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THE EFFECT OF PHOSPHATE AND PHOSPHATE ANALOGS ON THE ENZYMIC ACTIVITY OF PYRIDOXAL RECONSTITUTED PHOSPHORYLASE.

IOWA STATE UNIVERSITY, PH.D., 1978

The effect of phosphate and phosphate analogs on the enzymic activity of pyridoxal reconstituted phosphorylase

by

Richard Fred Parrish

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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In Charge of Major Work

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For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1978

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DEDICATION

To My Wife, Jean

ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
Glucose-1-P	glucose-1-phosphate
Glucose-6-P	glucose-6-phosphate
NADP	nicotinamide adenine dinucleotide phosphate
pyridoxal-5'-P	pyridoxal-5'-phosphate
TCA	trichloroacetic acid

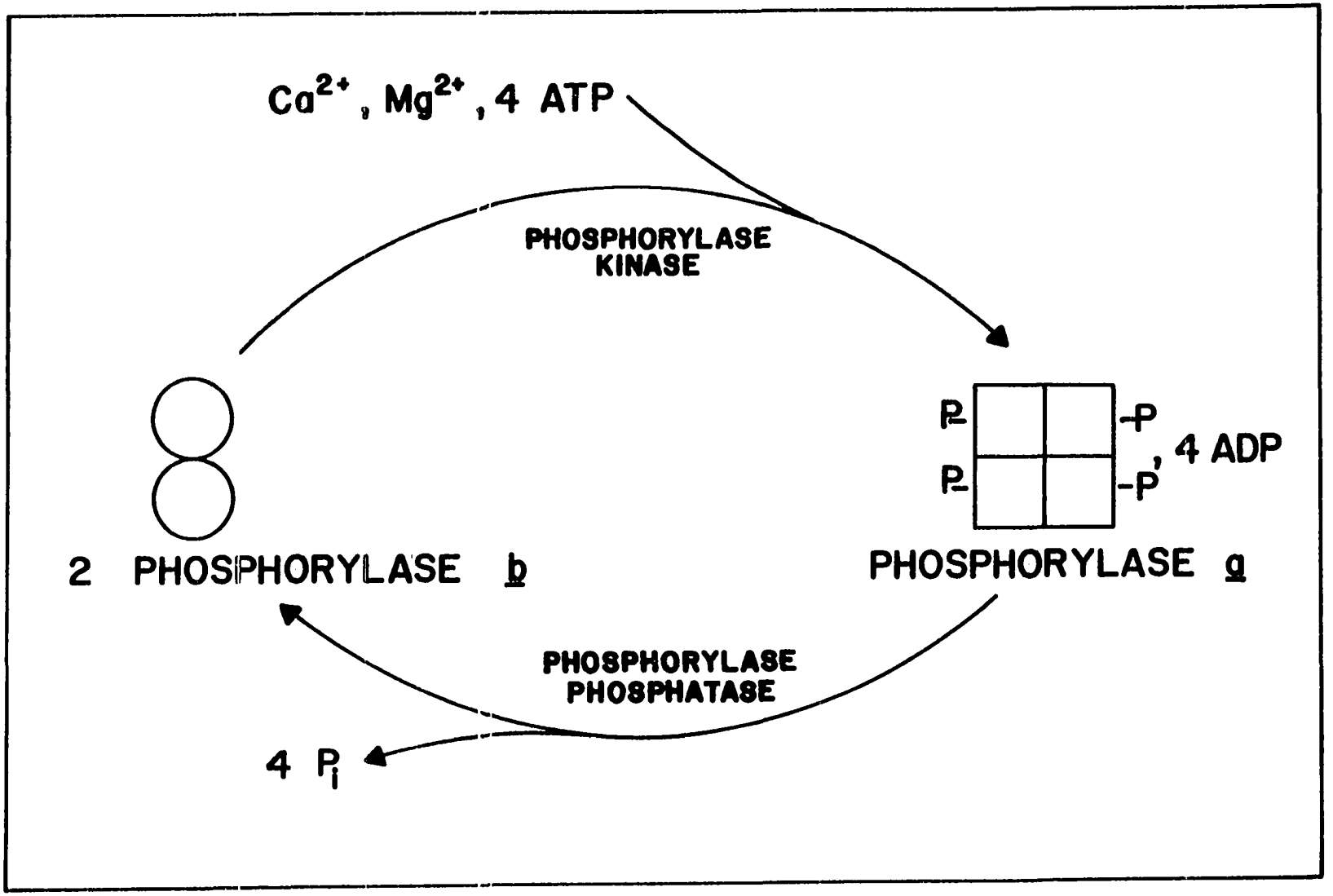
INTRODUCTION

Phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase E.C.2.4.1.1) occupies an important position in the metabolism of glycogen. This enzyme catalyzes the reaction:

$$\text{phosphate} + \text{glycogen}_n \rightleftharpoons \text{glucose-1-P} + \text{glycogen}_{n-1}$$

where glycogen_n and glycogen_{n-1} represent glucose polymers with n and n-1 glucosyl residues respectively. The crystalline enzyme isolated from rabbit skeletal muscle has been the most extensively studied. It exists in two distinct forms. Phosphorylase b is catalytically inactive in the absence of AMP (Green and Cori, 1943). Phosphorylase a, however, is catalytically active in the absence of AMP (Green et al., 1942). The interconversion between these two forms is mediated by the phosphorylation-dephosphorylation reactions shown in Figure 1. (Krebs and Fischer, 1956; Graves et al., 1960). These phosphorylation-dephosphorylation reactions are an important mechanism for the control of the enzymic activity of phosphorylase and have been found in such diverse species as amphibians (Metzger et al., 1968), insects (Stevenson and Wyatt 1964), fungus (Tellez-Inon and Torres, 1970) and yeast (Fosset et al., 1971), but not in

Figure 1. The interconversion reactions of phosphorylase.


Ca^{2+}, Mg^{2+}, 4 ATP
PHOSPHORYLASE KINASE
2 PHOSPHORYLASE b
PHOSPHORYLASE a
-P
-P
, 4 ADP
PHOSPHORYLASE PHOSPHATASE
4 P_i

either plants (Lee,1960) or bacteria (Schwartz and Hofnung, 1967).

Pyridoxal-5'-phosphate was isolated from rabbit skeletal muscle phosphorylase after treatment of the enzyme with either perchloric or trichloroacetic (Baranowski et al., 1957). Milder methods for the resolution of the coenzyme from the protein were subsequently developed and an apoprotein was isolated. This apoprotein was devoid of catalytic activity. Ultracentrifuge studies indicated that the apoprotein sedimented as a mixture of molecular weight species, while the holoenzyme sedimented as a single molecular weight species. Reconstitution of the apoprotein with pyridoxal-5'-P resulted in a recovery of the enzymic activity and a return to the original quaternary structure (Illingworth et al.,1958).

The reaction of sodium borohydride with phosphorylase showed that an imine double bond between pyridoxal-5'-P and the ε-amino group of a lysine residue was reduced. Surprisingly, this reduced enzyme species retained 60 per cent of the enzymic activity of the native enzyme (Fischer et al., 1958). In all other pyridoxal-5'-P requiring enzymes, the imine double bond was required for enzymic activity, and

reduction with sodium borohydride resulted in a total loss of enzymic activity (Snell and DiMari, 1970). The catalytic activity of the reduced phosphorylase posed a question concerning the role of pyridoxal-5'-P in phosphorylase. Did the coenzyme participate directly in catalysis, or was it simply acting as a conformational determinant that allowed catalysis in a different portion of the molecule?

A variety of techniques have clearly demonstrated a conformational role for pyridoxal-5'-P in phosphorylase. Apophosphorylase was much less stable than the holoenzyme (Hedrick et al., 1966; Shaltiel et al., 1969b). Removal of the coenzyme resulted in quaternary structure differences between the native and apoprotein (Illingworth et al., 1958). Tritium-hydrogen exchange studies demonstrated that more amino acid residues were exposed to solvent by apophosphorylase than by the native enzyme (Weissharr and Palm, 1972). The pattern of alkylation of protein sulfhydryl groups was altered by removal of the coenzyme (Avramovic-Zikic et al., 1970). Finally, native and apophosphorylase responded differently to substrate directed effectors of the interconverting enzymes phosphorylase kinase and phosphorylase phosphatase (Graves et al., 1975). However, removal of the

coenzyme did not lead to a general disordering of the protein since there was little difference between the native and apoprotein when the secondary structures were compared by optical activity techniques (Johnson and Graves, 1966; Hedrick, 1966).

No direct evidence for a catalytic role for pyridoxal-5'-P in phosphorylase has been reported. Lerch and Fischer (1975) compared the primary sequence of the phosphorylation site and the pyridoxal-5'-P binding site in phosphorylases isolated from yeast and mammals. The highly conserved sequence of the pyridoxal-5'-P site relative to the phosphorylation site was utilized as an argument that the coenzyme had a catalytic function in phosphorylase. The main thrust in attempts to establish a catalytic function for the coenzyme has been the synthesis of analogs of pyridoxal-5'-P and the testing of the ability of these analogs to bind to apophosphorylase and to restore catalytic activity. It was hoped that a critical function group would be found, and that studies involving this modified functional group would lead to an understanding of the role of pyridoxal-5'-P in phosphorylase. Table I lists the analogs of pyridoxal-5'-P that have been tested for ability to reactivate

Table I. Analogs of pyridoxal-5'-phosphate that have been tested for ability to bind and restore catalytic activity to apophosphorylase

Analog tested	Position modified	Binding	Per cent activity
Pyridoxine-5'-phosphate	4	NO	0[a]
Pyridoxamine-5'-phosphate	4	NO	0[a]
4'-Deoxypyridoxine-5'-phosphate	4	NO	0[a]
Pyridoxic acid 5'-phosphate	4	NO	0[a]
Isopyridoxal-5'-phosphate	4,5	NO	0[a]
2-Norpyridoxal-5'-phosphate	2	YES	65[a]
3'-O-methylpyridoxal-5'-phosphate	3	YES	25[a]
6-methylpyridoxal-5'-phosphate	6	YES	8[a]
Pyridoxal-5'-phosphate-N-oxide	1	?	?[b]
N-Methylpyridoxal-5'-phosphate	1	NO	0[a]

[a]Shaltiel et al., 1969b.

[b]Fischer et al., 1970.

Table I. (continued)

Analog tested	Position modified	Binding	Per cent activity
3'-O-methylpyridoxal-5'-phosphate-N-oxide	1,3	YES	0[c]
5'-Deoxypyridoxal	5	YES	0[d]
Pyridoxal	5	YES	0[d]
5'-Carboxymethyl deoxypyridoxal	5	YES	0[a]
Pyridoxal-5'-sulfate	5	YES	0[a]
α-Methylpyridoxal-5'-phosphate	5	YES	0[e]
Homopyridoxal-5'-phosphate	5	YES	0[e]
Pyridoxal-5'-phosphate monomethyl ester	5	YES	0[c]
Pyridoxal-5'-phosphate cyanoethyl ester	5	YES	0[e]
5'-Deoxypyridoxal phosphonic acid	5	YES	4-8[e]
Pyridoxal-5'-methylenephosphonic acid	5	YES	25[f]

[c] Pfeuffer et al., 1972a.

[d] Illingworth et al., 1958.

[e] Graves and Wang, 1972.

[f] Vidgoff et al., 1974.

apophosphorylase.

Several conclusions may be drawn after inspection of Table I. The free aldehyde at the 4'-position was necessary for coenzyme binding, since any modification at this position resulted in compounds that did not bind to the apoprotein. However, once the coenzyme was bound to the protein, reduction with sodium borohydride did not destroy enzymic activity and it, therefore, was highly unlikely that this functional group participated in catalysis. Modifications at the 2, 3 and 6 positions resulted in compounds that exhibited diminished activity after reconstitution with apophosphorylase and thus these positions were eliminated as being essential for catalysis. The role of the ring nitrogen remained unclear. Pyridoxal-5'-phosphate-N-oxide reconstituted phosphorylase demonstrated enzymic activity, but it was later reported that this analog was converted to pyridoxal-5'-P (Pfeuffer et al., 1972a). N-methyl-pyridoxal-5'-phosphate did not bind to apophosphorylase, while 3'-O-methyl-pyridoxal-5'-phosphate-N-oxide bound to apophosphorylase, but the reconstituted protein demonstrated no enzymic activity. Modifications at the 5'-position have been the most intensely studied. Of all the proteins obtained after

reconstitution of apophosphorylase with pyridoxal-5'-P analogs modified at the 5'-position, only 5'-deoxypyridoxal phosphonic acid and pyridoxal-5'-methylenephosphonic acid reconstituted phosphorylase demonstrated enzymic activity. Therefore, it was concluded that in order for a pyridoxal-5-P analog to manifest enzymic activity after reconstitution with apophosphorylase, the analog must possess an aldehyde at the 4-position and a covalently bound 5'-phosphate or phosphonate. However, a role for the coenzyme was still undefined.

Pyridoxal-5'-phosphate monomethyl ester reconstituted phosphorylase has been demonstrated to have many properties in common with the native enzyme. The ultraviolet and visible spectra, as well as the fluoresence spectra of pyridoxal-5'-P and pyridoxal-5'-phosphate monomethyl ester reconstituted phosphorylase were identical (Ehrlich et al., 1971). When native and pyridoxal-5'-phosphate monomethyl ester reconstituted phosphorylase were reduced with sodium borohydride, fluoresence measurements indicated that both proteins were perturbed by acidification in exactly the same way. (Ehrlich et al., 1971). There was no removal of pyridoxal-5'-phosphate monomethyl ester by L-cysteine in the absence

of deforming buffers (Pfeuffer et al., 1972b). Finally, native and pyridoxal-5'-phosphate monomethyl ester reconstituted phosphorylase exhibited similar tritium-hydrogen exchange patterns (Weissharr and Palm, 1972). Esterification of the 5'-phosphoryl group of pyridoxal-5'-P resulted in the loss of an ionizable hydrogen with a pKa of 6.2, and since pyridoxal-5'-phosphate monomethyl ester reconstituted phosphorylase was catalytically inactive, it was proposed that the 5'-phosphoryl group of pyridoxal-5'-P participated in a proton transfer reaction during catalysis (Pfeuffer et al., 1972b).

Although the 5'-phosphoryl group of the coenzyme is absent in pyridoxal, it has been previously demonstrated that: a) after reconstitution of apophosphorylase with pyridoxal, the resulting protein possessed a quaternary structure similar to the native pyridoxal-5'-P containing protein (Illingworth et al., 1958); b) apophosphorylase bound glycogen as tightly as the native enzyme (Kastenschmidt et al., 1968); c) the heterotropic interactions between AMP and glucose-1-P found in the native protein were also manifested by the pyridoxal reconstituted protein (Kastenschmidt et al., 1968). Thus the lack of enzymic activity reported

for pyridoxal reconstituted phosphorylase could not be explained by the inability of the protein to bind either substrates or nucleotide activator, but must have been the result of the absence of the covalently bound 5'-phosphoryl group of the coenzyme.

Wada and Morino (1964) reported that the apoprotein of glutamic-oxalacetic transaminase catalyzed the reversible conversion of pyridoxamine and oxaloacetate into L-aspartate and pyridoxal. The striking feature of this reaction was that the enzymes isolated from rabbit liver and E. coli. were shown to have an absolute dependence on the presence of inorganic phosphate for the expression of enzymic activity. When coupled with the previously described ability of pyridoxal reconstituted phosphorylase to bind substrates and nucleotide activator, this suggested that pyridoxal reconstituted phosphorylase might demonstrate catalytic activity if the experiments were performed in the presence of phosphate or a phosphate analog. This hypothesis received support when preliminary experiments (Graves, 1974), indicated that pyridoxal reconstituted phosphorylase possessed catalytic activity when glycogen degradation was measured in the presence of arsenate. Therefore, this investigation of the

properties of pyridoxal reconstituted phosphorylase in the presence of phosphate and phosphate analogs was undertaken. It was thought the information gained from the study of this enzyme form, in which the 5'-phosphoryl group of the native coenzyme was absent, would help to elucidate the role of the 5'-phosphoryl group of pyridoxal-5'-P in native phosphorylase.

MATERIALS AND METHODS

Pyridoxal was purchased from Sigma and was demonstrated to be free of pyridoxal-5'-P contamination as described later. Imidazole was recrystallized three times from ethyl acetate. Shellfish glycogen was purified by the method of Anderson and Graves (1973). (^{14}C)glycogen and disodium fluorophosphate were generous gifts of Dr. James A. Thomas and Ms. Betty Yan. All other materials were the highest quality available and were used without further purification.

Crystalline rabbit skeletal muscle phosphorylase b was prepared by the method of Fischer and Krebs (1962) and the residual AMP was removed by treatment with acid washed Norite A. A ratio of A_{260}:A_{280} of 0.53 or less was considered satisfactory. Apophosphorylase a and b were prepared by the method of Graves et al., (1975). The apoprotein routinely contained less than 0.1 per cent residual pyridoxal-5'-P, as measured by activity. Rabbit skeletal muscle phosphorylase phosphatase was prepared according to Hurd et al., (1966), through the dialysis and lyophilization step.

Pyridoxal reconstituted phosphorylase b was prepared by incubating apophosphorylase b (3-4mg/ml) in 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol pH 6.8 with a 50-fold

excess of pyridoxal for 35 minutes at 37° C. An equal volume of saturated, neutral ammonium sulfate was added and after centrifugation, the precipitate was dissolved in a minimal amount of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol ph 6.8 and dialyzed against this buffer in the cold. Crystallization occured after addition of AMP and magnesium chloride to a final concentration of 0.001 and 0.01 M respectively. The crystalline enzyme was collected by centrifugation, dissolved in a minimum amount of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, ph 6.8 and treated with acid washed Norite A to remove bound nucleotide. Pyridoxal reconstituted phosphorylase is less stable than the native enzyme. This instability could be minimized by storing the enzyme in the cold at a protein concentration greater than 30 gm/ml. Under these conditions, the enzyme usually retained more than 90 per cent of the original activity for at least 4 days. Whenever the enzymic activity of the material was less than 90 per cent of the original, the solution was discarded and a new enzyme preparation initiated.

Pyridoxal reconstituted phosphorylase a was prepared from pyridoxal reconstituted phosphorylase b by the method

of Krebs (1966), utilizing rabbit skeletal muscle phosphorylase b kinase (Brostrom et al., 1971). When preparing radioactively labeled pyridoxal reconstituted (^{32}P)phosphorylase, (γ-^{32}P)ATP was utilized (Glynn and Chappell, 1964).

Concentrations of native and pyridoxal reconstituted phosphorylase were measured spectrophotmetrically at 280 nm (Kastenschmidt et al., 1968). The concentration of phosphorylase phosphatase was measured by dissolving a weighted amount of lyophillized material in a known amount of buffer.

When activity comparisons between native and pyridoxal reconstituted phosphorylase were determined, apophosphorylase was reconstituted with either a 5-fold excess of pyridoxal-5'-P or a 50- fold excess of pyridoxal.

Phosphorylase activity in the direction of glycogen synthesis was measured by the method of Illingworth and Cori (1953), or by the incorporation of (^{14}C)glucose into glycogen form (^{14}C)glucose-1-P, employing the filter paper assay of Thomas et al., (1968). When the activity was measured in the presence of a phosphate analog, the concentration is indicated in the appropriate figure legend. When the time courses for the activity of pyridoxal reconstituted phosphorylase were determined, the amount of inorganic phosphate

liberated was measured by the method of Fiske and Subbarow (1925). Phosphorylase activity in the direction of glycogen degradation was measured according to Helmreich and Cori (1964a). This method was also used to determine the Km value for glycogen for pyridoxal reconstituted phosphorylase. When glycogen degradation was measured in the presence of arsenate (Helmreich and Cori, 1964b), maleate was substituted for β-glycerophosphate as the buffering anion.

Phosphorylase phosphatase activity was measured by the release of (^{32}p)phosphate from pyridoxal reconstituted (^{32}p) phosphorylase a by the following general method: 200 μliters of the reaction mixture containing pyridoxal reconstituted (^{32}p)phosphorylase a was added to 200 μliters of ice cold 10 per cent TCA; 100 μliters of BSA (20 mg/ml) was added and after 15 minutes on ice, the precipitated protein was removed by filtration through a glass wool plugged pipette; 200 μliters was removed and added to 10 ml of Bray's solution (Bray, 1960) and the amount of (^{32}p)phosphate released was measured in a Packard Tri Card Liquid Scintillation Counter.

The inactivation of pyridoxal reconstituted phosphorylase b by L-cysteine was measured by the following general method. Pyridoxal reconstituted phosphorylase was diluted

with 0.1 M β-glycerophosphate, 0.1 M L-cysteine, pH 6.8 to a final concentration of 2-4 mg/ml. The exact concentrations are given in the appropriate figure legend. After an appropriate incubation time at 30° C, an aliquot was removed and diluted 1:10 with 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol pH 6.8 and the enzymic activity was immediately measured according to Illingworth and Cori (1953) in the presence of 0.0075 M phosphite.

RESULTS

Enzymic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen synthesis

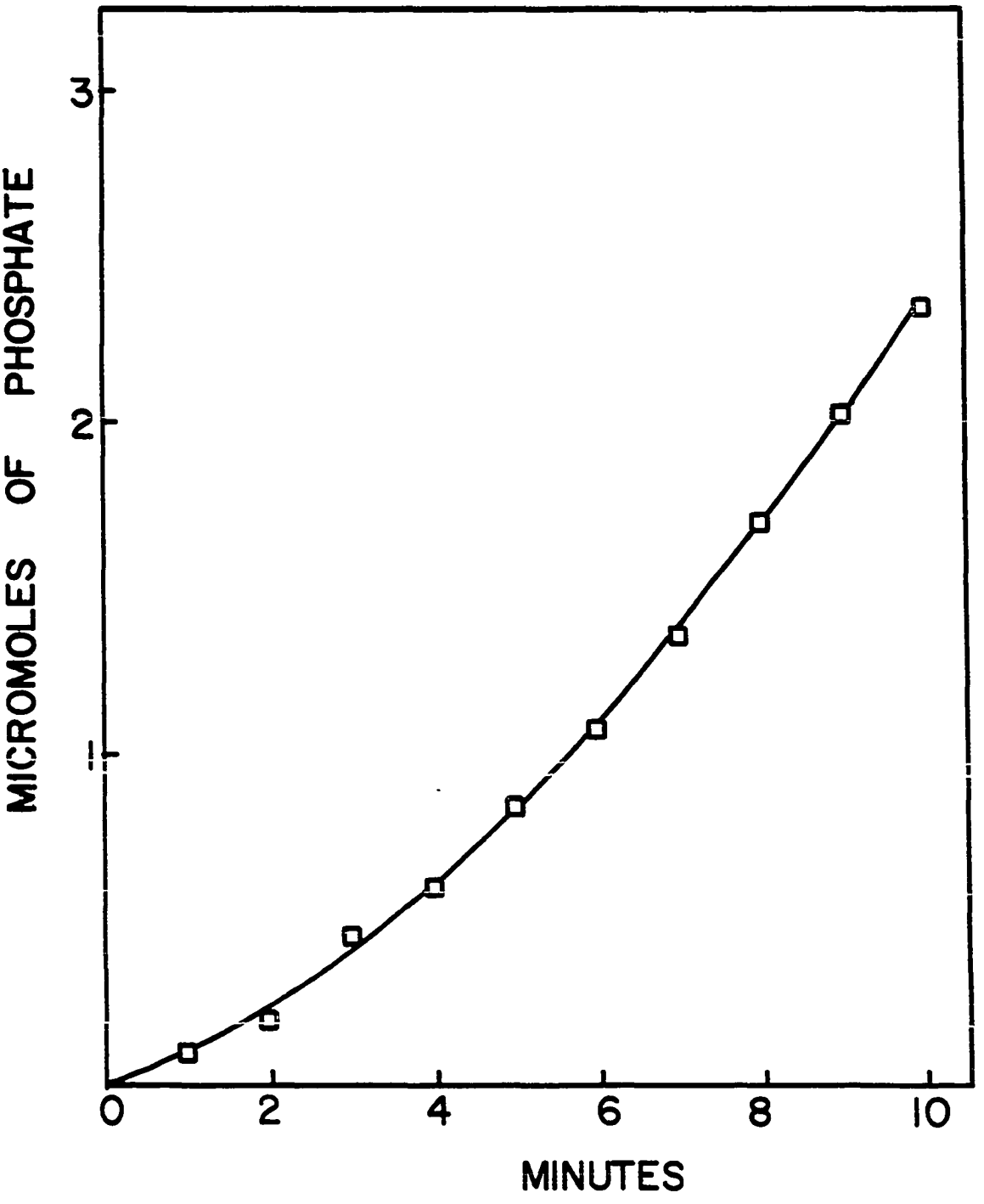
The majority of studies of phosphorylase utilize enzymic activity measurements in the direction of glycogen synthesis. In order to test for the enzymic activity of pyridoxal reconstituted phosphorylase, high substrate and enzyme concentrations were employed, since the enzymic activity, if present, would have to have been much less than that of the native enzyme. If the enzymic activity had been similar to that of the native enzyme, the activity would have been detected by previous investigators (Illingworth et al., 1958; Shaltiel et al, 1969b). Figure 2 demonstrates the enzymic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen synthesis. In the linear portion of the time course, the activity represents 4 per cent of the activity of the native enzyme. The most striking feature, other than the activity itself, was the lag in the generation of product, inorganic phosphate.

Elimination of pyridoxal-5'-P contamination as the source of the enzymic activity of pyridoxal reconstituted phosphorylase

If the enzymic activity of pyridoxal reconstituted

Figure 2. Enzymic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen synthesis.

The reaction mixture at pH 6.8 and 30°C consisted of 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.05 M glucose-1-P, 1% glycogen and enzyme (150 μg/ml). At the indicated times, 400 μliters was removed and the phosphate liberated was measured as described in the MATERIALS AND METHODS section.


MICROMOLES OF PHOSPHATE
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phosphorylase resulted from the presence of pyridoxal-5'-P in the pyridoxal, then the time course of activity for the enzyme obtained after incubation of apophosphorylase with limited amounts of pyridoxal-5'-P should show a lag. Figure 3 compares the time course for the activity of apophosphorylase reconstituted with pyridoxal and with sufficient pyridoxal-5'-P to activate 8 per cent of the apoprotein. The protein resulting from reconstitution of apophosphorylase with pyridoxal-5'-P showed no lag in the generation of product while the pyridoxal reconstituted protein demonstrated the expected lag. Shaltiel et al., (1969b) demonstrated that pretreatment of pyridoxamine-5'-phosphate or pyridoxine-5'-phosphate with apophosphorylase removed contaminating amounts of pyridoxal-5'-P and eliminated the apparent ability of these compounds to activate apophosphorylase. Apophosphorylase was incubated with a 50-fold excess of pyridoxal as described in the Materials and Methods section, and then chromatographed over Sephadex G-25 to separate the protein from the excess pyridoxal. The apophosphorylase treated pyridoxal and pyridoxal that had not been pretreated with apophosphorylase were used to reconstituted apophosphorylase and the specific activities of the resulting proteins were

Figure 3. Time course for the enzymic activity of apophosphorylase reconstituted with pyridoxal and pyridoxal-5'-P.

Apophosphorylase b (2.0 mg/ml) was incubated for 30 minutes at 37°C in solutions consisting of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 1.7×10^{-3} M pyridoxal (O), or 3.3×10^{-6} M pyridoxal-5'-P (O). 200 μliters of each solution was added to 1.8 ml of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, pH 6.8. 2.0 ml of 0.032 M glucose-1-P, 0.002 M AMP, 2% glycogen, pH 6.8 was added, incubated at 30°C, and at the indicated times, 400 μliters was removed and the amount of phosphate liberated was measured as described in the MATERIALS AND METHODS section.

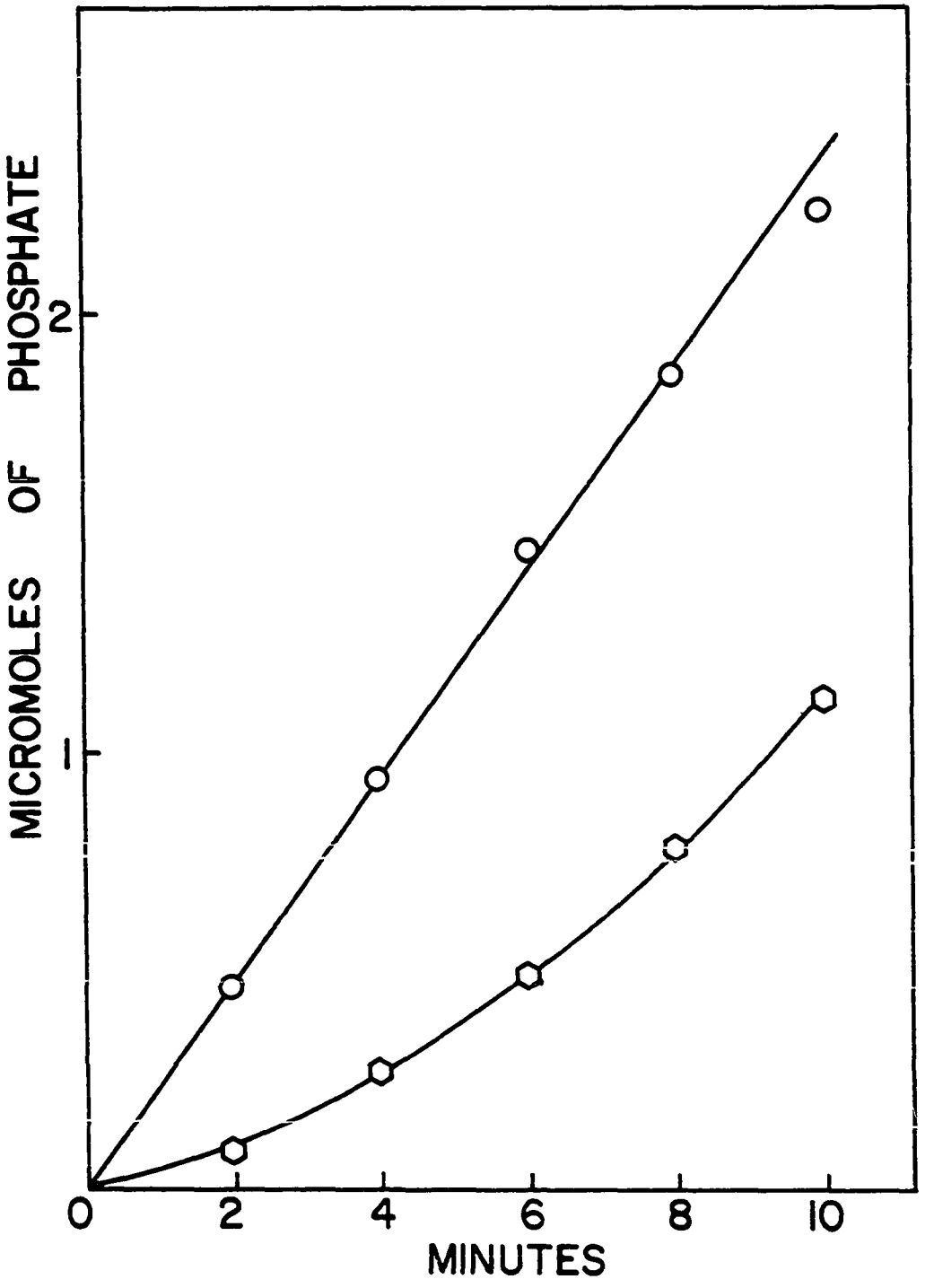
MICROMOLES OF PHOSPHATE
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Table II. The effect of pretreatment of pyridoxal with apophosphorylase on the specific activity of pyridoxal reconstituted phosphorylase.

Pyridoxal utilized in reconstitution	Specific activity (μmoles/min/mg)
Treated with apoenzyme	2.22
Not treated with apoenzyme	2.20

Pyridoxal reconstituted phosphorylase was prepared by incubating apophosphorylase with either pyridoxal or pyridoxal that had been pretreated with apophosphorylase. The enzymic activity of the resulting proteins was measured at 30°C and pH 6.8 in a total volume of 400 μliters of solution that consisted of 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 1% glycogen, 0.05 M glucose-1-P and enzyme (78 μg). After a 10 minute incubation, the amount of phosphate liberated was measured as described in the MATERIALS AND METHODS section. When the specific activity was calculated, no correction was made for the nonlinear time dependence of the product formation.

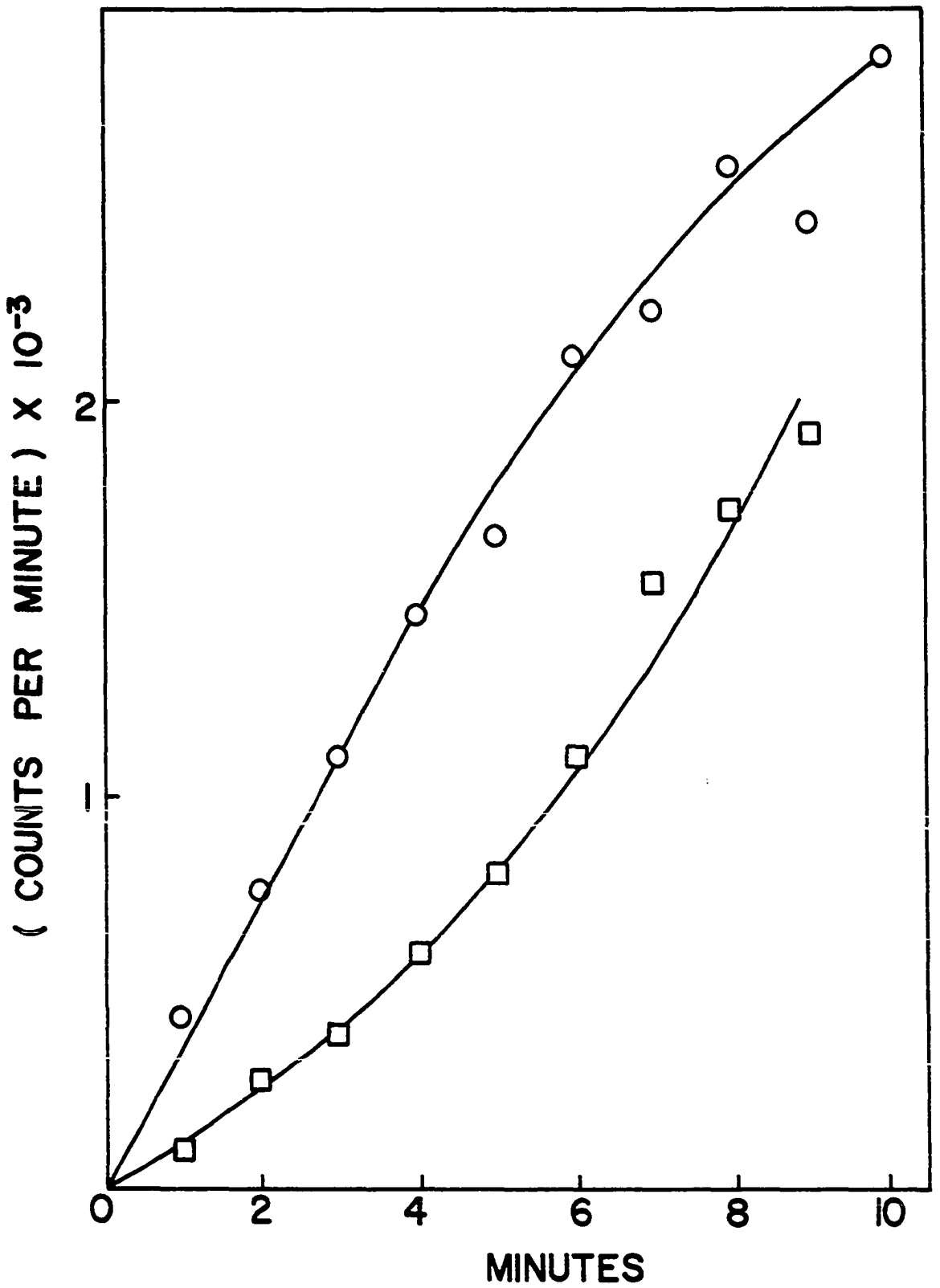
determined. If the enzymic activity of pyridoxal reconstituted phosphorylase resulted from contaminating amounts of pyridoxal-5'-P in the pyridoxal, this pretreatment should have removed all, or at least a large part, of the enzymic activity of the protein resulting from reconstitution with the apophosphorylase treated pyridoxal. Table II demonstrates that there was no such reduction in enzymatic activity. Apotryptophanase has been utilized to measure the concentration of pyridoxal-5'-P (Gunsalus et al., 1955). When apotryptophanase was tested with a perchloric acid extract of pyridoxal reconstituted phosphorylase, no pyridoxal-5'-P could be detected. In control experiments, 0.5 per cent contamination of pyridoxal by pyridoxal-5'-P could easily be detected. These experiments demonstrate that the enzymic activity observed for pyridoxal reconstituted phosphorylase could not have come from contaminating amounts of pyridoxal-5'-P in either the pyridoxal or the apophosphorylase preparation.

Explanation of the lag in product formation

Generation of an activator during the pyridoxal reconstituted phosphorylase catalyzed reaction would lead to the observed nonlinearity in the time course of product

Figure 4. Enzymic activity of pyridoxal reconstituted phosphorylase in the presence and absence of added phosphate.

The assay mixture contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, enzyme (165 μg/ml), 0.1 μC (^{14}C)glucose-1-P, pH 6.8 and phosphate, 0.0075 M (O), or no added phosphate (□). At the indicated times, 20 μliters was removed and the radioactivity incorporated into glycogen was measured as described in the MATERIALS AND METHODS section.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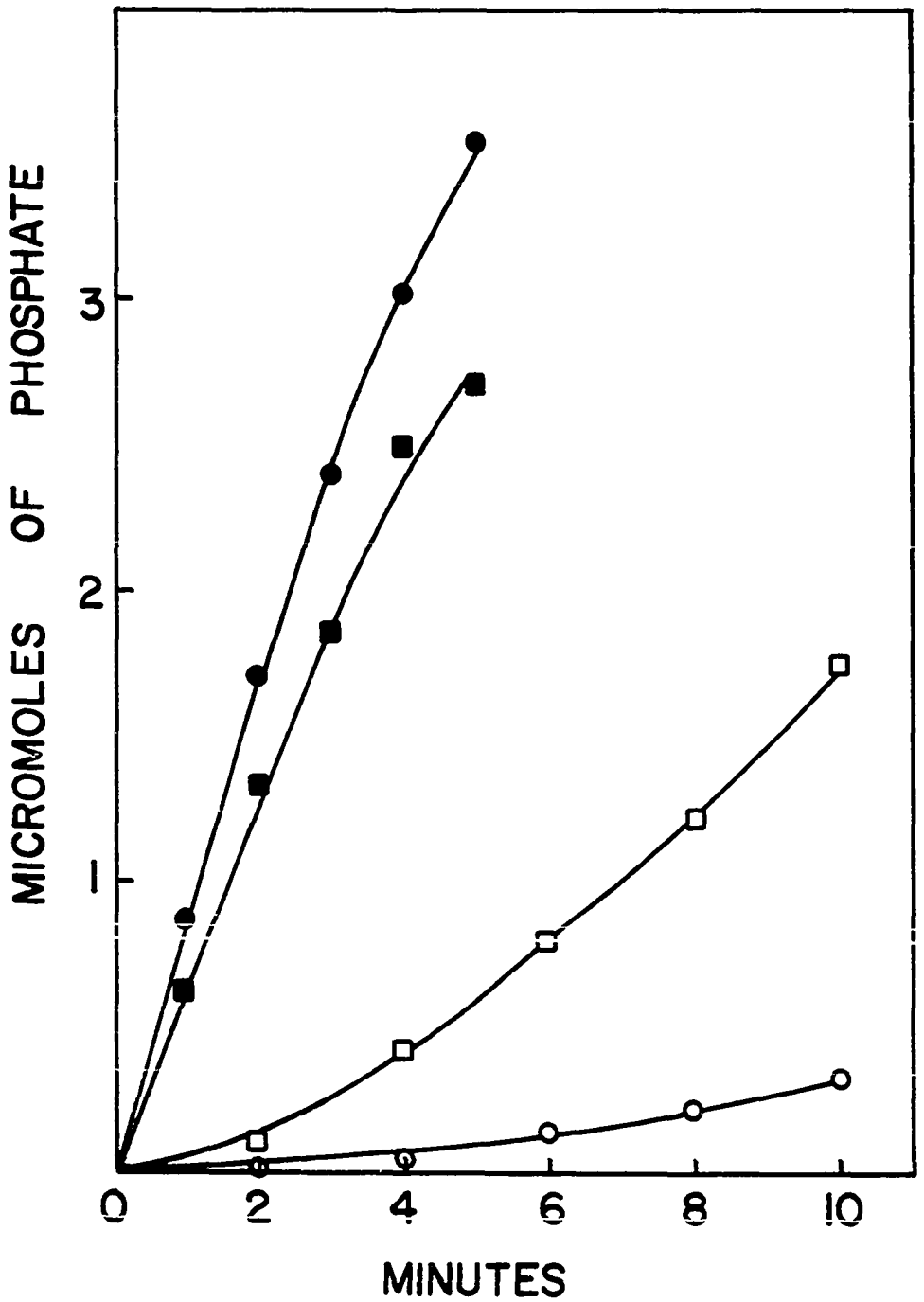
formation. Inorganic phosphate, the product, was the only substance increasing in concentration during the reaction. Therefore, the enzymic activity of pyridoxal reconstituted phosphorylase in the presence of added phosphate was determined. The normal colorimetric assay for phosphate release from glucose-1-P could not be used since the added phosphate would have resulted in unacceptably high background. This problem could be avoided by measuring the incorporation of (^{14}C)glucose into glycogen from (^{14}C)glucose-1-P. The time course for the activity of pyridoxal reconstituted phosphorylase in the presence and absence of added phosphate is shown in Figure 4. In the presence of added phosphate there was no lag, while in the absence of added phosphate, there was a lag.

Both the subtrate, glucose-1-P, and the buffer, B-glycerophosphate contained small amounts of phosphate, and it seems reasonable to conclude that contaminating amounts of phosphate were responsible for the initial enzymic activity of pyridoxal reconstituted phosphorylase. This activity in turn generated more phosphate from glucose-1-P, which generated more activity. This procedure continued until sufficient phosphate was generated to saturate the enzyme. At

Figure 5. Enzymic activity of native and pyridoxal reconstituted phosphorylase in the presence and absence of barium.

The reaction mixture at 30°C and pH 6.8 contained either native phosphorylase 25 µg/ml (closed symbols) or pyridoxal reconstituted phosphorylase 233 µg/ml (open symbols) and 0.075 M glucose-1-P, 0.001 M AMP, 1% glycogen, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol and no barium (□,■) or 0.05 M barium (●,○). At the indicated times, 400 µliters was removed and the amount of phosphate liberated was measured as described in MATERIALS AND METHODS, with the following additional step: The precipitated barium sulfate produced by the acidification with sulfuric acid was removed by centrifugation and the clear liquid was used in the phosphate assay.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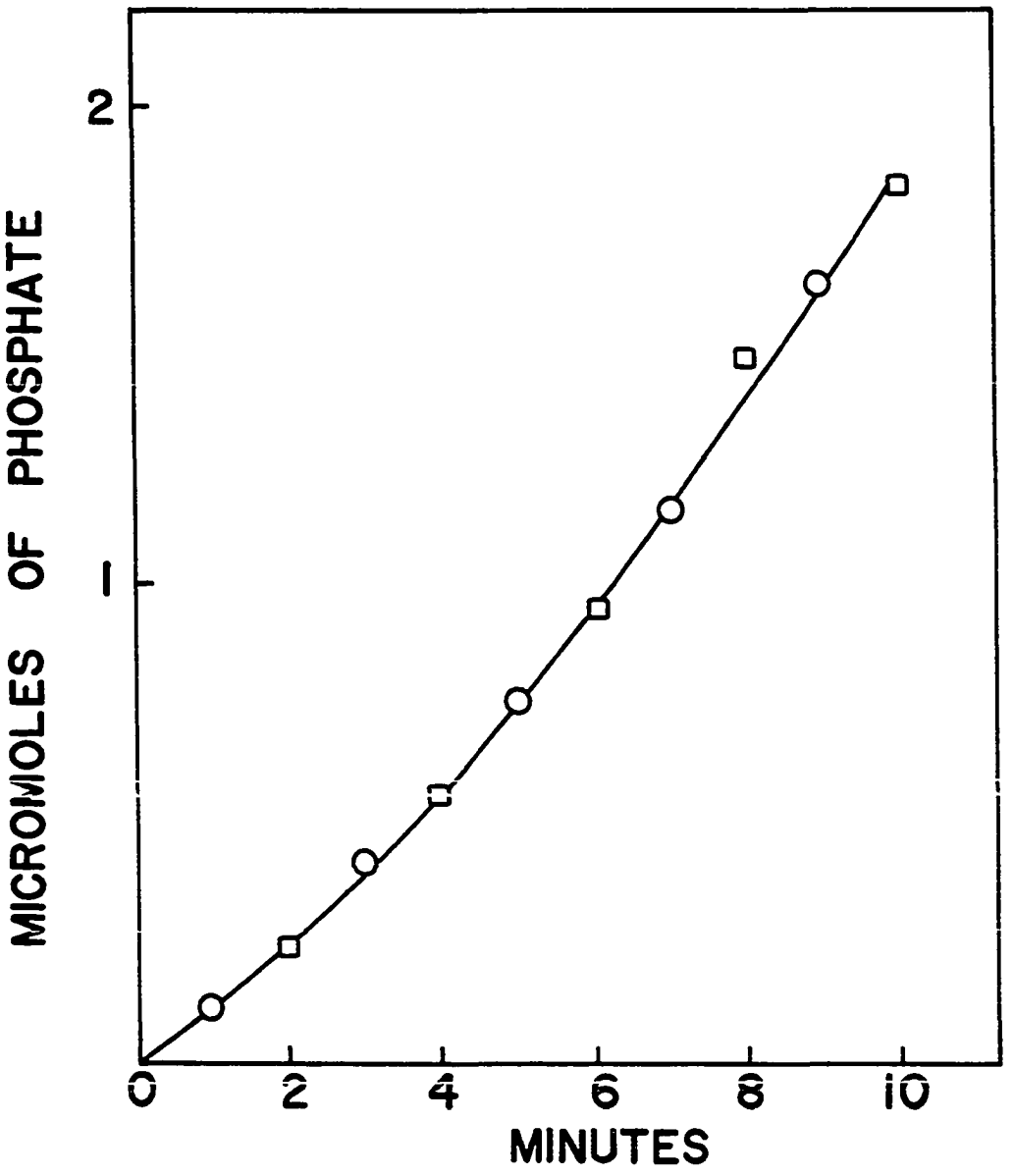
MINUTES

this time, the generation of product became linear with respect to time. The obvious test for this hypothesis was the removal of the initial phosphate. Inclusion of barium in the assay mixture resulted in an 85 per cent loss of activity for pyridoxal reconstituted phosphorylase, while there was an 18 per cent stimulation of the activity of the native enzyme. This is shown in Figure 5. Thus it was concluded that pyridoxal reconstituted phosphorylase possessed enzymic activity in the presence of inorganic phosphate.

The possibility remained that the lag was due to the formation of pyridoxal-5'-P from the phosphate in solution and the protein bound pyridoxal. To test this, pyridoxal reconstituted phosphorylase was incubated in the presence of AMP glycogen and glucose-1-P until equilibrium had been achieved. The enzymic activity of this preincubated enzyme was compared to the activity of an equivalent amount of pyridoxal reconstituted phosphorylase that had not been preincubated in the presence of substrates, but to which was added the amount of phosphate calculated to have been present in the preincubated sample at equilibrium. If there had been formation of pyridoxal-5'-P during the many catalytic events that the preincubated enzyme had undergone, there should have been a

Figure 6. The effect of preincubation in the presence of all substrates on the enzymic activity of pyridoxal reconstituted phosphorylase.

Pyridoxal reconstituted phosphorylase (3 mg/ml) was incubated with 1.3% glycogen, 0.0013 M AMP, 0.026 M glucose-1-P for 1 hour at 30° C. 100 µliters was removed and added to 1.1 ml of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, pH 6.8. An equal volume of a solution containing 0.1 M glucose-1-P, 2% glycogen, 0.002 M AMP pH 6.8 was added and at the indicated times, 400 µliters was removed and the amount of phosphate liberated was measured as described in the MATERIALS AND METHODS section. The phosphate concentration at equilibrium was calculated to be 0.019 M (□). 100 µliters of pyridoxal reconstituted phosphorylase (15 mg/ml) was added to 400 µliters of 0.026 M phosphate pH 6.8. 100 µliters was immediately removed, diluted and assayed as described above (○).


MICROMOLES OF PHOSPHATE
2
1
0
2
4
6
8
10
MINUTES

pronounced stimulation of the enzymic activity of this sample relative to the sample that had not been preincubated in the presence of substrates. Figure 6 demonstrates that there was no difference in the activity between the preincubated enzyme and the enzyme that had not been preincubated. This eliminated the possibility that the activation results from a covalent bond formation between the enzyme or the coenzyme and the phosphate required for activity. It also eliminated the possibility that the lag resulted from a slow substrate induced conformational change.

Enzymic activity of pyridoxal reconstituted phosphorylase in the presence of phosphate analogs

The enzymic activity of pyridoxal reconstituted phosphorylase in the presence of noncovalently bound phosphate prompted a search for other phosphate analogs that might promote enzymic activity. Table III shows the effects of several anions on the enzymic activity of native and pyridoxal reconstituted phosphorylase. With the exception of nitrate and bicarbonate, Table III also shows the second ionization constant for all anions tested. The structural similarity between phosphite, fluorophosphate, thiophosphate, pyrophosphate and phosphate is quite obvious. Nitrate has

Table III. The effect of phosphate analogs on the enzymic activity of pyridoxal reconstituted phosphorylase

Anion Added	pKa_2	Specific Activity	
		Pyridoxal[a]	Native[b]
None	-	2.4	60
Phosphite	6.6[c]	9.1	56
Fluorophosphate	4.8[d]	7.6	60
Nitrate	-	1.9	62
Sulfate	1.9[c]	2.0	60
Bicarbonate	6.4[c]	0.4	57
Thiophosphate	6.2[d]	0.2	62
Pyrophosphate	1.5[c]	0	61

[a]micromoles of phosphate per minute per mg of protein. The reaction mixture at 30°C and pH 6.8 contained pyridoxal reconstituted phosphorylase (54 µg), 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.075 M glucose-1-P, 0.001 M AMP, 1% glycogen and the indicated phosphate analogs at a concentration of 0.01M. After a 5 minute incubation, the phosphate liberated was determined as described in the MATERIALS AND METHODS section. For purposes of comparison, the activity resulting from the phosphate present in the buffer and substrate was defined as no additions. No correction was made for the nonlinear time dependence of the product formation.

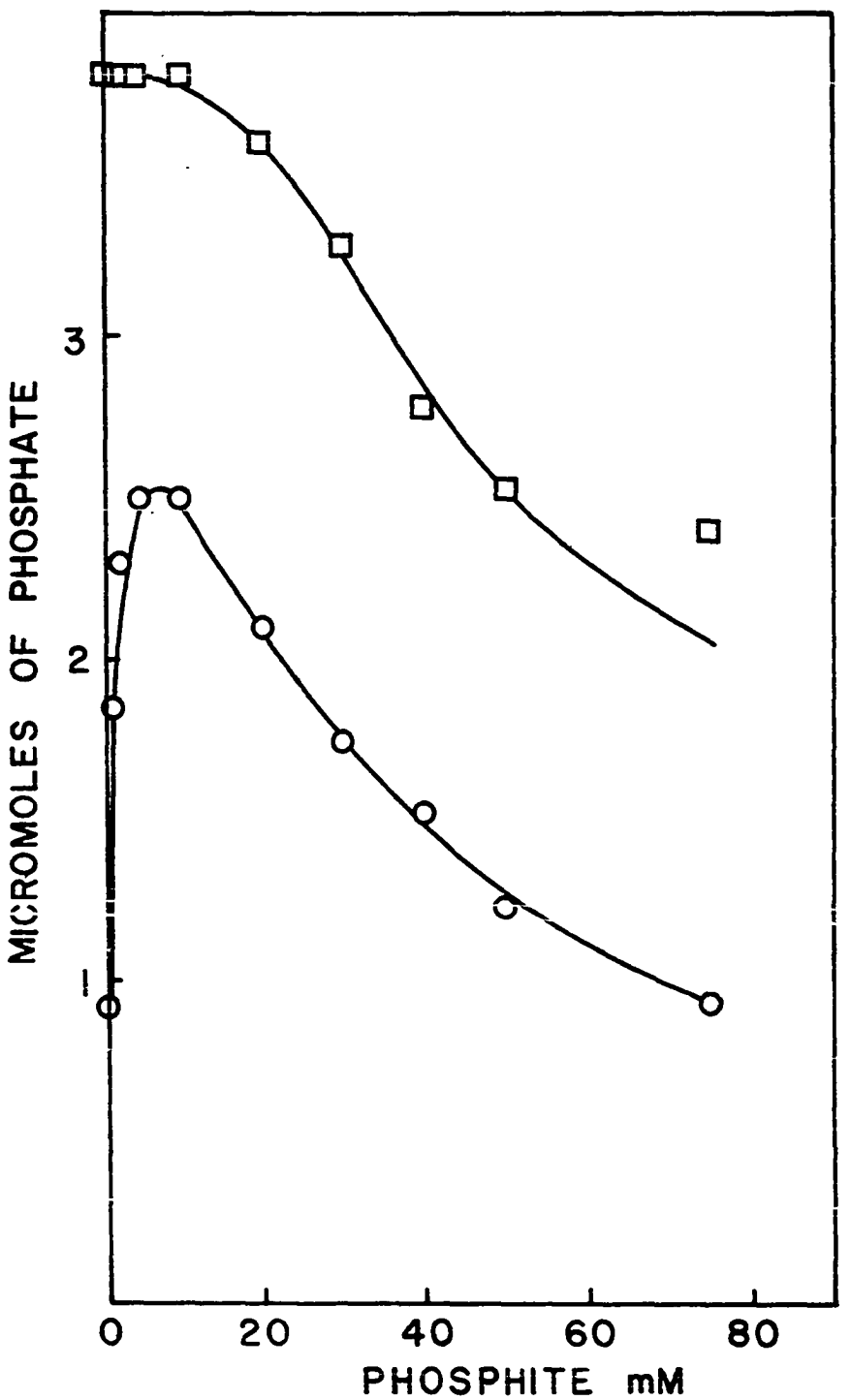
[b]Conditions were identical to a. except that the reaction mixture contained native phosphorylase (4.9 µg).

[c]Weast (1974).

[d]Van Wazer (1958).

Figure 7. Enzymic activity of native and pyridoxal reconstituted phosphorylase as a function of the phosphite concentration.

The reaction mixture at pH 6.8 in a total reaction volume of 400 µliters contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen and the indicated amounts of phosphite with native phosphorylase 8.9 µg (□); or pyridoxal reconstituted phosphorylase 60 µg (○). After a 5 minute reaction time at 30°C, the liberated phosphate was measured as described in the MATERIALS AND METHODS section.


MICROMOLES OF PHOSPHATE
3
2
1
0
20
40
60
80
PHOSPHITE mM

been proposed as a transition state analog of phosphate (Milner-White and Watts, 1971). Sulfate has a tetrahedral structure similar to phosphate, while bicarbonate has a pKa near neutrality. Phosphite and fluorophosphate stimulated the enzymic activity of pyridoxal reconstituted phosphorylase above the value that could be obtained with the control. Activity of the control was due to the presence and formation of inorganic phosphate in the system. Nitrate and sulfate had a slight inhibitory effect, but the anions bicarbonate, thiophosphate, and pyrophosphate were effective inhibitors of the enzymic activity of the pyridoxal reconstituted enzyme. The anions had no significant effect on the enzymic activity of the native enzyme.

Effects of phosphite on the enzymic activity of pyridoxal reconstituted phosphorylase

The effects of different concentrations of phosphite on the enzymic activity of native and pyridoxal reconstituted phosphorylase is shown in Figure 7. Pyridoxal reconstituted phosphorylase activity was stimulated at concentrations of phosphite that had no effect on the activity of the native enzyme. Above 15 mM phosphite, both native and pyridoxal reconstituted phosphorylase activity was inhibited. The time

course for the activity of pyridoxal reconstituted phosphorylase in the presence and absence of 0.0075 M phosphite is shown in Figure 8. There was no lag in the generation of phosphate in the presence of phosphite. The enzymic activity of pyridoxal reconstituted phosphorylase in the presence of 0.0075 M phosphite represented 8 per cent of the enzymic activity of the native enzyme. Figure 9 shows the protein concentration dependence of the enzymic activity of pyridoxal reconstituted phosphorylase in the presence and absence of 0.0075 M phosphite. The enzymic activity shows a linear relationship with respect to protein concentration in the presence of phosphite, while in the absence of phosphite there was a marked nonlinearity. This nonlinearity is especially pronounced in the 3-10 microgram range where the enzymic activity of native phosphorylase is normally measured. Figure 10 demonstrates that at the phosphite concentrations where the enzymic activity of pyridoxal reconstituted phosphorylase is stimulated, the effect is on the maximal velocity of the reaction, with little or no effect on the binding of substrate, glucose-1-P. Figure 11 demonstrates that in the concentration range where phosphite inhibits the enzymic activity of pyridoxal reconstituted

Figure 8. Time course for the enzymic activity of pyridoxal reconstituted phosphorylase in the presence and absence of added phosphite.

The reaction mixture at pH 6.8 and 30° C contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 1% glycogen, 0.001 M AMP, 0.016 M glucose-1-P, enzyme (150 µg/ml) and phosphite, 0.0075 M (□), or no phosphite (○). At the indicated times, 400 µliters was removed and the liberated phosphate measured as described in the MATERIALS AND METHODS section.

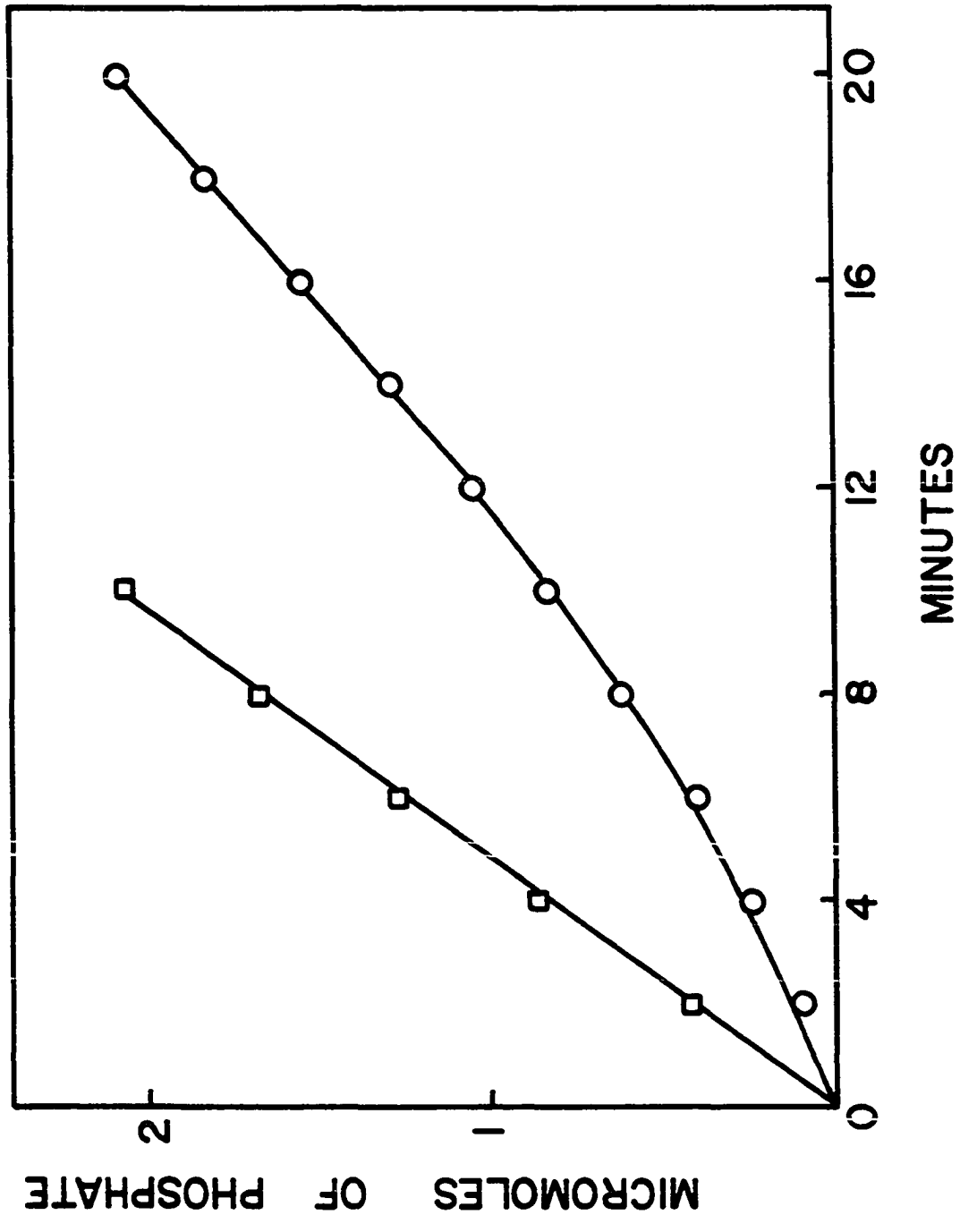

MICROMOLES OF PHOSPHATE
2
1
0
4
8
12
16
20
MINUTES

Figure 9. Protein concentration dependence of the enzymic activity of pyridoxal reconstituted phosphorylase in the presence and absence of phosphite.

The reaction mixture at 30° C and pH 6.8 in a total volume of 400 µliters consisted of 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 1% glycogen, 0.016 M glucose-1-P and phosphite 0.0075 M (□); or no phosphite (○); and the indicated amounts of enzyme. After a 5 minute reaction time, the liberated phosphate was measured as described in the MATERIALS AND METHODS section.

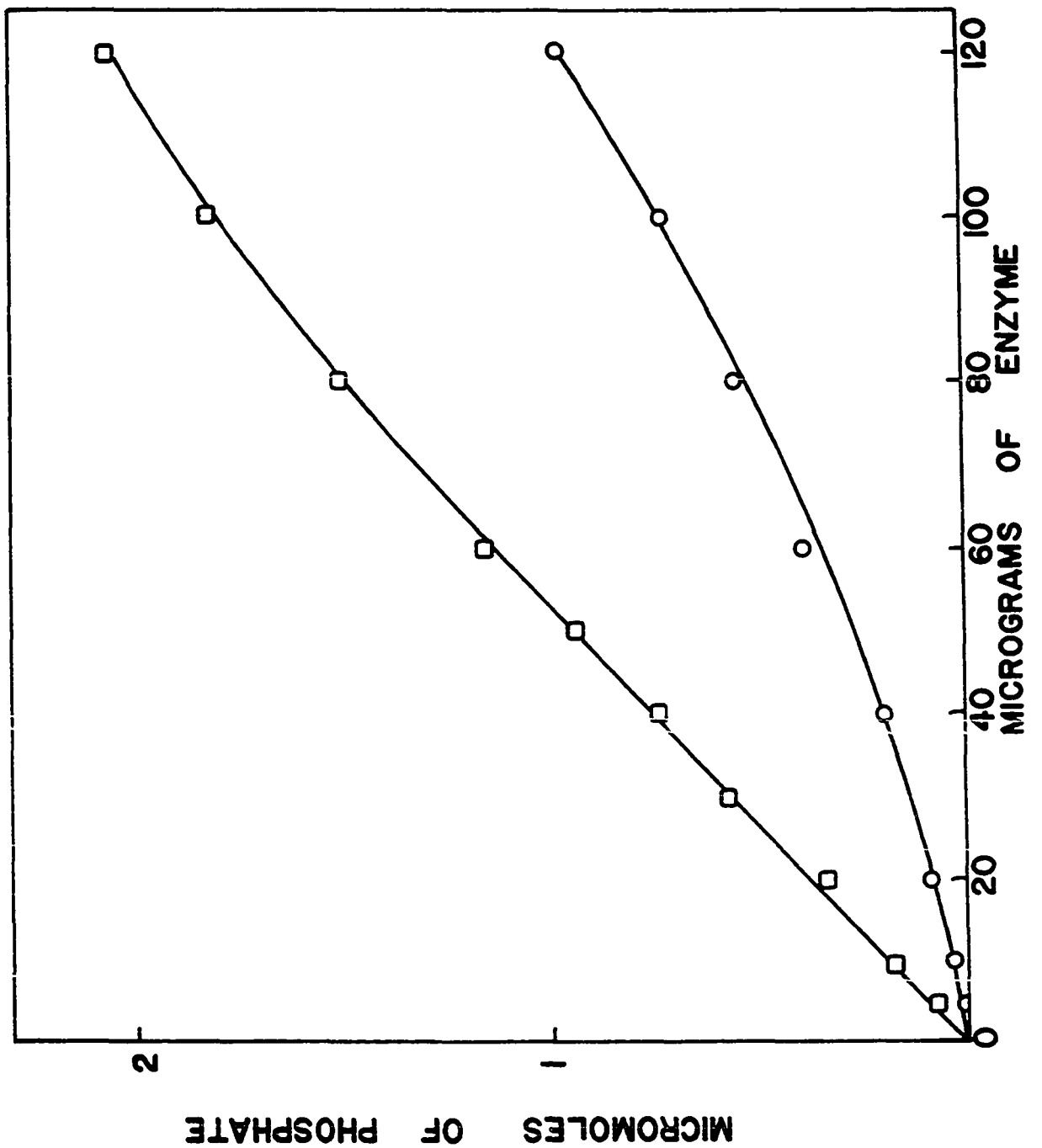

MICROMOLES OF PHOSPHATE
2
1
0
20
40
60
80
100
120
MICROGRAMS OF ENZYME

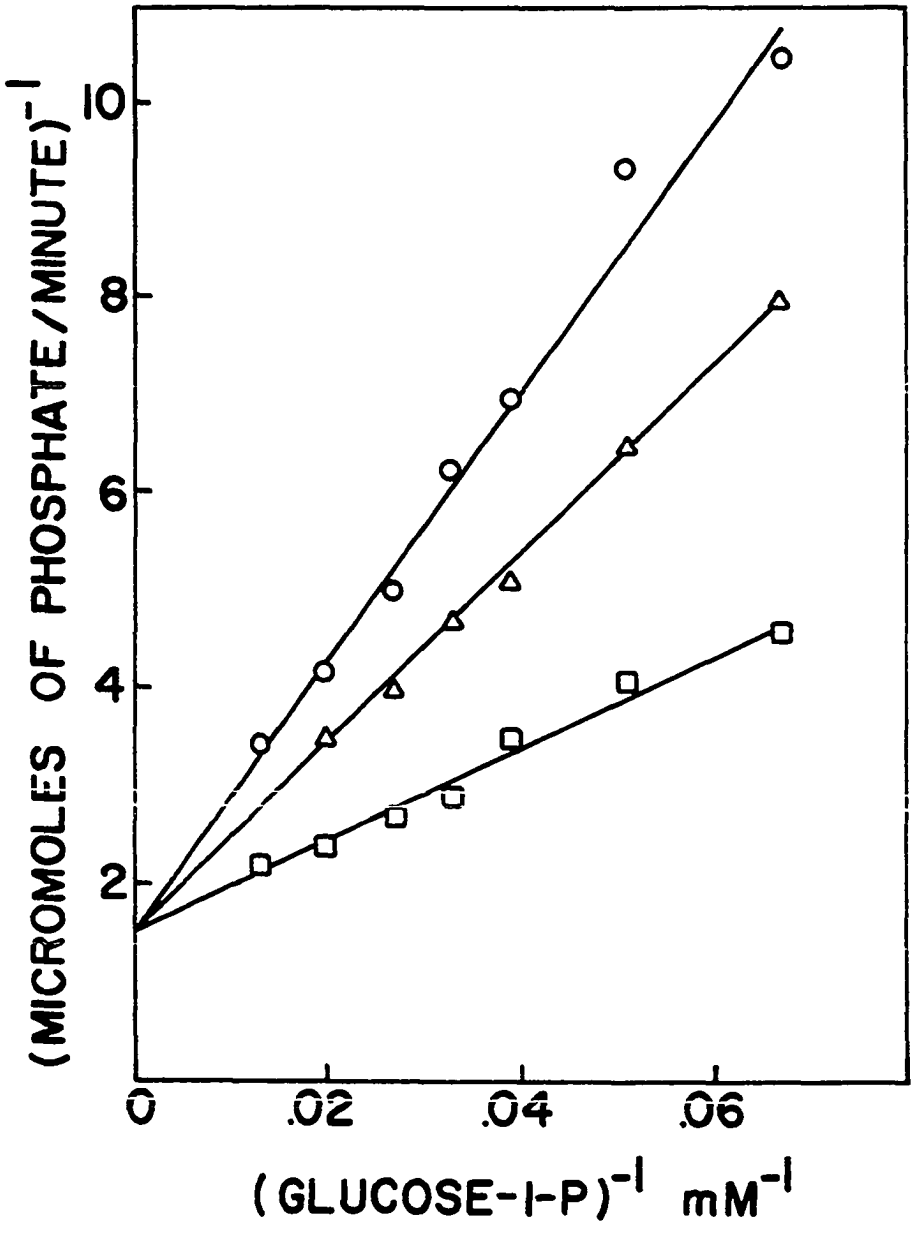
Figure 10. Double reciprocal plot for phosphite activation of pyridoxal reconstituted phosphorylase.

The reaction mixtures at 30° C and pH 6.8 consisted of enzyme (52 μg), 1% glycogen, 0.001 M AMP, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, glucose-1-P at the indicated concentrations and phosphite 0.0075 M (□), 0.00075 (○).

(MICROMOLES OF PHOSPHATE/MINUTE)$^{-1}$
(GLUCOSE-1-P)$^{-1}$ mM^{-1}
-.04
-.02
0
.02
.04
.06
2
4
6
8
10

Figure 11. Double reciprocal plot for phosphite inhibition of pyridoxal reconstituted phosphorylase.

The reaction mixture at 30° C and pH 6.8 consisted of enzyme (52 µg), 1% glycogen, 0.001 M AMP, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, glucose-1-P at the indicated concentrations and phosphite 0.0075 M (□), 0.025 M (△), 0.037 M (○).


(MICROMOLES OF PHOSPHATE/MINUTE)$^{-1}$
10
8
6
4
2
0
.02
.04
.06
(GLUCOSE-1-P)$^{-1}$ mM^{-1}

phosphorylase, the effect was on the binding of the substrate.

There was a remote possibility that phosphite was being oxidized to phosphate during the activity measurements. This conversion would mimic enzymic activity when the colorimetric assay for phosphate release from glucose-1-P was utilized. Figure 12 demonstrates the pyridoxal reconstituted phosphorylase catalyzed incorporation of (^{14}C)glucose into glycogen form (^{14}C)glucose-1-P in the presence and absence of 0.0075 M phosphite. Incorporation of radioactivity into glycogen was linear in the presence of phosphite but demonstrated a lag in the absence of phosphite. The results are similar to those seen in Figure 8 where the colorimetric phosphate assay was used to measure enzymic activity. Thus the possibility that phosphite oxidation was mimicking the enzymic activity of pyridoxal reconstituted phosphorylase was eliminated.

Since linear progress curves were seen with pyridoxal reconstituted phosphorylase in the presence of phosphite, kinetic studies of the enzyme were undertaken. Table lV shows the apparent kinetic constants calculated for pyridoxal reconstituted phosphorylase and compares them to the literature values for the native enzyme. The only significant

Figure 12. Pyridoxal reconstituted phosphorylase catalyzed incorporation of (^{14}C)glucose into glycogen from (^{14}C)glucose-1-P in the presence and absence of phosphite.

The reaction mixture contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, enzyme (165 µg/ml), 0.1 uC (^{14}C)glucose-1-P, pH 6.8, and phosphite 0.0075 M (O), no phosphite (□). At the indicated times, 20 µliters was removed and the radioactivity incorporated into glycogen was measured as described in the MATERIALS AND METHODS section.

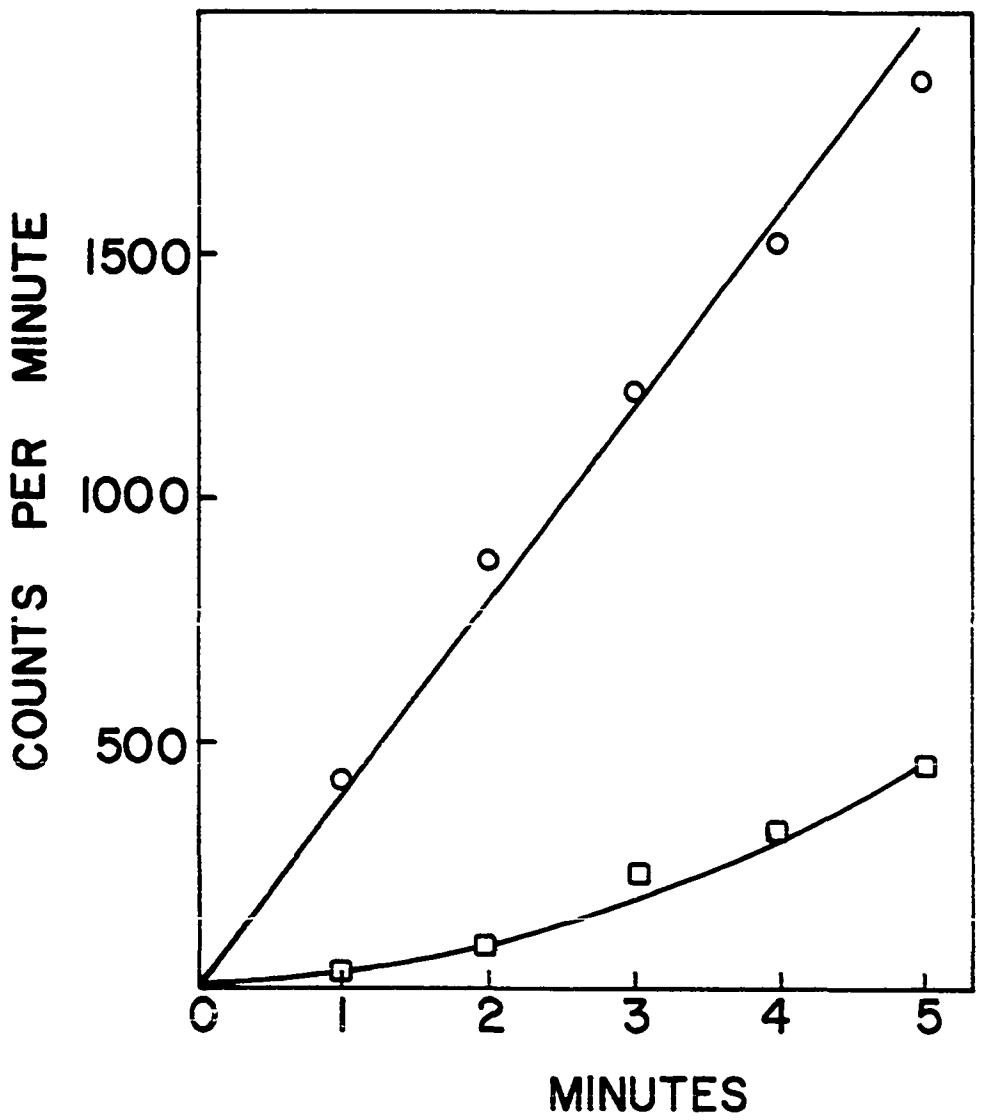

COUNTS PER MINUTE
1500
1000
500
0
1
2
3
4
5
MINUTES

Table IV. Properties of pyridoxal reconstituted and native phosphorylase b

Parameter	Pyridoxal reconstituted	Native
Km phosphite	9×10^{-4} M	-
Km AMP	2.5×10^{-5} M	$3\text{-}8 \times 10^{-5}$ M[a]
Km glucose-1-P	2.8×10^{-2} M	7×10^{-3} M[b]
Km glycogen	0.01%[c]	0.02%[d]
Cooperative binding of AMP	YES	YES[a]
Specific activity	3.4[e] (8.7%)	39[f]
Maximal velocity	12[g] (19%)	65[h]

[a]Kastenschmidt et al., (1968); Helmreich and Cori (1964a)

[b]Engers et al. (1969).

[c]Measured by the method of Helmreich and Cori (1964a).

[d]Anderson et al. (1973).

[e]Measured in the presence of 0.016 M glucose-1-P, 1% glycogen, 0.001 M AMP, 0.0075 M phosphite, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, pH 6.8.

[f]Measured according to e in the absence of phosphite

[g]Extrapolated from plots of $(\text{velocity})^{-1}$ vs $(\text{glucose-1-P})^{-1}$, in the presence of 0.0075 M phosphite.

[h]Extrapolated from plots of $(\text{velocity})^{-1}$ vs $(\text{glucose-1-P})^{-1}$, but in the absence of phosphite.

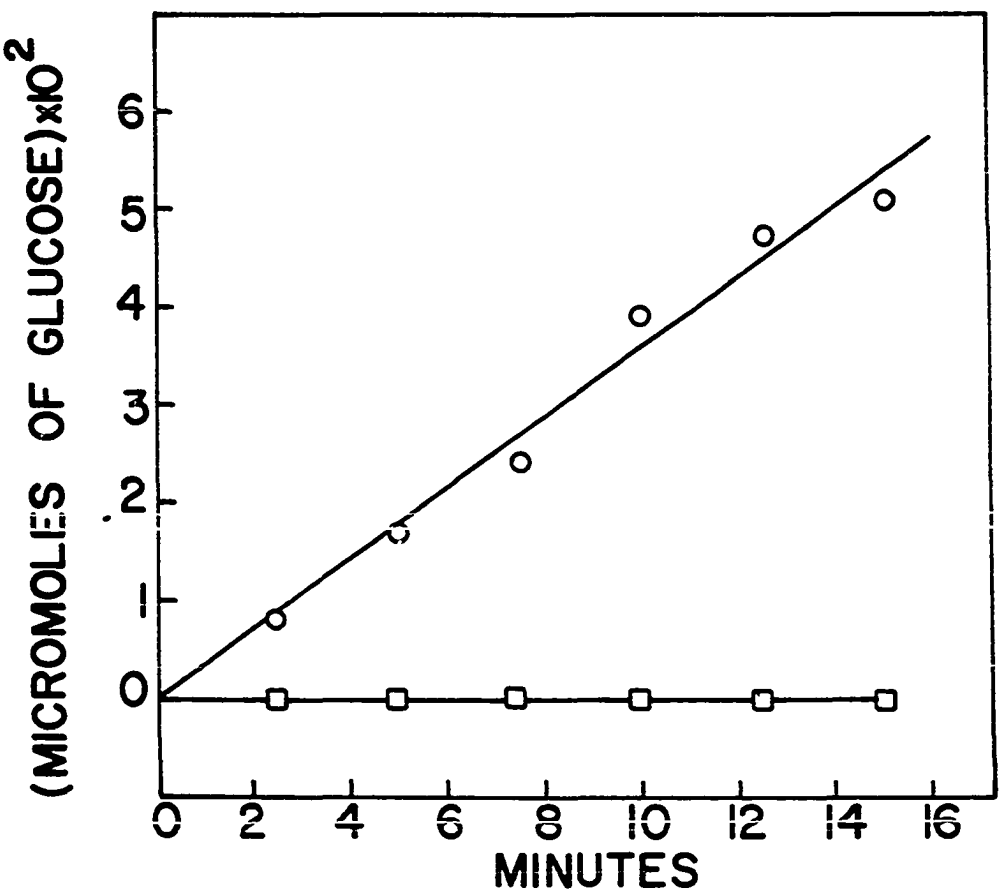
change was the apparent 4-fold increase in the Km for glucose-1-P exhibited by the Pyridoxal reconstituted enzyme. AMP has been previously reported to bind cooperatively to pyridoxal reconstituted phosphorylase with a binding constant of 6×10^{-5} M (Kastenschmidt et al., 1968).

Enzymic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen degradation

Since native phosphorylase can catalyze both the synthesis and degradation of glycogen, it was important to establish this capacity for pyridoxal reconstituted phosphorylase. When the enzymic activity was measured in the presence of 0.02 M phosphate, (Helmreich and Cori, 1964a) the enzymic activity of pyridoxal reconstituted phosphorylase was 6 per cent of that of the native enzyme. A slight modification of the method of Helmreich and Cori (1964b) was utilized to establish that arsenate could also function as a substrate in the pyridoxal reconstituted phosphorylase catalyzed degradation of glycogen. 0.05 M maleate was used instead of B-glycerophosphate as the assay buffer. This eliminated the possibility that small quantities of phosphate were responsible for the observed enzymic activity shown in Figure 13. When the enzymic activity was measured in the

Figure 13. Enzymic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen degradation.

The reaction mixture at pH 6.2 and 30° C contained pyridoxal reconstituted phosphorylase (500 µg/ml) (O) or apophosphorylase (500 µg/ml) (□) and 0.05 M maleate, 0.1 M arsenate, 0.001 M AMP, 2% glycogen. At the indicated times, 200 µliters was removed and the liberated glucose determined according to Helmreich and Cori (1964b).

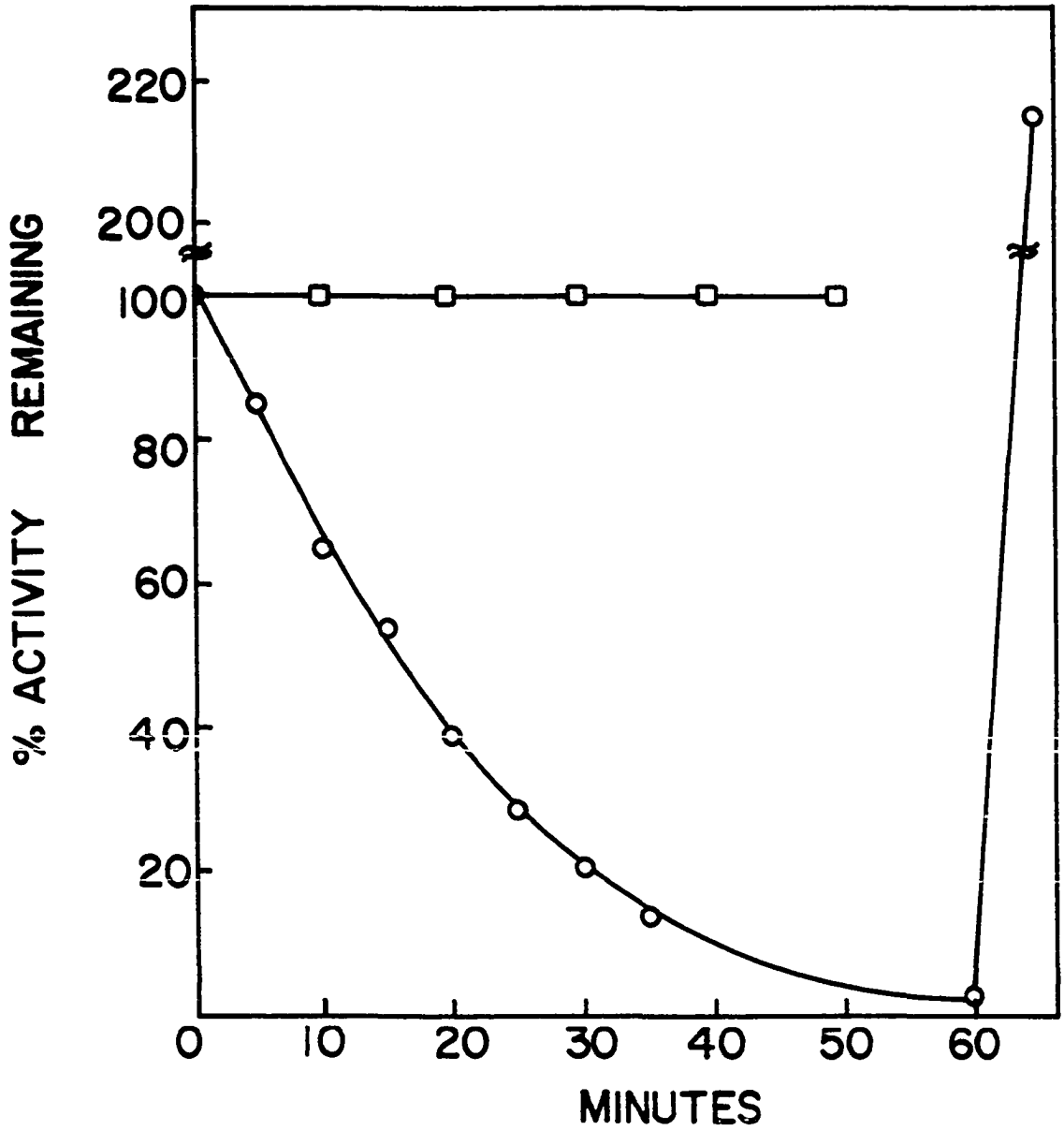

(MICROMOLES OF GLUCOSE)xIO^2
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
MINUTES

presence of 0.1 arsenate, pyridoxal reconstituted phosphorylase exhibited 3 per cent of the activity of the native enzyme. The apoprotein from which the pyridoxal reconstituted phosphorylase was prepared possessed no catalytic activity. Thus it may be concluded that pyridoxal reconstituted phosphorylase can catalyze both the synthesis and degradation of glycogen in the presence of an activator anion.

Since phosphite could activate pyridoxal reconstituted phosphorylase in the direction of glycogen synthesis, there was also the possibility that phosphite could serve as a substrate for native or pyridoxal reconstituted phosphorylase in the degradation of glycogen. The normal assay procedure could not be employed because it had been demonstrated by Robertson and Boyer (1956) that glucose-6-phosphite was not utilized by glucose-6-phosphate dehydrogenase, one of the enzymes in the coupled assay system for phosphorylase activity in the direction of glycogen degradation. However, after incubation of (^{14}C)glycogen with either native or pyridoxal reconstituted phosphorylase in the presence of AMP and phosphite, the only radioactive material observed was glycogen. Although this is not unequivocal evidence, it suggests that

Figure 14. Enzymic activity of pyridoxal reconstituted phosphorylase a and b in the presence of L-cysteine.

The reaction mixture at pH 6.8 and 30° C in a total volume of 2.0 ml contained 0.1 M β-glycerophosphate, 0.1 M L-cysteine, pyridoxal reconstituted phosphorylase a (3.2 mg/ml) (□), or pyridoxal reconstituted phosphorylase b (3.0 mg/ml) (○). At the indicated times, 100 μliters was removed and the remaining enzymic activity measured as described in the MATERIALS AND METHODS section. After 60 minutes of incubation, 100 μliters of pyridoxal-5'-P was added to the tube containing pyridoxal reconstituted phosphorylase b, and after a 5 minute incubation, 100 μliters was removed and treated as described above.


% ACTIVITY REMAINING
220
200
100
80
60
40
20
0
10
20
30
40
50
60
MINUTES

phosphite does not serve as a substrate for either native or pyridoxal reconstituted phosphorylase in the direction of glycogen degradation.

The effect of L-cysteine on the activity of pyridoxal reconstituted phosphorylase

Pfeuffer et al., (1972b) reported a 25 per cent loss of pyridoxal from pyridoxal reconstituted phosphorylase b after 4 hours at 0° C in the presence of L-cysteine but in the absence of the normal deforming buffers required for the resolution of pyridoxal-5'-P from native phosphorylase. Figure 14 demonstrates the time dependent inactivation of pyridoxal reconstituted phosphorylase b in the presence of L-cysteine. The inactivation was shown to be the result of resolution of pyridoxal from the protein and not from denaturation since addition of pyridoxal-5'-P resulted in a rapid return and increase in enzymic activity. Figure 14 also demonstrates that pyridoxal reconstituted phosphorylase a was not inactivated by L-cysteine under conditions where pyridoxal reconstituted phosphorylase b was completely inactivated. Table V demonstrates the ability of other aldehyde trapping agents to inactivate pyridoxal reconstituted phosphorylase b. Only hydroxylamine was effective as L-cysteine. Table VI

Table V. Inactivation of pyridoxal reconstituted phosphorylase b by aldehyde trapping reagents.

Reagent	% Activity remaining
L-cysteine	30
Hydroxylamine·Hcl	29
Hydrazine·2HCl	89
Semicarbazide·HCl	99
Isonicotinic acid hydazide	90

The reaction mixtures at 30° C and pH 6.8 consisted of 0.1 M β-glycerophosphate, enzyme (2.4 mg/ml) and the indicated reagents at a concentration of 0.1 M. After a 15 minute incubation period, 100 µliters was removed and the remaining enzymic activity was measured as described in the MATERIALS AND METHODS section.

Table VI. The effect of substrates and other small molecules on the inactivation of pyridoxal reconstituted phosphorylase by L-cysteine.

Substance added	Concentration	% Activity remaining
None		11
glucose	10 mM	18
glucose-1-P	10 mM	24
glycogen	1%	64
glycogen + glucose-1-P	1% 10 mM	64
AMP	1 mM	83
AMP + glucose-1-P	1 mM 10 mM	91
glycogen + AMP	1% 1 mM	100
glucose-6-P	10 mM	100

The reaction mixtures at pH 6.8 and 30° C contained, 0.04 M β-glycerophosphate, 0.03 M L-cysteine, enzyme (2 mg/ml) and the indicated concentrations of added materials. After a 40 minute incubation, 100 μliters was removed and the remaining enzymic activity was measured as described in the MATERIALS AND METHODS section.

shows that AMP, glucose-6-P and glycogen inhibited the resolution of pyridoxal from pyridoxal reconstituted phosphorylase b, but glucose and glucose-1-P had little if any protective effect. These results are very similar to those observed for the resolution of pyridoxal-5'-P from the native enzyme, with the one important exception that no deforming buffer was necessary with pyridoxal reconstituted phosphorylase b (Shaltiel et al., 1966; Hedrick et al., 1969; Shaltiel et al., 1969a). The sulfhydryl reagents D-cysteine, 2-mercaptoethanol, dithiothreitol, thioglycolic acid, glutathione and 2-aminoethanethiol did not resolve pyridoxal from pyridoxal reconstituted phosphorylase.

Since L-cysteine resolved the coenzyme from pyridoxal reconstituted phosphorylase b, the effect of L-cysteine on the reconstitution of apophosphorylase with pyridoxal was investigated. Table VII demonstrates that when apophosphorylase a was reconstituted with a 100-fold excess of pyridoxal in the presence of 0.01 M L-cysteine, the activity observed was only 25 per cent of that observed when 2-mercaptoethanol was included as the thiol reagent. In the original report of the lack of enzymic activity for pyridoxal reconstituted phosphorylase a (Illingworth et al., 1958).

Table VII. The effect of L-cysteine on the reconstitution of apophosphorylase a with pyridoxal

Sulfhydryl reagent	Specific activity (μMoles/min/mg)
2-mercaptoethanol	1.76
L-cysteine	0.46

100 μliters of apophosphorylase a (3.7 mg/ml) in 0.02 M β-glycerophosphate, pH 6.8 containing either 0.02 M 2-mercaptoethanol or 0.02 M L-cysteine was added to 100 μliters of 0.005 M pyridoxal and incubated 30 minutes at 37° C. 1.6 ml of 0.02 M β-glycerophosphate, 0.02 M L-cysteine, pH 6.8, was added to the incubation tube containing L-cysteine, while 1.6 ml of 0.02 M β-glycerophosphate, 0.02 M 2-mercaptoethanol pH 6.8 was added to the incubation tube containing 2-mercaptoethanol. 200 μliters was immediately removed from each dilution and added to 200 uliters of a solution consisting of 0.032 M glucose-1-P, 0.001 M AMP, 1% glycogen, pH 6.8. After a 5 minute incubation at 30° C, the amount of phosphate liberated was measured as described in the MATERIALS AND METHODS section. In calculating the specific activity, no correction was made for the nonlenear time dependence of the product formation.

0.01 M L-cysteine was included in the reconstitution of apophosphorylase a with a 100-fold excess of pyridoxal. The inclusion of L-cysteine in the reconstitution medium may have contributed to the reported lack of enzymic activity of pyridoxal reconstituted phosphorylase. These results indicate that L-cysteine should never be employed in the preparation or activity measurements of pyridoxal reconstituted phosphorylase.

Effect of phosphate analogs on the resolution of pyridoxal from pyridoxal reconstituted phosphorylase b

The inability of L-cysteine to resolve pyridoxal-5'-P from native phosphorylase b under conditions where pyridoxal reconstituted phosphorylase b was rapidly inactivated, suggested that the 5'-phosphoryl group was important in blocking resolution. Therefore, the effect of several phosphate analogs on the resolution of pyridoxal from pyridoxal reconstituted phosphorylase was tested. Table VIII shows that all phosphate analogs tested inhibited resolution.

Activity of pyridoxal reconstituted phosphorylase in the presence of fluorophosphate and pyrophosphate

Preliminary experiments indicated that, like phosphite, the presence of 0.0075 M fluorophosphate in the assay mixture

Table VIII. The effect of phosphate analogs on the inactivation of pyridoxal reconstituted phosphorylase b by L-cysteine

Anion added	% activity remaining
None	18
Phosphite	32
Fluorophosphate	33
Thiophosphate	36
Pyrophosphate	72

The reaction mixture at pH 6.8 and 30° C contained pyridoxal reconstituted phosphorylase b (2.7 mg/ml), 0.1 M L-cysteine, 0.1 M β-glycerophosphate and the indicated phosphate analogs at a concentration of 0.01 M. After a 20 minute incubation, 100 μliters was removed and the remaining activity was measured as described in the MATERIALS AND METHODS section.

resulted in linear product vs. time plots. Therefore, the apparent kinetic constants for pyridoxal reconstituted phosphorylase in the presence of phosphite and fluorophosphate were compared. The apparent binding constant for phosphite was 0.9 mM while that for fluorophosphate was 2 mM. The maximal velocity in the presence of saturating phosphite was 10.2 μmoles of phosphate per minute per mg of protein, while in the presence of saturating fluorophosphate the maximal velocity was 9.7 μmoles of phosphate per minute per mg of protein. Thus it may be concluded that phosphite and fluorophosphate have nearly identical capacities to activate pyridoxal reconstituted phosphorylase.

An investigation of the mode of inhibition of pyridoxal reconstituted phosphorylase by pyrophosphate was undertaken because there was no effect on the native enzyme at a concentration of pyrophosphate that completely inhibited the pyridoxal enzyme. Pyrophosphate exhibited competitive kinetics with respect to both phosphite and glucose-1-P with apparent K_i values of 0.03 and 0.3 mM respectively (Figures 15 and 16). Since it appeared that pyrophosphate could interact strongly with the pyridoxal enzyme, direct binding studies were done to determine if inhibition could be related to the binding of

Figure 15. Double reciprocal plot of pyrophosphate inhibition of pyridoxal reconstituted phosphorylase.

The reaction mixture at pH 6.8 contained enzyme (64 µg), 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 1% glycogen, 0.016 M glucose-1-P, phosphite at the indicated concentrations, and pyrophosphate at 0 (△), 0.0003 M (●), 0.0006 M (○), 0.0012 M (□).

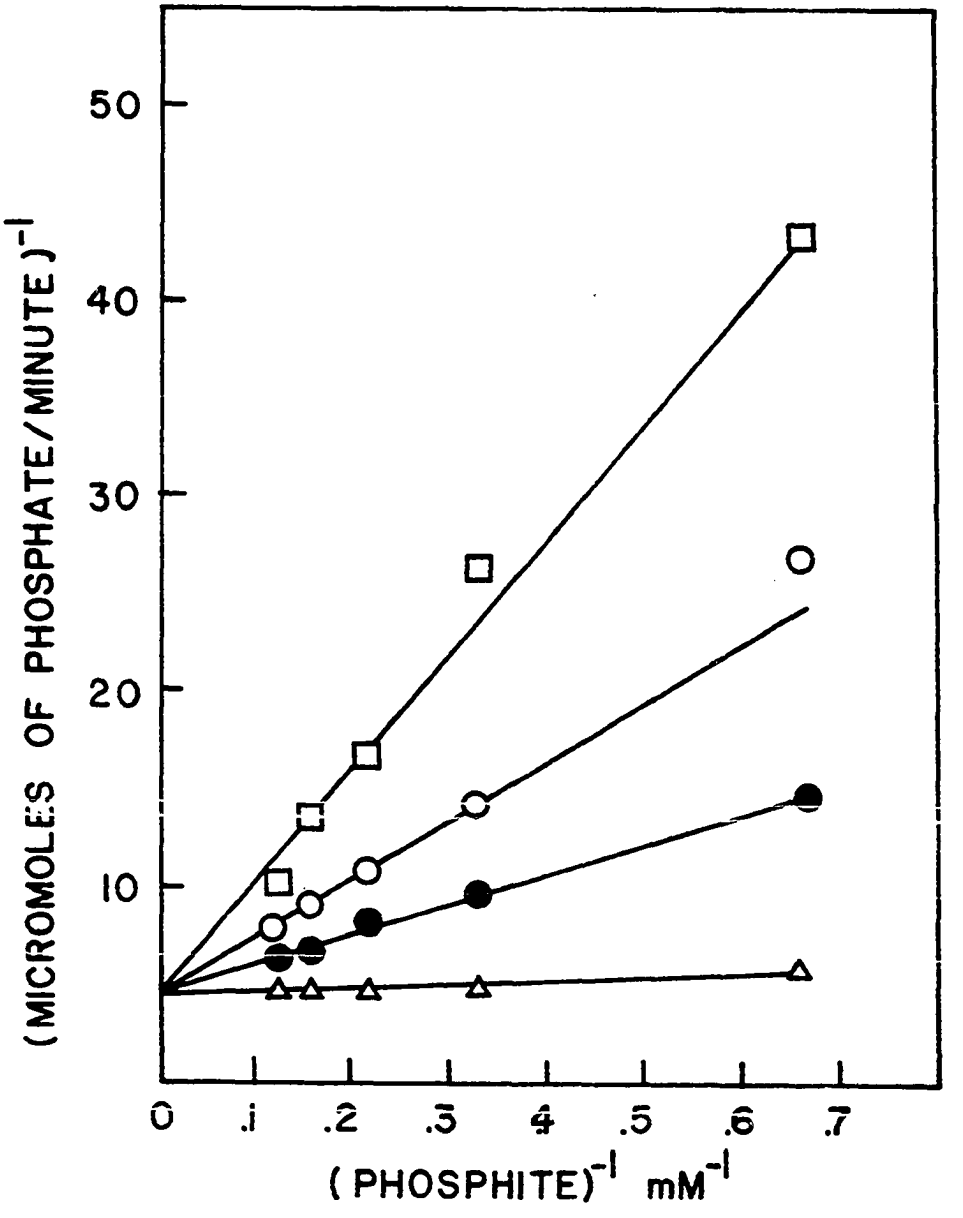
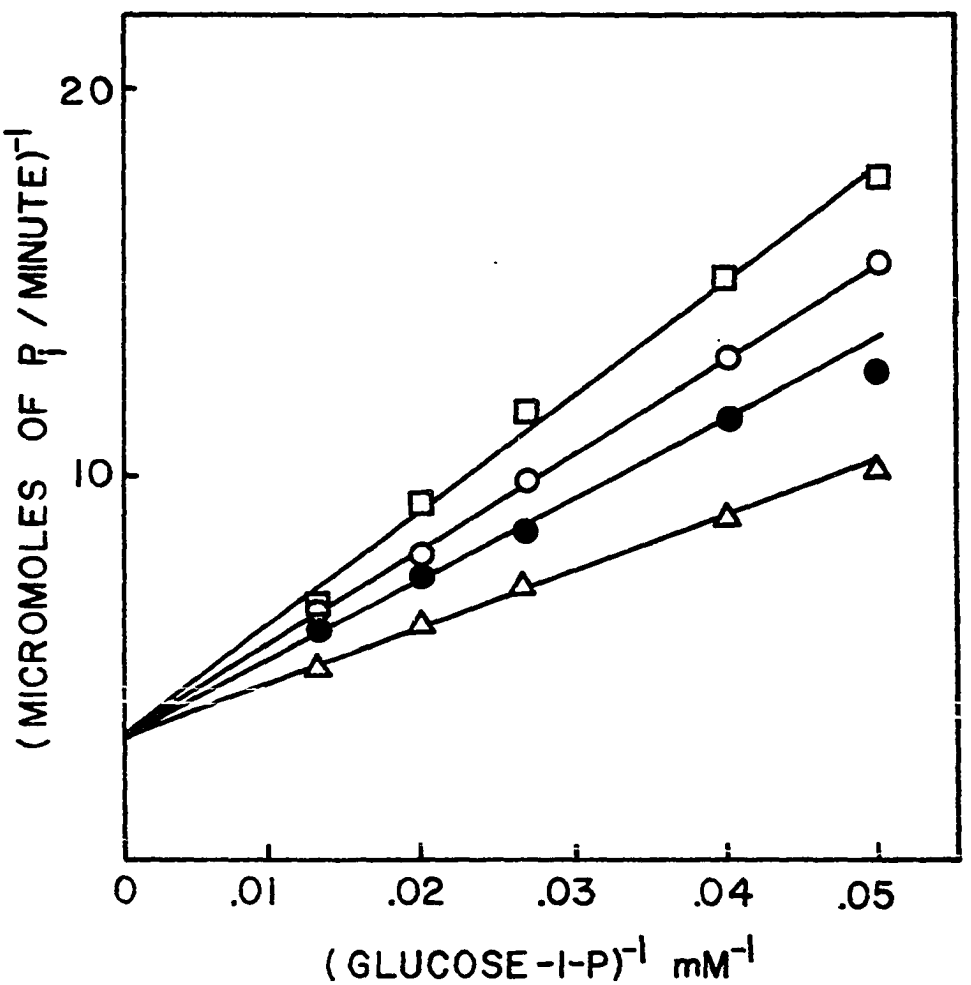

50
40
30
20
10
0
.1
.2
.3
.4
.5
.6
.7
(MICROMOLES OF PHOSPHATE/MINUTE)$^{-1}$
(PHOSPHITE)$^{-1}$ mM^{-1}

Figure 16. Double reciprocal plot of pyrophosphate inhibition of pyridoxal reconstituted phosphorylase.

The reaction mixtures at pH 6.8 consisted of enzyme (22 µg), 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 1% glycogen, 0.0075 M phosphite, glucose-1-P at the indicated concentrations and pyrophosphate at 0 (△), 0.0001 M (●), 0.0002 M (○), 0.0003 M (□).


20
10
0
.01
.02
.03
.04
.05
(MICROMOLES OF P_i / MINUTE)$^{-1}$
(GLUCOSE-1-P)$^{-1}$ mM^{-1}

1 or 2 moles of pyrophosphate per monomer of pyridoxal reconstituted phosphorylase. The results of such measurements are shown in Figure 17, from which it was determined that 0.9 moles of pyrophosphate bound per mole of pyridoxal reconstituted phosphorylase monomer with a binding constant of 8.5×10^{-5} M.

Stimulation of the dephosphorylation of pyridoxal reconstituted phosphorylase a by glucose-6-P and glucose

Glucose-6-P and glucose have been demonstrated to stimulate the phosphorylase phosphatase catalyzed dephosphorylation of native phosphorylase a. This stimulation was shown to result from the binding of glucose-6-P or glucose to the substrate, phosphorylase a and not to the catalyst, phosphorylase phosphatase (Martensen et al., 1973). However, Graves et al. (1975) subsequently observed that glucose-6-P and glucose had no effect on the phosphorylase phosphatase catalyzed dephosphorylation of apophosphorylase a. This indicated that pyridoxal-5'-P possessed an important role in the interconversion process. Since ultracentrifuge studies (Illingworth et al., 1958) indicated that the quaternary structure of pyridoxal reconstituted phosphorylase was very similar to that of the native enzyme, the effect of

Figure 17. Binding of (^{32}P)pyrophosphate by pyridoxal reconstituted phosphorylase.

The reaction mixture at pH 6.8 in a total volume of 0.2 ml, contained enzyme (0.2 mg), 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP and the indicated concentrations of (^{32}P)pyrophosphate. Binding was measured by the ultrafiltration method of Paulus (1969).

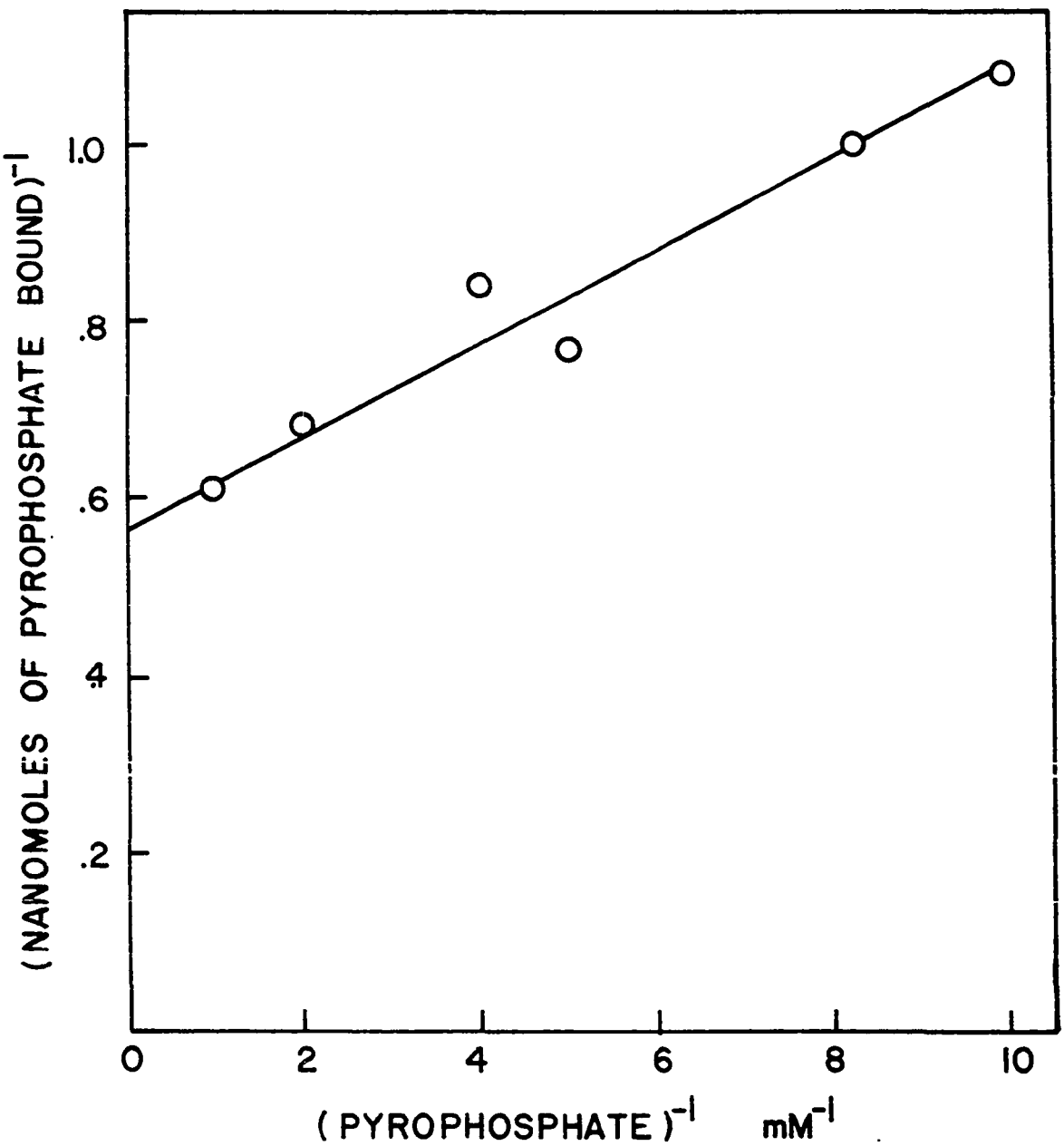

(NANOMOLES OF PYROPHOSPHATE BOUND)$^{-1}$
1.0
.8
.6
.4
.2
0
2
4
6
8
10
(PYROPHOSPHATE)$^{-1}$ mM^{-1}

Figure 18. The effect of glucose-6-P on the dephosphorylation of pyridoxal reconstituted phosphorylase *a* in the presence and absence of phosphate.

The reaction mixture at pH 7.5 consisted of pyridoxal reconstituted phosphorylase *a* (2.5 mg/ml), phosphorylase phosphatase (50 µg/ml), 0.025 M TRIS, 0.015 M 2-mercaptoethanol and no additions (□), 0.0075 M phosphite (■), 0.01 M glucose-6-P (○), 0.0075 M phosphite, 0.01 M glucose-6-P (●). At the indicated times 200 µliters was removed and added to 200 µliters of 10% TCA, and the (^{32}P) phosphate liberated was measured as described in the MATERIALS AND METHODS section.

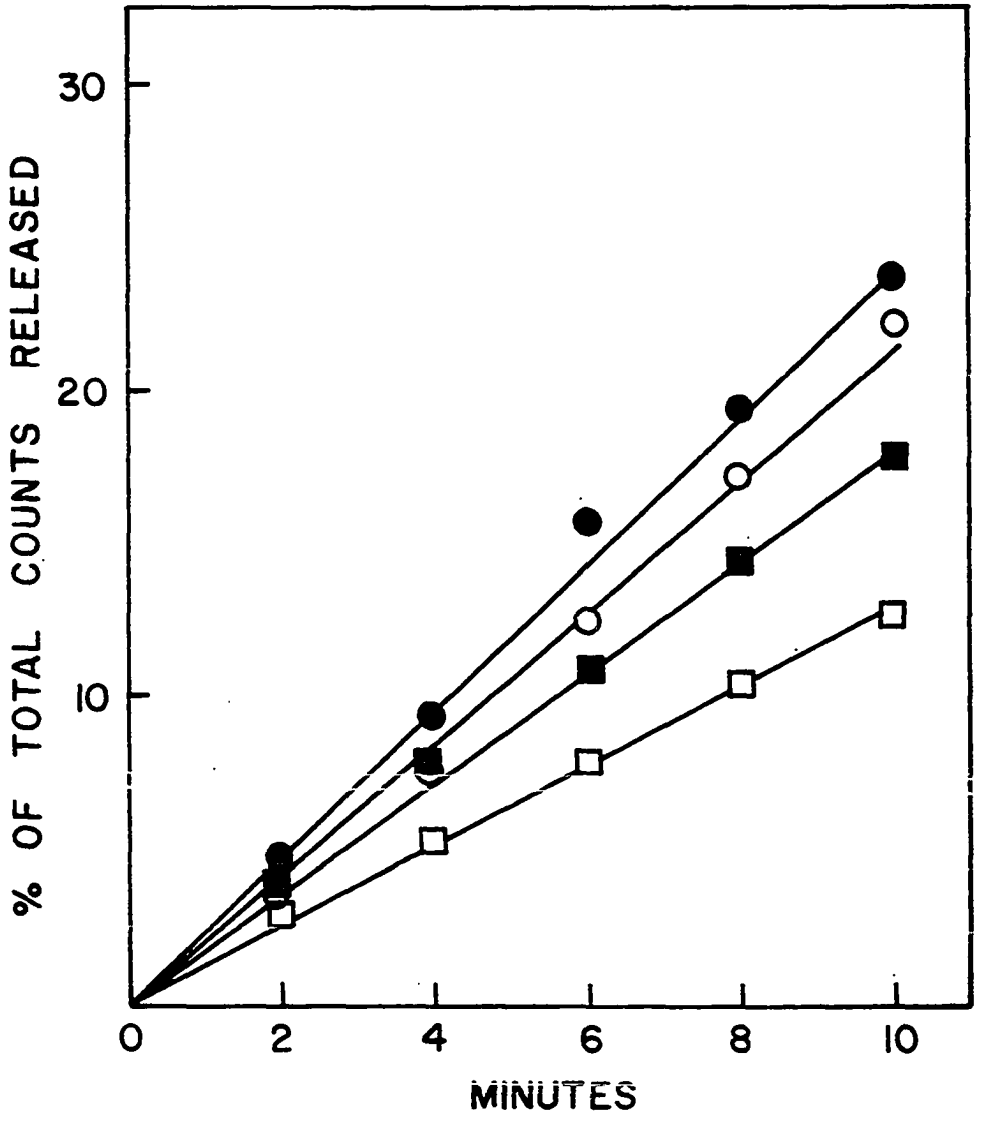
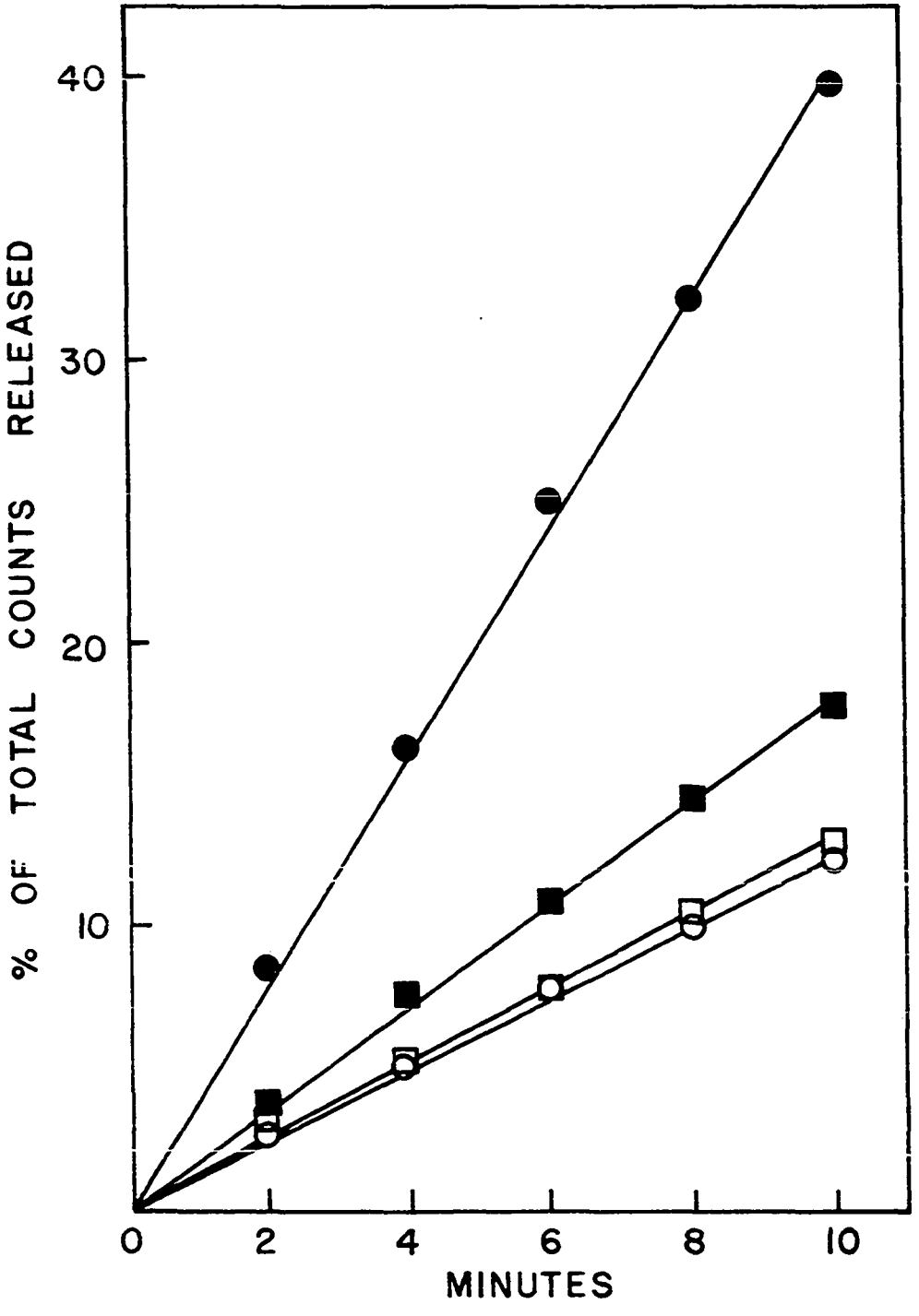

% OF TOTAL COUNTS RELEASED
30
20
10
0
2
4
6
8
10
MINUTES

Figure 19. The effect of glucose on the dephosphorylation of pyridoxal reconstituted phosphorylase a in the presence and absence of phosphite.

The reaction mixture at pH 7.5 consisted of pyridoxal reconstituted phosphorylase a (2.5 mg/ml), phosphorylase phosphatase (50 µg/ml), 0.025 M TRIS, 0.015 M 2-mercaptoethanol and no additions (□), 0.0075 M phosphite (■), 0.01 M glucose (○), 0.0075 M phosphite, 0.01 M glucose (●). At the indicated times, 200 µliters was removed and added to 200 µliters of 10% TCA and the (^{32}P) phosphate liberated was measured as described in the MATERIALS AND METHODS section.


40
30
20
10
% OF TOTAL COUNTS RELEASED
0
2
4
6
8
10
MINUTES

glucose-6-P and glucose on the dephosphorylation of pyridoxal reconstituted phosphorylase a was investigated. Figure 18 demonstrates that in the presence or absence of added phosphite, glucose-6-P stimulated the dephosphorylation of pyridoxal reconstituted phosphorylase a. Figure 18 also demonstrates that phosphite stimulated the dephosphorylation of pyridoxal reconstituted phosphorylase a. Figure 19 demonstrates that in the absence of phosphite, glucose had no effect on the dephosphorylation of pyridoxal reconstituted phosphorylase a. In the presence of phosphite, however, there was a marked stimulation of dephosphorylation. These results indicate that the 5'-phosphoryl group of pyridoxal-5'-P is not essential for expression of the stimulation of the dephosphorylation of pyridoxal reconstituted phosphorylase a when glucose-6-P is the activator. However, the lack of stimulation of dephosphorylation of pyridoxal reconstituted phosphorylase a by glucose, suggests that the 5'-phosphoryl group is required when glucose is the activator of dephosphorylation.

DISCUSSION

The experimental data demonstrates that pyridoxal reconstituted phosphorylase possesses the capability to catalyze both the synthesis and the degradation of glycogen. This catalysis occurs in spite of the absence of a covalently bound phosphoryl group at the 5'-position of the coenzyme. However, a non-covalently bound activator anion is required for this catalysis. Phosphate, phosphite and arsenate can be utilized as the activator anion. Phosphite, at concentrations that produced the maximal activation of pyridoxal reconstituted phosphorylase, had no effect on the enzymic activity of the native enzyme. Conversely, pyrophosphate, at concentrations that had no effect on the enzymic activity of the native enzyme, completely inhibited the enzymic activity of the pyridoxal reconstituted enzyme. The simplest explanation for these observations is that there is a site available in pyridoxal reconstituted phosphorylase that is not available in the native enzyme. Again, the simplest explanation is that phosphate, and phosphate analogs, bind at the site in pyridoxal reconstituted phosphorylase where the 5'-phosphoryl group of pyridoxal-5'-P would bind in the native enzyme. When this site is occupied by a suitable anion

activator, pyridoxal reconstituted phosphorylase manifests enzymic activity. There is precedence in the literature for this interpretation. Glucose-6-phosphate dehydrogenase can catalyze the oxidation of glucose, although poorly. This activity was stimulated 2-fold by phosphate, 3-fold by sulfate and 14-fold by bicarbonate. These anions were competitive inhibitors with respect to glucose-6-P. This was interpreted to mean that the anions bound at the site in the enzyme where the phosphoryl group of glucose-6-P would bind. The binding of the anion caused a conformational change in the protein that placed glucose in a favorable position with respect to NADP so that catalysis would be stimulated (Anderson et al., 1968; Anderson and Nordlie, 1968). Glucose phosphate isomerase, the enzyme that catalyzes the reversible conversion of glucose-6-P to fructose-6-P, does not convert fructose to glucose under normal conditions. This conversion of fructose to glucose does occur in the presence of arsenate. It was postulated that an ester-like complex was formed between fructose and arsenate that was similar to the natural substrate and allowed catalysis to occur (Lagunas, 1970). 2,3-diphosphoglycerate dependent phosphoglyceromutase catalyzes the conversion of 3-phosphoglycerate to

2-phosphoglycerate. In the absence of 2,3-diphosphoglycerate, no reaction occurred. If arsenate was added to the reaction mixture, enzymic activity was manifested. These data indicated that arsenate and 3-phosphoglycerate could substitute for the cofactor (2,3-diphosphoglycerate) and allow catalysis to occur (Grisolia and Cascales, 1966). Ray and Long (1976), in studies with phosphoglucomutase, demonstrated that the rate constant for phosphate transfer to xylose approached the value for the natural substrate, glucose-6-P, in the presence of phosphite. In the absence of added phosphite, the rate constant for phosphate transfer to xylose was 4 orders of magnitude smaller. The explanation invoked to explain this phenomenon was that phosphite bound at the site on the enzyme where the phosphoryl group of glucose-6-P would normally bind. The xylose, phosphite, enzyme complex was sufficiently similar to the complex between glucose-6-P and enzyme, that catalysis could occur. Finally, as previously mentioned, Wada and Morino (1964) reported that the apoprotein of glutamic-oxalacetic transaminase catalyzed the reversible conversion of pyridoxamine and oxaloacetate into L-aspartate and pyridoxal. The enzymes isolated from E. coli. and rabbit liver demonstrated a complete dependence on

phosphate for the expression of enzymic activity. Thus, in some instances, non-covalently bound phosphate or a phosphate analog and a non-phosphorylated analog of a phosphorylated substrate or cofactor could substitute for the phosphorylated substrate or cofactor and result in enzymic activity. This appears to be the situation for pyridoxal reconstituted phosphorylase.

The question must then be asked, why was the enzymic activity of pyridoxal reconstituted phosphorylase previously not observed? An unequivocal answer to this question cannot be given. However, all enzymic activity measurements were determined in the direction of glycogen synthesis. The failure to observe the enzymic activity under these conditions can probably be attributed to a combination of the following properties of pyridoxal reconstituted phosphorylase: a) the inherent lower enzymic activity of pyridoxal reconstituted phosphorylase relative to the native enzyme; b) the dependence of the enzymic activity of pyridoxal reconstituted phosphorylase on the presence of non-covalently bound phosphate or phosphate analog; c) the lag in the generation of product when only limiting amounts of phosphate are present in the assay; d) the non-linear dependence of enzymic

activity on enzyme concentration in the absence of added activator anion; e) the inclusion of L-cysteine in the reconstitution and assay buffers; f) the higher apparent binding constant for glucose-1-P of pyridoxal reconstituted phosphorylase relative to the native enzyme.

Further interpretation of the data requires a comparison of the properties of fluorophosphate and phosphite and the effects of these compounds on the properties of pyridoxal reconstituted phosphorylase. The Van der Walls radii of hydrogen and fluorine differ by less than 10% (Cotton and Wilkinson, 1966). The phosphorous-oxygen bond length in phosphite and fluorophosphate is identical. The oxygen-oxygen bond angle in phosphite is 110° while the same angle in fluorophosphate is 114°, a difference of less than 4% from the true tetrahedral angle of 109° found in phosphate (Handlovic, 1969; Berndt and Sylvester, 1972). Thus the overall tetrahedral structure of phosphite and fluorophosphate is nearly identical. The data in Table III show the pKa values for the second ionozation of phosphite and fluorophosphate to be 6.6 and 4.8 respectively. Therefore, the question of the ionization state of the activating anion must be addressed. At pH 6.8, fluorophosphate is 99% in the dianionic state, while

phosphite is 60% in the dianionic form. The similar structure of phosphite and fluorophosphate then suggest that if the monoanion was the activating species, the binding constant for fluorophosphate should be approximately 40-fold higher than that for phosphite. But if the dianion is the activating species, the binding constants should be similar. The latter situation was what was experimentally observed. Further evidence in support of the dianion as the activating species comes from the effect of phosphite and fluorophosphate on the resolution, by L-cysteine, of pyridoxal from the pyridoxal enzyme. If the anion form of fluorophosphate and phosphite protects against inactivation, similar protective effects would be seen at equivalent concentrations of the two anions. But, if the monoanionic species protected against inactivation by L-cysteine, a much higher concentration of fluorophosphate, relative to phosphite, would be required for equivalent protection. As can be seen in Table VIII, 10 mM phosphite and fluorophosphate provided equivalent protection against inactivation, thus supporting the hypothesis that it is the dianionic form of the anions that activates pyridoxal reconstituted phosphorylase. By analogy then, this suggests that the 5'-phosphoryl group of pyridoxal-5'-P in native phosphorylase is

in the dianionic form during catalysis. Support for this interpretation is provided by the nuclear magnetic resonance studies of Feldmann and Helmreich (1976) that showed that deprotonation of the monovalent form of the 5'-phosphoryl group of enzyme bound pyridoxal-5'-P occurred in the presence of AMP and arsenate.

Unfortunately, the data obtained with pyridoxal reconstituted phosphorylase cannot be utilized to draw a definitive conclusion regarding the ability of the 5'-phosphoryl group of pyridoxal-5'-P to function as a proton transfer agent in the native enzyme. But the very low pKa for fluorophosphate, coupled with its ability to activate pyridoxal reconstituted phosphorylase suggest that fluorophosphate does not accept a proton during the reaction catalyzed by pyridoxal reconstituted phosphorylase, and by analogy, a similar role in native phosphorylase must be considered unlikely.

The lack of effect on native phosphorylase at concentrations of pyrophosphate that completely inhibited the enzymic activity of pyridoxal reconstituted phosphorylase suggested that pyrophosphate bound at the site in pyridoxal reconstituted phosphorylase where the 5'-phosphoryl group of

pyridoxal-5'-P bound in the native enzyme. Since only one molecule of pyrophosphate bound per molecule of pyridoxal reconstituted phosphorylase, the competitive kinetics demonstrated between pyrophosphate and both the activator anion and substrate, suggested that these two sites were in close proximity in pyridoxal reconstituted phosphorylase. By analogy then, this suggests that the glucose-1-P binding site and the binding site for the 5'-phosphoryl group of pyridoxal-5'-P are also in close proximity in the native enzyme. The fact that the kinetically derived binding constants and the binding constant calculated from radioactive pyrophosphate binding do not agree, does not contradict this interpretation. Purich and Fromm (1972) demonstrated that competitive kinetics were seen when one molecule of inhibitor blocks both substrate binding sites in a random mechanism. The apparent inhibition constants are complex and contain a term for the other substrate, as well as, other rate constants. Since experimental conditions varied, the apparent inhibitor constants would not be expected to be identical. It must be stated, however, that the kinetic experiments with pyridoxal reconstituted phosphorylase cannot discriminate between the proposed proximity of the two sites

or the binding of pyrophosphate at another site with a concomitant change in the conformation of the protein that effects the binding of both the substrate and the activator anion. Only X-ray crystallographic studies can verify the proximity of the substrate and coenzyme in native phosphorylase. Unfortunately, the position of pyridoxal-5'-P has not yet been found in the native enzyme (Fletterick et al., 1976).

The role of pyridoxal-5'-P in the enzymatically catalyzed interconversion of phosphorylase a and b is an area that has received only scant attention. A relationship between the phosphorylatable serine and pyridoxal-5'-P was first demonstrated by the observation that under the conditions where pyridoxal-5'-P was resolved from phosphorylase b, the coenzyme could not be removed from phosphorylase a (Shaltiel et al., 1966). A more subtle effect of pyridoxal-5'-P on the interconversion reactions was demonstrated by Graves et al., (1975). In this study it was demonstrated that glucose-6-P, a substrate directed activator of the phosphorylase phosphatase catalyzed dephosphorylation of phosphorylase a, (Martensen et al., 1973) was without effect on the dephosphorylation of apophosphorylase a. The effects

of glucose-6-P and glucose on the phosphorylase phosphatase catalyzed dephosphorylation of pyridoxal reconstituted phosphorylase *a* must be considered against this background. Apparently the 5'-phosphoryl group of pyridoxal-5'-P is not required for the stimulatory effect of glucose-6-P on dephosphorylation. This is clear from the observation that glucose-6-P stimulated the dephosphorylation of pyridoxal reconstituted phosphorylase *a*. Apparently the basic ring structure of pyridoxal supplies sufficient conformational integrity to the protein so that glucose-6-P can function as an activator. Martensen *et al.*, (1973) demonstrated that glucose stimulated the dephosphorylation of phosphorylase *a* by binding to the substrate, phosphorylase *a*, and not to the catalyst, phosphorylase phosphatase. The lack of stimulatory effect of glucose on the dephosphorylastion of pyridoxal reconstituted phosphorylase *a* indicates that the 5'-phosphoryl group of pyridoxal-5'-P is required in order for glucose to stimulate the phosphorylase phosphatase catalyzed reaction. This requirement for the 5'-phosphoryl group of the coenzyme may have important physiological significance. An increase in the concentration of glucose has been proposed as the trigger mechanism that converts the

liver from a glycogen degrading organ, to a glycogen storage organ (Soskin, 1940). The rate of glycogen degradation is dependent on the concentration of liver phosphorylase a (Hers et al., 1970)., while the rate of glycogen synthesis is dependent on the concentration of active liver glycogen synthase (DeWulf and Hers, 1967). The link between degradation and synthesis of glycogen is thought to be the inhibition of glycogen synthase phosphatase by liver phosphorylase a (Stalmans et al., 1971). When the concentration of phosphorylase a falls below a certain level, the inhibition of glycogen synthase phosphatase is released and glycogen synthase is converted from the inactive to the active form (Stalmans et al., 1974a). Glucose has been demonstrated to stimulate phosphorylase phosphatase catalyzed dephosphorylation of liver phosphorylase a (Stalmans et al., 1970). This has led to the proposal, that in biochemical terms, glucose acts as a trigger mechanism by stimulation of phosphatase activity, thus disrupting the balance between phosphorylase kinase and phosphorylase phosphatase activities. The subsequent release of glycogen synthase phosphatase inhibition converts glycogen synthase from the inactive to active form and allows glycogen synthesis to occur (Stalmans

et al., 1974b). It is of course, dangerous to draw conclusions about the liver enzyme from observations on the muscle enzyme. In this case it appears to be justified since glucose stimulated the dephosphorylation of liver and muscle phosphorylase a even though under physiological conditions, there is no free glucose in muscle (Kipnis et al., 1959). The lack of stimulatory effect of glucose on the dephosphorylation of pyridoxal reconstituted phosphorylase a suggests that in the absence of the 5'-phosphoryl group of pyridoxal-5'P, the glucose stimulation of liver phosphorylase phosphatase cannot occur and this would effect the ability of the liver to switch back and forth from synthesis to degradation of glycogen. Thus a heretofore unobserved role for pyridoxal-5'-P, and in particular, the role of the 5'-phosphoryl group in the regulation of interconversion of phosphorylase a to phosphorylase b is indicated.

One of the objectives of this investigation was to determine if pyridoxal reconstituted phosphorylase could be utilized as a model system to study the role of pyridoxal-5'-P in native phosphorylase. A good model system should have many properties in common with the original system under study. It must also be sufficiently different from the

original system so that effects on the model system can be interpreted with respect to the original system. Pyridoxal reconstituted phosphorylase retained the cooperative binding of AMP and the heterotropic interactions between AMP and glucose-1-P demonstrated by the native enzyme. (Kastenschmidt et al., 1968). More importantly, this study has demonstrated that pyridoxal reconstituted phosphorylase does exhibit enzymic activity. However, the dependence of the enzymic activity on the presence of an anion activator is markedly different from the native enzyme. If it is accepted that the anion activator binds to the site in pyridoxal reconstituted phosphorylase where the 5'-phosphoryl group of pyridoxal-5'-P binds in the native enzyme, then the potential exists for the utilization of pyridoxal reconstituted phosphorylase to serve as model system to probe the role of the 5'-phosphoryl group in the native enzyme. However, it remains to be seen if this interpretation is correct and if suitable experiments can be designed to discriminate between a catalytic role or a conformational role for the coenzyme.

The phosphorylase phosphatase catalyzed dephosphorylation of phosphorylase a is stimulated by both glucose-6-P and glucose. The dephosphorylation of pyridoxal

reconstituted phosphorylase is stimulated by glucose-6-P but not by glucose. The similarity and difference required of a model system is satisfied in this instance and therefore, pyridoxal reconstituted phosphorylase *a* appears to be a suitable model system to study the role of the 5'-phosphoryl group of pyridoxal-5'-P in the phosphorylase phosphatase catalyzed conversion of phosphorylase *a* to phosphorylase *b*.

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