Reaction of Phosphofructokinase with Maleic Anhydride, Succinic Anhydride, and Pyridoxal 5′-Phosphate*

Kosaku Uyeda

ABSTRACT: Phosphofructokinase was treated with reagents which are known to react with amino groups of proteins, namely, maleic anhydride, succinic anhydride, and pyridoxal 5′-phosphate. All the reagents inactivated the enzyme. Fructose 6-phosphate, fructose 1,6-diphosphate, and adenosine monophosphate protected the enzyme against the inactivation by succinylation while adenosine triphosphate–Mg^{2+} did not.

The maleylation and succinylation led to the dissociation of phosphofructokinase to protein derivatives with a molecular weight of approximately 80,000. The reaction with pyridoxal 5′-phosphate resulted in the formation of a Shiff base and revealed the existence of three types of amino groups which differ in the reactivity toward the reagent. At relatively low concentrations of pyridoxal 5′-phosphate the phosphofructokinase was modified such that the degree of its inhibition by adenosine triphosphate was altered. The enzyme was dissociated to a 7S protein by the treatment with a higher concentration of pyridoxal 5′-phosphate. It was found that the antibody against the native phosphofructokinase reacted to form a precipitin with higher molecular weight maleyl, succinyl, and 5′-phosphopyridoxyl enzymes, but did not form precipitin with the derivatives with molecular weight of 80,000.

Sulfhydryl groups of phosphofructokinase have been shown by various investigators (Engelhardt and Sakov, 1943; Uyeda and Racker, 1965; Paetkau and Lardy, 1967; Younathan *et al.*, 1968; Kemp and Forest, 1968; Frenkel, 1968; Froede *et al.*, 1968) to be important for the enzymic activity. Other functional groups essential for the activity, however, have not been investigated in phosphofructokinase of rabbit muscle. It is the purpose of the present work to study the effect of reagents which are known to react with protein amino groups on the enzymic activity and on the protein structure. The reagents employed for the chemical modification of phosphofructokinase were maleic anhydride, succinic anhydride, and pyridoxal 5′-phosphate. The reagents have been used by a number of investigators (Klotz and Keresztes-Nagy, 1963; Hass, 1964; Anderson *et al.*, 1966; Kaldor and Weingach, 1966; Butler *et al.*, 1967; Rippa *et al.*, 1967; Shapiro *et al.*, 1968; Sia and Horecker, 1968) to block amino groups in proteins and peptides.

Material and Methods

DEAE-cellulose was purchased from Brown Co., Berlin, N. H., and purified according to the procedure described previously (Uyeda and Rabinowitz, 1965). Maleic anhydride and succinic anhydride were purchased from Calbiochem. Radioactive maleic anhydride-2,3-^{14}C was the product of Nuclear-Chicago Corp.

All the other chemicals were obtained from commercial sources as described previously (Uyeda and Racker, 1965).

Phosphofructokinase was purified from rabbit skeletal muscle according to the procedure of Ling *et al.* (1965) with a slight modification as follows. In step 3 of their preparation, the dialyzed enzyme often contained precipitate which lacked phosphofructokinase activity. The precipitate was removed by centrifugation and discarded. The supernatant solution was then applied to a DEAE-cellulose column (2.5 × 30 cm), about two times the size used by Ling *et al.* (1965), and the chromatography was carried out as described (Ling *et al.*, 1965).

The preparations of phosphofructokinase had the specific activities of 137–155 units/mg. In some preparations the enzyme was crystallized twice according to the procedure of Parmeggiani *et al.* (1966). Some preparations of the enzyme were analyzed for homogeneity with disc electrophoresis according to the procedure of Davis (1964). The electrophoresis of the phosphofructokinase (50–200 μg of protein) was performed in 4.5% acrylamide gel, and the electrode buffer was in Tris-glycine buffer at pH 8.3. The sample was layered with an equal volume of a mixture containing 0.2 M sucrose and the electrode buffer. A current of 4 mA/gel was applied for about 45 min at room temperature. The gel was stained in 1% Amido Black in 7% acetic acid for overnight. The stained gel was destained electrophoretically in the same acetic acid solution.

The phosphofructokinase preparation was analyzed with the disc electrophoresis immediately after the preparation. All the preparations showed a single band with R_m values, relative mobility, of 0.35–0.41 under the conditions employed. As the enzyme preparation was aged, however, the protein tended to precipitate on top of the upper gel and failed to penetrate into the gel.

Reaction with Maleic Anhydride or Succinic Anhydride. The phosphofructokinase (20 mg) was dialyzed for 4 hr at 2° against 500 ml of 0.05 M $NaHCO_3$, 0.2 mM EDTA (pH 8.0), and 10 mM 2-mercaptoethanol unless otherwise noted. All the succinylation reactions were carried out in the same

* From the Veterans Administration Hospital and the Biochemistry Department, The University of Texas Southwestern Medical School, Dallas, Texas. *Received December 20, 1968.* This work was supported by Research Grant GM16258 from National Institute of Health, U. S. Public Health Service.

buffer except 2-mercaptoethanol was omitted. The dialyzed enzyme had specific activities of 137–158 units/mg. The enzyme was diluted to 5 mg/ml with the mixture of $NaHCO_3$–EDTA–2-mercaptoethanol.

Weighed amounts of solid maleic anhydride or succinic anhydride were added in small increments to the enzyme solution, and the reaction mixture was stirred continuously with a magnetic stirrer at 2°. The pH of the reaction mixture was maintained between 8 and 9 with addition of 1 N KOH. The reaction was continued for 45 min. At the end of the reaction the enzyme solution was dialyzed overnight against 500 ml of 0.05 M potassium phosphate–0.1 mM EDTA (pH 7.0).

When maleic anhydride or succinic anhydride in less than 1 mg was used, an acetone solution of the compound was made immediately before use and aliquots of the solution were added to the reaction mixture.

Reaction with Pyridoxal 5'-Phosphate. The reaction mixture contained in a final volume of 1 ml, $NaHCO_3$ (50 mM, pH 8), EDTA (0.2 mM), and the phosphofructokinase (0.5 mg), which was dialyzed as described above. An aliquot of pyridoxal 5'-phosphate (0.01 M) was added to the reaction mixtures and incubated at room temperature for 20 min. A fresh aqueous solution of $NaBH_4$ (40 μl of 0.2 M) was then added to the reaction mixture and incubated at 0° for 20 min. The reduced reaction mixture was dialyzed overnight against Tris-phosphate (0.05 M pH 8.0) and EDTA (0.2 mM). The amount of 5'-phosphopyridoxyl groups substituted on the enzyme was determined from the absorbance at 322 mμ and using the molar extinction coefficient of 9000 (Dempsey and Snell, 1963).

Determination of Enzymic Activities. The activity of phosphofructokinase was determined at 28° in a reaction mixture containing in a final volume of 1 ml, 50 mM Tris-Cl (pH 8.2), 1 mM EDTA, 1.5 mM fructose 6-phosphate, 5 mM $MgCl_2$, 4 mM ammonium sulfate, 0.5 mM dithiothreitol, 0.19 mM NADH, and 0.01 mg/ml of bovine serum albumin and auxiliary enzymes. The auxiliary enzymes were added as described by Parmeggiani *et al.* (1966). The reaction was initiated with addition of phosphofructokinase and the rate of the reaction was determined with a Gilford recording spectrophotometer. A unit of the enzymic activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of product/min under the assay conditions. Specific activity is expressed as units per milligram of protein.

The inhibition of phosphofructokinase by ATP was determined as described previously (Uyeda and Racker, 1965) except the concentration of $MgCl_2$ was maintained at 3 mM.

Ultracentrifugation. Ultracentrifugation was carried out in a Spinco Model E ultracentrifuge. The sedimentation velocity experiments were done in a 30-mm double-sector centerpiece with a ANE rotor at 44,770 rpm. The sedimentation equilibrium was performed according to the procedure of Yphantis (1964) using a 12-mm double-sector cell and sapphire windows in an AND rotor. The ultracentrifugation was at 29,500 rpm at 18–20° for 24 hr. The protein concentration of the sample was between 0.6 and 1 mg per ml and the fringe displacement was determined with a Nikon comparator.

Antibody against Phosphofructokinase. A goat was injected weekly with the purified phosphofructokinase (10 mg each) in Freund's incomplete adjuvant (Difco). At the end of the third week a sample of blood was withdrawn and centrifuged to remove red cells. The serum was found to contain antibodies against phosphofructokinase as determined by precipitin tests using a capillary diffusion and double-immunodiffusion techniques. Although the titer of antibodies has not been determined accurately, 0.1 mg of phosphofructokinase was inhibited 97% with approximately 25 μl of the serum.

Determination of Protein. Protein was determined in 0.1 N NaOH from the absorbancy at 283 mμ using the absorption coefficient of 1.09 l./g cm (Paetkau *et al.*, 1968).

Results

Reaction with Maleic Anhydride. Phosphofructokinase was reacted with varying concentrations of maleic anhydride and the extent of inactivation was determined. The results as summarized in Table I show that 0.26 and 1.3 mM maleic anhydride caused 50 and 93% inactivation of the enzyme, respectively. A complete inactivation of the activity was obtained with higher concentrations (20 mM) of the reagent.

TABLE I: Inactivation by Maleic Anhydride.[a]

Maleic Anhydride (mM)	Act. (ΔA/min)	% Inhibn
0	0.140	0
0.26	0.072	49
0.52	0.042	69
0.78	0.030	79
1.04	0.018	87
1.3	0.010	93

[a] The reaction mixture contained in a final volume of 1 ml, $NaHCO_3$ (0.05 M at pH 8.0) and the dialyzed phosphofructokinase (0.5 mg). A solution of maleic anhydride (25 mM) in acetone was prepared immediately before use and given aliquots of the solution were added. The reaction was carried out as described in Methods. Aliquots of reaction mixture were assayed for the enzymic activity without dialysis. No loss of the activity was observed in a control with acetone.

An attempt was made to see if the inactivation by maleic anhydride is reversible. The maleyl enzyme, which was inactivated to 95%, was incubated in potassium acetate at pH 4.6 for 45 min at 0°, and the solution was neutralized to pH 8. Under these conditions, however, no recovery of the activity was observed. The presence of dithiothreitol in the reactivation mixture did not aid in the recovery of the enzymic activity.

A possible protection by substrates and effectors against maleic anhydride was tested. The enzyme was reacted with 0.7 mM maleic anhydride in the presence of 5 mM each of ATP and $MgCl_2$, fructose 6-phosphate, fructose 1,6-diphosphate, AMP, or P_i. The inhibition was approximately 60% in the absence of the effector. None of the compounds offered any significant protection against the inactivation under these conditions.

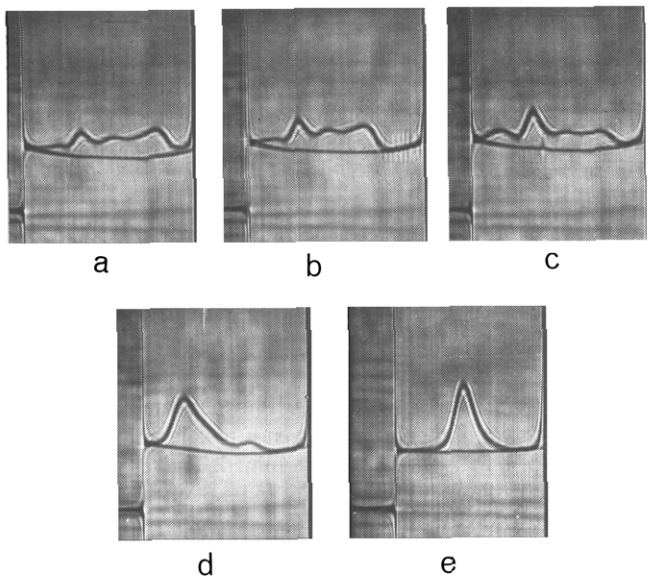

FIGURE 1: Schlieren patterns of phosphofructokinase and maleyl phosphofructokinase. The sedimentation patterns were determined with 0.8 ml of the sample containnig 4.5 mg/ml of protein in 0.05 M Tris-phosphate–0.2 mM EDTA at pH 8.0. The measurements were made with a Spinco Model E ultracentrifuge at a rotor speed of 44,770 rpm at an average temperature of 19°. The samples were treated as described in Methods with the following concentrations of maleic anhydride and the specific activities: (a) 0.0 mM, 127 U/mg; (b) 5.2 mM, 20 U/mg; (c) 10.4 mM, 7.1 U/mg; (d) 20.8 mM, 0 U/mg; and (e) 90 mM, 0 U/mg. The photographs were taken after (a) 42, (b) 34, (c) 43, (d) 81, and (e) 135 min after reaching 44,770 rpm.

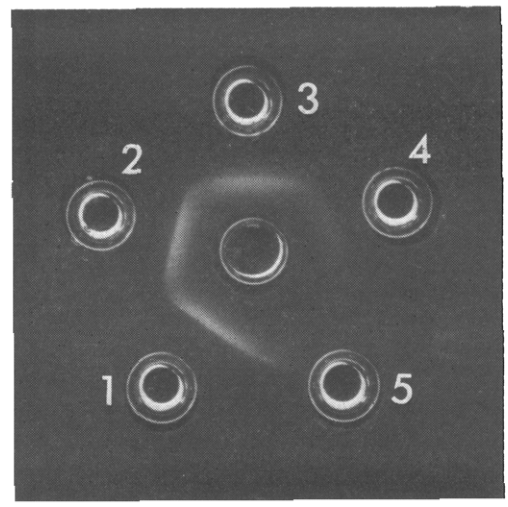

FIGURE 2: Immunoreactivity of maleyl phosphofructokinase. Aliquots (10 μl) of the maleyl phosphofructokinase samples described in Figure 1 were placed in the outer wells and the goat serum (10 μl) in the center of an agar gel immunoplate (Hyland). The plate was allowed to stand for 24 hr at room temperature. Wells 1–5 correspond to the maleyl phosphofructokinase samples of a–e in Figure 1.

Sia and Horecker (1968) have shown recently that aldolases, transaldolase, and fructose diphosphatase could be dissociated to subunits by the maleic anhydride treatment. It was of interest to see if the same treatment causes the dissociation of phosphofructokinase. The enzyme was treated with different concentrations of solid maleic anhydride and the enzymic activity as well as molecular weight of maleyl phosphofructokinase was determined. The results are shown in Figure 1. When phosphofructokinase (5 mg/ml) was reacted in the presence of 2-mercaptoethanol with 5.2 mM maleic anhydride, equivalent to two times the total lysine[1] in the protein, an 85% loss of the enzymic activity occurred, but no significant change in the sedimentation pattern (Figure 1b) from that of the native enzyme was observed (Figure 1a). The sedimentation coefficients of the three major peaks in these samples were estimated as approximately 13, 19.6, and 28 S. The reaction with 10 and 20 mM maleic anhydride caused a nearly complete loss of activity, and the decrease in the amounts of 19.6S and 28S proteins and the corresponding increase in 7S protein occurred (Figure 1c,d). Treatment with a higher concentration (90 mM) of maleic anhydride yielded a dissociated enzyme. The schlieren pattern of the dissociated enzyme (Figure 1e) showed a slightly skewed peak indicating contamination by aggregates. Disc electrophoresis revealed a major diffused band and a minor band. The molecular weight of the dissociated enzyme was determined by sedimentation equilibrium according to the method of Yphantis (1964). A plot of log (fringe displacements) *vs.* (distances from the rotational axis, X) showed a slight deviation from the linearity at the bottom of the cell suggesting the presence of aggregates. Nevertheless, the linear portion of the curve covered about 90% of the total measurement from the meniscus to the bottom of the cell and was used for the calculation. Assuming a partial specific volume of 0.728 ml/g (Paetkau and Lardy, 1967), the molecular weight of the maleyl phosphofructokinase was estimated as 77,000 and 80,000 in the absence and in the presence of 1 M KCl, respectively. By other methods, such as gel filtration on agarose 1.5 M (Bio-Rad Corp.) and disc electrophoresis on 7.5% gel, the molecular weight was determined as approximately 80,000, which is in agreement with the ultracentrifugal data.

The number of maleyl groups attached with the enzyme was determined with maleic anhydride-2,3-^{14}C. When phosphofructokinase (4.95 mg, 13 mμmoles) was reacted with 11 and 100 μmoles of maleic anhydride-2,3-^{14}C under the conditions described in Methods, approximately 20 and 90 moles, respectively, of maleyl residues per mole of enzyme[2] were found. Under these experimental conditions the maleyl enzyme with 20 moles of maleyl groups was inactive, with no significant change in the sedimentation pattern. The result seems to suggest that those lysine residues are essential for the enzymic activity, assuming that maleic anhydride reacts only with lysine. Moreover, the observation that the maleyl phosphofructokinase with 90 moles of maleyl residues is dissociated to the above subunit suggests that some of those 70 residues may be essential for holding the subunits together.

The immunoreactivity of the maleyl derivatives of phosphofructokinase against the goat antibody was examined and the result is shown in Figure 2. The outer wells of the immunodiffusion plates contained the samples whose sedimentation

[1] 180 lysines/mole of phosphofructokinase (Paetkau *et al.*, 1968).

[2] The value of 3.8×10^5 was used in all calculations as the molecular weight for the enzyme (Paetkau and Lardy, 1967).

TABLE II: Inactivation by Succinic Anhydride.[a]

Succinic Anhydride (mM)	Act. (ΔA/min)	% Inhibn
0	0.153	0
0.1	0.136	11
0.25	0.098	36
0.5	0.055	64
0.75	0.041	73
1.0	0.028	86

[a] The reaction mixture contained in a final volume of 1 ml, 0.48 mg of dialyzed phosphofructokinase and 0.05 M $NaHCO_3$ (pH 8.5). Aliquots of acetone solution of succinic anhydride (25 mM) were added to the reaction mixtures and the reaction was carried out for 30 min at 2°. The control sample contained 40 μl of acetone. The control showed no inactivation. An aliquot of the reaction mixture was diluted in 0.1 M Tris-phosphate (pH 8) and assayed for the enzymic activity.

TABLE III: Protection by Substrates and Effectors against Inactivation by Succinic Anhydride.[a]

Compounds	Act. (ΔA/min)	% Inhibn
No succinic anhydride	0.162	0
None	0.053	67
ATP, $MgCl_2$	0.017	90
Fructose 6-phosphate	0.149	8
Fructose 1,6-diphosphate	0.092	43
AMP	0.091	44
Orthophosphate	0.052	68

[a] The reaction mixtures and the conditions are the same as in Table II, except for the addition of 5 mM substrates or effectors and 0.75 mM succinic anhydride.

patterns are shown in Figure 1. The results show that the antibody forms precipitin with the maleyl phosphofructokinase as with the native phosphofructokinase. The maleyl phosphofructokinase which is fully dissociated to the 77,000 protein (Figure 1e), however, failed to form the precipitin (Figure 2, the well 5). Moreover, a correlation seems to exist between the amount of the precipitin and the decrease in the protein which corresponds to native phosphofructokinase. There is no correlation between the enzymic activity and immunoreactivity of the maleyl phosphofructokinase. For example, the maleyl enzymes with the specific activities of 20 and 7.1 units per mg formed the same amount of the precipitin as the native enzyme (Figure 2, wells 2 and 3).

Reaction with Succinic Anhydride. The results of the treatment of phosphofructokinase with varying concentrations of succinic anhydride are summarized in Table II. As shown in the table, the phosphofructokinase is inactivated to 86% with 1 mM succinic anhydride under the experimental conditions. The degree of the inactivation by succinic anhydride was comparable with that with maleic anhydride (Table I). Difference between these two reagents became apparent, however, when protection by the effectors against the inactivation by succinic anhydride was examined. As shown in Table III, fructose 6-phosphate protects the enzyme against the succinylation. As discussed above, all these effectors were ineffective in protecting against the inactivation by maleic anhydride unlike succinic anhydride. This may be an indication that both the double bond and the anhydride group of maleic anhydride react with more than one group on the enzyme, such as a thiol, in addition to the ϵ-amino group. Succinic anhydride, on the other hand, may react only with the amino groups.

In order to see if succinic anhydride causes dissociation of phosphofructokinase, the enzyme (5 mg) was reacted with 10, 25, and 50 mM succinic anhydride. Under these conditions all the succinyl phosphofructokinase preparations were dissociated as judged from the sedimentation patterns. The schlieren patterns of the succinyl phosphofructokinase, prepared by reacting with 50 mM succinic anhydride, show a single symmetrical peak similar to that of maleyl phosphofructokinase (Figure 1).

The molecular weight of the phosphofructokinase derivative in Tris-phosphate at pH 8 was determined as 78,000 by the sedimentation equilibrium method assuming the partial specific volume of 0.728 ml/g (Paetkau and Lardy, 1967). In the presence of 1 M KCl, the value of 82,500 was obtained for the molecular weight. The results show that the molecular weights of the succinyl phosphofructokinase in the presence and the absence of KCl are comparable with those of maleyl phosphofructokinase.

The dissociated succinyl phosphofructokinase failed to react with the antibody to form precipitin as in the case of dissociated maleyl phosphofructokinase.

Reaction with Pyridoxal 5'-Phosphate. In order to obtain more direct evidence that lysine residues of the enzyme are involved in the enzymic activity, pyridoxal 5'-phosphate, a more specific reagent for ϵ-amino group of lysine residue, was used as a possible inhibitor of the enzyme activity.

Phosphofructokinase was reacted with pyridoxal 5'-phosphate, and reduced with $NaBH_4$ to covalently link phosphopyridoxyl group to the enzyme. Figure 3 illustrates the results of the experiment. Figure 3A shows the spectra of the phosphofructokinase, of pyridoxal 5'-phosphate, and of the product of the reaction. The difference spectrum of the product formed by the reaction before the reduction shows the absorption maxima at 432, 324, and 280 mμ and a minimum at 377 mμ (Figure 3B). The product shows the characteristic spectrum of a Shiff base formed by the interaction of ϵ-amino group of lysine in the protein and pyridoxal 5'-phosphate (Jenkins and Singer, 1957). Moreover, the spectrum of the reduced product (Figure 3C) of the Shiff base shows an absorption maximum at 322 mμ which agrees with that of 5'-phosphopyridoxyl enzyme (Fischer *et al.*, 1962). Similar spectra have been obtained recently by Shapiro *et al.* (1968) by treatment of muscle aldolase with pyridoxal 5'-phosphate.

In order to correlate the extent of the reaction with pyridoxal 5'-phosphate and the loss of the enzymic activity, the enzyme was treated with different concentrations of pyridoxal 5'-phosphate. The reacted enzyme was then reduced with $NaBH_4$ and the enzymic activity was determined. The

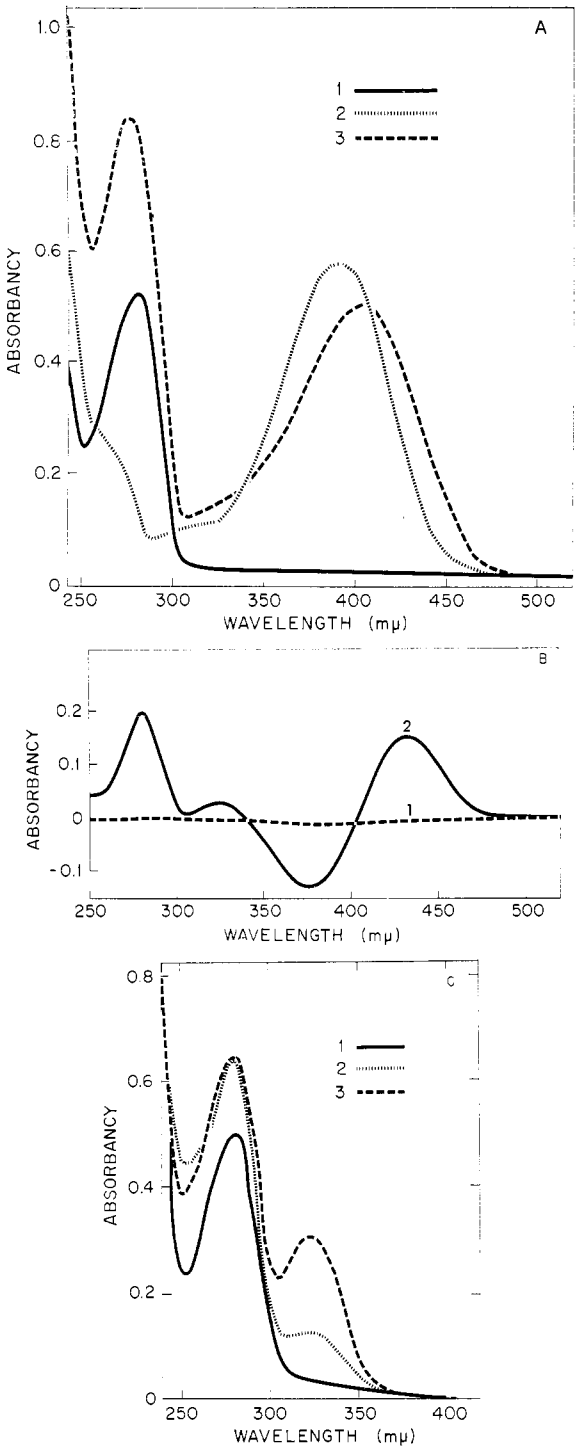

FIGURE 3: Spectral changes accompanying the pyridoxal 5′-phosphate treatment of phosphofructokinase. The reaction mixture contained in a final volume of 1 ml: phosphofructokinase (1.2 mg), pyridoxal 5′-phosphate (0.1 mM), $NaHCO_3$ (0.05 M), and 2-mercaptoethanol (0.01 M) at pH 8. (A) Spectra of the enzyme (1), pyridoxal 5′-phosphate (2), and the product formed by mixing the enzyme and pyridoxal 5′-phosphate (3). The difference spectra (B) were determined using tandem cells. One side of the cell contained phosphofructokinase (1.2 mg/ml) and the other contained pyridoxal 5′-phosphate (0.1 M in $NaHCO_3$–2-mercaptoethanol). Both the sample and reference cells contained the same components. The difference spectra (B) were determined before (1) and after (2) mixing of the sample cell against the reference. Part C is the spectra of the products formed after reduction with $NaBH_4$, and dialysis as described in Methods. The reduced and dialyzed enzymes were after treatment with (1) 0, (2) 0.1, and (3) 0.7 mM of pyridoxal 5′-phosphate.

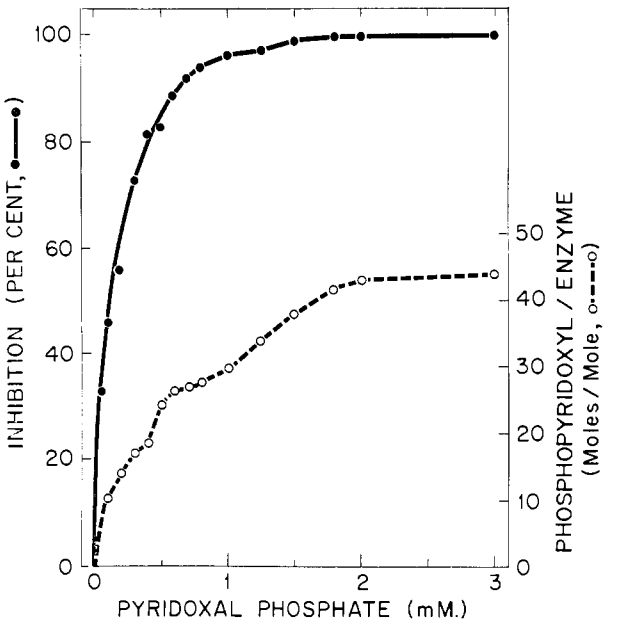

FIGURE 4: Inactivation by pyridoxal 5′-phosphate. The reaction mixture contained in a final volume of 1 ml: phosphofructokinase (0.5 mg), indicated concentrations of pyridoxal 5′-phosphate, and $NaHCO_3$ (0.05 M) at pH 8. The $NaBH_4$ reduction, dialysis, the determination of the enzymic activity, and the number of phosphopyridoxyl residues were carried out as described in Methods.

number of 5′-phosphopyridoxyl groups on the enzyme after dialysis was determined from the absorbance at 323 mμ and using the molar extinction coefficient of 9000 (Dempsey and Snell, 1963). The results of the experiment are shown in Figure 4. The results in the figure suggest the existence of at least three kinds of lysine residues in the enzyme. The first 18–20 lysine residues react readily with pyridoxal 5′-phosphate which results in 80% loss of the enzyme activity. The reaction of the next 10 lysine residues with pyridoxal 5′-phosphate abolishes the enzymic activity up to 96%. The remainder of the lysine, about 15 residues, is the least reactive. Under these conditions the maximum of 44 lysine residues reacted with pyridoxal 5′-phosphate which corresponds to about 24% of the total lysine residues in the phosphofructokinase. Treatment with a higher concentration of pyridoxal 5′-phosphate has not been attempted. Pyridoxal is less effective since the treatment of 0.7 mg of phosphofructokinase with 1 mM pyridoxal inactivated the enzyme only 20% under the similar conditions.

The effect of various substrates and effectors against the inhibition by pyridoxal 5′-phosphate was next examined. As shown in Table IV, a significant protection by fructose 6-phosphate and fructose 1,6-diphosphate was observed before and after the reduction by $NaBH_4$. ATP and Mg^{2+} were completely ineffective against the inactivation. These results are in agreement with those obtained with the succinic anhydride treatment (Table III), which suggests a possible involvement of the lysine residues for the binding of fructose 6-phosphate and fructose 1,6-diphosphate, but not ATP and Mg^{2+}.

The inhibition by pyridoxal 5′-phosphate is considerably less before than after the reduction by $NaBH_4$. The reason for this may be that the Shiff base formation by the reaction,

TABLE IV: Inhibition by Pyridoxal 5′-Phosphate and Protection by Substrates.[a]

Compound (mM)	% Inhibition	
	Before $NaBH_4$	After $NaBH_4$
No pyridoxal-phosphate	0	0
None	38	86
ATP (5), $MgCl_2$ (5)	36	86
Fructose 6-phosphate (5)	0	42
Fructose 1,6-diphosphate (5)	10	41
AMP (5)	28	56
Phosphate (10)	42	78

[a] The reaction was carried out as described in Methods, and 0.5 mM pyridoxal 5′-phosphate was used. The enzymic activity after the reduction was determined without dialysis.

TABLE V: Reaction with Pyridoxal 5′-Phosphate and Possible Release of ATP Inhibition.[a]

Pyridoxal Phosphate (mM)	Fructose 6-Phosphate (mM)	Activity (ΔA/min)	
		ATP (1 mM)	ATP (3 mM)
0	0	0.136	0.002
0.15	0	0.096	0.023
0.15	5	0.148	0.029
0.25	0	0.063	0.026
0.25	5	0.106	0.042

[a] The reaction with pyridoxal 5′-phosphate and $NaBH_4$ reduction was carried out with 0.5 mg/ml of phosphofructokinase as described in Methods. The enzymic activity in the presence of 1 and 3 mM ATP was determined as described under ATP inhibition assay in Methods. Aliquots of 2 μl of the reaction mixtures except for the control sample (1 μl) were used for the assay. A control without $NaBH_4$ reduction showed 100% inhibition by 3 mM ATP under the identical conditions.

before reduction, is reversed during the assay of the enzymic activity. This conclusion is supported by the observation that the rate of the enzymic reaction with the enzyme which had not been reduced showed a considerable lag period during the assay. The lag period, however, was shortened or eliminated after the reduction. Similar reversibility of pyridoxal 5′-phosphate inactivation of aldolase has been reported by Shapiro *et al.* (1968).

A possible difference in the reactivity of lysine residues at the active site and an "allosteric site" of phosphofructokinase toward pyridoxal 5′-phosphate was investigated. The enzyme was reacted with 0.15 and 0.25 mM of pyridoxal 5′-phosphate in the presence and the absence of fructose 6-phosphate. The treated enzyme was then assayed in the ATP inhibition assay for the determination of possible release of the inhibition. The results of the experiment are summarized in Table V. The enzyme lost approximately 50% of the activity with increasing pyridoxal 5′-phosphate but the inactivation could be prevented to some extent by fructose 6-phosphate. The modified enzyme showed at least tenfold increase in the activity in the presence of 3 mM ATP compared with the native enzyme. The ATP sensitivity, however, was the same in the presence and the absence of fructose 6-phosphate. Although the release of the ATP inhibition was not complete, the result suggests some differences in the reactivity of lysine residues at the active site and the "allosteric site" toward pyridoxal 5′-phosphate.

In order to see if the pyridoxal 5′-phosphate treatment causes any change in the molecular weights of the phosphofructokinase, the enzyme (5 mg) was reacted with 0.2 and 3 mM pyridoxal 5′-phosphate, then reduced with $NaBH_4$, and dialyzed overnight. As a control the enzyme was treated the same way as the samples except pyridoxal 5′-phosphate was omitted. Under these conditions the specific activity of the control enzyme was 54 units/mg, a 50% loss of the enzymic activity. The phosphofructokinase which had been reacted with 0.2 and 3 mM pyridoxal 5′-phosphate showed the specific activities of 43 and 0.3 units per mg, respectively. The corresponding contents of 5′-phosphopyridoxyl groups in the sample were determined as 3.5 and 33 moles per mole of enzyme. The schlieren patterns of the 5′-phosphopyridoxyl phosphofructokinase are shown in Figure 5. The peaks observed in the control sample correspond approximately to those of the native phosphofructokinase, namely, 13, 20, and 28 S. The phosphofructokinase sample with 3.5 moles of phosphopyridoxyl groups show essentially the same components as in the control except for a significant decrease in the concentration of 28S protein. The sedimentation pattern of the modified phosphofructokinase with 33 moles of phosphopyridoxyl groups (Figure 5c,d) is significantly altered from that of the native phosphofructokinase and shows a considerable dissociation to 7S and 13S components, the major peak corresponding to the former.

Immunoreactivities of the 5′-phosphopyridoxyl phosphofructokinases which were prepared as described in Figure 4 were tested with the goat antibody. The phosphofructokinase derivatives with 10–17 5′-phosphopyridoxyl residues show a single precipitin band which is indistinguishable from that of the native phosphofructokinase. More than 20 5′-phosphopyridoxyl residues per mole of enzyme (Figure 6, 6–15), however, resulted in the formation of two precipitin bands. The outer band is same as that formed with the native and phosphofructokinase derivatives with less than 20 5′-phosphopyridoxyl groups. The new inner precipitin band, however, appeared to be due to a smaller derivative of the phosphofructokinase derivative. Based on the sedimentation pattern obtained with phosphofructokinase containing 33 residues of 5′-phosphopyridoxyl group where the majority of the protein showed the molecular weight of 7 S, the species which formed the inner precipitin band may correspond to the protein. The observation that at the junction of the wells 6 and 10 of Figure 6 the inner band merged into the outer band of 6 indicates the "partial identity" of the antigen. Thus the inner band is not due to an extraneous protein, but it is closely related to the phosphofructokinase or its 5′-phosphopyridoxyl derivatives. As shown in the immunopatterns 11–15, the phosphopyri-

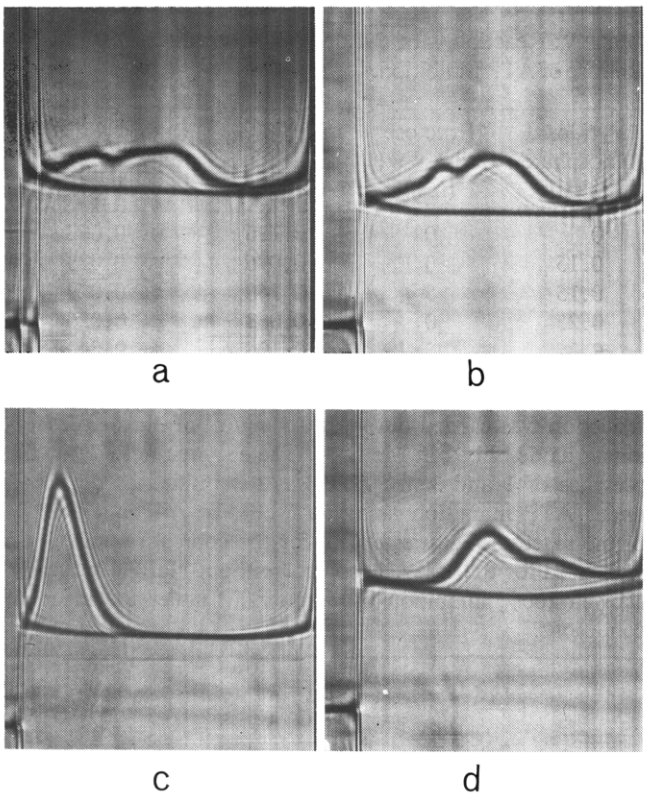

FIGURE 5: Schlieren patterns of phosphopyridoxyl phosphofructokinase. The reaction components and the conditions are described in the text. The concentrations of phosphopyridoxyl residues per mole of enzyme were (a) 0, (b) 3.5, and (c, d) 33 moles. The ultracentrifugation was carried out as in Figure 1. The exposures were taken at (a) 18, (b) 25, (c) 30, and (d) 125 min after reaching the speed.

doxyl enzyme containing greater than 30 residues resulted in diffused and faint bands indicating weaker precipitin reactions with the antibody.

Discussion

The data presented here provide evidence for the involvement of amino groups of lysine residues for the activity of phosphofructokinase. The evidence for the existence of the essential amino groups for its activity is (1) a complete inactivation by maleic anhydride, succinic anhydride, and pyridoxal 5′-phosphate, (2) the protection by fructose 6-phosphate against inhibition by succinic anhydride and pyridoxal 5′-phosphate. It is interesting to note that ATP–Mg^{2+}, however, is completely ineffective in the protection. The result suggests that the amino groups are not essential for the binding of ATP–Mg^{2+}.

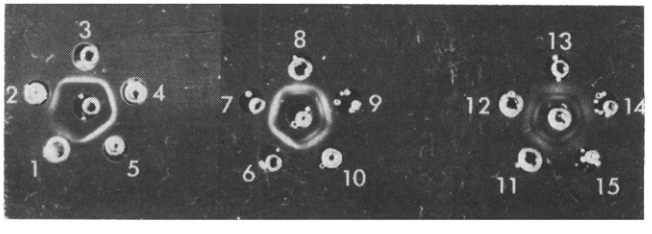

FIGURE 6: Immunoreactivity of phosphopyridoxyl phosphofructokinase. The phosphopyridoxyl phosphofructokinase samples described in Figure 4 were used. Samples (10 μl) of the phosphofructokinase derivatives are pipetted in the outer wells and the goat serum (10 μl) in the center of an agar immunoplate. The concentrations of phosphopyridoxyl groups in moles per mole of the enzyme were (1) and (2) 0, (3) 10, (4) 14, (5) 17, (6) 19, (7) 24, (8) 26, (9) 27, (10) 28, (11) 30, (12) 34, (13) 38, (14) 41, and (15) 42. A considerable amount of precipitate formed in the samples 1 and 2 after dialysis.

It has not been shown conclusively that the maleic anhydride reacts specifically with lysine residues of phosphofructokinase. Possibilities exist that these anhydrides may react with thiol and *o*-tyrosyl groups (Riordan and Vallee, 1964) of the enzyme in addition to ϵ-amino groups of lysine. One would expect, however, the derivatives of these groups (Riordan and Vallee, 1964) to be unstable under the experimental conditions employed.[3] Moreover, it has been reported recently by Butler *et al.* (1967) that maleic anhydride is specific for amino groups of proteins and peptides. The more conclusive evidence for the involvement of ϵ-amino group of lysine in phosphofructokinase activity is the observation that pyridoxal 5′-phosphate, a more specific reagent for lysine, forms a product with the characteristic absorption spectrum for a Shiff base (Figure 3) and results in a concomitant loss of the enzymic activity.

In the experiments designed to determine the number of lysine residues which are essential for the activity, it was found that the substitution of about 30 5′-phosphopyridoxyl residues per mole of enzyme resulted in a complete loss of the activity. This is equivalent to 17% of total lysine residues in the protein based on the value of 180 per mole of the enzyme (Paetkau *et al.*, 1968). In agreement with this result was the observation that the complete loss of activity occurred with 20 moles of maleyl-^{14}C groups substituted per mole of the enzyme. This is equivalent to about 11% of total lysine residues or 1.2 lysines/subunit assuming the enzyme consists of 16 subunits (Paetkau *et al.*, 1968). The loss of the enzymic activity with the substitution of a relatively few maleyl, succinyl, or 5′-phosphopyridoxyl groups suggests that the lysine residues which are essential for the enzymic activity are exposed on the surface of the enzyme molecule.

It is not known whether all these reagents react with the same lysine residues in the molecule. The observation that the inactivation of the enzyme by both succinic anhydride and pyridoxal 5′-phosphate was protected by fructose 6-phosphate suggests that these reagents reacted with the same lysine residues in the molecule.

The molecular weights of various forms of phosphofructokinase from rabbit muscle were summarized recently by Paetkau *et al.* (1968). Dissociated 5′-phosphopyridoxyl phosphofructokinase has the molecular weight of about 190,000 which agrees with the value obtained for the enzyme in 0.8 M urea at pH 5.8 and 0.8 mM fructose 1,6-diphosphate (Paetkau *et al.*, 1968). Based on the high-speed sedimentation equilibrium measurements, the molecular weights of succinyl and maleyl phosphofructokinase were calculated as 78,000. This value may not be accurate, because the same partial specific volume for the enzyme of 0.728 ml/g (Paetkau and Lardy, 1967) was used for the calculation. The partial specific volume of the modified proteins, in fact, may be smaller than that of

[3] It is possible that more extensive dialysis may cause a complete desuccinylation of tryosyl residue resulting in reactivation of the enzyme. This possibility could not be tested because even the native enzyme lost over 90% of the activity by the extensive dialysis.

the enzyme, as in the case of succinyl aldolase. Hass (1964) reported a partial specific volume of 0.704 ml/g for the succinyl aldolase and 0.745 ml/g for the native aldolase. If a similar difference in the partial specific volumes of the modified phosphofructokinase and the native enzyme occur, the molecular weight of the former would be significantly less than 78,000. Until the partial specific volume and its molecular weight of the modified enzyme are known, we cannot decide on the number of subunits in the protein.

Pyridoxal 5′-phosphate is found to be a useful reagent for the determination of the lysine residues in phosphofructokinase with different reactivity. There appeared to be three breaks in the titration curve of phosphofructokinase with pyridoxal 5′-phosphate (Figure 4). The initial 18 lysine residues, equivalent to 1.2/subunit, are highly reactive and the reaction of these residues results in over 80% inactivation of the enzymic activity. The second group of lysines, about 10 residues, is essential for the remainder of the enzymic activity. These results may indicate that the lysine groups which are involved in the enzymic activity are exposed on the surface of the enzyme molecule. The third group of the lysines consists of about 15 residues, which is less reactive. The low reactivity with the reagent may be caused by a steric hindrance effect due to the presence of pyridoxal 5′-phosphate or some other groups on the protein.

An interesting observation is that the reaction with a relatively low concentration of pyridoxal 5′-phosphate results in an alteration in the ATP sensitivity. The results indicate the difference in the reactivity of lysine residues at "allosteric" and at active sites toward pyridoxal 5′-phosphate. We have reported previously (Uyeda and Racker, 1965) a similar change in the ATP sensitivity of phosphofructokinase by aging and chymotrypsin treatment. More recently, Salas *et al.* (1968) showed that the yeast phosphofructokinase could also be desensitized by the chymotrysin treatment.

Immunoreactivity of the modified phosphofructokinase is of interest. The phosphofructokinase with a relatively few maleyl groups shows the positive precipitin reaction with the goat antibody against phosphofructokinase (Figure 2). However, increasing the number of substituents results in the phosphofructokinase derivative with negative precipitin reaction. Correlating the sedimentation patterns of the maleyl phosphofructokinase and succinyl phosphofructokinase and the immunoreactivity, it is possible that the reactivity with the antibody requires a phosphofructokinase protein with a molecular weight of at least 190,000. The dissociated maleyl or succinyl phosphofructokinase with molecular weights of 78,000 shows no precipitin. On the other hand, the subunits with a molecular weight of 190,000 appear to show the positive precipitin reaction. This is based on the observation that phosphopyridoxyl phosphofructokinase shows two precipitin bands (Figure 6), one of which corresponds to that of native phosphofructokinase, and the second band shows a faster rate of diffusion suggesting a smaller size. Judging from the schlieren patterns (Figure 5), this smaller subunit of the phosphopyridoxyl phosphofructokinase has an approximate molecular weight of 190,000.

Acknowledgment

The author thanks Mrs. Nancy Whissen for her skillful technical assistance.

References

Anderson, B. M., Anderson, C. D., and Churchich, J. E. (1966), *Biochemistry 5*, 2893.

Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1967), *Biochem. J. 103*, 78p.

Davis, E. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dempsey, W. B., and Snell, E. E. (1963), *Biochemistry 2*, 1414.

Engelhardt, V. A., and Sakov, N. E. (1943), *Biokhimiya 8*, 9.

Fischer, E. H., Forrey, A. W., Hedrick, J. L., Hughes, R. C., Kent, A. B., and Krebs, E. G. (1962), *in* Proceedings of the International Symposium on Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., Fasella, P. M., Braunstein, A., and Rossi-Fanelli, A., Ed., Rome.

Frenkel, R. (1968), *Federation Proc. 27*, 339.

Froede, H. C., Ahlfors, C. E., and Mansour, T. E. (1968), *Federation Proc. 27*, 339.

Hass, L. F. (1964), *Biochemistry 3*, 535.

Jenkins, W. T., and Singer, I. W. (1957), *J. Am. Chem. Soc. 79*, 2655.

Kaldor, G., and Weingach, S. (1968), *Federation Proc. 25*, 641.

Kemp, R. G., and Forest, P. B. (1968), *Biochemistry 7*, 2596.

Klotz, I. M., and Keresztes-Nagy, S. (1963), *Biochemistry 2*, 445.

Ling, K.-H., Marcus, F., and Lardy, H. A. (1965), *J. Biol. Chem. 240*, 1893.

Paetkau, V., and Lardy, H. A. (1967), *J. Biol. Chem. 242*, 2035.

Paetkau, V. H., Younathan, E. S., and Lardy, H. A. (1968), *J. Mol. Biol. 33*, 721.

Parmeggiani, A., Luft, J. H., Love, D. S., and Krebs, E. G. (1966), *J. Biol. Chem. 241*, 4625.

Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.

Rippa, M., Spanio, L., and Pontremoli, S. (1967), *Arch. Biochem. Biophys. 118*, 48.

Salas, M. L., Salas, J., and Sols, A. (1968), *Biochem. Biophys. Res. Commun. 31*, 731.

Shapiro, S., Enser, M., Pugh, E., and Horecker, B. L. (1968), *Arch. Biochem. Biophys. 128*, 554.

Sia, C. L., and Horecker, B. L. (1968), *Biochem. Biophys. Res. Commun. 31*, 731.

Uyeda, K., and Rabinowitz, J. C. (1965), *J. Biol. Chem. 240*, 1701.

Uyeda, K., and Racker, E. (1965), *J. Biol. Chem. 240*, 4682.

Younathan, E. S., Paetkau, V., and Lardy, H. A. (1968), *J. Biol. Chem. 243*, 1603.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.