Effect of Phosphate Analogues on the Activity of Pyridoxal Reconstituted Glycogen Phosphorylase†

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ABSTRACT: Pyridoxal reconstituted phosphorylase was demonstrated to possess enzymatic activity in the synthesis and degradation of glycogen. The activity was dependent on the presence of a noncovalently bound anion activator. In the direction of glycogen synthesis in the absence of added anion activator, a lag period was observed in production formation. The lag was related to the generation of phosphate, the activator anion, from glucose 1-phosphate. Inorganic phosphite eliminated the lag and resulted in an assay in which the rate of product formation was linear with respect to both time and enzyme concentration. At saturating substrate concentrations, the pyridoxal enzyme in the presence of phosphite was estimated to have 19% of the maximal velocity of the native enzyme. Various other phosphate analogues were tested with pyridoxal reconstituted phosphorylase. Fluorophosphate stimulated the enzymatic activity of pyridoxal reconstituted phosphorylase almost as well as phosphite, while pyrophosphate was found to be a potent competitive inhibitor of phosphite activation and glucose-1-P binding. One mole of pyrophosphate was bound per mole of pyridoxal reconstituted phosphorylase *b* monomer with a K_d of 8.5×10^{-5} M. Pyrophosphate, fluorophosphate, phosphite, and thiophosphate inhibited the resolution of the cofactor from the enzyme. From these experiments, it was suggested that the phosphate analogues bound at a site in the pyridoxal enzyme where the 5′-phosphoryl group of pyridoxal 5′-phosphate binds in the native enzyme, and that the activator anion is in the dianionic state during catalysis.

Pyridoxal 5′-phosphate was demonstrated by Baranowski et al. (1957) to be the cofactor present in phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1). Removal of the cofactor from phosphorylase *b* resulted in the loss of enzymatic activity. After incubation of the apoprotein with pyridoxal-5′-P,[1] both the enzymatic activity and the structural properties were recovered (Illingworth et al., 1958). The subsequent discovery of Fischer et al. (1958) that an imine double bond between pyridoxal-5′-P and the ε-amino group of a lysine residue in phosphorylase could be reduced by sodium borohydride with little loss of enzymatic activity placed phosphorylase in a unique position among pyridoxal-5′-P requiring enzymes. All others functioned by transamination, and reduction with sodium borohydride destroyed all enzymatic activity (Snell and DiMari, 1970). This then posed a question concerning the role of pyridoxal-5′-P in phosphorylase. Did the cofactor participate directly in catalysis by some new mechanism, or was it simply acting as a biological cement to hold the protein in the correct conformation for catalysis to occur in another portion of the molecule?

Several different experimental techniques clearly demonstrated a conformational role for pyridoxal-5′-P in phosphorylase. It has been found that apophosphorylases *b* and *a* are less stable than their holoenzymes, and their quaternary structures are significantly different from the native enzyme forms (Hedrick et al., 1966; Shaltiel et al., 1969b). Weisshaar and Palm (1972) were able to show differences between the native and apoprotein with respect to tritium–hydrogen exchange. Graves et al. (1975) found that removal of the coenzyme influenced enzymatic interconversion. Substrate directed effectors which influenced reactions with the native enzyme had little or no effect on the apoenzyme. Removal of the cofactor, however, 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 probed with optical activity techniques (Johnson and Graves, 1966; Hedrick, 1966).

In contrast to the evidence for a conformational role for the cofactor, definitive evidence for a catalytic function has been lacking, but it should be pointed out that all α-glucan phosphorylases studied contain pyridoxal 5′-phosphate. Furthermore, Lerch and Fischer (1975) compared the primary sequence of the phosphorylation site and pyridoxal-5′-P site in rabbit and yeast phosphorylase. The primary sequence of the pyridoxal-5′-P site was indeed highly conserved, while the sequence of the phosphorylation site was much less so. This was interpreted to mean that a catalytic role for the cofactor is likely. The search for a catalytic role for pyridoxal-5′-P in phosphorylase has been pursued mainly through the synthesis of analogues of the native cofactor and the testing of the ability of these analogues to bind to apophosphorylase and to restore catalytic activity. A list containing most of the analogues tested with apophosphorylase can be found in articles by Graves and Wang (1972) and Shaltiel et al. (1969b). From these studies it was concluded that the functional groups at the 2, 3, 4, and 6 positions of pyridoxal-5′-P were not required for catalysis, while the involvement of the ring nitrogen remained unclear. With the notable exception of 5′-deoxypyridoxalmethylenephosphonic acid (Fischer et al., 1970; Vidgoff et al., 1974) and 5′-deoxypyridoxalphosphonic acid (Graves and Wang, 1972), all other modifications of the cofactor at the 5′ position have resulted in proteins reported to be catalytically inactive. Thus, it was concluded that the 5′ position was critical for activity, and on the basis of studies with pyridoxal 5′-phosphate monomethyl ester reconstituted phosphorylase, a proton transfer

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[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; pyridoxal-5′-P, pyridoxal 5′-phosphate; glucose-1-P, glucose 1-phosphate; glucose 6-P, glucose 6-phosphate.

mechanism was proposed as the function for the 5′-phosphoryl group (Pfeuffer et al., 1972a; Ehrlich et al., 1971; Weisshaar and Palm, 1972). This concept has been supported by recent nuclear magnetic resonance spectroscopic measurements (Feldmann and Helmreich, 1976).

Pyridoxal reconstituted phosphorylase appeared to be a good system to test possible roles for the 5′-phosphoryl group of pyridoxal-5′-P in the native protein. Studies by Kastenschmidt et al. (1968) with apophosphorylase *b*, pyridoxal, and 5′-deoxypyridoxal reconstituted phosphorylase *b* suggested that the inactivity of these enzyme forms could not be explained by the inability to bind substrates or nucleotide activator. Inactivity of pyridoxal or 5′-deoxypyridoxal enzyme forms must be related to the absence of the 5′-phosphoryl group of the cofactor. In this paper, we report that pyridoxal reconstituted phosphorylase possesses enzymatic activity in the presence of noncovalently bound phosphate, phosphite, fluorophosphate, and arsenate. We have utilized pyridoxal reconstituted phosphorylase as a model system to investigate the role of the 5′-phosphoryl group in the native protein and the results suggest that the 5′-phosphoryl group is in the dianionic form during catalysis, and that the binding sites for pyridoxal-5′-P and the substrate, glucose-1-P, are in close proximity.

Materials and Methods

Rabbit skeletal muscle phosphorylase *b* was prepared according to Fischer and Krebs (1962). Apophosphorylase *b* was prepared by the method of Graves et al. (1975). The apoenzyme routinely retained less than 0.1% of the activity of the native enzyme. Apotryptophanase was purchased from Sigma Chemical Co. Purified disodium fluorophosphate and [^{14}C]-glycogen were generous gifts of Ms. Betty Yan and Dr. James Thomas, respectively. Shellfish glycogen was purified by the method of Anderson and Graves (1973). All other materials were the highest quality commercially available.

Pyridoxal reconstituted phosphorylase *b* was prepared by incubation of apophosphorylase *b* (3–4 mg/mL in 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, pH 6.8) with a 50-fold excess of pyridoxal at 37 °C for 35 min. 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 several changes of this buffer, in the cold. Crystallization occurred after the addition of AMP and magnesium chloride to final concentrations of 0.001 and 0.01 M, respectively. The crystalline enzyme was collected by centrifugation and dissolved in 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol (pH 6.8) and treated with acid-washed Norit A to remove bound nucleotide. The Norit A treated enzyme was stored at a concentration greater than 30 mg/mL in the cold. Under these conditions, the enzyme retained at least 90% of the initial activity after 4 days. When the enzyme activity fell below 90% of the original, the enzyme solution was discarded. Pyridoxal reconstituted phosphorylase *a* was prepared from pyridoxal reconstituted phosphorylase *b*, by the method of Krebs (1966) employing phosphorylase kinase (Brostrom et al., 1971).

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 from [^{14}C]glucose-1-P, with the filter paper assay of Thomas et al. (1968). When the activity was measured in the presence of phosphate or a phosphate analogue, the concentrations are 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).

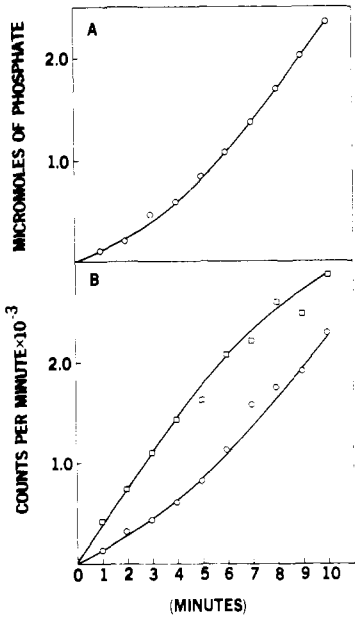

FIGURE 1: (A) Enzymatic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen synthesis. The assay mixture contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.05 M glucose-1-P, 1% glycogen, and enzyme (150 μg/mL) (pH 6.8). The reaction was initiated by the addition of enzyme, and at the indicated times 400 μL was removed, and the phosphate liberated was measured as described under Materials and Methods. (B) Enzymatic activity of pyridoxal reconstituted phosphorylase in the presence and absence of added inorganic 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), [^{14}C]glucose-1-P (0.1 μCi) (pH 6.8), and added phosphate 0.0075 M (□), or no added phosphate (○). At the indicated times, 20 μL was removed, and the radioactivity incorporated into glycogen was determined according to Thomas et al. (1968).

Phosphorylase activity in the direction of glycogen degradation was measured according to the method of Helmreich and Cori (1964a). Glycogen degradation in the presence of arsenate was measured essentially by the method of Helmreich and Cori (1964b). For comparisons of the activity of pyridoxal reconstituted phosphorylase and native enzymes, apophosphorylase was reconstituted with either a 50-fold excess of pyridoxal or a 5-fold excess of pyridoxal-5′-P. Protein concentration was measured spectrophotometrically at 280 nm (Kastenschmidt et al., 1968). Tryptophanase activity was measured according to Gunsalus et al. (1955).

Pyrophosphate binding to pyridoxal reconstituted phosphorylase was done by ultrafiltration by the procedure of Paulus (1969).

Results

Enzymatic Activity of Pyridoxal Reconstituted Phosphorylase in the Direction of Glycogen Synthesis. Most studies of phosphorylase utilize enzymatic activity measurements in the direction of glycogen synthesis. To test for the enzymatic activity of pyridoxal reconstituted phosphorylase, high substrate and enzyme concentrations were employed because the enzymatic activity, if present, must have been much less than that of the native protein, or it would have been detected by previous investigators (Illingworth et al., 1958; Shaltiel et al., 1969b). Figure 1A demonstrates the enzymatic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen synthesis. In the linear portion of the time course, the

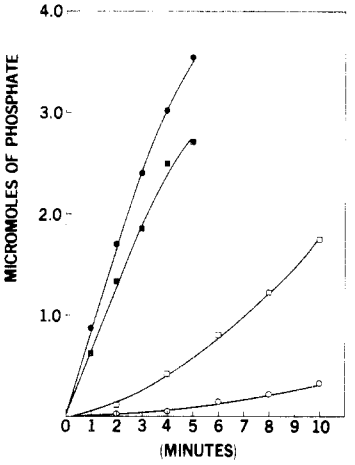

FIGURE 2: The enzymatic activity of native and pyridoxal reconstituted phosphorylase in the presence and absence of barium. The reaction mixtures at 30 °C and pH 6.8 contained either native phosphorylase (25 μg/mL) (closed symbols) or pyridoxal reconstituted phosphorylase (233 μg/mL) (open symbols) and 0.075 M glucose-1-P, 0.001 M AMP, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 1% glycogen, and no barium chloride (■,□) or 0.05 M barium chloride (●,○). At the indicated times, 400 μL was removed, and the amount of phosphate liberated was measured according to Fiske and Subbarow (1925), with the following additional step. The precipitated barium sulfate produced by the acidification with sulfuric acid was removed by centrifugation, and the remaining clear liquid was used to determine the amount of phosphate liberated.

TABLE I: Effect of Phosphate Analogues on the Activity of Native and Pyridoxal Reconstituted Phosphorylase

Anion added	pK_{a_2}	Specific activity[a] 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]	0.0	61

[a] Micromoles of phosphate per minute per milligram of protein; the reaction mixture contained pyridoxal reconstituted phosphorylase (54 μg), 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.075 M glucose-1-P, 1% glycogen, and the indicated phosphate analogues at a concentration of 0.01 M. After incubation for 5 min at 30 °C, the phosphate liberated was measured according to Fiske and Subbarow (1925). For comparison purposes, the activity resulting from the phosphate contamination in the substrate and buffer was defined as no additions, and no correction was made for the nonlinear time dependence of the product formation. [b] Conditions were identical with *a* except that the reaction mixture contained native phosphorylase (4.9 μg). [c] Weast (1974). [d] Van Wazer (1958).

activity represents 4% of the activity of the native enzyme. The most striking feature, other than the enzymatic activity, was the lag in the generation of product, inorganic phosphate.

The possibility that the observed activity was the result of contaminating amounts of pyridoxal-5′-P in the pyridoxal or in the apo preparation had to be eliminated. 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 reactivate apophosphorylase. When similar pretreatment experiments were performed with apophosphorylase and a 50-fold excess of pyridoxal, no change in the specific activity was observed when apophosphorylase was reconstituted with either pretreated or nonpretreated pyridoxal. Crystallized pyridoxal reconstituted phosphorylase and native phosphorylase were treated with perchloric acid to remove the cofactor (Baranowski et al., 1957). By using apotryptophanase (Gunsalus et al., 1955), no pyridoxal-5′-P could be detected in the material liberated from the pyridoxal reconstituted enzyme, while the material liberated from the native enzyme contained the expected amount of pyridoxal-5′-P. In control experiments, a 0.5% contamination of pyridoxal by pyridoxal-5′-P could easily be detected. Finally, limited amounts of pyridoxal-5′-P were added to the apoenzyme to yield 8% of the activity of the native enzyme. When this material was assayed for enzymatic activity, there was no lag in the generation of phosphate. These experiments demonstrate that the enzymatic activity measured for pyridoxal reconstituted phosphorylase could not have come from contaminating amounts of pyridoxal-5′-P in either the pyridoxal or the apoenzyme preparation.

The lag in the product formation was next examined. Generation of an activator during the reaction would lead to the observed nonlinearity in the product formation. The only substance increasing in concentration during the reaction was inorganic phosphate. Therefore, the activity in the presence of added phosphate was determined. However, 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 of added phosphate was linear, while the activity in the absence of added phosphate showed a lag (Figure 1B).

Both the substrate and the buffer β-glycerophosphate contained small amounts of phosphate, and it therefore seems reasonable to conclude that contaminating amounts of phosphate were responsible for the initial activity. This activity in turn generated phosphate from glucose-1-P, which generated more activity until there was sufficient phosphate to saturate the enzyme. At this time, linear progress curves resulted. The obvious test for this hypothesis was the removal of the initial phosphate. Inclusion of barium in the assay mixture resulted in an 85% loss of activity of pyridoxal reconstituted phosphorylase, while there was an 18% stimulation of the activity of the native enzyme (Figure 2). Thus it was concluded that pyridoxal reconstituted phosphorylase possessed enzymatic activity, but phosphate was required for activity.

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 activity of this preincubated enzyme was compared with the activity of an equivalent amount of pyridoxal reconstituted phosphorylase that had not been preincubated. 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 pronounced stimulation of the enzymatic activity relative to the sample that had not been preincubated. Both the preincubated and nonpreincubated enzymes manifested a lag in product formation, but there was no difference in the activity of the preincubated sample relative to the enzyme that had not been preincubated in the presence of substrates. This eliminated the possibility that the activation resulted from a covalent bond formation between the enzyme or cofactor and the phosphate required for activity.

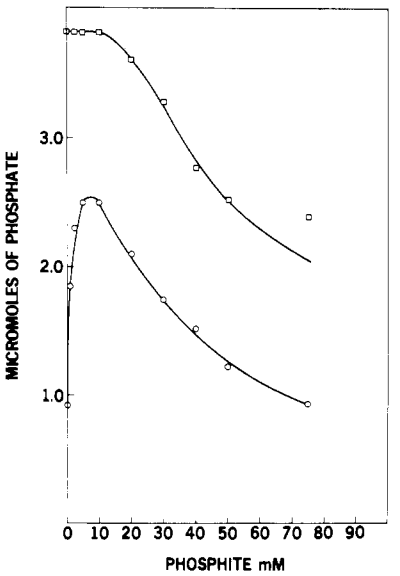

FIGURE 3: The enzymatic activity of native and pyridoxal reconstituted phosphorylase as a function of the phosphite concentration. The reaction mixture at pH 6.8 in a total volume of 400 μL contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, and the indicated amounts of phosphite and native phosphorylase (8.9 μg) (□), or pyridoxal reconstituted phosphorylase (60 μg) (○). After a 5-min incubation time at 30 °C, the liberated phosphate was measured according to Fiske and Subbarow (1925).

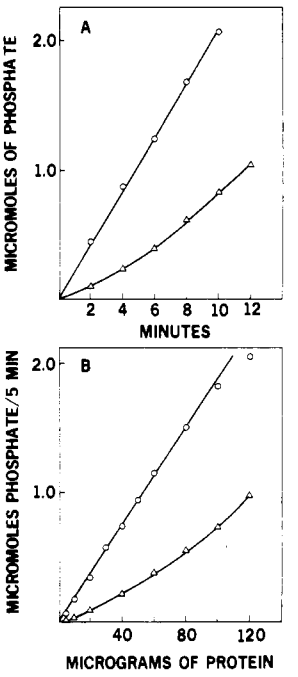

FIGURE 4: (A) Enzymatic activity of pyridoxal reconstituted phosphorylase in the presence and absence of added phosphite. The reaction mixture contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 1% glycogen, 0.001 M AMP, 0.016 M glucose-1-P, enzyme (150 μg/mL), and phosphite, 0.0075 M (○), or no phosphite (△). At the indicated times 400 μL was removed, and the liberated phosphate was measured according to Fiske and Subbarow (1925). (B) Protein concentration dependence of the enzymic activity of pyridoxal reconstituted phosphorylase in the presence and absence of added phosphite. The reaction mixture in a total volume of 400 μL at pH 6.8 contained β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, and phosphite, 0.0075 M (○), or no phosphite (△), and the indicated amounts of enzyme. After a 5-min incubation at 30 °C, the liberated phosphate was measured according to Fiske and Subbarow (1925).

Activity of Pyridoxal Reconstituted Phosphorylase in the Presence of Phosphate Analogues. The enzymatic activity of pyridoxal reconstituted phosphorylase in the presence of noncovalently bound phosphate prompted a search for phosphate analogues that might promote enzymatic activity (Table I). Inorganic phosphite, fluorophosphate, thiophosphate, and pyrophosphate were used because of their structural features and pK_a values. Nitrate was used because it has been proposed as a transition state analogue of phosphate (Milner-White and Watts, 1971). Sulfate was examined because it has a tetrahedral structure similar to phosphate while bicarbonate possessed an ionization constant near neutrality. Phosphite and fluorophosphate stimulated the enzymatic activity of pyridoxal reconstituted phosphorylase above the value that could be obtained with the control. Activity of the control is 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 activity of the pyridoxal enzyme. These compounds at the concentrations employed had no significant effect on the activity of native phosphorylase *b*.

Effect of Phosphite on the Enzymatic Activity of Pyridoxal Reconstituted Phosphorylase. The effect of phosphite on the activity of native and pyridoxal reconstituted phosphorylase is shown in Figure 3. Above 1.5×10^{-2} M phosphite, both native and pyridoxal reconstituted phosphorylase were inhibited. Subsequent experiments demonstrated that, at these phosphite concentrations, competitive kinetics were found between phosphite and the substrate, glucose-1-P, for native phosphorylase *b*. The time course for the activity of pyridoxal reconstituted phosphorylase in the presence and absence of 7.5×10^{-3} M phosphite is shown in Figure 4A. There was no lag in the generation of phosphate in the presence of phosphite, while the lag was present in the absence of phosphite. The activity in the presence of phosphite under these conditions represented 8% of the activity of the native enzyme. Figure 4B shows the protein concentration dependence of the enzymatic activity in the presence and absence of 7.5×10^{-3} M phosphite. The 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-μg range where the activity of native phosphorylase would normally be measured.

There was a remote possibility that phosphite was being oxidized to phosphate during the assay measurements. This conversion would then mimic enzymic activity when the colorimetric assay for phosphate release was utilized. When the activity of pyridoxal reconstituted phosphorylase was measured by the incorporation of [^{14}C]glucose into glycogen from glucose-1-P, the time course in the presence of phosphite was linear, but the time course in the absence of phosphite demonstrated a lag in radioactive label incorporation. The incorporation of radioactivity into glycogen exactly paralleled the release of phosphate from glucose-1-P and thus eliminated the possibility that phosphite was being oxidized to phosphate.

Because linear progress curves were seen with pyridoxal reconstituted phosphorylase in the presence of phosphite, kinetic studies of the enzyme were undertaken. Table II shows the apparent kinetic constants calculated for pyridoxal reconstituted phosphorylase and compares them with the literature values for the native enzyme. One significant change was the apparent fourfold higher apparent K_m for glucose-1-P exhibited by the pyridoxal reconstituted protein. Other K_m values were changed little. From our double reciprocal plots of pyridoxal reconstituted and native phosphorylase, it was

TABLE II: Properties of Pyridoxal Reconstituted and Native Phosphorylase *b*.

Parameters	Pyridoxal reconstituted	Native
K_m phosphite	9×10^{-4} M[a]	
K_m AMP	2.5×10^{-5} M[b]	$3\text{-}8 \times 10^{-5}$ M[g]
K_m glucose-1-P	2.8×10^{-2} M[c]	7×10^{-3} M[h]
K_m glycogen	0.01%[d]	0.02%[i]
Cooperative binding of AMP	Yes	Yes[g]
Sp act.	3.4[e] (8.7%)	39[j]
Max velocity	12[f] (19%)	65[k]

[a] Measured in the presence of 0.075 M glucose-1-P, 1% glycogen, 10^{-3} M AMP, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol (pH 6.8), and concentrations of phosphite between 0.00075 and 0.0075 M. [b] Measured in the presence of 0.016 M glucose-1-P, 1% glycogen, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.0075 M phosphite (pH 6.8), and concentrations of AMP between 10^{-5} and 10^{-4} M. [c] Measured in the presence of 10^{-3} M AMP, 1% glycogen, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.0075 M phosphite (pH 6.8), and concentrations of glucose-1-P between 0.0075 and 0.075 M. [d] Measured utilizing the coupled assay of Helmreich and Cori (1964a). [e] Measured in the presence of 0.016 M glucose-1-P, 1% glycogen, 10^{-3} M AMP, 0.0075 M phosphite, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, pH 6.8. [f] Extrapolated from plots of $(\text{velocity})^{-1}$ vs. $(\text{glucose-1-P})^{-1}$ from *c*. [g] Kastenschmidt et al. (1968); Helmreich and Cori (1964a). [h] Engers et al. (1969). [i] Anderson et al. (1973). [j] Measured according to *e* in the absence of phosphite. [k] Extrapolated from $(\text{velocity})^{-1}$ vs. $(\text{glucose-1-P})^{-1}$ under the conditions of *c* but in the absence of phosphite.

estimated that the pyridoxal enzyme has 20% of the activity of native phosphorylase *b*.

Since phosphite could activate pyridoxal reconstituted phosphorylase, there was also the possibility that phosphite could serve as a substrate in the place of inorganic phosphate in the degradation. 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 employed in the coupled assay system for phosphorylase activity. Therefore, enzymatic cleavage of radioactive glycogen by phosphite was attempted. However, after incubation of [^{14}C]glycogen with either native or pyridoxal reconstituted phosphorylase in the presence of phosphite and AMP, no radioactive phosphorus-containing compound could be detected. These data indicate that phosphite does not serve as a substrate for either native or pyridoxal reconstituted phosphorylase in the direction of glycogen degradation.

Enzymatic Activity of Pyridoxal Reconstituted Phosphorylase in the Direction of Glycogen Degradation. Since native phosphorylase can catalyze effectively both the synthesis and degradation of glycogen, it was important to establish this capacity for the pyridoxal reconstituted system. Also, using an assay based on degradation allowed us to test the effect of arsenate on the activity of the pyridoxal enzyme. When the enzymatic activity was measured at a phosphate concentration of 0.02 M, pyridoxal reconstituted phosphorylase had an activity 6% of the native enzyme. A slight modification of the method of Helmreich and Cori (1964b) was utilized to establish that arsenate could function with pyridoxal reconstituted phosphorylase in the degradation of glycogen (Figure 5). In this case, 0.05 M maleate was used as the buffer instead of β-glycerophosphate. When the activity was measured in the presence of 0.1 M arsenate, the pyridoxal reconstituted enzyme exhibited 3% of the activity of the native enzyme. The apoenzyme from which the pyridoxal reconstituted enzyme was

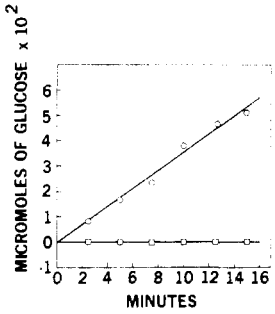

FIGURE 5: Enzymatic activity of pyridoxal reconstituted phosphorylase in the direction of glycogen degradation. The reaction mixture at pH 6.2 and 30 °C contained pyridoxal reconstituted phosphorylase, 500 μg/mL (○), or apophosphorylase, 500 μg/mL (□), and 0.05 M maleate, 0.1 M arsenate, 0.001 M AMP, and 2% glycogen. At the indicated times, 200 μL was removed, and the liberated glucose was determined according to Helmreich and Cori (1964b).

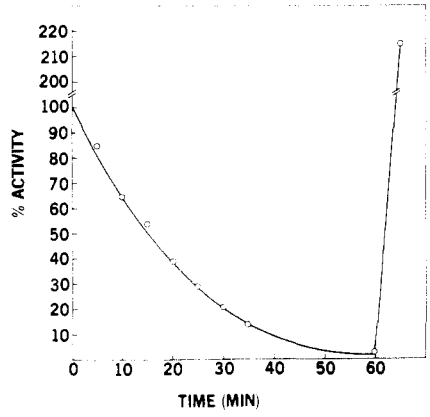

FIGURE 6: Enzymatic activity of pyridoxal reconstituted phosphorylase in the presence of L-cysteine. The reaction mixture at 30 °C contained 0.1 M β-glycerophosphate, 0.1 M L-cysteine, and enzyme (3 mg/mL) (pH 6.8) in a total volume of 2.0 mL. At the indicated times, 100 μL was removed and added to 900 μL of ice-cold 0.04 M β-glycerophosphate–0.03 M 2-mercaptoethanol (pH 6.8), and the remaining enzymatic activity was measured in the presence of 0.0075 M phosphite as described under Materials and Methods. After 60 min, 100 μL of pyridoxal-5′-P was added, and after a 5-min incubation, the enzymic activity of a 100-μL aliquot was measured.

prepared possessed no enzymatic activity. Thus, it may be concluded that pyridoxal reconstituted phosphorylase can catalyze both the synthesis and degradation of glycogen, and that a phosphorus-containing anion is not essential for the activity.

The Effect of L-Cysteine on the Activity of Pyridoxal Reconstituted Phosphorylase. Pfeuffer et al. (1972b) reported a 25% loss of cofactor from pyridoxal reconstituted phosphorylase *b* in 4 h at 0 °C in the presence of L-cysteine and the absence of the normal deforming buffers required for the resolution of the cofactor from native phosphorylase. The buffer employed by Illingworth et al. (1958) for reconstitution of apophosphorylase *a* with pyridoxal contained L-cysteine, and this might have been a contributing factor to their report of no activity for the reconstituted enzyme. We have repeated the reconstitution of apophosphorylase *a* with pyridoxal and have found that, in the presence of 1×10^{-2} M L-cysteine, the concentration used by Illingworth et al. (1958), the enzymatic activity was only 25% of that obtained when 2-mercaptoethanol was used as the thiol reagent. Figure 6 shows the time-dependent inactivation of pyridoxal reconstituted phosphorylase in the presence of L-cysteine. The inactivation was shown to be the result of resolution of pyridoxal from the protein and not

TABLE III: The Effect of Phosphate Analogues on the Inactivation of Pyridoxal Reconstituted Phosphorylase by L-Cysteine.[a]

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

[a] The reaction mixture at pH 6.8 and 30 °C contained pyridoxal reconstituted phosphorylase *b* (2.7 mg/mL), 0.1 M β-glycerophosphate, 0.1 M L-cysteine, and the indicated phosphate analogues at 0.01 M. After a 20-min incubation, an aliquot was removed and the remaining enzymic activity was measured as described in Materials and Methods. The presence of AMP and glycogen in the assay mixture ensured that no further inactivation occurred during the activity measurements.

denaturation since addition of pyridoxal-5′-P resulted in a rapid return and increase in enzymatic activity. Activities greater than 100% are expected because pyridoxal-5′-P reconstituted phosphorylase has a higher activity than pyridoxal phosphorylase. Hydroxylamine, but not other aldehyde-trapping reagents, could rapidly inactivate pyridoxal reconstituted phosphorylase *b*. Sulfhydryl reagents including D-cysteine and 2-mercaptoethanol were ineffective as resolving agents. Glycogen, AMP, and glucose-6-P, but not glucose-1-P, had pronounced inhibitory effects on resolution. It should be stressed that no "deforming buffer" was required for this resolution. Both native phosphorylase *b* and pyridoxal reconstituted phosphorylase *a* were completely stable under these conditions. These results are very similar to those reported for the native enzyme, with the important exception that no deforming buffer was required (Shaltiel et al., 1966, 1969a; Hedrick et al., 1969).

Effect of Phosphate Analogues on the Resolution of Pyridoxal from Pyridoxal Reconstituted Phosphorylase. The inability of L-cysteine to resolve the cofactor from native phosphorylase under conditions where pyridoxal reconstituted phosphorylase was rapidly inactivated suggested that the 5′-phosphoryl group was important in blocking resolution. Therefore, the effect of several phosphate analogues on the cysteine resolution of the cofactor from pyridoxal reconstituted phosphorylase was tested (Table III). All the analogues tested inhibited the 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 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. When measured under the conditions given in Table II, the apparent binding constant for phosphite was 0.9 mM while that for fluorophosphate was 2 mM. The maximal velocity in the presence of phosphite was 10.2 μmol of P_i min^{-1} (mg of protein)$^{-1}$, while in the presence of fluorophosphate the maximal velocity was 9.7 μmol of P_i min^{-1} (mg of protein)$^{-1}$. 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

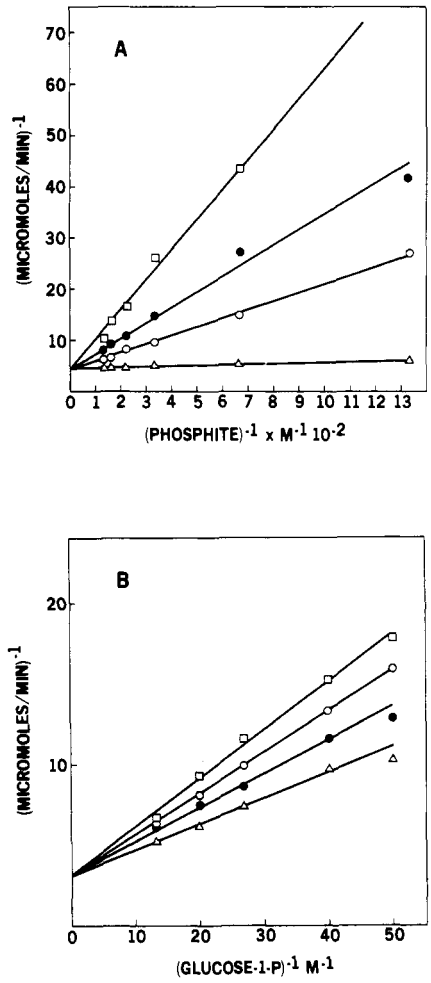

FIGURE 7: Double reciprocal plots of pyrophosphate inhibition of pyridoxal reconstituted phosphorylase. The reaction mixtures at pH 6.8 consisted of pyridoxal reconstituted phosphorylase, 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, and 1% glycogen. (A) Enzyme, 64 μg, 0.016 M glucose-1-P, phosphite at the indicated concentrations, and pyrophosphate at 0 (△), 0.0003 M (○), 0.0006 M (●), and 0.0012 M (□). (B) Enzyme, 22 μg, 0.0075 M phosphite, glucose-1-P at the indicated concentrations, and pyrophosphate at 0 (△), 0.0001 M (●), 0.0002 M (○), and 0.0003 M (□).

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 7A and 7B). 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 1 or 2 molecules of pyrophosphate per monomer of pyridoxal reconstituted phosphorylase. The results of such measurements are shown in Figure 8, from which it was determined that 0.9 mol of pyrophosphate bound per mol of pyridoxal reconstituted phosphorylase monomer with a binding constant of 8.5×10^{-5} M.

Discussion

The experimental data presented in this paper clearly demonstrate that, in the presence of noncovalently bound phosphate or an appropriate phosphate analogue, pyridoxal reconstituted phosphorylase possesses enzymic activity. Phosphate analogues, at concentrations that had dramatic effects on the activity of pyridoxal reconstituted phosphorylase, had no effect on the native enzyme. The simplest explanation for these observations is that phosphate or the phosphate an-

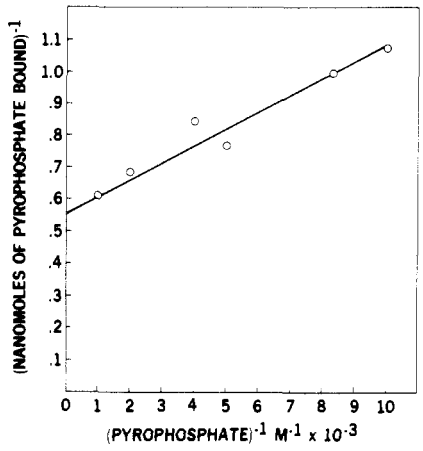

FIGURE 8: Binding of [^{32}P]pyrophosphate by pyridoxal reconstituted phosphorylase. The reaction mixture at pH 6.8 in a total volume of 0.2 mL contained pyridoxal reconstituted phosphorylase (0.2 mg), 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, and the indicated concentrations of [^{32}P]pyrophosphate. Binding was measured by the ultrafiltration method of Paulus (1969).

alogues bound at the site in pyridoxal reconstituted phosphorylase where the 5′-phosphoryl group of pyridoxal-5′-P would bind in the native enzyme.[2] Definite proof of this interpretation will come only from x-ray crystallographic studies, but there is precedence in the literature for this interpretation. Noltman (1972) describes several systems involving phosphorylated sugars where arsenate and the dephosphorylated sugar can result in enzymatic activity. Ray and Long (1976), in studies with phosphoglucomutase, have demonstrated that the rate constant for phosphate transfer to xylose, in the presence of phosphite, approaches the value for the natural substrate glucose-6-P, while in the absence of phosphite, the rate constant for phosphate transfer to xylose is 4 orders of magnitude smaller. Wada and Morino (1964) reported that the apoprotein of glutamic-oxaloacetic 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 *Escherichia coli* were shown to have an absolute dependence on the presence of inorganic phosphate for the expression of enzymatic activity. Thus, it seemed that, in certain instances, noncovalently bound phosphate or phosphate analogues and a nonphosphorylated analogue of a phosphorylated substrate or cofactor could substitute for the phosphorylated substrate or cofactor and result in enzymatic activity.

In previous literature reports pertaining to the inactivity of pyridoxal reconstituted phosphorylase, all activity measurements were performed in the direction of glycogen synthesis. The previous failures to observe the enzymatic activity of pyridoxal reconstituted phosphorylase under these conditions can probably be attributed to a combination of the following properties of the enzyme: (a) the inherent lower enzymatic activity of pyridoxal reconstituted phosphorylase; (b) the dependence of the activity on the pressure of noncovalently bound phosphate or phosphate analogues; (c) the lag in the generation of product when only limiting amounts of phosphate were present; (d) the nonlinear dependence of enzymatic activity on enzyme concentration in the absence of activating anions; (e) the inclusion of L-cysteine in the buffers; (f) the higher apparent binding constant of pyridoxal reconstituted phosphorylase for glucose-1-P.

Further interpretation of the data presented in this paper 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 Waals radii of hydrogen and fluorine differ by less than 10% (Cotton and Wilkinson, 1966). The phosphorus–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 I show the pK_a values for the second ionization 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 suggests 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 would 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 dianion 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 III, 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) and Feldmann and Hull (1977), which 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 in this communication 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 pK_a for fluorophosphate, coupled with its ability to activate pyridoxal reconstituted phosphorylase, suggests that fluorophosphate does not accept a proton during the reaction catalyzed by pyridoxal reconstituted phosphorylase. By analogy, this suggests that a proton transfer involving the 5′-phosphoryl of pyridoxal-5′-P in native phosphorylase may not occur.

The effects of pyrophosphate on pyridoxal reconstituted phosphorylase were quite surprising since at the concentrations used no effect is seen on the native enzyme. The kinetic studies indicated that pyrophosphate was competitive with respect to both the substrate and the anion activator phosphite. Two apparent K_I values were calculated. The radioactive binding studies indicated that only one molecule of pyrophosphate bound per monomer of phosphorylase. These results are not contradictory and can be explained by a model where one molecule of pyrophosphate encompasses both the glucose-1-P

[2]Experiments done with inactive pyridoxal phosphate monoethyl ester reconstituted phosphorylase *b* support this view. This enzyme form cannot be reactivated by phosphate presumably because the phosphate position is blocked by the inactive monomethyl ester of phosphate (E. Helmreich, personal communication).

and -5′-P sites of the coenzyme. Purich and Fromm (1972) showed that competitive kinetics are seen by the binding of one molecule of inhibitor to the two substrate sites in a random mechanism. The inhibition constants are complex and contain terms for the second substrate as well as other rate constants. Thus, it would not be expected that the apparent inhibition constants would be equal and comparable to a K_d evaluated by direct binding. The data can be explained if the two sites are close but these kinetic results do not eliminate a mechanism where the two sites are apart. In this case, binding at one site would have to mutually exclude the binding of glucose-1-P and the activator anion. The true explanation awaits the elucidation of the three-dimensional structure of phosphorylase. Binding sites for substrate (Fletterick et al., 1976) have been specified, but the pyridoxal-5′-P site has not.

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References

Anderson, R. A., and Graves, D. J. (1973), *Biochemistry 12,* 1895.

Anderson, R. A., Parrish, R. F., and Graves, D. J. (1973), *Biochemistry 12,* 1901.

Baranowski, T., Illingworth, B., Brown, D. H., and Cori, C. F. (1957), *Biochim. Biophys. Acta 25,* 16.

Berndt, A. F., and Sylvester, J. M. (1972), *Acta Crystallogr., Sect. B 28,* 2191.

Brostrom, C. O., Hunkeler, F. L., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1961.

Cotton, F. A., and Wilkinson, G. (1966), Advanced Inorganic Chemistry, 2nd ed, New York, N.Y., Interscience, pp 114–115.

Ehrlich, J., Feldmann, K., Helmreich, E., and Pfeuffer, T. (1971), Joint Biochemical Symposium USSR–GDR, May 3–7, Schloss Reinhardsbrunn, Akademieverlag, Berlin.

Engers, H. D., Bridger, W. A., and Madsen, N. B. (1969), *J. Biol. Chem. 244,* 5936.

Feldmann, K., and Helmreich, E. J. M. (1976), *Biochemistry 15,* 2394.

Feldmann, K., and Hull, W. E. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 856.

Fischer, E. H., Kent, A. B., Synder, E. R., and Krebs, E. G. (1958), *J. Am. Chem. Soc. 80,* 2906.

Fischer, E. H., and Krebs, E. G. (1962), *Methods Enzymol. 5,* 369.

Fischer, E. H., Pocker, A., and Saari, J. C. (1970), *Essays Biochem. 6,* 23.

Fiske, D. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.

Fletterick, R. J., Sygusch, J., Sample, H., and Madsen, R. B. (1976), *J. Biol. Chem. 251,* 6142.

Graves, D. J., Carlson, G. M., Skuster, J. R., Parrish, R. F., Carty, T. J., and Tessmer, G. W. (1975), *J. Biol. Chem. 250,* 2254.

Graves, D. J., and Wang, J. L. (1972), *Enzymes, 3rd Ed. 7,* 435.

Gunsalus, I. C., Courtland, C. G., and Stamer, J. R. (1955), *Methods Enzymol. 2,* 238.

Handlovic, M. (1969), *Acta Crystallogr., Sect. B 25,* 227.

Hedrick, J. L. (1966), *Arch. Biochem. Biophys. 114,* 216.

Hedrick, J. L., Shaltiel, S., and Fischer, E. H. (1966), *Biochemistry 5,* 2117.

Hedrick, J. L., Shaltiel, S., and Fischer, E. H. (1969), *Biochemistry 8,* 2422.

Helmreich, E., and Cori, C. F. (1964a), *Proc. Natl. Acad. Sci. U.S.A. 51,* 131.

Helmreich, E., and Cori, C. F. (1964b), *Proc. Natl. Acad. Sci. U.S.A. 52,* 647.

Illingworth, B., and Cori, G. T. (1953), *Biochem. Prep. 3,* 1.

Illingworth, B., Jansz, H. S., Brown, D. H., and Cori, C. F. (1958), *Proc. Natl. Acad. Sci. U.S.A. 44,* 1180.

Johnson, G. F., and Graves, D. J. (1966), *Biochemistry 5,* 2906.

Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E. (1968), *Biochemistry 7,* 3590.

Krebs, E. G. (1966), *Methods Enzymol. 8,* 543.

Lerch, K., and Fischer, E. H. (1975), *Biochemistry 14,* 2009.

Milner-White, E. J., and Watts, D. C. (1971), *Biochem. J. 122,* 727.

Noltman, E. (1972), *Enzymes, 3rd Ed. 6,* 272.

Paulus, H. (1969), *Anal. Biochem. 32,* 91.

Pfeuffer, T., Ehrlich, J., and Helmreich, E. (1972a), *Biochemistry 11,* 2125.

Pfeuffer, T., Ehrlich, J., and Helmreich, E. (1972b), *Biochemistry 11,* 2136.

Purich, D. L., and Fromm, H. J. (1972), *Biochim. Biophys. Acta 276,* 563.

Ray, W. L. J., and Long, J. W. (1976), *Biochemistry 15,* 3993.

Robertson, H. E., and Boyer, P. D. (1956), *Arch. Biochem. Biophys. 62,* 380.

Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5,* 2108.

Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1969a), *Biochemistry 8,* 2429.

Shaltiel, S., Hedrick, J. L., Pocker, A., and Fischer, E. H. (1969b), *Biochemistry 8,* 5189.

Snell, E. S., and DiMari, S. J. (1970), *Enzymes, 3rd Ed. 2,* 335.

Thomas, J. A., Schlender, K. K., and Larner, J. (1968), *Anal. Biochem. 25,* 486.

Van Wazer, J. R. (1958), Phosphorus and Its Compounds, Vol. 1, New York, N.Y., Interscience, pp 379–821.

Vidgoff, J. M., Pocker, A., Hullar, T. L., and Fischer, E. H. (1974), *Biochem. Biophys. Res. Commun. 57,* 1166.

Wada, H., and Morino, Y. (1964), *Vitam. Horm. (N.Y.) 22,* 411.

Weast, R. C., Ed. (1974), Handbook of Chemistry and Physics, 55th ed, Cleveland, Ohio, Chemical Rubber Co., p D-91.

Weisshaar, H. D., and Palm, D. (1972), *Biochemistry 11,* 2146.