Effects of Temperature and Activating Cations on the Fluorescence of Pyruvate Kinase*

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ABSTRACT: The fluorescence emission and polarization spectra of rabbit muscle pyruvate kinase were examined under a variety of conditions. The tryptophyl contribution to the fluorescence of the enzyme in the presence of activating cations, KCl and $MnCl_2$, is reduced when compared to the fluorescence in tetramethylammonium chloride, a nonactivating cation. This confirmed a previous conclusion of F. J. Kayne and C. H. Suelter (*J. Am. Chem. Soc. 87*, 897 (1965)) that the environment of one or more tryptophyl residues was modified as the result of the interaction of activating cations or when the temperature of an aqueous solution was lowered from 30 to 3°. The polarization, being quite low especially in the spectral region between 250 and 300 mμ, was increased by the addition of activating cations or when the solution temperature is lowered to 3°. The evidence indicates that adding KCl and $MnCl_2$ or lowering the solution temperature of pyruvate kinase leads to a structure in which the rotation of the tryptophyl residues is restricted.

Pyruvate kinase (EC 2.7.1.40), regardless of source of isolation (Boyer, 1953), requires both monovalent and divalent cations for catalytic activity. Studies of the interactions of these cations with rabbit muscle pyruvate kinase by a variety of techniques (Suelter *et al.*, 1966; Mildvan and Cohn, 1965, 1966; Sorger *et al.*, 1965) indicates that the enzyme–metal complex has a conformation which is different from that of free enzyme. As a result of this conformational transition, a blue shift in the protein spectrum was observed, which was interpreted to mean that one or more protein tryptophyl residues were brought into an aqueous environment (Kayne and Suelter, 1965 and unpublished data). A similar spectral shift was observed when the temperature of an aqueous solution was lowered from 30 to 0°. Since tryptophyl fluorescence emission is markedly affected by various spectral perturbants (C. H. Suelter and G. Weber, 1967, unpublished data), a study of the fluorescent properties of muscle pyruvate kinase as affected by the presence of cations or by a change in solution temperature was conducted.

Experimental Section

Muscle pyruvate kinase was prepared and characterized according to procedures previously described (Kayne and Suelter, 1965). The fluorescent measurements were made in Professor Gregorio Weber's laboratory with a spectrophotofluorimeter designed and built by him (Weber and Young, 1964). The polarization spectra were obtained with the instrument described by Weber and Bablouzian (1966). The cuvet compartments of both instruments were maintained at constant temperature by means of a circulating water bath. The temperature was determined with a Tri-R thermistor thermometer.

Results

Previous studies of the absorption spectrum of pyruvate kinase lead to the conclusion that the environment of certain tryptophyl residues was modified as a result of the interaction of activating cations, K^+ or Mn^{2+}, or when the solution temperature was changed (Kayne and Suelter, 1965 and unpublished data; Suelter *et al.*, 1966). Similar studies examining the fluorescence emission properties of pyruvate kinase are reported here. Figures 1 and 2 present the fluorescence emission spectrum of pyruvate kinase at the same amplification in the presence of 0.101 M TMA^+,[1] a nonactivating cation, and in the presence of 0.1 M KCl plus 10^{-3} M $MnCl_2$, activating cations, respectively. As suggested by Weber and Young (1964), the emission spectra obtained when protein is excited at 280, 292, and 297 mμ were recorded to yield the same relative fluorescence at 375 mμ. That is, the spectra are essentially normalized to give the same relative tryptophyl fluorescence. Under these conditions the difference between the emission spectrum obtained at 280-mμ excitation and that obtained at 292-mμ excitation represents tyrosyl fluorescence with a peak at 303 mμ. Such difference emission spectra are also given in Figures 1 and 2.

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[1] Abbreviations used: TMA^+, tetramethylammonium cation; TMACl, tetramethylammonium chloride; *q*, quantum yield.

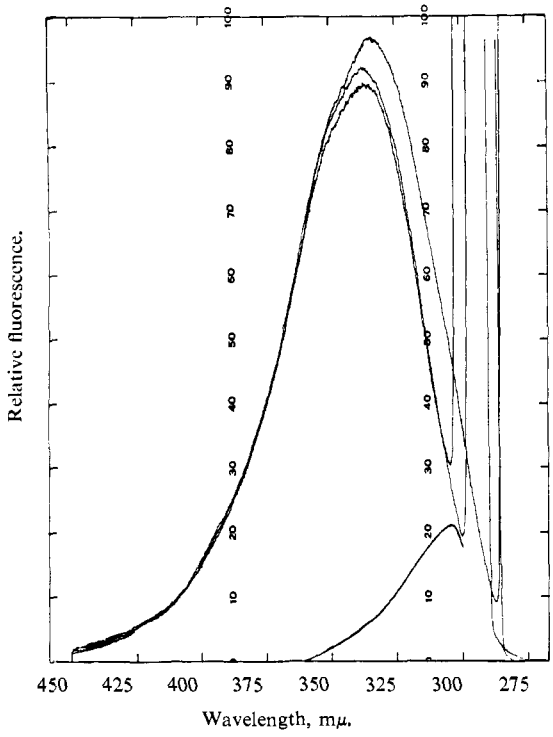

FIGURE 1: The fluorescence emission spectra of pyruvate kinase excited at 280 (upper spectrum), 292, and 297 mμ. The small curve in lower right-hand corner represents the difference in emission when excited at 280 and 292 mμ. Conditions: pyruvate kinase that had previously been passed over Sephadex (G-25) in 5×10^{-3} M Tris·HCl (pH 8.6), 0.9 mg/ml, TMACl (0.101 M), and Tris·HCl (0.05 M, pH 7.8); temperature 30°.

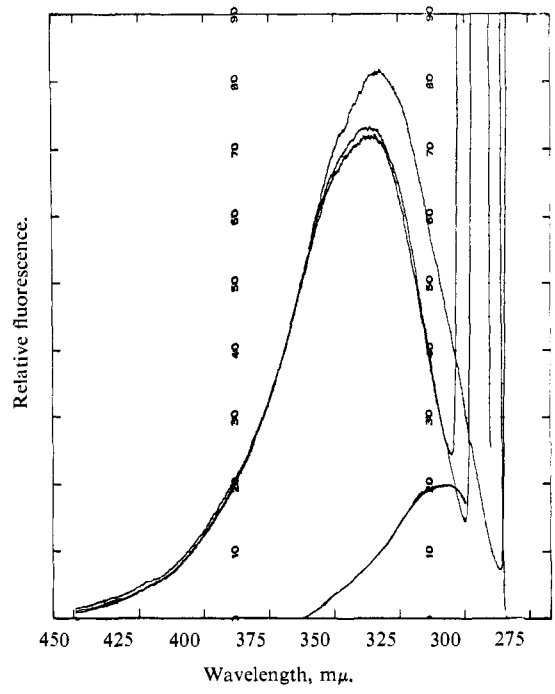

FIGURE 2: The fluorescence emission spectra of pyruvate kinase excited at 280 (upper spectrum), 292, and 297 mμ. The small curve in lower right-hand corner represents the difference in emission when excited at 280 and 292 mμ. Conditions: pyruvate kinase that had previously been passed over Sephadex (G-25) in 5×10^{-3} M Tris·HCl (pH 8.6), 0.9 mg/ml, KCl (0.1 M), $MnCl_2$ (10^{-3} M), and Tris·HCl (0.05 M, pH 7.8); temperature 30°.

Figure 3 presents the algebraic difference between the emission of pyruvate kinase in TMACl and the emission of enzyme in KCl and MnCl both excited at 280 mμ. This emission difference spectrum being essentially independent of exciting wavelength resembles the protein emission spectrum with a broad maximum in the range of 330–350 mμ and a half-band width of 68 mμ. Both of these latter properties are typical of tryptophyl emission. A detailed analysis of the emission spectra of Figures 1 and 2 and the difference emission spectrum of Figure 3 is presented in Table I. The most pronounced difference between the spectra of Figures 1 and 2 is the reduced quantum yield of the protein examined in the presence of activating cations as portrayed by the difference emission spectrum of Figure 3. The quantum yield of fluorescence of a protein in which tryosine and tryptophan contribute has been defined by Weber and Young (1964) as q(protein) = q(Tyr) + q(Trp), where q(Tyr or Trp) = quanta emitted as tyrosyl or tryptophyl fluorescence/quanta absorbed by protein. Since the tyrosyl fluorescence of the enzyme in the presence and absence of activating cations is essentially identical (Table I), the reduction in the quantum yield of enzyme in the presence of activating cations is reflected in the tryptophyl fluorescence.

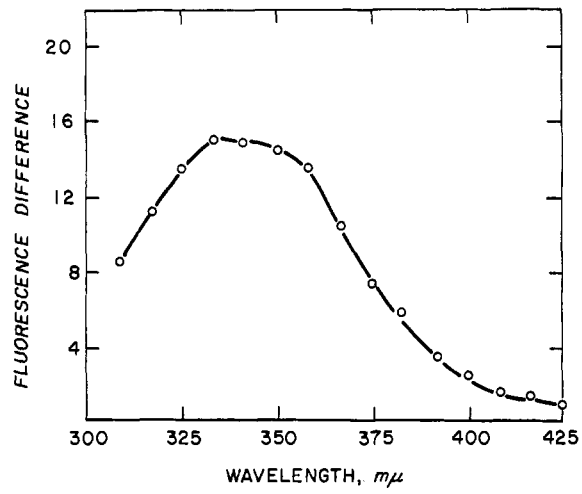

FIGURE 3: The difference between the fluorescence emission of pyruvate kinase in the presence of 0.101 M TMACl (Figure 1, upper curve) and pyruvate kinase in the presence of 0.1 M KCl and 10^{-3} M $MnCl_2$ (Figure 2, upper curve).

TABLE I: Fluorescence Spectral Constants and Quantum Yields of Pyruvate Kinase at 30° in the Presence and Absence of Activating Cations and at 3° in the Absence of Activating Cations.

	Excitation (280 mμ)						Excitation (297 mμ)		
Pyruvate Kinase	λ_{max}[a]	$\lambda_{-1/2}$[a]	$\lambda_{+1/2}$	(q)Tyr	(q)Trp	(q)Protein	λ_{max}	$\lambda_{-1/2}$	$\lambda_{+1/2}$
0.101 M TMACl, 30°[b]	334	304	369	0.027	0.174	0.200	336	312	372
0.1 M KCl–10^{-3} M $MnCl_2$, 30°[b]	334	304	369	0.023	0.142	0.165	336	312	372
0.1 M TMACl, 3°	334	308	370	—	—	—	333	316	376[c]
Fluorescence Difference (Figure 3)	330–350	305	375	—	—	—	330–345[d]	311[d]	370[d]

[a] λ_{max} is the maximum of emission. $\lambda_{-1/2}$ is the wavelength of half-intensity of emission to the short-wave side of λ_{max}. $\lambda_{+1/2}$ is the wavelength of half-intensity of emission to the long-wave side of λ_{max}. The quantum yields were based on tryptophan emission assuming $q = 0.2$. [b] Pyruvate kinase (1.2 mg/ml)–Tris, pH 7.5, 0.05 M. [c] Excitation, 295 mμ. [d] Not shown in Figure 3.

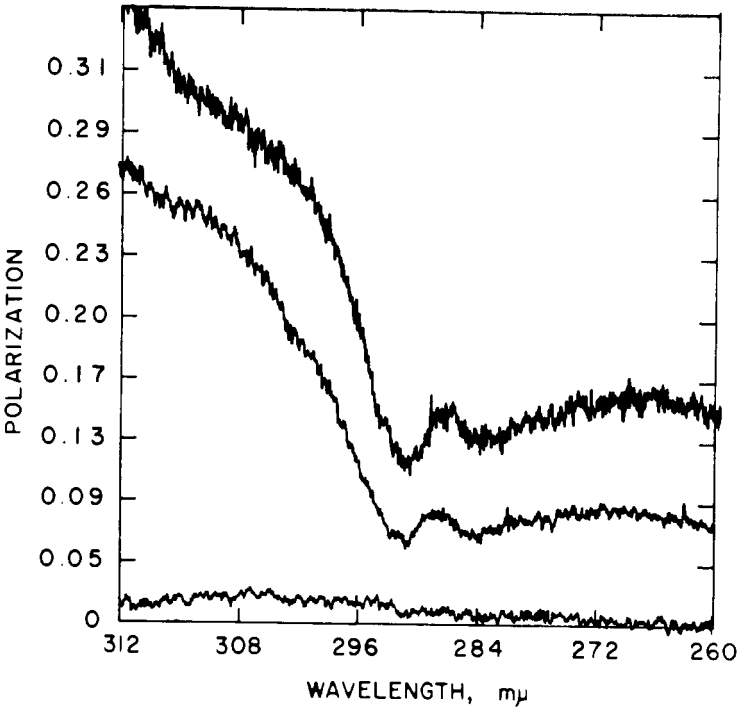

FIGURE 4: Fluorescence polarization spectra of pyruvate kinase in the presence of 0.1 M KCl–10^{-3} M $MnCl_2$ (upper spectrum) and in the presence of 0.101 M TMACl (center spectrum). The lower tracing is obtained with the exciting polarizer positioned to give $I_{||} = I_{\perp}$ or $p = 0$. Conditions: pyruvate kinase that had previously been passed over Sephadex (G-25) in 5×10^{-3} M Tris·HCl (pH 8.6, 1.08 mg/ml), and TMA cacodylate (0.05 M, pH 7.8); temperature, 28°.

The polarization spectra of pyruvate kinase in the presence of activating cations, $MnCl_2$ and KCl, are presented in Figure 4. The polarization is greater at all exciting wavelengths than the polarization observed in TMACl, a nonactivating cation. A blue shift of 2–3 mμ is also observed in the polarization spectra of the cation-activated enzyme. A similar shift in the polarization spectra and increase in polarization is observed when the temperature of an aqueous solution is lowered from 28 to 3° (Figure 5).

Figure 6 shows the polarization spectra of pyruvate kinase in 0.101 M TMACl at 30° in 0, 1.5, 3.0, and 5.0

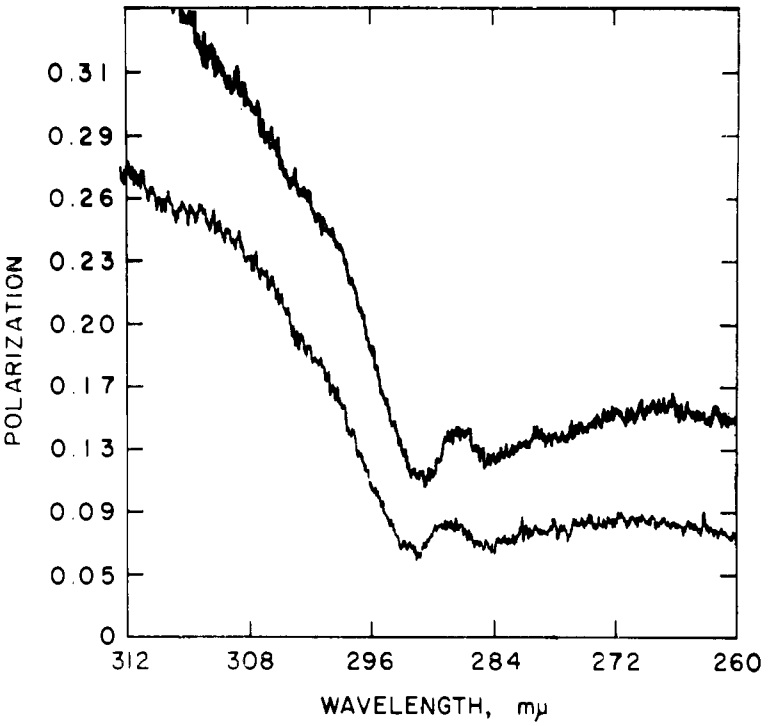

FIGURE 5: Fluorescence polarization spectra of pyruvate kinase at 3 (upper spectrum) and 28° (lower spectrum). Conditions: pyruvate kinase that had previously been passed over Sephadex (G-25) in 5 × 10^{-3} M Tris·HCl (pH 8.6), 1.08 mg/ml, TMA cacodylate (0.05 M, pH 7.8), and TMACl (0.1 M).

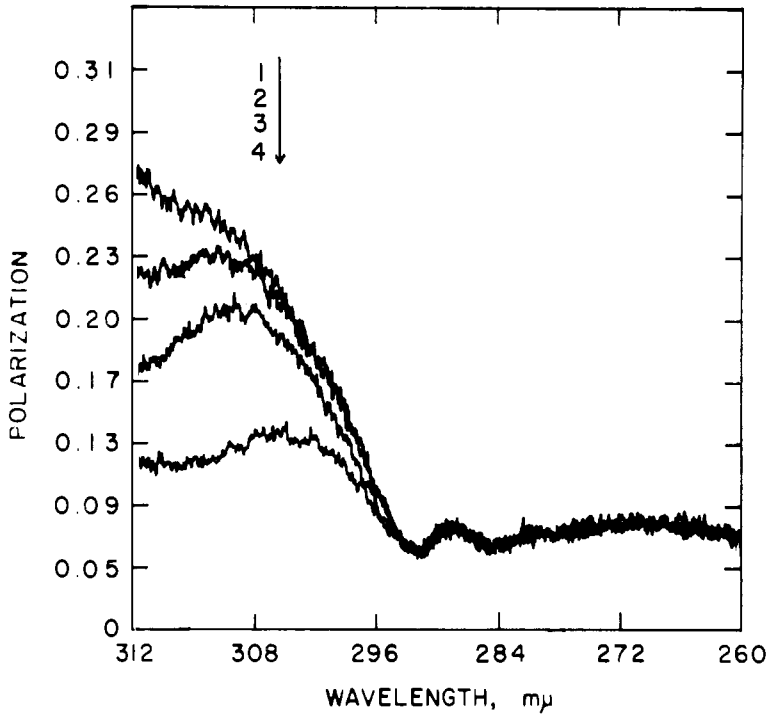

FIGURE 6: Fluorescence polarization spectra of pyruvate kinase in the presence of increasing concentrations of urea. Conditions: pyruvate kinase that had previously been passed over Sephadex (G-25) in 5 × 10^{-3} M Tris·HCl (pH 8.6), 1.08 mg/ml, TMACl (0.1 M), and TMA cacodylate (0.05 M, pH 7.8), temperature, 28°. Spectrum 1, no urea; spectrum 2, 1.5 M urea; spectrum 3, 3 M urea; spectrum 4, 5 M urea.

M urea. The polarization remains constant at all concentrations of urea from 260- to 300-mμ excitation. A reduction in polarization was only observed in the spectral region between 300 and 312 mμ as the concentration of urea was increased.

Discussion

As documented in the introduction, all evidence accumulated to date indicates that muscle pyruvate kinase undergoes a conformational transition as a result of the interaction with activating cations or substrates or when the temperature of an aqueous solution is lowered from 28 to 3°. As a result of this transition an ultraviolet difference spectrum is observed which is typical of a tryptophyl perturbation. The changes in the fluorescence emission of the enzyme when measured under the same conditions are in agreement with the above data. The fluorescence emission difference spectrum (Figure 3) has a broad maximum between 330 and 350 mμ. Such emission is characteristic of tryptophyl fluorescence (C. H. Suelter and G. Weber, unpublished data), confirming the perturbation of one or more tryptophyl residues during the conformational transitions.

Previous analysis of the protein difference spectrum suggested that one or more tryptophyl residues were transferred from a nonaqueous to an aqueous environment during the conformational transition (Kayne and Suelter, 1965). From such a transition one would expect an increase in fluorescence at 350 mμ and a decrease at 332 mμ if no other changes in yield or spectra take place in the remaining tryptophan residues. To reconcile the observed changes (decrease in quantum yield without a change in the emission spectrum, Figure 3), it is necessary to assume changes in the fluorescence characteristics of other tryptophyl residues besides those most responsible for the spectroscopic changes. Nevertheless, the correspondence between the spectrophotometric and the fluorometric observations confirms the existence of at least two different enzyme forms rather than changes in a unique tryptophan residue affected in the process (Kayne and Suelter, 1965 and unpublished data).

The polarization spectra show that essentially the same changes occur when enzyme in TMACl is brought from 28 to 3° (Figure 5) and when TMA^+ is replaced by K^+ and Mn^{2+} (Figure 4). Again the finding may be explained by the existence of at least two different forms that interconvert. The polarization changes may be described as consisting of: (1) a general increase in polarization over the whole range of exciting light, and (2) a shift of 2–3 mμ of the maxima and minima toward shorter wavelengths. The latter change indicates that shifts in the relative positions of the electronic transitions in tryptophan are occurring. The former necessitates the assumption that the tryptophan residues have a local freedom of rotation at 30° in TMACl which they lose when the temperature is lowered to 3° or when TMACl is replaced by KCl and MnCl. To a good approximation this freedom of rotation may be characterized by the mean cosine square of the angle that the emission oscillator would sweep during the lifetime of the excited state. This is given by $(1/P - 1/3) = (1/P_0 - 1/3)(1/(3/2\ \overline{\cos^2}\ \theta - 1/2))$ (Perrin, 1929).

If we consider two forms (A and B) of an enzyme, the equation may be written as $(1/P_A - 1/3)/(1/P_B - 1/3) = 1/\beta$. The parameter β may be taken as a measure of the relative increase in freedom in B as compared to A. If $\beta = 1$, no extra freedom in B as compared to A. If $\beta \rightarrow 0$, complete freedom in B as compared to A. It is interesting that in the conformational transitions brought about by temperature changes or interactions of activating cations β remains essentially independent of exciting wavelength, while in the denaturation brought about by urea (Figure 6) β is strongly dependent on wavelength. Since there is a considerable region of the spectrum (Figure 6) for which $\beta = 1$, the results cannot be interpreted as being due to the increased freedom of rotation of tryptophans but as a result of shifting of electronic transitions of different polarization with respect to each other. Yet the enzyme is completely dissociated into subunits at 4.0 M urea (Steinmetz and Deal, 1966). Thus assuming that the lifetime of the excited states do not change, the tryptophyl residues of the subunits of pyruvate kinase still have essentially the same rotational restrictions as those of native enzyme. It is interesting that the polarization of pyruvate kinase in 0.101 M TMACl at 30° (Figure 4) is also essentially identical with that of dissociated lactic dehydrogenase (Anderson and Weber, 1966).

Brewer and Weber (1966) reported that the addition of Mg^{2+} to yeast enolase resulted in an increase in polarization at all exciting wavelengths between 240 and 300 mμ. A general increase in polarization was also observed with pyruvate kinase after the addition of activating cations (Figure 4) or when the solution temperature was lowered (Figure 5). This increase, apparent at all exciting wavelengths, indicates less freedom in the rotation of the tryptophyl residues suggesting the conversion to a more compact molecule during the transformation. Sedimentation data are also in support of a more compact protein molecule following the interaction of activating cations with yeast enolase (Brewer and Weber, 1966) or pyruvate kinase (F. J. Kayne and C. H. Suelter, 1967, unpublished data).

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Binding of Metabolites by Phosphofructokinase*

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ABSTRACT: The binding of metabolites by skeletal muscle phosphofructokinase has been studied by the gel filtration technique. Adenosine 5′-diphosphate (ADP), adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP), and AMP were bound competitively with dissociation constants of 0.5, 0.6, and 1.8 μM, respectively. Competition studies using a variety of compounds indicated a high specificity for adenine derivatives at this binding site. The dissociation constant for cyclic 3′,5′-AMP was decreased in the presence of fructose-6-P and fructose-1,6-diP. At pH 6.95, the dissociation constant for fructose-6-P with P-fructokinase was 17 μM; the constant decreased at higher pH and in the presence of AMP, inorganic phosphate, and ammonium ion. Adenosine 5′-triphosphate (ATP) inhibited the binding of fructose-6-P and this inhibition was released by inorganic phosphate. Extrapolation of the data for the binding by P-fructokinase of fructose-6-P, AMP, ADP, and cyclic 3′,5′-AMP to maximum binding indicated a molar binding unit of 90,000 g for these compounds, but 3 moles of ATP was found to be bound by this amount of enzyme. Dissociation constants for the ATP binding sites were in the range of 5–15 μM. Citrate increased the affinity of P-fructokinase for ATP and decreased the affinity of the enzyme for AMP and fructose-6-P. The results of the equilibrium binding studies are discussed in relation to the kinetic behavior of P-fructokinase.

Phosphofructokinase represents one of the most complex enzymes in regard to the regulation of its activity by interaction with a variety of compounds. The kinetics of P-fructokinase from a great number of sources have been studied extensively (Mansour, 1963; Passonneau and Lowry, 1962, 1963; Atkinson and Walton, 1965; Lowry and Passonneau, 1966; Uyeda and Racker, 1965; Underwood and Newsholme, 1965; Viñuela *et al.*, 1963). This work has indicated that under appropriate conditions the enzyme is inhibited by a substrate for the reaction, adenosine 5′-triphosphate (ATP),[1] and by citrate and Mg^{2+}. The activity is enhanced by the other substrate, fructose-6-P, by the products of the reaction, fructose 1,6-diphosphate and ADP, and by AMP, cyclic 3′,5′-AMP, inorganic phosphate, and various cations. The sigmoid response of the enzyme to fructose-6-P has prompted Monod *et al.* (1965) to include P-fructokinase in the growing list of allosteric enzymes. Recently, Lowry and Passonneau (1966), in a thorough study of brain P-fructokinase, have suggested that there are at least seven and possibly 12 kinetically important binding sites for substrates, inhibitors, and deinhibitors. Garfinkel (1966), from a computer analysis of the kinetic data of Passonneau and Lowry (1962, 1963) for skeletal muscle P-fructokinase, has indicated that 1 mole of enzyme binds 2 moles of fructose-6-P, 3 moles of ATP, 3 moles of AMP, 5 moles of inorganic phosphate, and 1 mole of citrate.

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[1] Abbreviations used in this work: AMP, ADP, and ATP, adenosine 5′-mono-, -di-, and -triphosphates; cyclic 3′,5′-AMP, adenosine 3′,5′-monophosphate; IMP and ITP, inosine 5′-mono-, and -triphosphates; UMP and UTP, uridine 5′-mono-, and -triphosphates; XMP, xanthosine 5′-monophosphate; CMP, cytidine 5′-monophosphate; GMP, guanosine 5′-monophosphate; glycero-P, β-glycerophosphate; FDP, fructose diphosphate.