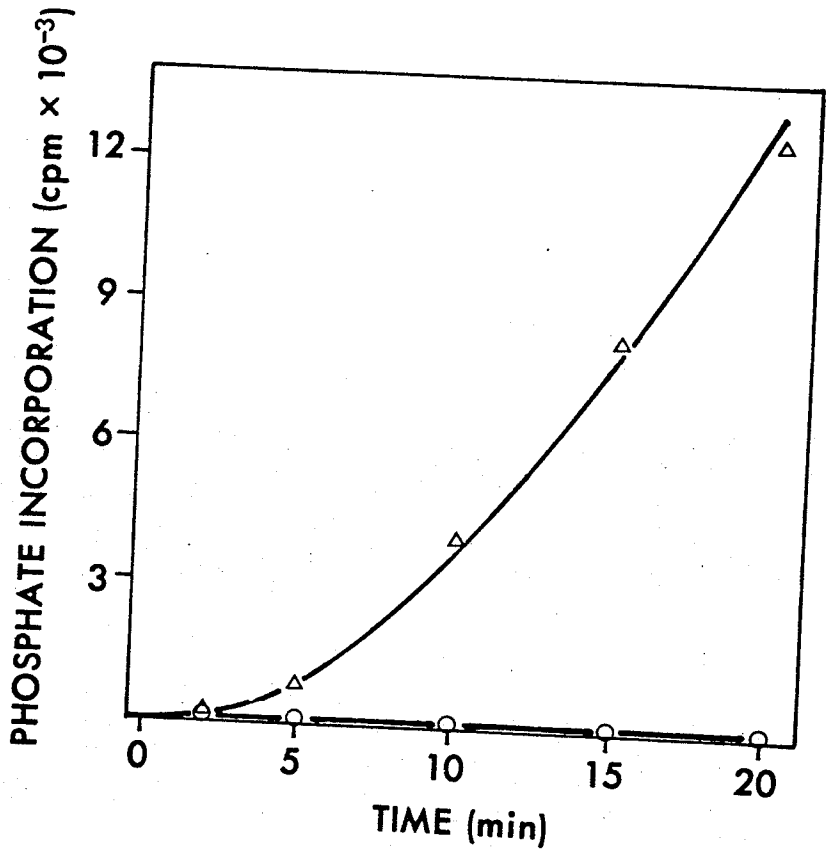

Fig. 20. Effect of EGTA on the activity of nonactivated phosphorylase kinase in the presence of ethanol. Nonactivated phosphorylase kinase (0.34 g/ml) was assayed in the presence of 0.1 mM Ca^{2+} (Δ) or 1 mM EGTA (O). Ethanol was present at a concentration of 0.85 M. Other details are given in "Methods". Samples were removed at the different times for the determination of ^{32}P incorporated into protein.

activity in the presence 0.85 M ethanol. This observation indicates that even though organic solvents stimulate phosphorylase kinase they do not release the enzyme from its Ca^{2+} dependency.

4. Possible Physiological Stimulators of Phosphorylase Kinase Activity

Since phosphorylase kinase has been found to be partly membrane-bound and the membrane-bound form has a higher pH 6.8/8.2 activity ratio (153, 231, 246), the possibility was considered that phospholipids may activate the enzyme. Table 4 lists different substances tried as possible activators of nonactivated phosphorylase kinase. Neither the saturated fatty acids, phospholipids, nor the nonionic detergents Triton X-100 and Tween 20 serve as activators of the kinase. However, the unsaturated fatty acids, palmitoleic and oleic acids, stimulate the kinase activity almost three-fold when used at a concentration of 2 mM. Because ethanol activates phosphorylase kinase and the fatty acids were dissolved in ethanol the actual fold of stimulation achieved by the unsaturated fatty acids was difficult to quantitate. Hence, the sodium derivative of oleic acid was used to make an aqueous suspension of this fatty acid and thereby investigate more closely its stimulatory effect. Fig. 21 shows the stimulatory effect of oleic acid in the concentration range 0 to 4 mM. It can be seen that a 6-fold stimulation is obtained by this fatty acid at a concentration of 0.8 mM. Because of the insolubility of the fatty acid in aqueous solution, the actual effective stimulatory concentration may be less than 0.8 mM.

It has been reported (231) that membrane-bound phosphorylase kinase exhibits a pH 6.8/8.2 ratio of 0.5. A comparably high pH 6.8/8.2 ratio of 0.4 was found when nonactivated phosphorylase kinase (pH 6.8/8.2 ratio initially 0.04) was assayed in the presence of 1 mM oleic

TABLE 4. Effect of Fatty Acids, Phospholipids and Nonionic Detergents on the Catalytic Activity of Nonactivated Phosphorylase Kinase[a].

Addition	Stimulation (fold)
none	1.00
lauric acid	0.70
myristic acid	0.73
palmitic acid	0.85
stearic acid	1.19
arachidic acid	1.27
palmitoleic acid	2.52
oleic acid	2.61
phosphatidyl choline	0.72
mixed phospholipids	0.87
triton X-100	1.04
tween 20	0.96

[a]The fatty acids and phospholipids were tested at 2 mM, the detergents at 0.5%. Stock solutions of all the substances tested were made in ethanol. This means that upon addition of each substance to the basic reaction mixture (see "Methods"), 0.34 M ethanol was introduced into the assay. The slight stimulation caused by this concentration of solvent is taken into account in calculating fold of stimulation. The concentration of phosphorylase kinase was 0.52 μg/ml.

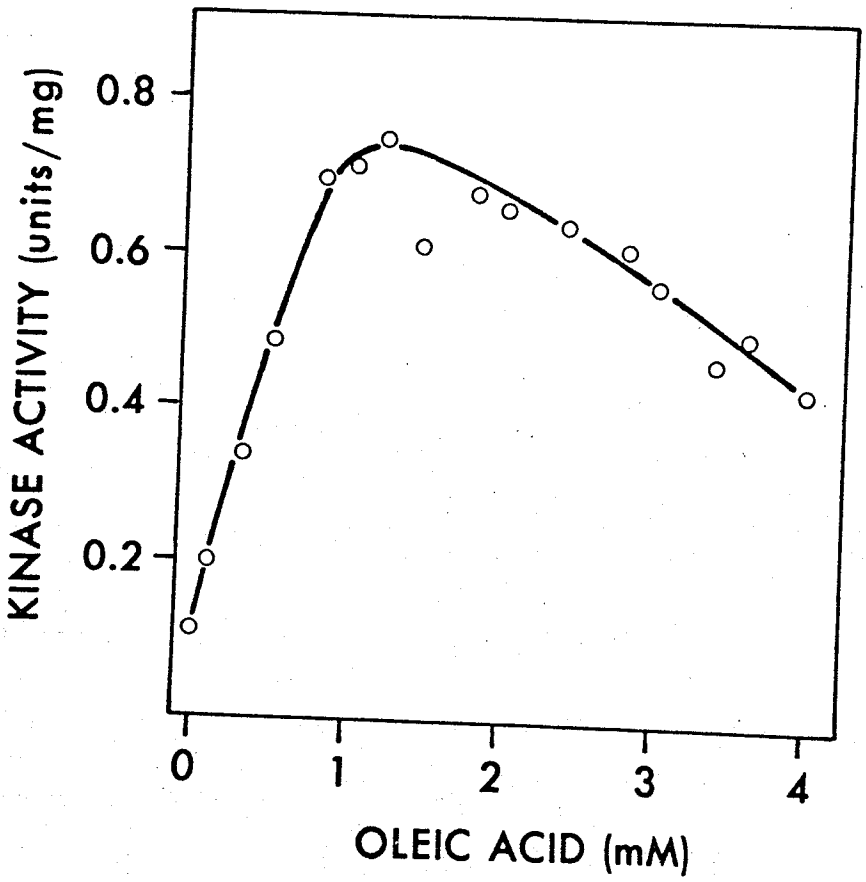

Fig. 21. Stimulation of nonactivated phosphorylase kinase by oleic acid. Phosphorylase kinase (0.52 μg/ml) was assayed in the basic reaction mixture as indicated in "Methods". Oleic acid (as its salt, sodium oleate) was added at the concentrations shown directly to each assay tube. Aliquots were removed after 5 min for the determination of ^{32}P incorporation.

acid. It therefore seems that unsaturated fatty acids may be important physiological activators of phosphorylase kinase.

V. DISCUSSION

Rabbit skeletal muscle phosphorylase kinase can be activated by the phosphorylation of specific sites on the enzyme. Such phosphorylation can be catalyzed either autocatalytically or by the cAMP-dependent protein kinase (185). Recent detailed studies on the autocatalytic mechanism revealed that 7 to 9 moles of phosphate per monomeric unit of the kinase can be incorporated with the resultant enzyme activation being as high as 200-fold (7 , 8) . The protein kinase-catalyzed reaction on the other hand was shown to involve the incorporation of 2 moles of phosphate per monomeric unit with enzyme activation being 50-fold (4). Both subunits A and B were found to be phosphorylated by either mechanism (4, 6, 7, 8).

The present results indicate that phosphorylase kinase can be activated by the protein kinase-catalyzed reaction to higher levels than previously observed. Phosphorylase kinase apparently contain two sets of phosphorylation sites. These sites are phosphorylated at different rates by protein kinase. As a result the protein kinase-catalyzed activation of phosphorylase kinase at high concentrations of Mg^{2+} can conveniently be analyzed in two phases. In the first phase only two sites per monomeric unit of phosphorylase kinase become significantly phosphorylated. One of these sites is rapidly labelled and is on subunit B, the other become phosphorylated at a slower rate and is located on subunit A. Enzyme activation reaches 50-fold in this phase and seems to correlate best with the rapid phosphorylation of the single site on subunit B. This phase of phosphorylase kinase activation can be studied separately since only the above two sites are phosphorylated when the reaction is catalyzed at low

concentrations of Mg^{2+}. Earlier studies (4) analyzed this phase only and agree with the findings here. In the second phase, up to 5 to 7 additional sites per monomeric unit of the kinase become phosphorylated at a slower rate. These sites are located almost exclusively on subunit A and their phosphorylation is responsible for a further increase in the enzyme activity by 135-fold. It is therefore clear that the protein kinase-catalyzed activation of phosphorylase kinase results from the phosphorylation of sites on both subunits A and B. These conclusions are supported by the finding of Hayakawa et al. (6) who observed that a slow further activation of phosphorylase kinase can be correlated with the phosphorylation of subunit A. Lincoln and Corbin (150) have also recently demonstrated that up to 6 moles of phosphate could be incorporated into phosphorylase kinase by the catalytic subunit of the cAMP-dependent protein kinase. They did not analyse the distribution of these phosphates between subunits A and B.

Phosphorylase kinase activated by the protein kinase-catalyzed reaction at high Mg^{2+} concentration is similar to phosphorylase kinase that is autoactivated in many respects. These include enzyme activity and phosphate content as well as the distribution of these phosphates between subunits A and B. These results indicate that activation by the two different mechanisms may result from the phosphorylation of similar if not identical sites. Such a hypothesis is supported by the finding that only about 7 phosphates per monomeric unit of phosphorylase kinase were incorporated in 80 min when activation by the two different mechanisms is allowed to proceed simultaneously. The observation that no further phosphorylation by protein kinase occurs when the maximally autophosphorylated phosphorylase kinase is used as substrate reinforces the argument for a

common set of phosphorylation sites. The use of synthetic peptides to study the substrate specificity of protein kinase (182, 247-250) and phosphorylase kinase (181, 182, 183) does not discount the possibility of the two kinases having overlapping specificity in the natural substrate. In fact, it was demonstrated that a peptide derived from phosphorylase and containing the convertible seryl residue could be phosphorylated by both protein kinase and phosphorylase kinase (181, 182). However native phosphorylase is not a substrate for protein kinase (181, 227). Hence protein conformation is an important determinant in dictating enzyme specificity, as previously emphasized (247, 251). It is therefore postulated that the high concentration of Mg^{2+} alters the conformation of phosphorylase kinase in such a way as to render the second phase phosphorylation sites more accessible to the catalytic action of protein kinase.

The activation of phosphorylase kinase resulting from the phosphorylation of specific sites on the enzyme can be reversed by two homogeneous preparations of phosphoprotein phosphatases from rabbit liver. These phosphatases were initially found to be very similar catalytically (13). Results in the present study support such a conclusion.

Phosphorylase kinase labelled to various extents were used as substrates for the phosphoprotein phosphatases. The cAMP-dependent protein kinase could readily catalyze the incorporation of 2 moles of phosphate per monomeric unit into phosphorylase kinase. One of these phosphates is in subunit A, the other in subunit B. Under similar experimental conditions the phosphorylation reaction could be terminated when approximately 1 phosphate per monomeric unit of the kinase is incorporated. In this case subunit B contains approximately 0.70 moles and subunit A 0.30 moles of phosphate. When these two different phosphorylated forms of phosphorylase

kinase were used as substrates for the phosphoprotein phosphatases, subunit B was shown to be rapidly dephosphorylated. This was paralleled by an equally rapid deactivation of the kinase and almost complete reversal to the nonactivated state (less than 10% of the activated activity remained). Prolonged exposure to the phosphatase also results in the slow release of radioactivity from subunit A. These results are in general agreement with those of Cohen and coworkers (10) . These investigators have partially purified two phosphoprotein phosphatases: phosphatase A, which specifically dephosphorylates subunit A and phosphatase B, specific for subunit B (12) . The pattern of dephosphorylation of activated phosphorylase kinase by the homogeneous phosphatases seem to agree with that observed when phosphatase B is used (12) . Thus it appears the pure phosphatases are similar to or the same as phosphatase B.

When highly phosphorylated forms of phosphorylase kinase were used as substrates for the phosphoprotein phosphatases, only a partial deactivation of the enzyme occurred. Such highly phosphorylated forms of phosphorylase kinase were prepared either by the autocatalytic reaction or by the cAMP-dependent protein kinase reaction catalyzed at high concentrations of Mg^{2+} (see "Methods"). These phosphorylated kinases contained between 5 to 10 moles of phosphate per monomeric unit with most of these phosphates being on subunit A. The dephosphorylation of these kinases involved a very rapid dephosphorylation of subunit B and a slower but significant dephosphorylation of subunit A. In fact the dephosphorylation of subunit B was as rapid as for the case of kinase containing only 1 or 2 phosphates. Unlike the latter case, however, subunit B dephosphorylation resulted in only a 50% inactivation of phosphory-

lase kinase. Complete reversal of the activated kinase to the nonactivated state also depends on the dephosphorylation of subunit A. The phosphoprotein phosphatases did not seem to make a distinction between the two phosphorylated forms of the kinase. Phosphorylase kinase phosphorylated either autocatalytically or by protein kinase at high Mg^{2+} concentrations were equally good substrates for the phosphatases. The subunit dephosphorylation patterns for the two forms were similar and only a partial deactivation resulted from the dephosphorylation of either subunit A or subunit B alone. These findings further support the hypothesis that the same or similar sites are phosphorylated during the autoactivation or the protein kinase-catalyzed activation in the presence of high Mg^{2+} concentrations.

The regulation of phosphorylase kinase by phosphorylation-dephosphorylation reactions seems to be very complex indeed. Previous studies by Cohen and coworkers (4 , 10) have led to the suggestion that phosphorylase kinase activation results from the phosphorylation of subunit B. However, when the correlation of phosphorylase kinase activity and subunit phosphorylation in the autophosphorylation reaction (7) and the cAMP protein kinase-catalyzed reaction at high concentrations of Mg^{2+} (9) are examined, it appears that the additional phosphorylation of subunit A also contributes to further enzyme activation. However, it is not clear from the earlier studies (4 , 7 , 10) whether phosphorylation of subunit B is always required for phosphorylase kinase to exist in the activated form. As mentioned above, the highly phosphorylated forms of the kinase still possessed about 50% of their initial activated activity when most of the phosphate was released from subunit B by the phosphatases. Hence, it seems that the phos-

phorylation of subunit B is not an absolute requirement for phosphorylase kinase to remain in or possibly achieve the activated state. The regulatory role postulated for subunit A, that is, the control of enzyme activity by "second site phosphorylation" (10) has not been reproduced using the reconstituted system. The phosphorylation state of subunit A did not influence the rate of dephosphorylation of subunit B. The latter was dephosphorylated equally rapidly whether subunit A contained 0.20, 1.40 or 4.30 moles of phosphate. Such independent dephosphorylation of the subunits raises the possibility of regulating phosphorylase kinase activity by the separate phosphorylation-dephosphorylation of subunits A and B. The rapid activation of phosphorylase kinase that seems to correlate with an equally rapid phosphorylation of subunit B by the protein kinase-catalyzed reaction at low Mg^{2+}, the almost exclusive phosphorylation of subunit A and the further activation of the enzyme observed at high Mg^{2+} as well as the existence of separate phosphatases for subunits A and B all argue in favor of such a hypothesis.

Besides phosphorylation results in the present study indicate that phosphorylase kinase can be activated to high levels in a non-covalent manner. Previously it was shown that the nonactivated kinase could be stimulated to small extents by glycogen, heparin (14), Ca^{2+} (5, 18, 19), Mg^{2+} (16, 252), high pH (1) and neutral salts (17). These activations do not involve covalent modification of the enzyme. High concentrations of various organic solvents were also shown to activate the nonactivated phosphorylase kinase. With some of these solvents the final extent of the enzyme activation is comparable to that achieved by phosphorylation. For instance, a 45-fold activation of the kinase was obtained in the presence of 1.72 M ethanol at pH 6.8. This level of

activation is similar to that obtained when two specific sites on phosphorylase kinase are phosphorylated by the cAMP-dependent protein kinase. The organic solvent activation of the enzyme does not seem to involve covalent modification since the activation depends on the presence of the solvent in the assay medium. Removal of the solvent results in the kinase activation being largely reversed.

It does not seem too likely that the stimulation of the non-activated kinase may be explained by the binding of the organic solvent to a particular stereospecific site on the kinase molecule. The very high concentrations of the effective solvents required as well as the variability in structure among the solvents support such an argument. Instead it is thought that the change in physical properties of the reaction medium induces in phosphorylase kinase a new conformation of higher intrinsic activity. The observation that the enzyme activity is also enhanced by organic solvents when protein substrates other than phosphorylase b are used suggests that the effect of the solvents is at least partly on the kinase molecule. This suggestion does not exclude the possibility that the organic solvents can influence the structure of phosphorylase b. In fact the solvents probably do have some effect on phosphorylase b since it has been demonstrated that 10% methanol can effectively prevent the cold inactivation of phosphorylase b (253).

The mechanism of phosphorylase kinase activation by organic solvents is only partly understood. However, the organic solvent-activated kinase and the phosphorylated kinase are similar both in their catalytic activities and in their response to pH. This suggests that similar conformational changes are induced in the enzyme by the two different methods of activation.

The characteristic pH activity curve of nonactivated phosphorylase kinase (1 ; also Fig. 17A) seem to suggest that the active conformation of the enzyme depends on the change in ionization state of a few key residues on the enzyme. Hence, the activation of phosphorylase kinase by protein phosphorylation or by high concentrations of organic solvents may also result from the perturbation of the ionization state of these residues.

The observation that phosphorylase kinase can be activated to very high levels by a noncovalent mechanism suggests that endogenous effectors which can noncovalently activate the enzyme may be present in cells. A preliminary search for such factors has been carried out by examining the ability of various lipids to activate phosphorylase kinase. These studies have met only with limited success. Among the fatty acids and phospholipids tested, only two monounsaturated fatty acids, palmitoleic and oleic acids, are activating. Phosphorylase kinase is only activated about 6-fold by these fatty acids compared to 45-fold achieved by ethanol. Nevertheless, the observation indicates that lipids with certain special structural features may activate the kinase.

The increased conversion of phosphorylase b to phosphorylase a during the electrical stimulation of skeletal muscle is not accompanied by an activation of phosphorylase kinase (20, 21, 22) . It has therefore been postulated that the activation of the nonactivated form of phosphorylase kinase by Ca^{2+} is responsible for the coupling between muscle contraction and glycogenolysis. However, it has also been suggested (4, 197) that if all the phosphorylase kinase in skeletal muscle is present in the nonactivated state its activity, even at saturating amounts of Ca^{2+}, will not be high enough to account for the rapid activation of phosphorylase observed in isometrically stimulated frog

sartorius muscle (230). These apparently paradoxical suggestions may be reconciled if indeed there is an endogenous factor (or factors) in muscle cells capable of activating phosphorylase kinase to a high level non-covalently.

A significant portion of phosphorylase kinase activity has been shown to be associated with the particulate fraction, termed glycogen particles, of muscle extract (176, 232). Recent studies (231) indicate that the particulate enzyme has a pH 6.8/8.2 activity ratio of 0.5 and may be bound to the sarcoplasmic reticulum. The possibility should therefore be examined that the particulate fraction contains an endogenous activator for phosphorylase kinase. Another likely candidate for such an activator may be the feedback regulator of Ho and Sutherland (254). This regulator has been suggested to be a fatty acid and shown to enhance the autoactivation of phosphorylase kinase (254).

The observation that oleic and palmitoleic acids, but not saturated fatty acids of the same chain length, can serve as activators of phosphorylase kinase appear to be similar to a few other cases of enzyme activation by fatty acids. For example, guanylate cyclase of fibroblasts is activated 1.5, 6.9 and 8.6 fold by stearic, oleic and elaidic acids, respectively (255). C_{55}-isoprenoid alcohol phosphokinase from Staphylococcus aureus can be markedly activated by oleic acid but not by stearic acid (256). It has been suggested that fatty acids interact with enzymes and other proteins as amphiphyles. For long chain fatty acids, introduction of a double bond apparently enhance their ability to act as amphiphyles (255). Since only a limited number of fatty acids and related compounds have been tested for phosphorylase kinase activating properties, a more detailed analysis of the relationship between fatty acid structure and enzyme

activation is not feasible.

The findings in the present study have helped to further elucidate as well as point out that the regulation of phosphorylase kinase is very involved indeed. Both covalent and non-covalent mechanisms seem to be major and alternative means of controlling the enzyme activity. Phosphorylase kinase can be activated to high levels by a noncovalent mechanism. However, it seems that the presence of one or more types of endogenous activator molecules may be required for this mode of activation to be of significance in tissues. The covalent mechanism of activation is under hormonal control and has many complexities of its own. The phosphorylation and dephosphorylation of phosphorylase kinase seem geared to control the enzyme activity by increments. Phosphorylation of the enzyme at 2 sites per monomeric unit results in a 50-fold activation. The increase in enzyme activity seem to correlate best with the phosphorylation of a single site on subunit B. This form of the enzyme can be totally deactivated by a dephosphorylation of subunit B. A further 135-fold activation occurs when additional sites are phosphorylated almost exclusively on subunit A. This highly phosphorylated form of the enzyme is deactivated by the dephosphorylation of both subunits A and B. Dephosphorylation of either subunit alone results in only a partial deactivation of the enzyme. These observations indicate that phosphorylase kinase activity can possibly be regulated by the separate phosphorylation and dephosphorylation of subunits A and B. The discovery of distinct phosphatases for subunits A and B (12) supports such a hypothesis.

VI. REFERENCES

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