THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 10, Issue of May 25, pp. 2268-2279, 1966
Printed in U.S.A.

Kinetic Evidence for Multiple Binding Sites on Phosphofructokinase*

(Received for publication, September 7, 1965

OLIVER H. LOWRY AND JANET V. PASSONNEAU

From the Department of Pharmacology and the Beaumont-May Institute of Neurology, Washington University, St. Louis, Missouri 63110

SUMMARY

For brain phosphofructokinase at pH 8, with noninhibitory levels of adenosine triphosphate, the Michaelis constants for ATP (0.1 mM) and fructose-6-P (0.04 mM) are each independent of the concentration of the second substrate.

Inhibition of P-fructokinase by ATP is highly susceptible to many influences. In addition to the specific effects of NH_4^+, inorganic orthophosphate, AMP, fructose-6-P, and fructose diphosphate, ATP inhibition is affected by pH, the concentration of Mg^{2+}, dimercaptopropanol, and ethylenediaminetetraacetate.

At pH 8, with a high constant level of Mg^{2+}, the velocities can be quantitatively accounted for, over a wide range of levels of both substrates, by a formulation involving two inhibitor sites for ATP and three deinhibitor sites for fructose-6-P, none of which influences the substrate site Michaelis constants for ATP or fructose-6-P.

Free ATP is much more inhibitory than $MgATP^{2-}$. Because of this, and because Mg^{2+} itself is inhibitory, the relationship between Mg^{2+} concentration and activity is complex. At pH 8 an Mg^{2+}:ATP ratio of about 2:1 is optimal except at very low ATP levels; at pH 7 much higher Mg^{2+}:ATP ratios are required for maximal rates. Inhibition by Mg^{2+} is antagonized by AMP.

NH_4^+, P_i, and AMP all increase activity, particularly at inhibitory levels of ATP. The effects are synergistic. At pH 7 with almost complete inhibition by ATP, the results of adding various combinations of the three activators fit a formulation in which the catalytic activity increases progressively as one, two, or three of the activators are bound to the molecule. The formulation requires that the presence of any two of the activators increases the affinity of the enzyme for the third.

ADP and cyclic 3′,5′-AMP appear to act in the same manner as AMP, presumably at the same site. K^+ appears to act in the same manner as NH_4^+ but does not increase enzyme activity to the same degree.

At pH 8 and 27°, P-fructokinase loses half its activity in 4 sec and 95% in 2 min. It can be protected by very low levels of either substrate or either product or by AMP or sulfate. The half-life is doubled by 3×10^{-10} M fructose diphosphate.

The kinetic findings have been interpreted to indicate the presence of not less than seven substrate, inhibitor, and deinhibitor sites (and possibly as many as 12). It would appear that these sites are so arranged that the addition of one inhibitor (ATP) makes it easier to add the other (citrate) and harder to add the deinhibitors, whereas addition of any two deinhibitors makes it easier to add the third and harder to add either inhibitor.

* Supported in part by Grants P-78 from the American Cancer Society and NB-01352 and NB-00434 from the United States Public Health Service.

The rate of conversion of glucose 6-phosphate to pyruvate via the Embden-Meyerhof pathway appears to be controlled at the point of fructose-6-P phosphorylation. Evidence to this effect has been obtained for muscle (1–4), schistosomes (5), flukes (6), yeast (7–9), liver (10), heart (11), diaphragm (12), brain (13), red cells (14, 15), ascites tumor cells (16), kidney cortex, and several solid tumors (17).

It seems probable that the strange kinetic properties of P-fructokinase account for the control mechanism. Lardy and Parks (18) found that adenosine triphosphate at high concentrations inhibits muscle P-fructokinase, and they suggested that ATP might exert control *in vivo*. Inhibition by ATP has also been found for P-fructokinase from schistosomes (5), liver flukes (19), yeast (20, 21), liver (22), bacteria, plant materials, and a wide variety of mammalian tissues (23). A second potent inhibitor is citrate (24–27), which is synergistic with ATP. Thus accumulation of adequate levels of ATP and citrate could shut down the P-fructokinase step.

It was reported earlier (28) that the ATP inhibition can be overcome by small increases in ADP, AMP, and inorganic orthophosphate, all of which must increase in the cell whenever use of ATP exceeds rate production. Likewise NH_4^+, which increases during anoxia in certain tissues, is a potent stimulator of P-fructokinase (22, 29). Three other ATP antagonists are fructose-6-P, 3′,5′-cyclic AMP (19, 28), and fructose diphosphate (28). The fact that both fructose-6-P and fructose diphosphate deinhibit creates a situation in which small deviations in fructose-6-P level can be rapidly corrected. Cyclic AMP may not be of significance for P-fructokinase in the mammal, but it is probably an important regulatory factor in certain lower forms such as the fluke (19). In spite of the length of the list of activators and deinhibitors, high specificity is shown by the fact that among

the common nucleotide mono- and diphosphates only 5′-adenyl derivatives are active.

Attempts to make a definitive study of the kinetics of P-fructokinase are complicated by the following interesting difficulties. (These difficulties apply specifically to this enzyme from brain, but are probably shared by other mammalian P-fructokinases.) (*a*) There appear to be a multiplicity of inhibitor and deinhibitor sites (20, 26, 28, 30). (*b*) One substrate is an inhibitor; the other substrate and both products are deinhibitors. Fructose diphosphate is active at such low concentrations that rate can depend more on how fast fructose diphosphate is removed than on how much enzyme is present. (*c*) Under some conditions the inhibitor constant for ATP is lower than the Michaelis constant. (*d*) Mg^{2+} is moderately inhibitory, but ATP combined with Mg^{2+} is much less inhibitory than free ATP. Consequently, changes in ATP can have opposing inhibitory and deinhibitory effects which are difficult to disentangle. (*e*) Trace amounts of a variety of substances can affect enzyme activity; *e.g.* 0.2 mM SO_4^{2-} or 0.01 mM ethylenediaminetetraacetate has distinct effects. (*f*) The enzyme is exceedingly unstable in the absence of protecting agents. At pH 8, for example, 50% of the activity is lost in 4 sec, and activity is lost even more quickly at pH 7. Protecting substances alter the kinetic parameters. (*g*) With few exceptions, the concentration of each of the inhibitors or deinhibitors influences the apparent dissociation constants for all the rest.

Because of these problems, it cannot be claimed that the results to be presented establish exact mechanisms for P-fructokinase kinetics, but they make it seem inescapable that there exists in the enzyme a complex system of interacting binding sites for substrates, inhibitors, and deinhibitors.

MATERIALS AND METHODS

Enzyme Preparations—The studies were performed with partially purified P-fructokinase from sheep brain. All preparative operations, unless noted, were carried out at 0–4°, and centrifugation was performed at 10,000 × *g* for 15 to 20 min. In calculating yield, mechanical losses such as entrapment of fluid in precipitates have been disregarded. Precipitates were dissolved and dilutions were made in 0.1 M phosphate buffer (4:1 ratio of K_2HPO_4 and NaH_2PO_4) containing 1 mM EDTA and 0.25 mM 5′-AMP. The early steps are adapted from Ling, Byrne, and Lardy (31). Values given for enrichment and yield are cumulative.

Sheep brain (5.8 kg) was homogenized in a Waring Blendor with 4 volumes of 0.02 M Tris-HCl, pH 8, containing 0.06 M $MgCl_2$. After centrifugation, the supernatant fluid (2.5-fold enrichment, 80% yield) was brought to an ethanol concentration of 20% (v/v) at 0° to −5°. The resultant precipitate was suspended in 2 liters of the phosphate diluent and centrifuged, and the supernatant fluid was extracted by shaking three times with 2 volumes of ethyl ether and three times with 2 volumes of petroleum ether (8.1-fold enrichment, 56% yield). The volume was brought to 2.1 liters (1% protein) and protamine sulfate was added, first to 0.3% concentration (precipitate discarded), then to 0.65%. This second precipitate was suspended in 360 ml of the phosphate diluent and centrifuged. In the supernatant fluid P-fructokinase, enrichment was 29-fold with 22% yield. The protamine precipitation was repeated; the fraction not precipitating at 0.24% was discarded and that precipitating at 1% was saved (48-fold enrichment, 11% yield). To this solution (46 ml) were added 28 ml of 0.8% $Ca_3(PO_4)_2$ gel (Sigma; gel to protein ration, 0.5:1). Most of the activity was adsorbed and was then eluted with 24 ml of 0.2 M phosphate, pH 7.5, containing 1 mM AMP (60-fold enrichment, 8% yield). The gel treatment was repeated by diluting the eluate with an equal volume of 0.8% gel. Since phosphate concentration was now higher, the activity did not adsorb until a second volume of 0.8% gel was added to the first supernatant fluid. Three successive eluates, made with 24 ml each of the 0.2 M phosphate solution, were combined and precipitated with 3 M $(NH_4)_2SO_4$ at pH 7.5 (106-fold enrichment, 5% yield). This was then fractionated with $(NH_4)_2SO_4$ at pH 7.5. The fraction soluble in 1.4 M (7.5 ml total volume) and precipitating at 2.0 M is designated Preparation A (238-fold enrichment, 4% over-all yield). A second, smaller Preparation B was made in a similar manner, but in this case the richest fraction was that which precipitated between 1.0 and 1.4 M $(NH_4)_2SO_4$. Both preparations had activity of about 18 moles per kg of protein per min at pH 8 and 27° under optimal conditions. This activity is 6 times that of a brain P-fructokinase preparation of Muntz (32) and is 12 to 15% of that of pure enzyme from skeletal muscle (33, 34) or heart (35). The preparations were stored in the phosphate diluting fluid at −80° with not more than 30% loss in 6 months in spite of frequent thawing and refreezing.

Rate Measurements—The rate measurements were made in the Farrand model A fluorometer at about 27°. The conditions of assay are unusually critical. Since both products can accelerate the reaction, their concentrations must be kept low, either by rapid removal or by measuring rates with very small total product formation. Fluxes must also be kept small in order to measure rates at the very low substrate levels necessary for demonstration of certain kinetic features. Either the DPNH used in the assay system must be carefully freed of 5′-AMP, which is a common contaminant,[1] or the DPNH level must be kept very small. For these reasons, if the assays had been carried out in the spectrophotometer, which requires much higher reaction rates and DPNH levels than the fluorometer, and if unpurified DPNH had been employed, some of the kinetic properties of P-fructokinase might not have been observed. Since both NH_4^+ and sulfate can enhance activity at levels below 1 mM, auxiliary enzymes must be freed of $(NH_4)_2SO_4$; otherwise this also will obscure kinetic properties.

Usually the reaction was carried out in the presence of crystalline aldolase (Sigma), triose-P isomerase, glycero-P dehydrogenase, P-glucose isomerase (all from Boehringer), and 0.008 mM DPNH. Sufficient amounts of the enzymes were used to convert 95% of added fructose diphosphate to glycero-P within 1 min (half-time, 10 to 20 sec). Since 2 moles of DPNH are oxidized per mole of fructose diphosphate formed, initial velocities could be obtained during the phosphorylation of as little as 1 μmole of fructose-6-P per liter. This kept ADP from accumulating to a disturbing degree, but fructose diphosphate concentration is a more critical factor. The steady state level depends chiefly upon aldolase activity. Since this enzyme operates in the assay system with substrate well below K_m, the Michaelis-Menten equation reduces to $v = V(S)/K_m$. Therefore, the steady state substrate level $(S) = vK_m/V$. For muscle aldolase at 27° (either pH 7 or 8), $K_m = 3$ μM; commercial prep-

[1] Commercial DPNH preparations contain 5 to 15% 5′-AMP on a molar basis. For directions for removal of this with alkaline phosphatase, see Reference 13.

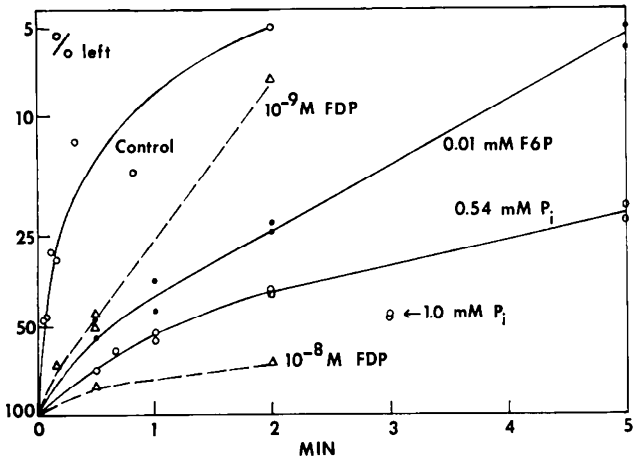

FIG. 1. Loss of activity of P-fructokinase in 0.02 M Tris buffer at pH 8. A stock dilution of enzyme (Preparation A) was made with 10 mM P_i, 0.1 mM AMP, and 0.1 mM EDTA present to stabilize activity. At zero time 1.2 μl of this (total protein, 0.04 μg) were added to 1 ml of solution in a fluorometer tube. (The high dilution reduced the concentration of stabilizers to almost negligible levels.) The control solution contained, in addition to the buffer, 7.5 mM $MgCl_2$, 0.01% bovine plasma albumin, and 0.008 mM DPNH. The other additions are as shown on the figure. *FDP*, fructose diphosphate; *F6P*, fructose-6-P. At the times indicated, 3 μl were added of a mixture of the four auxiliary enzymes (see "Materials and Methods"), which contributed sufficient $(NH_4)_2SO_4$ (0.7 mM) to stabilize activity thereafter. After another 10 sec, the reaction was started by adding 5 μl of a mixture of ATP, glucose-6-P, P_i, and 5'-AMP to give final concentrations of 0.1, 0.5, 2.5, and 0.1 mM, respectively. For zero time samples, P-fructokinase was added after the auxiliary enzyme mixture.

arations have exhibited values for V of about 10 μmoles per mg of protein per min. Therefore, with 20 μg of protein per ml, as used in most of these studies, V would be 200 μmoles per liter per min. At a P-fructokinase velocity of 1 μmole per liter per min, the fructose diphosphate steady state level would be (1/200) $\times$ 3 μM = 0.015 μM. This is satisfactory, but levels of 0.1 μM would accelerate the velocity significantly under certain conditions; consequently it was necessary to make certain that aldolase preparations retained full activity. As an added precaution, in almost every instance duplicate velocity measurements were made with two levels of P-fructokinase. If the higher level gave a disproportionately high rate, the measurements were repeated with lower enzyme levels, or the experiment was revised.

To study the effect of fructose diphosphate, the rate of formation of ADP, rather than of fructose diphosphate, was measured. Again the disappearance of DPNH was followed directly. In this case the system contained pyruvate kinase, lactic dehydrogenase, and P-pyruvate (36) as well as P-glucose isomerase. For control samples it was also necessary in certain cases to add aldolase to reduce the accumulation of fructose diphosphate. This is effective, even though triose phosphates are not removed, since at 27° the equilibrium constant is such (37) that 1 μM dihydroxyacetone-P and 1 μM glyceraldehyde-P are in equilibrium with about 0.02 μM fructose diphosphate.

RESULTS

Stabilizers—Brain P-fructokinase is exceedingly unstable in simple Tris or imidazole buffers, but may be stabilized by a number of substances, including both substrates and both products. A similar situation has been found for the enzyme from skeletal muscle (33) and heart (38). (The instability, particularly below pH 7, and the protective effect of divalent anions were described much earlier by Colowick (39), Utter (40), and Taylor (41).)

Instability was studied with brain P-fructokinase at high dilution in 0.02 M Tris-HCl at pH 8. Half of the activity was lost at 25° in 4 to 5 sec (Fig. 1). The decay rate was not strictly first order, but fell off with time as though the population of enzyme molecules were heterogeneous in this respect. At the end of 2 min only 5% of the original activity remained.

The most potent stabilizer (fructose diphosphate) increased the half-time 4-fold at a concentration of 10^{-9} M (Fig. 1), and more than 100-fold at 10^{-6} M (not shown). A number of substances were tested as possible protecting agents. In the case of the eight most potent, the concentrations required to double the inactivation half-time were: fructose diphosphate, 3×10^{-10} M; fructose-6-P, 5×10^{-7} M; ATP, 1×10^{-6} M; ADP, 3×10^{-6} M; glucose-6-P, 3×10^{-6} M; sulfate, 1×10^{-5} M; 5'-AMP, 1.5×10^{-5} M; and P_i, 3×10^{-5} M. All of these except glucose-6-P and 5'-AMP were reported by Ling *et al.* (33) to stabilize muscle P-fructokinase, and the first four in the list, as well as 5'-AMP, were found by Mansour (38) to reactivate the cardiac enzyme. If it is assumed that these substances act by combining with the enzyme, then the concentration of each which doubles the half-life is equal to or greater than the dissociation constant. These apparent dissociation constants are surprisingly small, particularly in the case of the substrates and products of the kinase. The effectiveness of glucose-6-P was not due to conversion to fructose-6-P. The same degree of protection was observed with Na_2SO_4 and with $(NH_4)_2SO_4$; ammonium acetate was inactive at 4 mM. Citrate (0.8 mM) and Mg^{2+} were almost without effect. EDTA at low concentrations (10^{-6} M) doubled or tripled the half-life, but higher concentrations were not more effective; consequently, EDTA is unable to give the prolonged protection characteristic of all eight substances above. An increase in ionic strength may accelerate inactivation, as shown by the following. Sufficient sulfate (0.5 mM) was added to prolong the half-life to 20 min. When, in addition, potassium acetate was added to give a concentration of 0.15 M, the half-life was reduced to 0.8 min. As reported by others (33, 38–41), inactivation is favored by lowering the pH. With brain enzyme (partially stabilized by sulfate), inactivation was 5 or 10 times faster at pH 7 in 0.02 M imidazole than in 0.02 M Tris-HCl at pH 8.

After inactivation of cardiac P-fructokinase at pH 5.8, Mansour (38) was able to obtain reactivation at pH 8 in the presence of the substrates or products of the enzyme. In the present instance there has been no sign of reactivation in the fluorometer tube even though ample quantities of the substrates, as well as P_i and AMP, were present. If inactivation is due to cleavage of a dimer, as Mansour's data indicate, then reactivation would not be anticipated at the extreme dilution of the present experiments. In fact, Mansour demonstrated a concentration effect under certain reactivation conditions.

Kinetic Constants for ATP and Fructose-6-P—It is necessary to distinguish between combinations of ATP and fructose-6-P at substrate sites and those at nonsubstrate sites; the terms "Michaelis constant" or "K_m" will be restricted to combinations at substrate sites.

The most complete studies were made at pH 8 rather than pH 7, because the inhibitory effects of ATP are so great at pH 7

that it is difficult to determine the Michaelis constant for uninhibited enzyme. The instability of the enzyme and the fact that the substrates act as stabilizers presented an additional problem. Without some nonsubstrate stabilizer, kinetic studies would be uninterpretable, since kinetic and stabilization phenomena would be intermingled. No stabilizer has been found which does not have some effect on the kinetics, but a compromise substance seemed to be sulfate, which is an excellent stabilizer, yet seems to have relatively minor effects on kinetic constants. Therefore, 2 mM Na_2SO_4 has been used in all experiments to be reported.

At pH 8 in 0.02 M Tris, the K_m for ATP is about 0.1 mM and is independent of fructose-6-P concentration (Figs. 2 and 3). The K_m for fructose-6-P is 0.04 mM and is similarly independent of ATP concentration. (Note that the same values for K_m are obtained directly from reciprocal plots, as from plots of the intercepts.) Thus the kinetic evidence indicates random order of addition without interaction between the two substrate sites. (In the case of the yeast enzyme, Viñuela, Salas, and Sols (20) found evidence of interaction since each substrate raised the Michaelis constant for the other.)

Although normal kinetics is observed at low ATP levels, inhibition becomes evident with ATP concentrations of 0.2 mM and above (Fig. 4). Inspection of Fig. 4, as well as consideration

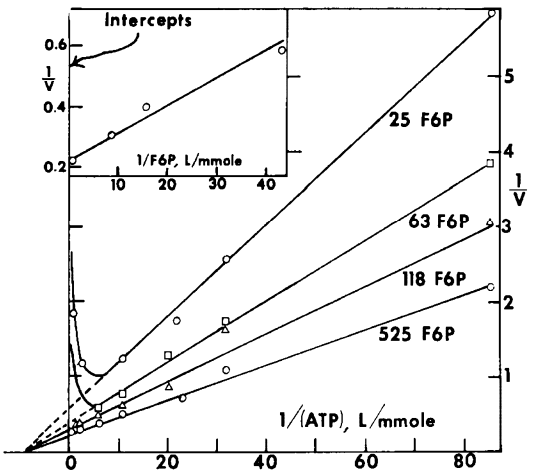

FIG. 2. P-Fructokinase activity at pH 8 as a function of noninhibitory ATP concentrations. The medium was 1 ml of 0.02 M Tris-HCl buffer containing 7.5 mM $MgCl_2$, 0.01% bovine plasma albumin, 2 mM Na_2SO_4, and 0.008 mM DPNH. The desired amount of ATP was added in a small volume; this was followed by 3 μl of a mixture of aldolase, triose-P isomerase, glycero-P dehydrogenase, and P-glucose isomerase to provide final concentrations of 20, 1.5, 7, and 1.5 μg per ml, respectively. The enzyme mixture was dialyzed before use to eliminate $(NH_4)_2SO_4$. A series of enzyme dilutions (Preparation A) were made beforehand in a stabilizing solution consisting of 10 mM phosphate buffer (pH 7.5), 0.1 mM AMP, 0.1 mM EDTA, and 0.02% bovine plasma albumin. For each assay, 1.5 μl of a suitable dilution were added to the reaction mixture. In this way any possible influence of the minute quantities of the components of the stabilizing solution would be held constant. Exactly 2 min after addition of P-fructokinase, the reaction was started by adding glucose-6-P. Rates were not allowed to exceed 1 μmole per liter per min. Fructose 6-phosphate (*F6P*) concentrations are micromolar, and were calculated from the equilibrium ratio for glucose-6-P to fructose-6-P at 25°, *i.e.* 3.35:1 (42). Velocities are calculated as moles per kg of protein per min. The K_m for ATP from the *larger part* of the graph is 0.104 mM; the K_m for fructose-6-P from the plot of the intercepts is 0.04 mM.

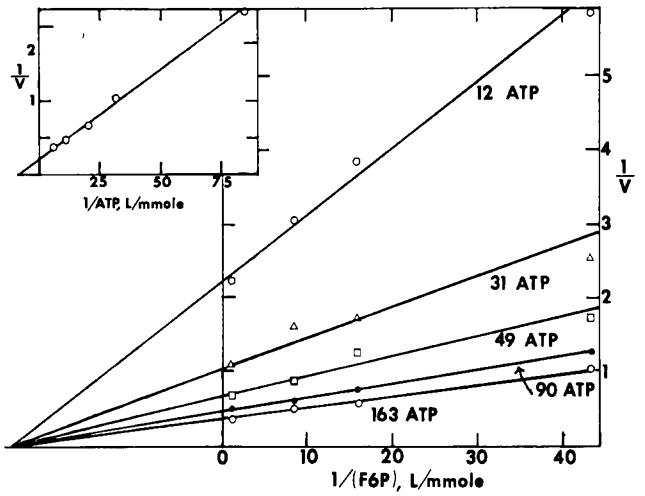

FIG. 3. P-Fructokinase activity at pH 8 as a function of fructose 6-phosphate (*F6P*) concentration. The conditions are described in Fig. 2. ATP concentrations are micromolar. K_m for fructose-6-P is determined to be 0.040 mM; K_m for ATP from the plot of the intercepts is 0.10 mM.

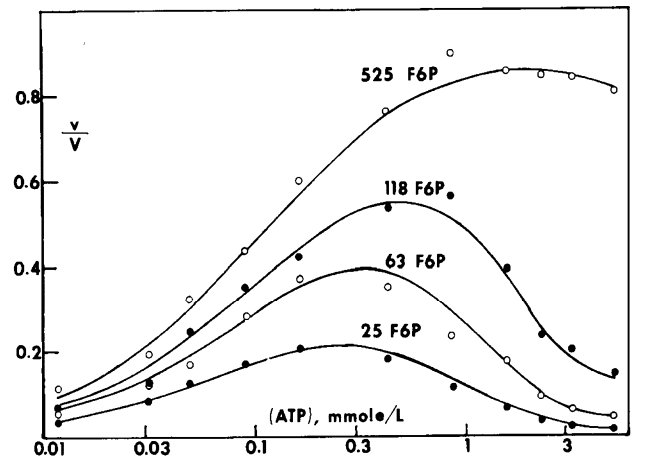

FIG. 4. P-Fructokinase activity as a function of ATP at inhibitory and noninhibitory levels. The conditions are those of Fig. 2. Velocity is plotted as a fraction of the maximum velocity with respect to a logarithmic plot of ATP concentration. Fructose 6-phosphate (*F6P*) concentrations are micromolar. The *points* are observed; the *curves* are calculated for

$$\frac{v}{V} = \frac{[250(\text{F-6-P})(\text{ATP})]}{[(1 + 25(\text{F-6-P})(1 + 10(\text{ATP}))]} \times \frac{[1 + 218(\text{F-6-P})^2 + 1070(\text{F-6-P})^3]}{[1 + 218(\text{F-6-P})^2 + 1070(\text{F-6-P})^3 + 0.85(\text{ATP}) + 1.39(\text{ATP})^2]}$$

where F-6-P denotes fructose 6-phosphate.

of earlier data, makes it clear that, when ATP is present at inhibitory levels, fructose-6-P not only can overcome this inhibition but must do so by acting at more than one site. Thus, at the highest level of ATP shown, a 20-fold increase in fructose-6-P results in a 50-fold increase in velocity. Fig. 3 has shown that with noninhibitory ATP levels reciprocal plots of velocity with respect to fructose-6-P concentration are linear even at high fructose-6-P levels. Therefore, if at higher levels fructose-6-P is adding to nonsubstrate sites, such addition must affect neither its own Michaelis constant nor the maximum velocity. Simi-

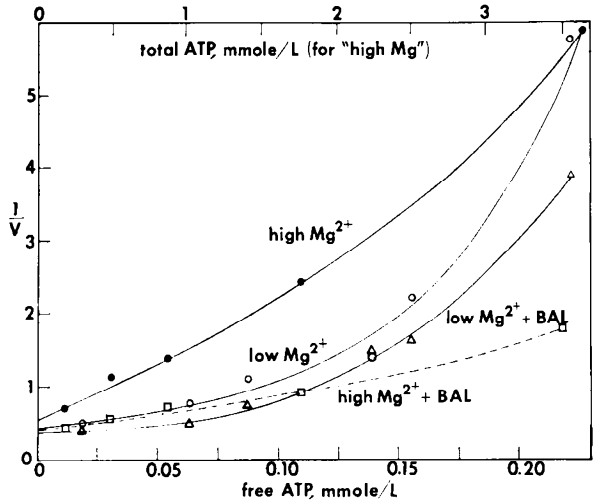

FIG. 5. Inhibition of P-fructokinase by free ATP and $MgATP^{2-}$. The analytical conditions were as described in Fig. 2 except that enzyme Preparation B was used. Fructose-6-P concentration was 0.025 mM. For the curves labeled *high* Mg^{2+}, Mg^{2+} concentrations were 2 mM plus 1.1 times ATP concentration. The *upper scale* applies to these curves. For the *low* Mg^{2+} curves, Mg^{2+} concentration was held constant at 0.2 mM. The *lower scale* applies to these curves. Free ATP was calculated on the basis of a stability constant for $MgATP^{2-}$ of 38,000 M^{-1} (43). 2,3-Dimercaptopropanol (*BAL*), when present, was 0.18 mM. Velocities are calculated as moles per kg of protein per min.

larly, Fig. 2 shows that the K_m for ATP is the same at all levels of fructose-6-P, whether deinhibitor sites are occupied by fructose-6-P or not. If the deinhibitor sites for fructose-6-P do not influence the substrate kinetic constants, it seems possible that ATP at inhibitory sites may result in over-all inhibition without affecting the Michaelis constants for either ATP or fructose-6-P.

Assuming this to be the case, the situation would be as follows.

$$\frac{v}{V} = \frac{\dfrac{(F)(A)}{K_F K_A}}{\left(1 + \dfrac{(F)}{K_F}\right)\left(1 + \dfrac{(A)}{K_A}\right)} \times \frac{1 + f(F)}{1 + f(F) + f(A)} \qquad (1)$$

where v, V, F, A, K_F, and K_A represent velocity, maximum velocity, fructose-6-P, ATP, and the Michaelis constants for fructose-6-P and ATP, respectively, and $f(F)$ and $f(A)$ represent functions of F and A. Equation 1 states that the velocity is equal to that expected for random order of addition in the absence of inhibitor multiplied by an inhibitor term.

The inhibitor term was calculated for all inhibited values of Fig. 4 by dividing each observed value by the value that would be expected in the absence of inhibition, *i.e.* that calculated from the first portion of Equation 1. Plots of these inhibitor terms against fructose-6-P, when extrapolated to zero fructose-6-P concentration, gave values for $(1 + f(A))$ at a series of ATP levels. These gave a reasonable fit to $f(A) = 0.85(A) + 1.4(A)^2$. Substitution of values for $f(A)$ back into the inhibitor terms gave a fit for $f(F) = 218F^2 + 1070F^3$.

In what follows it is assumed (*a*) that P-fructokinase can combine with 1 or 2 molecules of ATP at inhibitor sites to give Ea_1, Ea_2, and Eaa, and is completely inactive in each case; (*b*) that it can combine with 1, 2, or 3 molecules of fructose-6-P at deinhibitor sites to give Ef_1, Ef_2, Ef_3, Ef_1f_2, Ef_1f_3, Ef_2f_3 and $Efff$, and is in each case fully active (within the limits set by the Michaelis constants); and (*c*) that it cannot combine at the same time with both ATP at an inhibitor site and fructose-6-P at a deinhibitor site. According to these assumptions, the term $f(A)$ represents the ratio of the sum of all forms with ATP at inhibitor sites to free enzyme, E. By "free enzyme" is meant enzyme containing neither ATP nor fructose-6-P at other than substrate sites.

Thus

$$f(A) = \frac{Ea_1 + Ea_2 + Eaa}{E} = \frac{(A)}{K_{a_1}} + \frac{(A)}{K_{a_2}} + \frac{(A)^2}{K_{a_1}K_{a_2}^{a_1}} \qquad (2)$$

Since

$$\frac{(E)(A)}{(Ea_1)} = K_{a_1}$$

$$\frac{(E)(A)}{(Ea_2)} = K_{a_2}$$

and

$$\frac{(Ea_1)(A)}{(Eaa)} = K_{a_2}^{a_1}$$

From the evaluation above of $f(A)$, the sum of the two first order terms of Equation 2, $A/K_{a_1} + A/K_{a_2} = 0.85A$. The data do not permit separate evaluation of K_{a_1} and K_{a_2}, but if they should be equal they would each have the value of (2/0.85) mM = 2.4 mM. Similarly, the second order term of Equation 2, $(A)^2/K_{a_1}K_{a_2}^{a_1} = 1.4\,(A)^2$. If $K_{a_1} = 2.4$ mM, $K_{a_2}^{a_1} = (1/2.4 \times 1.4)$ mM = 0.3 mM; *i.e.* occupancy of one site increases the affinity for the other. A similar evaluation of $f(F)$ indicates that no K_f is small enough to be significant (smaller than 0.5 mM). If the three K_f^f values should be the same, each would be equal to 0.096 mM. In this event K_f^{ff} is calculated to be 0.097 mM; *i.e.* occupancy of one deinhibitor site makes addition easier at both of the other sites.

Comparison of observed and calculated values (Fig. 4) shows satisfactory fit, but of course this does not prove that the present formulation is the correct one; it is only one possibility.

There are several facts which urge caution in interpretation of the results so far presented. In these experiments Mg^{2+} was held constant at 7.5 mM. As will be shown below, Mg^{2+} is itself inhibitory and free ATP is much more inhibitory than $MgATP^{2-}$; consequently, as ATP was increased, there would be some decrease in Mg^{2+} as well as some disproportionate increase in free ATP. In addition, at pH 8 in Tris buffer, both 2,3-dimercaptopropanol (Fig. 5) and EDTA (not shown) can enhance activity, particularly at high ATP levels. This could mean that inhibitory metals were present in the reagents—a possibility that has proven difficult to rule out. On the other hand, the fact that trace amounts of relatively weak chelating agents are strong deinhibitors raises the possibility that 2,3-dimercaptopropanol and EDTA act on the enzyme itself.

In any event an experiment is presented (Fig. 5) which shows that under different circumstances there is less striking evidence of an exponential term for ATP inhibition; *i.e.* there is less deviation from linearity in a plot of ATP with respect to $1/v$. In this experiment, a constant Mg^{2+} excess of 2 mM over the ATP concentration was maintained. In addition a different enzyme preparation was used which was less sensitive to ATP inhibition.

(Differences in ATP sensitivity among different brain portions have been demonstrated before (23).)

Inhibition by Free ATP—In confirmation of the original observations of Lardy and Parks (18), when Mg^{2+} levels are less than those of ATP, kinase activity is greatly inhibited (Fig. 6). Thus ATP is much more inhibitory when free than when combined with Mg^{2+}. The figure shows, however, that $MgATP^{2-}$ is also inhibitory since inhibition does not continue to diminish as Mg^{2+} is increased to high levels. At pH 7.3, Mg^{2+}:ATP ratios of 5:1 or more are required for maximal activity (Fig. 6). The pH shift in Mg^{2+} requirement is far more than would be required to compensate for the change with pH in the ratio of ATP^{4-} to $HATP^{3-}$ if the problem were merely to bind the same total amount of ATP. This suggests that possibly free $HATP^{3-}$ is an exceedingly potent inhibitor. (At the Mg^{++} and ATP concentrations in the range of these studies, only $HATP^{3-}$, ATP^{4-}, and $MgATP^{2-}$ would be expected to be significant forms of ATP (43).)

Experiments with ATP variation at high and low Mg^{2+} levels permit a better assessment of the relative inhibitory capacity of free ATP and of $MgATP^{2-}$ (Fig. 5). With a constant *low* level of Mg^{2+} (0.2 mM), ATP was varied from 0.09 to 0.4 mM. In this case the levels of $MgATP^{2-}$ were too low to produce much inhibition and, except at the lowest levels of free ATP, were nearly constant (0.15 to 0.2 mM). A level of 0.09 mM free ATP produced 50% inhibition, whether or not 2,3-dimercaptopropanol was present. An exponential inhibitory function is evident. With a high level of Mg^{2+} (2 mM constant excess over ATP), 0.5 mM total ATP was required to produce 50% inhibition; with 2,3-dimercaptopropanol present, 1.3 mM ATP was required for the same inhibition (Fig. 5). The amount of free ATP in this case was probably negligible. (At the highest ATP concentration, it is calculated to be 0.04 mM, which, according to the figure, would produce only 30% inhibition.) Thus, under the conditions of these experiments, free ATP is 6 to 14 times more effective than $MgATP^{2-}$ as an inhibitor. Garfinkel has concluded from a computer analysis of earlier data for muscle enzyme that free ATP is 35 times more effective than $MgATP^{2-}$ (44).

Inhibition by Mg^{2+}—Mg^{2+} appears to be an inhibitor, at least at pH 8 (Fig. 6). Linear plots are obtained for $1/v$ with respect to Mg^{2+} concentration at high Mg^{2+} levels (not shown). The K_i at pH 8 is 4 to 5 mM and is little affected by fructose-6-P or ATP levels. Mg^{2+} inhibition is reduced by high levels of P_i (Table I). The possibility that this is due to direct Mg^{2+} binding has not been excluded. AMP, however, is an effective deinhibitor at low levels, and this antagonism to Mg^{2+} probably accounts for most of the enhancement by AMP at pH 8. (This is definitely not the case at pH 7.) NH_4^+ and fructose-6-P have little or no antagonism for Mg^{2+} inhibition (Table I).

Other Inhibitors—Ca^{2+}, like Mg^{2+}, is inhibitory. This has not been studied thoroughly, but at pH 8 under the conditions of Fig. 2 (in the presence of 7.5 mM Mg^{2+}, 0.09 mM ATP, and 0.12 mM fructose-6-P) a linear plot was obtained for Ca^{2+} concentration with respect to $1/v$, with 50% inhibition at 1.05 mM Ca^{2+} (not shown). Under these same conditions K_i for Mg^{2+} was found to be 4 mM. If Ca^{2+} and Mg^{2+} compete for the same site, K_i for Ca^{2+} may be calculated to be 0.37 mM, which would make it 10 times more inhibitory than Mg^{2+}. The possibility that the inhibition is due to competition for ATP has not been ruled out.

Zn^{2+} and Cu^{2+} are very inhibitory. At pH 8 the levels which inhibited 50% were 2×10^{-6} and 3×10^{-6} M, respectively. Muntz observed inhibition by Zn^{2+} and Cu^{2+}, but at much higher levels (32).

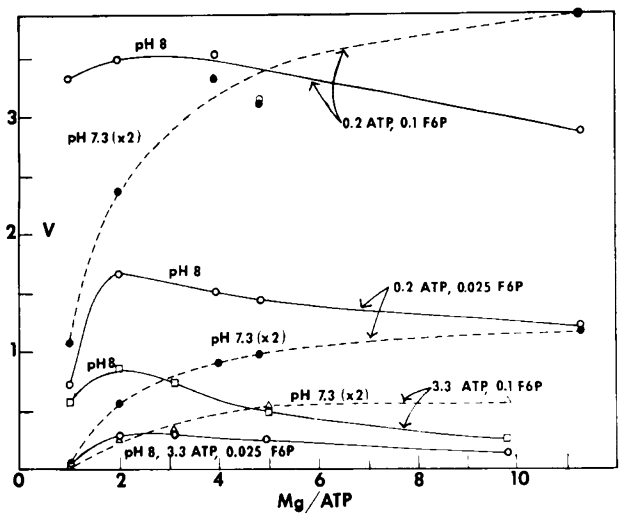

FIG. 6. Effect of Mg^{2+} concentration at pH 7.3 and pH 8 with various combinations of ATP and fructose 6-phosphate (*F6P*) concentration. The analytical conditions were similar to those of Fig. 2 except for an increase of buffer strength to 0.04 M and of bovine plasma albumin to 0.02%. At pH 7.3 the only change was substitution of 0.04 M imidazole-HCl for Tris-HCl. Enzyme Preparation B was used. ATP and fructose-6-P concentrations shown are millimolar. Velocity is recorded as moles per kg of protein per min. Note that the *abscissa* is the *ratio* of Mg^{2+} to ATP concentration and that the pH 7.3 curves have been plotted at twice the scale of the pH 8 curves.

TABLE I

Antagonists of inhibition by magnesium

Velocity measurements were made at pH 8 under the conditions of Fig. 2, with 0.1 mM ATP and 0.022 mM fructose-6-P (except as noted). When added, the concentrations of P_i, NH_4^+, and AMP were 10 mM, 3 mM, and 0.3 mM, respectively.

Addition	Reaction velocity		
	0.16 mM Mg^{2+} (lowest concentration)	1.5 mM Mg^{2+}	7.5 mM Mg^{2+}
	moles/kg × min	*% of lowest Mg^{2+} rate*	
None	1.26	64	27
P_i	2.32	103	82[a]
NH_4^+	4.25	76	40
AMP	1.98	95	75
P_i, NH_4^+	3.40	98	80
P_i, AMP	2.92	111	89
AMP, NH_4^+	4.60	117	89
P_i, NH_4^+, AMP	3.96	118	100
Fructose-6-P, 0.11 mM	3.58	52	28

[a] With 1 mM P_i, activity was 52% compared to that with 0.16 mM Mg^{2+}.

Effects of AMP, NH_4^+, and P_i—With ATP levels low enough to exert little or no inhibition, AMP, NH_4^+, and P_i are all able to increase activity (Figs. 7 and 8; Table II). The increased rates are due to a combination of lowered Michaelis constants for both substrates and increases in maximum velocities (Table II). With a combination of all three stimulators, the calculated

maximum velocity is increased almost 4-fold, and the velocity with both substrates at low level would be increased nearly 30-fold. It will be seen that NH_4^+ has the least effect on the Michaelis constant for ATP, but the greatest effect on V_{max}. The P_i in these experiments was added as K_2HPO_4. It is now known that part of the effect was due to the K^+. As mentioned above, much of the AMP effect at pH 8 is due to its ability to counteract inhibition by Mg^{2+}, and AMP exerts much less effect if an Mg^{2+}:ATP ratio of 2:1 is maintained.

When ATP was present at inhibitory levels, the three additions had in general even greater effects (Figs. 9 and 10; Table II). P_i was particularly effective in overcoming inhibition by ATP. Neither AMP nor NH_4^+ overcomes the nonlinearity of the Lineweaver-Burk plot for fructose-6-P in the presence of an inhibitory level of ATP (Fig. 9). When both were present, the plot was almost linear (not shown). In contrast, the presence of P_i confers linearity whether or not the other two additions are present.

Even P_i, however, did not abolish the exponential nature of ATP inhibition under these conditions (Fig. 10). NH^{4+} had almost no effect on K'_i for ATP, and AMP had very little influence, whereas P_i increased K'_i by a factor of 3 (Table II). (It is strange that P_i lowers the Michaelis constant for ATP, but raises its inhibitor constant.) The combined effect of all three additions would be to increase velocity 80-fold at high ATP and low fructose-6-P concentrations.

The data were analyzed, in the manner described earlier, for

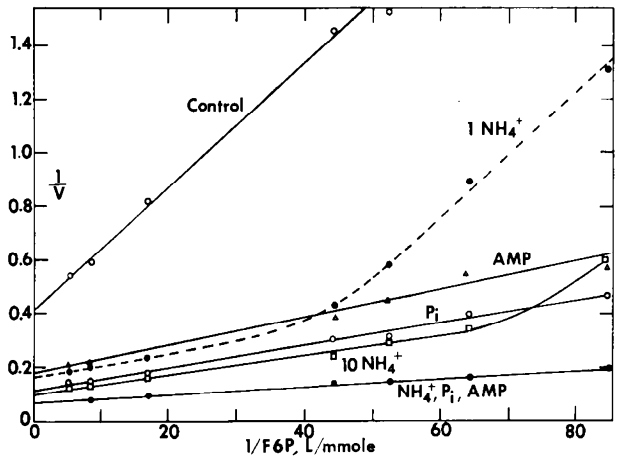

FIG. 7. Effect of NH_4^+, AMP, and P_i on velocity and Michaelis constants for fructose 6-phosphate (*F6P*). The ATP level was 0.09 mM. The concentrations, unless noted otherwise, were: AMP, 0.3 mM; P_i, 10 mM; NH_4^+, 10 mM. Curves for two levels of NH_4^+ concentration, 1 and 10 mM, are shown. The conditions of assay, enzyme preparation, and recording of velocity are the same as in Fig. 2. P_i was added as K_2HPO_4.

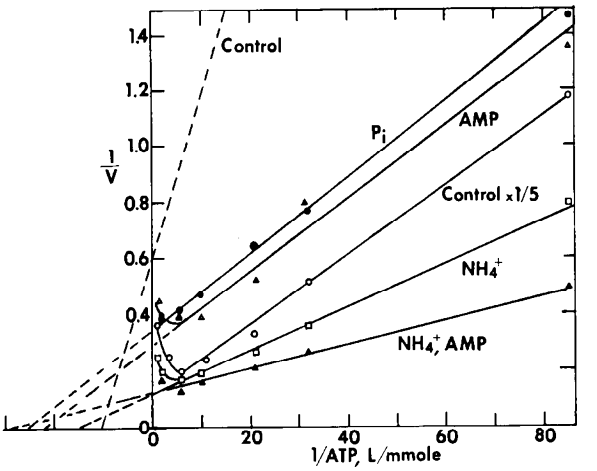

FIG. 8. Effect of NH_4^+ (10 mM), AMP (0.3 mM), and P_i (10 mM) on velocity and Michaelis constants for ATP. The fructose-6-P level was 0.027 mM. The conditions of assay, enzyme preparation, and recording of velocity are the same as in Fig. 2. P_i was added as K_2HPO_4.

TABLE II

Effect of AMP, P_i, and NH_4^+ on kinetic constants for P-fructokinase at pH 8

The analytical conditions and enzyme preparation were the same as for Fig. 2. Some of the data on which this table is based are shown in Figs. 7 through 10. The Michaelis constants for ATP (K'_A) and fructose-6-P (K'_F) are calculated from reciprocal plots of velocity with respect to concentration. In cases of nonlinearity, the values represent the concentrations that gave half of the extrapolated velocity for infinite concentration. The inhibitor constants K'_i represent the ATP concentrations which reduced activity to 50% of the extrapolated activity at zero ATP concentration (Fig. 10). In general the reciprocal of the velocity gave a linear plot with respect to $[ATP]^2$ (Fig. 10). V_{max} values were calculated from the intercepts of reciprocal plots for velocity with respect to fructose-6-P concentration with 0.09 mM ATP. These values were then multiplied by $(0.09 + K'_A)/K'_A$ to give theoretical values for both substrates at infinitely high levels. The functions $f(F)$ and $f(A)$ are explained in the text.

AMP	P_i	NH_4^+	K'_F		K'_A	K'_i	V_{max}	$f(F)$	$f(A)$
			0.09 mM ATP	4.3 mM	0.027 mM fructose-6-P	0.027 mM fructose-6-P			
mM	*mM*	*mM*	*μM*	*μM*	*μM*	*mM*	*moles/kg/min*		
			41	750	105	1.2	5.3	$218F^2 + 1070F^3$	$0.85A + 1.4A^2$
0.25			30	260	45	1.8	8.5	$220F + 300F^2$	$1.2A + 3.3A^2$
	10		37	95	41	3.9	12.8	$40F$	$0.4A$
		10	37	154	64	1.4	17.1	$160F + 750F^2$	$2.2A + 3.4A^2$
0.25	10		25	95	30	4.1	12.4		
0.25		10	13	128	33[a]	2.1	19.3		
	10	10	27	40	27[a]	4.2	14.8		
0.25	10	10	20	38	26[a]	3.8	18.5		

[a] NH_4^+ concentration was 3.3 mM.

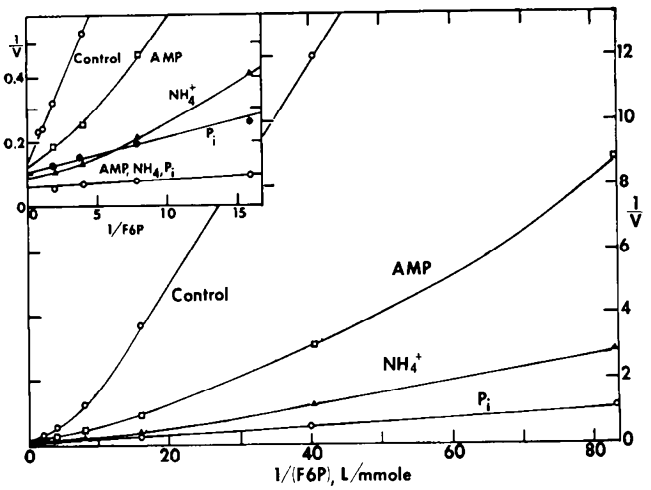

FIG. 9. Effect of NH_4^+ (10 mM), AMP (0.3 mM), and P_i (10 mM) on the velocity and apparent Michaelis constants for fructose 6-phosphate (*F6P*) at an inhibitory level of ATP (4.3 mM). The conditions of assay, enzyme preparation, and recording of velocity are the same as in Fig. 2. P_i was added as K_2HPO_4.

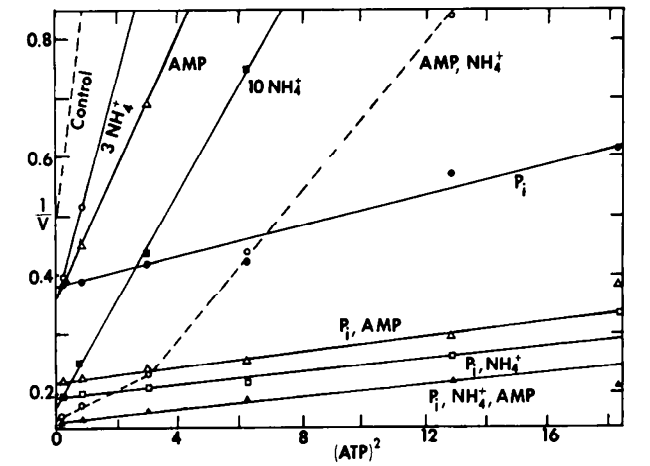

FIG. 10. Effect of NH_4^+, AMP, and P_i on the velocity with a low level of fructose-6-P and inhibitory levels of ATP. Except as noted otherwise, concentrations were: NH_4^+, 3 mM; AMP, 0.3 mM; and P_i, 10 mM. Fructose-6-P concentration was 0.027 mM. The conditions of assay, enzyme preparation, and recording of velocity are the same as in Fig. 2. P_i, when present, was added as K_2HPO_4.

the effects of each of the additives on the inhibitor and deinhibitor functions $f(F)$ and $f(A)$. The data are such as to warrant only tentative presentation (Table II). It would appear that neither AMP nor NH_4^+ alone has a favorable effect under these circumstances on $f(A)$, whereas NH_4^+ and, more especially, AMP increases the linear and squared terms of $f(F)$. (The deinhibition is so great that a cubed term would not have been detected in the data.) P_i, in contrast, has much more effect on $f(A)$ than on $f(F)$. (The effect on $f(A)$ is so large that also in this case higher order terms in $f(F)$ would not have been detected.)

As will be shown below, the apparent dissociation constants for NH_4^+, AMP, and P_i are raised by increasing ATP and lowered by increasing fructose-6-P. This is probably a factor in the shape of the curves of Figs. 7 and 8, since at higher ATP levels and lower fructose-6-P levels the concentrations of the three additions were not sufficient to give maximal effects.

From the experiments at pH 8, the effects of NH_4^+, P_i, and AMP appear to be additive. The nature of their interactions is brought out better at pH 7 under conditions of extreme inhibition by ATP. An experiment was conducted with all possible combinations of three relatively low levels of NH_4^+, P_i, and AMP (Table III). The over-all result from the 64 combinations gives an unmistakable picture of synergism among the three components. For example, 1 mM P_i alone raised the velocity only 8 mmoles per kg per min. When 1 mM P_i was added to 0.1 mM AMP, to 1 mM NH_4^+, or to both, velocities rose 41, 30, and 116 mmoles per kg per min, respectively.

TABLE III

Effect of AMP, NH_4^+, and P_i on P-fructokinase activity at pH 7 at high level of ATP

The basic medium was 0.04 M imidazole-HCl containing 2 mM Na_2SO_4, 0.02% bovine plasma albumin, 1.97 mM ATP, 4.2 mM $MgCl_2$, and 0.008 mM DPNH. The auxiliary enzymes and the incubation procedure were as described for Fig. 2. The fructose-6-P level was 0.027 mM. Kinase Preparation B was used. The velocities were calculated as described in the text.

AMP	NH_4^+	P_i	Velocity		AMP	NH_4^+	P_i	Velocity	
			Observed	Calculated				Observed	Calculated
mM	*mM*	*mM*	*mmoles/kg/min*		*mM*	*mM*	*mM*	*mmoles/kg/min*	
0	0	0	3	0	0.2	1	0	168	158
0	0	1	11	11	0.2	1	1	334	310
0	0	2	16	20	0.2	1	2	451	421
0	0	4	30	32	0.2	1	4	560	592
0.1	0	0	19	27	0.4	1	0	266	288
0.1	0	1	60	64	0.4	1	1	501	529
0.1	0	2	99	94	0.4	1	2	670	687
0.1	0	4	132	132	0.4	1	4	895	845
0.2	0	0	33	45	0.1	2	0	161	149
0.2	0	1	93	99	0.1	2	1	318	306
0.2	0	2	142	141	0.1	2	2	433	470
0.2	0	4	198	200	0.1	2	4	583	568
0.4	0	0	84	67	0.2	2	0	255	184
0.4	0	1	148	144	0.2	2	1	486	480
0.4	0	2	207	198	0.2	2	2	648	604
0.4	0	4	263	282	0.2	2	4	868	693
0	1	0	14	13	0.4	2	0	376	371
0	1	1	44	42	0.4	2	1	691	690
0	1	2	68	64	0.4	2	2	910	885
0	1	4	107	95	0.4	2	4	1190	1090
0	2	0	23	19	0.1	4	0	213	197
0	2	1	56	60	0.1	4	1	411	421
0	2	2	87	89	0.1	4	2	558	556
0	2	4	126	130	0.1	4	4	750	687
0	4	0	24	26	0.2	4	0	337	381
0	4	1	66	77	0.2	4	1	629	636
0	4	2	130	116	0.2	4	2	835	900
0	4	4	160	168	0.2	4	4	1098	1035
0.1	1	0	91	114	0.4	4	0	493	569
0.1	1	1	207	229	0.4	4	1	890	982
0.1	1	2	246	314	0.4	4	2	1155	1070
0.1	1	4	443	428	0.4	4	4	1480	1260

A coherent formulation for the results of this experiment was obtained by the following assumptions: (*a*) that when all three stimulators are present the enzyme (*E*) can exist with any combination of the three additives, *vis. m*, *n*, *p*, *mn*, *mp*, *np*, and *nmp* (where, *m*, *n*, *p*, *mn*, etc., represent enzyme combined with AMP, NH_4^+, P_i, both AMP and NH_4^+, etc.); (*b*) that the dissociation constants for each stimulator may be affected by the presence of one or both of the others; (*c*) that with the chosen levels of ATP and fructose-6-P the catalytic capacity of the enzyme is negligible unless it is combined with one or more of the stimulators; and (*d*) that the catalytic capacity of each of the combined forms of the enzyme can differ from the rest. These catalytic capacities will be represented by V_m, V_{mn}, V_{mnp}, etc. A fifth assumption, which is certainly not completely correct, is that the Michaelis constant for fructose-6-P is the same for all forms. Neglect of this tends to exaggerate differences in the catalytic capacities of the different forms.

Based on these assumptions and the mass law, the fraction of the enzyme existing in any given form (for example, *np*) is calculated as

$$np = \left[\frac{(N)(P)}{K_m K_p{}^n}\right] \Big/ \left[1 + \frac{(M)}{K_m} + \frac{(N)}{K_n} + \frac{(P)}{K_p} + \frac{(M)(N)}{K_m K_n{}^m} + \frac{(N)(P)}{K_m K_p{}^n} + \frac{(M)(P)}{K_m K_p{}^m} + \frac{(M)(N)(P)}{K_m K_n{}^m K_p{}^{mn}}\right]$$

where *M*, *N*, and *P* represent AMP, NH_4^+, and P_i, and the dissociation constants are defined as

$$K_p = \frac{(E)(P)}{(p)}, \qquad K_p{}^n = \frac{(n)(P)}{(np)}, \qquad K_p{}^{mn} = \frac{(mn)(P)}{(mnp)}, \text{ etc.}$$

After calculating the various forms of the enzyme we may write the velocity as

$$v = (mV_m + nV_n + pV_p + \ldots + mnpV_{mnp}) \qquad (3)$$

The various dissociation constants were calculated from Lineweaver-Burk plots with a certain amount of juggling to fit the over-all data pattern, since only three points were available in any given case. Consequently no great reliance should be placed on the absolute values. For AMP the values used in the calculation are: $K_m = K_m{}^n = K_m{}^p = 0.4$ mM; $K_m^{np} = 0.16$ mM. For NH_4^+ the values are: $K_n = K_n{}^m = K_m{}^p = 2.5$ mM; $K_n^{mp} = 1.0$ mM. For P_i the values are: $K_p = K_p{}^m = K_p{}^n = 6$ mM; $K_p^{mn} = 2.4$ mM. (Although there are 12 constants, only seven are needed since $K_m K_n{}^m = K_n K_m{}^n$, etc.) The values for the catalytic capacity of each form of the enzyme were calculated stepwise. First V_m, V_n, and V_p were obtained as the extrapolated maxima when each stimulator was added alone. Next the data for the sets with two added components were analyzed by first deducting the contributions to the velocity that would be attributed to the monosubstituted forms and then assigning the remaining velocity to the disubstituted form. There were nine values on which to base each of these estimates. Finally the process was repeated with the three additive sets. Here there were 27 values on which to base the estimate. Some measure of the consistency of the data is provided by the standard errors of the estimates as calculated from the 9 to 27 values in each case. The calculated activities (moles per kg of protein per min) are: $V_m = 0.16$; $V_n = 0.04$; $V_p = 0.08$; $V_{mn} = 0.48 \pm 0.08$; $V_{np} = 0.57 \pm 0.03$; $V_{mp} = 1.13 \pm 0.03$; $V_{mnp} = 4.22 \pm 0.08$.

By inserting these values in Equation 3, the velocities were calculated for each combination of AMP, NH_4^+, and P_i. The agreement between observed and calculated results (Table III) seems satisfactory. If the formulation has any validity, then these three stimulators must be attaching at different sites; otherwise each would raise the apparent dissociation constants for the other. Just the opposite is true: the presence of any two stimulators lowers the dissociation constant for the third. (There is some indication that the presence of one stimulator may favor the addition of a second, but the data are too fragmentary to settle this point.) The calculated increases in catalytic capacity appear to be too great to attribute to lowering Michaelis constants for fructose-6-P. At pH 8 (Table II), the K_m values are only moderately lower with combinations than with the individual stimulators alone. Rough estimates of the K_m values for fructose-6-P at pH 7 were 0.05, 0.09, and 0.08 mM with saturating levels of AMP, NH_4^+, and P_i, respectively. The level of ATP was 0.25 mM. If these values are also valid for 2 mM ATP, then in the experiment of Table III, with 0.027 mM fructose-6-P, reducing the K_m could not in itself increase rates more than 4-fold (0.09/(0.09 + 0.027)), whereas there is a calculated 30- to 100-fold difference in catalytic capacity between the singly and triply substituted forms of the enzyme.

TABLE IV

Effect of ATP and fructose-6-P on apparent dissociation constants for AMP, NH_4^+, and P_i from P-fructokinase at pH 7

The analytical conditions and the enzyme preparation were same as for Table III. $MgCl_2$ concentration was equal to 0.2 mM plus twice the ATP concentration. The apparent dissociation constants, *K'*, represent the concentrations giving half-maximal enhancement. In most cases linear plots were obtained for $1/\Delta v$ with respect to 1/concentration. The values in parentheses are from incomplete experiments.

ATP	Fructose 6-phosphate	Control	AMP		NH_4^+		P_i	
		v	V^a	*K'*	V^a	*K'*	V^a	*K'*
mM	*mM*	*mole/kg/min*	*moles/kg/min*	*mM*	*moles/kg/min*	*mM*	*moles/kg/min*	*mM*
0.025	0.025	0.36			2.15	0.7		
0.25	0.025	0.12	0.77	(0.03)	1.10	(1.0)	0.47	0.9
2.4	0.025	0.03	0.28	0.53	0.32	11.0	0.24	8.3
0.25	0.22	0.75	1.90	(0.02)	3.41	0.5	1.39	0.5
2.4	0.22	0.27	1.94	0.11	2.47	2.7	1.32	4.5

[a] These are the extrapolated velocities for infinite concentration of the substance in the heading.

Dissociation Constants for AMP, NH_4^+, and P_i.—The apparent dissociation constants for the kinase with AMP, NH_4^+, and P_i are markedly increased by ATP and lowered by fructose-6-P (Table IV). A similar effect of ATP on the 3',5'-cyclic AMP requirement for deinhibition was observed for the cardiac enzyme by Mansour (30). With a still lower ATP concentration (0.01 mM) than shown in Table IV and 0.22 mM fructose-6-P concentration, under somewhat different conditions (pH 7.0 imidazole, 0.15 M potassium acetate, 7.5 mM $MgCl_2$), an even lower dissociation constant for AMP (0.003 mM) was obtained. At pH 8 in Tris buffer, values similar to those at pH 7 were found for apparent dissociation constants of all three compounds, and

similar effects of ATP and fructose-6-P on the apparent constants were observed. The constant for NH_4^+ with both ATP and fructose-6-P at 0.25 mM agrees well with that reported by Muntz (0.4 mM), which was determined with 6 mM ATP and 9 mM fructose-6-P (32).

Other Stimulators—In addition to the three stimulators described, 3,'5'-cyclic AMP, ADP, fructose diphosphate, and K^+ can also increase activity. The first two appear to act in the same manner as AMP and probably at the same site. *Cyclic AMP* by itself gives about the same stimulation as 5'-AMP and adds little or nothing when 5'-AMP is already present, but does increase activity when NH_4^+ or P_i is present (Table V). The apparent dissociation constants for cyclic AMP have been found to be about half those of 5'-AMP under the same conditions, and they are similarly affected by ATP (footnotes to Table V).

ADP does not give the same degree of stimulation as AMP and is inhibitory at higher levels. ADP does not increase activity when AMP is present but can enhance activity when added to samples containing NH_4^+ or P_i (Table V).

Fructose Diphosphate—Alone, at low levels of ATP, fructose diphosphate can double activity, and the effect is decreased but not abolished if AMP (or in some cases P_i) is already present (Table V). At low ATP levels, under the conditions of Table V, a half-maximal effect was seen at 0.008 mM. If, however, the enzyme is inhibited by high levels of ATP, enhancement is much greater and much *lower* levels of fructose diphosphate are required (Table V and footnotes to the table). Similarly, with inhibition by 3 mM magnesium citrate (0.09 mM ATP and 0.02 mM fructose-6-P), fructose diphosphate increased activity about 3-fold (not shown) with a half-maximal effect at 0.0005 mM. Thus the activating effects of fructose diphosphate appear to be of a different nature in the presence and absence of inhibition. No experiments are available with fructose diphosphate added to AMP in the presence of inhibitory ATP levels to decide whether or not these two activators can supplement each other, but fructose diphosphate clearly can supplement P_i (Table V).

In confirmation of the observations of Muntz and Hurwitz (29), K^+ enhances activity. At pH 8, under the conditions of Fig. 2, with 0.09 mM ATP and 0.12 mM fructose-6-P, activity was about doubled with 20 mM K^+, and the effect was half-maximal with 5 mM K^+. The K^+ effect is similar to that of NH_4^+, but the maximal enhancement is only one-third as great. In the presence of 2 and 10 mM NH_4^+, 20 mM K^+ was without noticeable effect. This suggests that the two ions act in a similar manner, possibly at the same site.

Other Nucleotides—As Viñuela *et al.* (20) and Ramaiah, Hathaway, and Atkinson (21) showed for P-fructokinase from yeast, GTP is less inhibitory than ATP. Rather extensive studies with brain kinase and GTP may be summarized as follows. (*a*) At pH 8, with 0.1 mM GTP, the responses to P_i, NH_4^+, and AMP are almost the same as with 0.1 mM ATP (Table VI). (*b*) At pH 8, values of K'_m for fructose-6-P tend to be somewhat lower with GTP as substrate than with ATP. For example, in an experiment made under the conditions of Fig. 2, values of K'_m for fructose-6-P were 39 and 71 μM with GTP and ATP, respectively. When 10 mM NH_4^+ was also present, the corresponding values for K'_m were 16 and 38. (*c*) Even though GTP is ordinarily not inhibitory at pH 8, it is synergistic with citrate,

TABLE V

Effect of 3',5'-cyclic AMP, ADP, and fructose diphosphate on P-fructokinase activity in presence and absence of other activators

The analytical conditions for the experiments with cyclic AMP and ADP were the same as for Fig. 2. In the case of fructose diphosphate, the basic medium was the same but the reagent contained, in addition, 20 mM KCl and 0.4 mM P-pyruvate, and the auxiliary enzymes were pyruvate kinase (40 μg per ml), cardiac lactic dehydrogenase (20 μg per ml), and P-glucose isomerase (1.5 μg per ml). The presence of K^+ raises the control level of activity.

Test substance	Concentration	Substrates		Velocities observed with other additions to medium			
		ATP	Fructose-6-P	None	AMP, 0.03 mM	NH_4^+, 10 mM	P_i, 10 mM
	mM	*mM*	*mM*	*moles/kg/min*			
Cyclic AMP	0	3.8	0.11	0.8	2.5	4.2	5.4
	0.10[a]	3.8	0.11	3.0	3.1	7.8	6.3
	0	0.09	0.11	1.5	3.2	5.3	4.4
	0.05[b]	0.09	0.11	3.8	3.3	7.3	5.0
ADP	0	0.09	0.11	1.3	3.2	5.7	3.8
	0.05[c]	0.09	0.11	1.9	2.5	6.4	4.8
Fructose diphosphate	0	0.09	0.09	1.9	5.8	4.1	4.0
	0.4[d]	0.09	0.09	3.4	6.4	6.0	5.5[e]
	0	0.7	0.09	1.47[f]			6.0[f]
	0.1	0.7	0.09	3.70			6.0
	0	3.8	0.09	0.34[f]			2.8[f]
	0.1[g]	3.8	0.09	2.7			4.8

[a] Half-maximal enhancement of control rate was obtained with 0.02 mM cyclic AMP.

[b] Half-maximal enhancement of control rate was obtained with 0.008 mM cyclic AMP, whereas raising the concentration to 0.2 and 0.5 mM did not increase the rate above that shown for 0.05 mM.

[c] Peak effect was obtained at this concentration; levels above 0.1 mM inhibited (70% inhibition at 1 mM).

[d] Half-maximal enhancement of control rate was obtained with 0.008 mM fructose diphosphate.

[e] When AMP, NH_4^+, and P_i were all present, velocity was 8.8 and 8.9 moles per kg of protein per min, respectively, with and without fructose diphosphate.

[f] Aldolase, 20 μg per ml, was added, to remove most of the fructose diphosphate as it was formed (see "Materials and Methods").

[g] Half-maximal enhancement of control rate was obtained with 0.001 mM fructose diphosphate; there was no inhibition at 1 mM.

TABLE VI

Effects of P_i, NH_4^+, and AMP on P-fructokinase activity with GTP as substrate

The conditions and enzyme preparation were those of Fig. 2, with 7.5 mM Mg^{2+} and 0.12 mM GTP.

Fructose-6-P	Velocity observed with additions to medium				
	None	P_i, 10 mM	NH_4^+, 10 mM	AMP, 0.25 mM	All three[a]
mM	*moles/kg/min*				
0.012	0.65	2.13	1.66	1.75	5.4
0.118	1.69	6.8	7.8	4.6	12.5

[a] Added at the same concentrations as shown in columns to the left.

TABLE VII

P-fructokinase activity with six nucleoside triphosphates

Analytical conditions were those of Fig. 2 with 7.5 mM Mg^{2+} and 0.018 mM fructose-6-P.

Nucleoside triphosphate	V^a	K'_m	$K'_i{}^b$	v	
				1.6 mM XTP	1.6 mM XTP + ATP[c]
	moles/kg/hr	*mM*	*mM*	*moles/kg/hr*	
ATP	3.2	0.04	1.4		2.4
GTP	4.6	0.05	>5	4.6[d]	2.3[d]
ITP	1.0	0.02	2	0.5	2.3
UTP	2.5	0.06	1	1.2	0.4
CTP	2.2	0.06	>5	2.0	1.4
TTP[e]	1.1	0.25	>5	1.1	1.2

[a] Maximum velocity for XTP extrapolated to infinity, but fructose-6-P at only 0.018 mM.

[b] Concentration to give 50% inhibition.

[c] At 1 mM ATP.

[d] At 1.2 mM GTP.

[e] Based on fragmentary data.

just as in the case of ATP (26). Therefore, in the presence of citrate, distinct inhibition by GTP is observed. (*d*) At pH 7, in 0.04 M imidazole-HCl buffer, GTP becomes a strong inhibitor, particularly when the GTP concentration exceeds that of Mg^{2+}. At pH 7, to achieve the same degree of inhibition, about 3-fold higher levels of GTP than of ATP are required.

P-Fructokinase shows little specificity in regard to the phosphate donor. In the case of brain enzyme ITP (32), and in the case of muscle enzyme ITP, UTP (45), and CTP (31) have all been shown to phosphorylate fructose-6-P. Yeast kinase will utilize not only ATP and GTP but also ITP and CTP (21). Brain kinase can use any of these triphosphates and TTP as well (Table VII).

The Michaelis constants for ITP and UTP shown in the table may be compared with values of 0.07 mM and 0.033 mM, respectively, reported by Ling and Lardy for muscle enzyme (45).

Of the nucleotides tested, ITP and UTP are inhibitory at higher levels (Table VII). Inhibition of heart P-fructokinase by UTP was observed by Mansour (28). The addition of a moderately inhibitory level of ATP to samples containing the other nucleotides resulted in decreased rates in the case of GTP, UTP, and CTP, increased rates in the case of ITP, and little change in the case of TTP. The results suggest that a more elaborate study of these interactions might be rewarding.

In agreement with earlier reports for enzyme from skeletal muscle (28) and heart (30), 5′-AMP and 3′,5′-cyclic AMP are the only mononucleotides found to enhance brain P-fructokinase. Other nucleotides tested with the brain enzyme were 3′-AMP, 5′-GMP, 5′-IMP, 5′-CMP, 5′-UMP, and 5′-TMP.

DISCUSSION

Although the kinetics of brain P-fructokinase obviously has not been clearly worked out, certain features emerge and certain problems to be solved can be identified.

It is believed that a model to fit the present data would require, as a minimum, two substrate sites, one ATP inhibitor site, one citrate inhibitor site, one fructose-6-P deinhibitor site, and separate deinhibitor sites for NH_4^+, AMP, and P_i, a total of eight. It is possible that one of the deinhibitor sites might coincide with an ATP inhibitor site, but not more than one. The fructose-6-P deinhibitor site must be separate from those for the other three deinhibitors, since increasing fructose-6-P increases the apparent affinity for all three. In addition to these eight (or possibly seven) sites, the kinetics suggests that there is an additional ATP inhibitor site and two additional fructose-6-P deinhibitor sites, to which may have to be added a Mg^{2+} inhibitor site—a possible dozen! Uncertainties about the inhibitory capacities of ATP^{4-}, $HATP^{3-}$, and $MgATP^{2-}$, and about the possible influence of trace metals, make conclusions as to these additional sites insecure. Garfinkel has made a computer analysis (44) of our much less complete kinetic data for the muscle kinase (26, 28). His results indicate that each enzyme molecule can add 2 molecules of fructose-6-P, 3 of ATP, 3 of AMP, 4 of P_i, and 1 of citrate. He concludes that at least six binding sites are required but that there may be many more.

It was earlier shown that ATP and citrate are synergistic inhibitors, in that increase in ATP increases the apparent affinity for citrate and vice versa (26). Conversely, fructose-6-P decreases the apparent affinity for citrate. The present data show just the reverse situation in regard to the deinhibitors. ATP decreases apparent affinity for NH_4^+, P_i, and AMP, whereas fructose-6-P increases affinity for each. These results suggest a model (Fig. 11) in which inhibitor sites are so situated that occupancy by ATP or citrate tends to separate the two substrate sites from each other and also to separate the points of attachment of the deinhibitors. Conversely, occupancy of the deinhibitor sites draws the substrate sites closer together but separates the points of attachment of the inhibitors. Each site is represented as a two-point unit, but any arrangement that would result in a deformation in the right direction would suffice. It would be equally satisfactory to attribute the deformations to a push within a pocket or fold, rather than to a pull, in which case the addition of ATP, for example, would enlarge the pocket, making it easier for citrate to crowd in, etc.

The instability phenomenon described earlier in the paper is not to be confused with inhibition by ATP or citrate, even though both Mansour (38) and Ling, Marcus, and Lardy (33) found that the inactivated enzyme can be reactivated by appropriate treatment. If the brain kinase behaves like that from heart, the protection by various substances may be interpreted as prevention of cleavage into inactive subunits (38). It would be in-

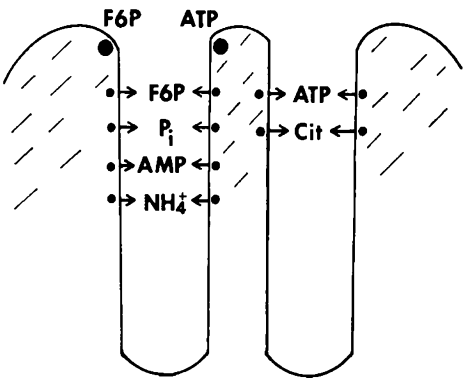

FIG. 11. Model for P-fructokinase. The *large closed circles* represent substrate sites. Inhibitor (*right*) and deinhibitor sites (*left*) are each represented as having two points for attachment. Access to these sites is conceived of as being from the front or back. *F6P*, fructose 6-phosphate; *Cit*, citrate.

structive if the apparent dissociation constants for protection could be related to some of the inhibitor or deinhibitor constants, in the hope that it would be unnecessary to postulate still more binding sites. Unfortunately, because of strong reciprocal effects of each active substance on binding of the others, the protection data, which were obtained with only one component present at a time, are not very satisfactory for comparison with kinetic constants. However, the values of the protection constants appear too small by several orders of magnitude to match with deinhibitor constants or Michaelis constants. Further study of the kinetics of protection should be rewarding.

The present results suggest several future lines of attack on the kinetics of P-fructokinase. (*a*) It might be a simplification to carry out ATP inhibition experiments in general with ATP in excess over Mg^{2+}. $MgATP^{2-}$, which is much less inhibitory than free ATP, could be kept low enough to be ignored, and the complication of Mg^{2+} inhibition would be eliminated. (*b*) More extensive kinetic studies with apparently simpler enzymes from lower forms (19, 20, 23) should be useful. Conceivably there exists a series of P-fructokinases in which one or another of the control features seen in the mammal may be absent. (*c*) Ultimately, kinetic studies must be supplemented with physical and chemical studies of pure enzyme, to see whether the various binding properties deduced from kinetic behavior are substantiated by direct analysis.

REFERENCES

1. Cori, C. F., in *A symposium on respiratory enzymes*, University of Wisconsin Press, Madison, 1941, p. 175.
2. Iwakawa, Y., *J. Biochem. (Tokyo)*, **36**, 191 (1944).
3. Engel'hart, V. A., and Sakov, N. E., *Biokhimiya*, **8**, 9 (1943).
4. Ozand, P., and Narahara, H. T., *J. Biol. Chem.*, **239**, 3146 (1964).
5. Bueding, E., and Mansour, J. M., *Brit. J. Pharmacol.*, **12**, 159 (1957).
6. Mansour, T. E., *J. Pharmacol. Exptl. Therap.*, **135**, 94 (1962).
7. Lynen, F., Hartmann, G., Netter, K. F., and Schuegraf, A., in G. E. W. Wolstenholme and C. M. O'Connor (Editors), *Ciba Foundation symposium on regulation of cell metabolism*, Little, Brown and Company, Boston, 1959, p. 256.
8. Hommes, F. A., *Arch. Biochem. Biophys.*, **108**, 36 (1964).
9. Ghosh, A., and Chance, B., *Biochem. Biophys. Res. Commun.*, **16**, 174 (1964).
10. Aisenberg, A. C., and Potter, V. R., *J. Biol. Chem.*, **224**, 1115 (1957).
11. Park, C. R., Morgan, H. E., Henderson, M. J., Regen, D. M., Cadenas, E., and Post, R. L., *Recent Progr. Hormone Res.*, **17**, 493 (1961).
12. Newsholme, E. A., and Randle, P. J., *Biochem. J.*, **80**, 655 (1961).
13. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W., *J. Biol. Chem.*, **239**, 18 (1964).
14. Minakami, S., Saito, T., Suzuki, C., and Yoshikawa, H., *Biochem. Biophys. Res. Commun.*, **17**, 748 (1964).
15. Tsuboi, K. K., and Fukunaga, K., *J. Biol. Chem.*, **240**, 2806 (1965).
16. Lonberg-Holm, K. K., *Biochim. Biophys. Acta*, **35**, 464 (1959).
17. Wu, R., *Biochem. Biophys. Res. Commun.*, **14**, 79 (1963).
18. Lardy, H. A., and Parks, R. E., Jr., in O. H. Gaebler (Editor), *Enzymes: of biological structure and function*, Academic Press, Inc., New York, 1956, p. 584.
19. Mansour, T. E., and Mansour, J. M., *J. Biol. Chem.*, **237**, 629 (1962).
20. Viñuela, E., Salas, M. L., and Sols, A., *Biochem. Biophys. Res. Commun.*, **12**, 140 (1963).
21. Ramaiah, A., Hathaway, J. A., and Atkinson, D. E., *J. Biol. Chem.*, **239**, 3619 (1964).
22. Passonneau, J. V., and Lowry, O. H., *Advan. Enzyme Regulation*, **2**, 265 (1964).
23. Lowry, O. H., and Passonneau, J. V., *Arch. Exptl. Pathol. Pharmakol.*, **248**, 185 (1964).
24. Parmeggiani, A., and Bowman, R. H., *Biochem. Biophys. Res. Commun.*, **12**, 268 (1963).
25. Garland, P. B., Randle, P. J., and Newsholme, E. A., *Nature*, **200**, 169 (1963).
26. Passonneau, J. V., and Lowry, O. H., *Biochem. Biophys. Res. Commun.*, **13**, 372 (1963).
27. Salas, M. L., Viñuela, E., Salas, M., and Sols, A., *Biochem. Biophys. Res. Commun.*, **19**, 371 (1965).
28. Passonneau, J. V., and Lowry, O. H., *Biochem. Biophys. Res. Commun.*, **7**, 10 (1962).
29. Muntz, J. A., and Hurwitz, J., *Arch. Biochem. Biophys.*, **32**, 137 (1951).
30. Mansour, T. E., *J. Biol. Chem.*, **238**, 2285 (1963).
31. Ling, K.-H., Byrne, W. L., and Lardy, H. A., *Methods Enzymol.*, **1**, 306 (1955).
32. Muntz, J. A., *Arch. Biochem. Biophys.*, **42**, 435 (1953).
33. Ling, K.-H., Marcus, F., and Lardy, H. A., *J. Biol. Chem.*, **240**, 1893 (1965).
34. Parmeggiani, A., and Krebs, E. G., *Biochem. Biophys. Res. Commun.*, **19**, 89 (1965).
35. Mansour, T. E., Wakid, N. W., and Sprouse, H. M., *Biochem. Biophys. Res. Commun.*, **19**, 721 (1965).
36. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193**, 481 (1951).
37. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
38. Mansour, T. E., *J. Biol. Chem.*, **240**, 2165 (1965).
39. Colowick, S. P., *Abstracts of the American Chemical Society Meeting, New York, 1947*, p. 56C.
40. Utter, M. F., *Federation Proc.*, **6**, 299 (1947).
41. Taylor, J. F., in W. D. McElroy and B. Glass (Editors), *Phosphorous metabolism, Vol. I*, The Johns Hopkins Press, Baltimore, 1951, p. 104.
42. Kahana, S. E., Lowry, O. H., Schulz, D. W., Passonneau, J. V., and Crawford, E. J., *J. Biol. Chem.*, **235**, 2178 (1960).
43. Burton, K., *Biochem. J.*, **71**, 388 (1959).
44. Garfinkel, D., in B. Chance (Editor), *Control of energy metabolism*, Academic Press, Inc., New York, in press.
45. Ling, K.-H., and Lardy, H. A., *J. Am. Chem. Soc.*, **76**, 2842 (1954).