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MULTIPLE INHIBITOR SITES FOR ATP ON MUSCLE PHOSPHOFRUCTOKINASE AS INFLUENCED BY A CHANGE OF pH A COMPUTER ANALYSIS OF "NON-LINEAR" KINETIC DATA*

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SUMMARY

Kinetic studies have been made of the pH-dependent inhibition of phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) by ATP. Non-linear relationships between the reciprocal of the initial velocity and concentration of ATP at inhibitory levels indicated the multiple existence of ATP inhibitor sites on phosphofructokinase. Since the conventional graphical method of measuring kinetic parameters from the ordinary Michaelis–Menten kinetic data fails to give decisive values for such non-linear kinetics, a computer method for obtaining the most plausible parameters based on statistical considerations was explored by means of a digital computer.

Application of this estimation method to the kinetic data obtained at various pH levels from 6.8 to 9.6 led to the following conclusions. (1) Multiplicity of the ATP inhibitor sites, or cooperativity of binding of ATP at the inhibitor sites, was found to be most pronounced at pH 7.3 and was reduced by a shift of pH to either side. The precipitous inhibition of the enzyme caused by a minute increase of ATP concentration at a lower pH may be due to a high cooperativity at the inhibitor sites. (2) Affinity of ATP for the inhibitor sites decreased markedly as the pH was raised from 6.8 to 8.0. This reduction of affinity may play a significant role in the removal of the ATP inhibition of the enzyme by raising the pH.

A physiological significance of these strange kinetic properties of phosphofructokinase in the cellular regulation of glycolysis is discussed.

INTRODUCTION

Phosphofructokinase (ATP D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) reaction is a rate-determining step of the glycolytic system fortified with

* Preliminary accounts of part of this work were presented before the 7th Intern. Congr. of Biochem., Tokyo, 1967, (Abstr. IV, p. 755) and the Seminar on Metabolic Control under the Japan–U.S. Cooperation Science Program, organized by D. E. ATKINSON and O. HAYAISHI, Kyoto, 1966 (Abstr., p. 22).

ATP, because an excess of ATP is inhibitory to the enzyme. Under such conditions, the overall rate of glycolysis could be enhanced by activating phosphofructokinase The activation of phosphofructokinase in such a state, *i.e.*, the reversal of the ATP-induced inhibition of phosphofructokinase from various sources, was found to be brought about by a number of substances including another substrate, Fru-6-*P*[1-12], and products[1,6,8,9,11,13,14] of the enzyme, nucleoside monophosphates[1,3-9,11-13,15-17,19], inorganic phosphate[1,5,7,9,11-13,17] and metal ions[9,20,21] On the contrary, citrate increases the degree of the inhibition induced by ATP[6,9,10,22-27] Such a modification of ATP-induced inhibition of the enzyme undoubtedly plays a key role in the regulation of cellular glycolytic rate Our recent studies using isolated rat diaphragm showed that raising the pH of the incubation medium was also effective in overcoming the inhibition and, as a result, stimulated the production of lactate by the intact-cell preparation as well as glycolytic rate of the ATP-added cell-free extracts[28]

Since the inhibition pattern of phosphofructokinase, sigmoidal rather than hyperbolic, is strikingly affected by minute changes of pH around the physiological value, it would appear that pH dependence of the ATP-induced inhibition of phosphofructokinase plays a significant role in the regulation of glycolysis in the cell[28]. Hence, an analysis of the "non-linear" kinetics of muscle phosphofructokinase in the presence of ATP and H^+ at inhibitory levels was undertaken on the basis of statistical considerations with the aid of a digital computer. The purpose of the present paper is to show kinetic evidence that binding of ATP at the inhibitor sites of the enzyme is profoundly affected by a change in pH

MATERIALS AND METHODS

Phosphofructokinase was extracted from rabbit muscle and partially purified according to the method of GATT AND RACKER[29]. Ketose-1-phosphate aldolase was also prepared from rabbit muscle as described by TAYLER[30] Other enzymes for assaying phosphofructokinase, including triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase, were purchased from Sigma Chemical Co. Phosphofructokinase activity was measured by estimating the rate of NADH oxidation in the presence of ketose-1-phosphate aldolase, triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase The rate of disappearance of NADH was followed at a wavelength of 340 mμ and a decrease in absorbance during the initial 2 min was taken as the initial velocity (v).

Fru-6-*P*, NADH, ATP (sodium salt) and Tris were obtained from Sigma Chemical Co ATP was freed of sodium by applying twice to an ion exchange column (Dowex 50). The acid eluate of free ATP was immediately neutralized with Tris and stored at $-20°$ before use. Contamination of this solution with a minute quantity of sodium was detected by flame photometric analysis. However, further treatment with ion exchanger was not undertaken because Na^+, at a concentration as high as 10 mM, exerted no influence upon the reaction velocity under the present experimental conditions (see also ref 34)

The fitting of the observed reaction velocities to the postulated kinetic equation was conducted in principle according to the method of least squares by means of a digital computer (NEAC 2203G, Nippon Electric Company) with a Fortran IV programming

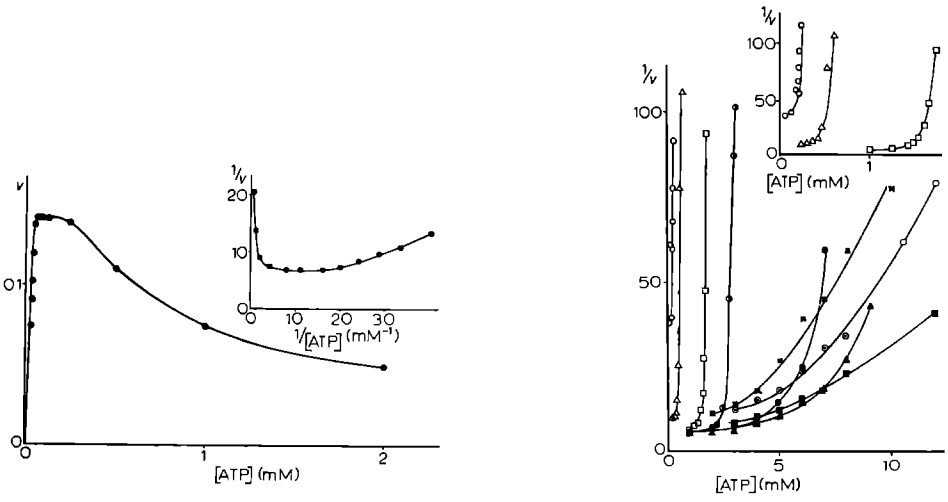

Fig 1 Phosphofructokinase activity at pH 7 6 as a function of ATP at inhibitory and non-inhibitory levels Phosphofructokinase preparation stored by suspending in 50% satd $(NH_4)_2SO_4$ at 0° was diluted 20-fold with 0 1 M Tris–HCl buffer (pH 7 6) Each cuvette contained 0 1 M Tris–HCl buffer (pH 7 6), 3 mM $MgCl_2$, 0 01 ml diluted phosphofructokinase, ATP (at a final concentration as indicated), enzyme mixture (ketose-1-phosphate aldolase, triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase, each equivalent to the amount capable of converting 1 μmole substrate per min) and 0 4 μmole NADH in a total volume of 1 5 ml The reaction was initiated by the addition of 0 025 mM Fru-6-*P* Decrease in absorbance at 340 mμ during the initial 2 min was plotted as initial velocity on the ordinate Temperature, 22° A double reciprocal plot is shown in the inset

Fig 2 Effects of pH on the kinetics of phosphofructokinase The conditions are those of Fig 1 The reaction was initiated by the addition of 0 05 mM Fru-6-*P* ○—○, pH 6 8, △—△, pH 7 0, □—□, pH 7 3; ◑—◑, pH 7 6, ●—●, pH 8 0, ▲—▲, pH 8 3, ■—■, pH 8 8, ○—○, pH 9 3, ×—×, pH 9 6 The inset shows replotting of the kinetic data at pH's 6 8–7 3 on a different scale

RESULTS

Kinetic pattern of phosphofructokinase in the presence of ATP at inhibitory levels

As shown in Fig 1, an increase in the concentration of ATP beyond optimal concentration (about 0 3 mM under the conditions in Fig. 1) caused an inhibition of the rate of phosphofructokinase reaction. As pointed out in our recent publication[28], the inhibition of muscle phosphofructokinase by excess ATP is characterized by the following two observations First, the inhibition is pH dependent, incubation of the enzyme at lower pH gives rise to stronger inhibition[5,9,28] Second, a sigmoidal inhibition pattern was obtained by plotting the initial velocities against ATP at its inhibitory levels In an attempt to study these particular properties of phosphofructokinase reaction further, the reaction velocity was estimated in the presence of an inhibitory concentration of ATP at various pH levels. Patterns illustrated in Fig. 2 were obtained by plotting reciprocals of the initial velocities as a function of ATP. These curves are characterized by their non-linearity which is in keeping with the sigmoidal form of velocity–ATP concentration relationship reported previously[28] The slope of the plots is so steep below pH 7 3 that the reciprocal of velocity would appear to be linearly related to ATP concentration in this pH range (Fig 2). However, the plots also prove

to be non-linear as seen in the inset in which they have been redrawn to a different scale, enlarged with respect to the x axis and shortened with respect to the y axis

If it is assumed that the inhibition caused by an excess amount of substrate is due to binding of substrate at any site (inhibitor site) other than the catalytic site, the non-linear relationship demonstrated in Fig. 2 would suggest that there are two or more inhibitor sites on the enzyme molecule, because a linear plot should be obtained for the single inhibitor site.

A kinetic equation conforming to the ATP inhibition of phosphofructokinase

A set of plots in Fig 2 suggests that the ability of the inhibitor site to absorb ATP was influenced by a change in hydrogen ion concentration If it is assumed that binding of ATP at the inhibitor sites is a highly cooperative process and independent of the binding at the catalytic site, the following equations are obtained for dissociation of the enzyme–ATP complexes

$$\begin{array}{lll} E + S & \rightleftharpoons ES_a & K_a \\ \left\{\begin{array}{l} E + S \\ ES_1 + S \\ \quad\cdot \\ ES_{n-1} + s \end{array}\right. & \begin{array}{l} \rightleftharpoons ES_1 \\ \rightleftharpoons ES_2 \\ \quad\cdot \\ \rightleftharpoons ES_n \end{array} & \begin{array}{l} K_{i-1} \\ K_{i-2} \\ \quad\cdot \\ K_{i-n} \end{array} \\ \hline E + nS & \rightleftharpoons ES_n & K_{i-1} \cdot K_{i-2} \cdot \quad . \quad K_{i-n} \\ \left\{\begin{array}{l} ES_a + S \\ ES_aS_1 + S \\ \quad\cdot \\ ES_aS_{n-1} + S \end{array}\right. & \begin{array}{l} \rightleftharpoons ES_aS_1 \\ \rightleftharpoons ES_aS_2 \\ \quad\cdot \\ \rightleftharpoons ES_aS_n \end{array} & \begin{array}{l} K_{i-1} \\ K_{i-2} \\ \quad\cdot \\ K_{i-n} \end{array} \\ \hline \begin{array}{l} ES_a + nS \\ ES_n + S \end{array} & \begin{array}{l} \rightleftharpoons ES_aS_n \\ \rightleftharpoons ES_aS_n \end{array} & \begin{array}{l} K_{i-1} \cdot K_{i-2} \quad K_{i-n} \\ K_a \end{array} \end{array}$$

where ES_a and ES_n represent the enzyme molecule occupied by ATP at its catalytic site and all of its inhibitor sites, respectively. n is a parameter related to multiplicity of the inhibitor sites (or cooperativity of binding at inhibitor sites, as discussed later)

In view of the fact that a greater increase in ATP concentration over the inhibitory levels resulted in an almost total inhibition of the enzyme activity (Figs. 1 and 2), it is reasonably assumed that no product could be formed from the enzyme molecule once its inhibitor site has been occupied. If this is the case, the following kinetic equation may be derived

$$v = \frac{V}{\left(1 + \frac{K_a}{s}\right)\left(1 + \frac{s^n}{K_i^n}\right)} \qquad (1)$$

where V is the maximum velocity and $K_i = \sqrt{K_{i-1} \cdot K_{i-2} \quad . \quad K_{i-n}}$. This equation may be transformed to the following one when the reaction velocity is determined in the presence of ATP at the inhibitory level

$$\log\left(\frac{V}{v} - 1\right) = n \log(s) + \log\,(K_i^n) \qquad (2)$$

because K_a/s in Eqn. 1 could be neglected under such conditions. Therefore, two kinetic parameters, n and K_i, could be obtained from the logarithmic plots of $(V/v - 1)$ against ATP. This affords a pattern equivalent to the empirical Hill plot Another parameter, V, which is required for calculating one variable, log $(V/v - 1)$, in Eqn 2,

should be determined from the experimental data as accurately as possible, because employment of a deviated value for the maximum velocity would result in an unreliable pattern.

If a linear relationship were maintained between the reciprocal of velocity and reciprocal of substrate concentration (or between the reciprocal of velocity and substrate concentration itself in the case of the substrate-inhibition patterns such as those in Fig 2), V could be estimated by determining the intersection point of the linear plot on the $1/v$ axis. A curvilinear relationship as observed for phosphofructokinase, however, makes it rather difficult to estimate V directly from the graphical expression of the kinetic data Therefore, a calculation method has been explored for estimating the most plausible V on the basis of statistical considerations as shown in the following section.

Computation method for the estimation of V and its reliability as verified by computer simulation studies

The principle of the estimation of V by calculation involves an assumption that the most linear Hill plot is obtained if the most plausible V is employed for calculating a variable, $\log (V/v - 1)$, *i.e.*, that the most plausible V is presumed to be the one that affords the minimum deviation from regression* based on the conventional methods of statistical analysis when the Hill plot is drawn by means of least-squares fitting. The calculation procedure is as follows. First, an arbitrary value, as small as possible, is taken as a V Using this tentative V the experimental data are fitted to Eqn 2 by the method of least squares and the deviation from regression is calculated Then the next value, slightly larger than the preceding one, is taken as V and the calculation procedure is repeated as before After many repetitions of this calculation, each successively employing a new value as V, a value is found which minimizes the deviation from regression This value should be the most plausible V according to the assumption described above This procedure demands a great deal of calculation which is made possible only by means of a computer

A computer experiment described below was then performed in an attempt to verify that this calculation procedure can provide reliable kinetic parameters.

Since Eqn 2 is a mere variant of Eqn 3, the calculation method presented above

* When y_i is linearly related to x_i, the sample regression equation is written as

$$y - \bar{y} = \frac{\Sigma(x_i - \bar{x})\,(y_i - \bar{y})}{\Sigma(x_i - \bar{x})^2}(x - \bar{x})$$

according to the principle of least-squares fitting. The sum of squares for the difference between the sample value y_i and the corresponding value of y estimated by the above equation is expressed as

$$\Sigma(y_i - \bar{y})^2 - \frac{(\Sigma(x_i - \bar{x})\,(y_i - \bar{y}))^2}{\Sigma(x_i - \bar{x})^2}$$

which, usually referred to as the deviation from regression (D R), affords a measure of the degree of deviation from linearity

R in Tables II–IV, termed the coefficient of multiple correlation, has been derived from D R according to the following equation

$$R = \sqrt{1 - \frac{\text{D R}}{\Sigma(y_i - \bar{y})^2}}$$

R measures the closeness of fit of the experimental data to the postulated equation

would afford valid kinetic parameters if concentration of the substrate(s) as well as the observed velocity (v), s depending on Eqn. 3, were determined without error

$$v = \frac{V}{1 + \left(\frac{s}{K_i}\right)^n} \tag{3}$$

In fact, however, although s can ordinarily be controlled quite precisely, v is subject to greater or lesser experimental error. In a simulation study, presented below, a "true" or "population" value for v was calculated by means of Eqn. 3 on the basis of s from $8 \cdot 10^{-4}$ to $2.6 \cdot 10^{-3}$ and the true parameters, $K_i = 10^{-3}$, $V = 100$ and $n = 5$ were computed These values are presented in the second column of Table I. A table of normally distributed random numbers was utilized to generate a normally distributed population of the values of v with the desired deviation around each of the ten "true" values of v. The magnitude of the error employed for the present study is approx 5% in the first case and 10% in the second case expressed as a coefficient of variation The mean and the actual coefficients of variation of 100 samples thus calculated are also shown in Table I. An actual experiment was simulated by having the computer withdraw a single value of v at random from each of the ten populations From each such set of ten "experimental" values, the computer then calculated the V, n and K_i according to the principle of calculation presented above. The average of a hundred values thus calculated for each kinetic constant is presented in Table II along with the

TABLE I

VALUES ASSUMED FOR COMPUTER EXPERIMENT

"Population" value of v was prepared according to the following equation,

$$v = \frac{V}{1 + \left(\frac{s}{K_i}\right)^n}$$

where it is assumed that $V = 100$, $K_i = 10^{-3}$ and $n = 5$ A hundred "sample" values for each "population" value of v were obtained with the aid of a table of normally distributed random numbers with smaller errors in Case 1 and larger errors in Case 2

s (mM)	"Population" value of v	Case 1 (with smaller errors)		Case 2 (with larger errors)	
		Mean of 100 "sample" value of v	Coefficient of variance* (%)	Mean of 100 "sample" value of v	Coefficient of variance* (%)
0.8	75.3	75.1	5.5	74.9	11.0
1.0	50.0	49.9	5.5	49.7	11.0
1.2	28.7	28.8	4.7	29.0	9.3
1.4	15.7	15.8	4.7	15.9	9.9
1.6	8.71	8.71	5.5	8.70	10.5
1.8	5.03	5.03	5.4	5.02	11.1
2.0	3.03	3.02	5.7	3.01	10.8
2.2	1.90	1.90	5.0	1.89	9.9
2.4	1.24	1.25	5.7	1.25	10.9
2.6	0.84	0.84	5.1	0.84	10.9

* Coefficient of variance = (standard deviation/mean of 100 samples) × 100

TABLE II

THE MEAN AND STANDARD ERRORS OF KINETIC PARAMETERS ESTIMATED FOR 100 SETS OF "SAMPLE" VALUES OF v

From each of 100 sets of the "sample" value of v, kinetic parameters, V, n, and K_i, were calculated by computer as described in the text (each set consists of ten "observations" as shown in Table I) The mean and the S E were calculated for 100 estimates The coefficient of multiple correlation (R) was also calculated for each set of "sample" values of v and the mean is listed in the last column as a measure of closeness of fit

Case No	V		n		$K_i \times 10^3$		R
	Mean	S E	Mean	S E	Mean	S E	
1	100 5	1 02	5 00	0 013	1 00	0 0035	0 999
2	103 8	2 12	4 99	0 025	0 99	0 0070	0 998

standard error of the mean The average value for coefficient of multiple correlation (R) is also shown in Table II as a measure of closeness of fit

The results of the computer analysis listed in Table II show that the calculated parameters based on the "experimental" data are in good agreement with the "true" parameters and thus may provide statistical evidence of the reliability of the computation method presented here for estimating the most plausible kinetic parameters from the non-linear kinetic data

Kinetic parameters of phosphofructokinase

By means of this computation method, kinetic parameters of phosphofructokinase concerning the inhibitor sites can be estimated from the kinetic patterns in Fig. 2 Fig. 3 presents the logarithmic plots of $(V/v - 1)$ against ATP drawn by the

TABLE III

KINETIC PARAMETERS ESTIMATED AT VARIOUS pH LEVELS

Figures in parentheses represent the fiducial limit with a significance level of 5% R is a measure of goodness of fit as indicated in the footnote, p 54

pH	V	$n \pm S E$	$K_i \pm S E$ (mM)	R
6 8	0 096	1 75 ± 0 14 (1 4– 2 1)	0 18 ± 0 06	0 992
7 0	0 253	8 02 ± 0 43 (6 8– 9.2)	0 40 ± 0 17	0 995
7 3	0 348	12 00 ± 1.03 (9 4–14 6)	1 5 ± 0 82	0 982
7 6	0 378	9 75 ± 0 52 (8 5–11 0)	2 3 ± 0 75	0 984
8 0	0 382	3 98 ± 0 12 (3 7– 4 3)	4 2 ± 0 72	0 998
8 3	0 404	2 42 ± 0 09 (2 2– 2 7)	4 7 ± 0 99	0 995
8 8	0 316	2 08 ± 0 04 (2 0– 2 2)	5 1 ± 0 52	0 999
9 3	0 308	1 82 ± 0 06 (1 7– 2 1)	3 3 ± 0 58	0 997
9 6	0 310	1 52 ± 0 11 (1 3– 1 8)	1 3 ± 0 52	0 988

method of least-squares based on the computed V which minimizes the deviation from regression. The kinetic parameters thus estimated are listed in Table III and depicted graphically in Fig. 4 as a function of pH. As is shown in Fig 4, n, which is related to multiplicity of inhibitor sites, is markedly affected by a change of pH from 6 8 to 8 0, with the maximum value as large as 12 at pH 7 3. It is of some interest that the n–pH curve is symmetrical in this pH range This fact may suggest that the ionic state of

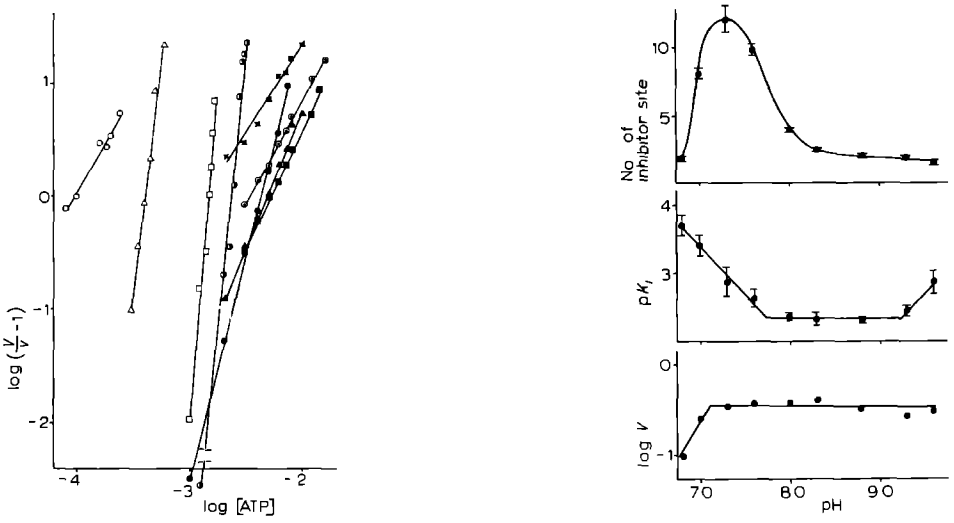

Fig 3 Logarithmic plots of $(V/v - 1)$ against ATP concentration at various pH levels Kinetic data in Fig 2, along with the most plausible V determined from them as described in text, were used for calculating the variables shown in abscissa and ordinate ○—○, pH 6 8, △—△, pH 7 0, □—□, pH 7 3, ◑—◑, pH 7 6, ●—●, pH 8 0, ▲—▲, pH 8 3, ■—■, pH 8 8, ○—○, pH 9 3, ×—×, pH 9 6

Fig 4 Plots of n, pK_i and log V as a function of pH Kinetic parameters in Table III were plotted against pH Perpendicular lines represent the standard error of the kinetic parameter The standard error of pK_i was calculated from those of K_i in Table III according to an equation,

$$\text{S E } (pK_i) = \log e \quad \frac{\text{S E } (K_i)}{K_i}$$

as suggested by WILKINSON[40]

the ionizable group(s) on the enzyme protein plays a significant role in the multiplicity (or cooperativity) of the inhibitor sites. It may be concluded that a strong inhibition of phosphofructokinase caused by a very minute increase of ATP concentration at the inhibitory level is due to such a great multiplicity (or cooperativity) of the inhibitor sites at lower pH levels

Affinity of ATP to the inhibitor sites, being expressed as pK_i in Fig 4, is also pH dependent over the range of pH from 6 8 to 8.0; an abrupt decrease in pK_i occurred upon a shift of pH toward alkaline values Thus, one of the most outstanding characteristics of the ATP-induced inhibition of phosphofructokinase, a marked reduction of the degree of inhibition by raising the pH, may be accounted for by this pronounced increase in K_i in response to a rise of pH

A relation of V to the pH is also shown in Fig 4 The discontinuities observed in pK_i– and log V–pH plots at pH's 7 8 and 9 2 and at pH 7 2 may have something to do with the pK of the ionizable group closely associated with the binding sites on the free enzyme and enzyme–substrate complexes, respectively, because pK's of substrates, ATP and Fru-6-P, differ significantly from these values

Effect of the concentration of Fru-6-P on the inhibition by ATP

Several investigators have reported that Fru-6-P is capable of overcoming the

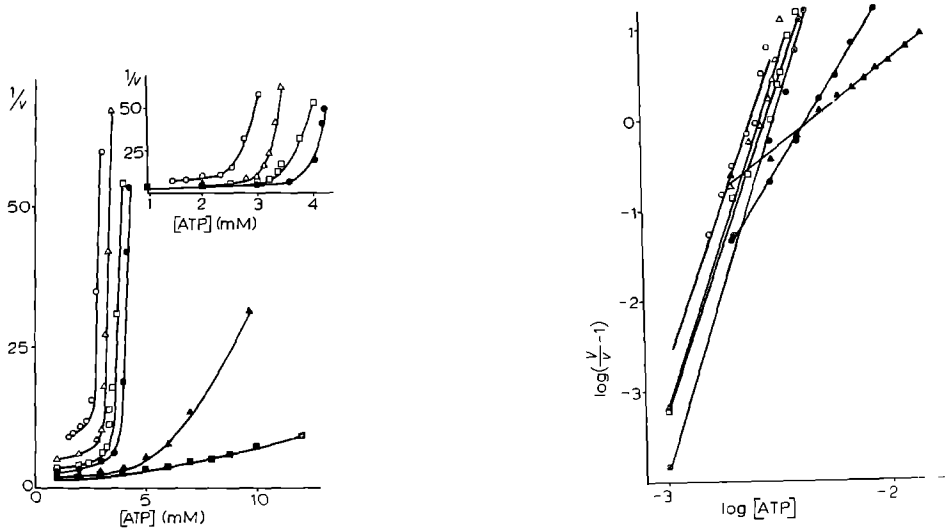

Fig 5 Effects of Fru-6-*P* concentration on the kinetics of phosphofructokinase The conditions are those in Fig 1 Fru-6-*P* concentration ○—○, 0 025 mM, △—△, 0 05 mM, □—□, 0 075 mM, ●—●, 0 1 mM, ▲—▲, 0 25 mM, ■—■, 0 5 mM The inset shows replotting of the kinetic data on a different scale

Fig 6 Logarithmic plots of $(V/v - 1)$ against ATP concentration at various conditions of Fru-6-*P* Kinetic data in Fig 5, along with the most plausible V estimated therefrom as described in text, were used for calculating the variables in abscissa and ordinate Fru-6-*P* concentration ○—○, 0 025 mM, △—△, 0 05 mM, □—□, 0 075 mM, ○—○, 0 1 mM, ●—●, 0 25 mM, ▲—▲, 0 5 mM

ATP-induced inhibition[1–12]. So, the rate of phosphofructokinase reaction in the presence of ATP at inhibitory levels was measured at a fixed pH (pH 7.6) with increasing concentration of Fru-6-*P* The results, shown in Fig 5, were analyzed by computer in the same manner as were the kinetic data in Fig 2 Fig 6 shows the logarithmic plots of $(V/v - 1)$ against ATP concentration drawn by the method of least-squares on the basis of the V estimated by computer The kinetic parameters

TABLE IV

EFFECT OF FRU-6-*P* CONCENTRATION ON KINETIC PARAMETERS

Figures in parentheses represent the fiducial limit with a significance level of 5% R is a measure of goodness of fit as indicated in the footnote, p 54

Concn of Fru-6-P (mM)	V	$n \pm SE$	$K_1 \pm SE$ *(mM)*	R
0 025	0 240	6 86 ± 0 65 (5 2–8 5)	2 4 ± 1 41	0 979
0 05	0 392	7 92 ± 0 44 (6 8–9 0)	2 7 ± 0 91	0 989
0 075	0.584	7 02 ± 0 37 (6 1–7.8)	2 9 ± 0 89	0 991
0 1	0 684	7 40 ± 0 46 (6 2–8 6)	3 1 ± 1 10	0 986
0 25	1 02	4 00 ± 0 24 (3 5–4 5)	4 4 ± 1 39	0 986
0 5	1 40	1 92 ± 0 07 (1 7–2 1)	4 8 ± 0 81	0 994

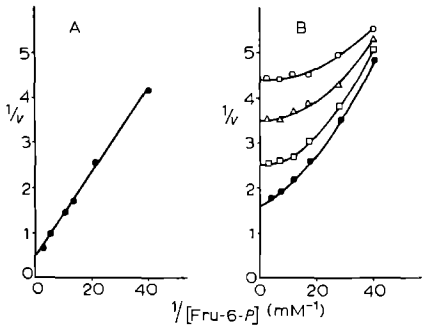

Fig 7 A. Double reciprocal plot of the V against Fru-6-P. B Double reciprocal plots of the initial velocity of phosphofructokinase reaction against Fru-6-P in the presence of much lower concentrations of ATP The conditions are those of Fig 1 ATP concentration ○—○, 0 024 mM, △—△, 0 03 mM, □—□, 0 04 mM, ●—●, 0 06 mM

determined are summarized in Table IV As is evident in Table IV, n was virtually uninfluenced by an increase of the concentration of Fru-6-P up to 0.1 mM, but a further increase resulted in a reduction. K_i steadily increased as the concentration of Fru-6-P increased from 0 025 to 0.5 mM. It would appear that there is a detectable interaction between the binding of ATP at the inhibitor site and binding of Fru-6-P to the enzyme molecule

When reciprocals of the V thus estimated are plotted against reciprocals of Fru-6-P, the linear relationship was obtained as shown in Fig. 7A POGSON AND RANDLE[10] also reported that a plot of $1/V$ against $1/s$ was linear for the rat heart enzyme. Fig 7A is considered to represent the effect of Fru-6-P on the reaction velocity of phosphofructokinase when a catalytic site for ATP has been saturated with an ATP molecule In an attempt to study the effect of Fru-6-P on an ATP-unsaturated enzyme, the initial velocities were measured as a function of the concentration of Fru-6-P with a series of ATP levels low enough to exert no inhibition (Fig. 7B) The non-linear double reciprocal plots presented in Fig 7B indicate a cooperative interaction of Fru-6-P with the enzyme As the concentration of ATP increases, however, the cooperative interaction tends to be reduced Therefore, the linear relationship between reciprocals of V and reciprocals of Fru-6-P (Fig 7A), in keeping with the experimental data in Fig 7B, makes it possible to assume that the adsorption of Fru-6-P at the allosteric site is prevented by the occupancy of the catalytic site for ATP.

Alternative reaction models

Eqn. 2, which has been used to obtain the kinetic parameters, was derived by assuming that binding of ATP at the inhibitor sites is a sufficiently cooperative process If no cooperativity is assumed for the binding of ATP, the situation would be accounted for by the following equation,

$$v = \frac{V}{\left(1 + \frac{K_a}{S}\right)\left(1 + \frac{s}{K_i}\right)^n} \qquad (4)$$

where it is also assumed that all of the inhibitor sites have the same intrinsic dissociation constant, K_i.

When steady-state kinetics are applied, the following expression is made possible on the basis of topological considerations first proposed by VOLKENSTEIN AND GOLDSTEIN[35].

$$E \underset{k_{-a}+k_p}{\overset{k_{+a}s}{\rightleftharpoons}} ES_a \underset{k_{-1}}{\overset{nk_{+1}s}{\rightleftharpoons}} nES_aS_1 \rightleftharpoons \underset{mk_{-1}}{\overset{(n-m+1)k_{+1}s}{\rightleftharpoons}} \frac{n!}{(n-m)!\,m!} ES_aS_m \rightleftharpoons \underset{nk_{-1}}{\overset{k_{+1}s}{\rightleftharpoons}} ES_aS_n$$

$ES_a \xrightarrow{k_p}$ product

where k represents the velocity constant at the appropriate reaction step A kinetic equation essentially identical to Eqn. 4 is then derived by defining $K_a = (k_{-a} + k_p)/k_{+a}$ and $K_i = k_{-1}/k_{+1}$. Though fitting of the kinetic data to Eqn. 4 was attempted by means of digital computer[36], the closeness of fit attained was far inferior to that in the case of fitting to Eqn 2 This may lend some support to the conclusion that the binding of ATP at the inhibitor sites is a highly cooperative process.

DISCUSSION

Since the reaction rate as well as kinetic patterns of phosphofructokinase are profoundly influenced by a variety of factors, the kinetic properties observed *in vitro* may involve some departure from those in living cells The present experiments were undertaken to observe the pH dependence of ATP inhibition of the enzyme and the involvement of effectors other than Fru-6-*P* was not studied Nevertheless, some components of the reaction mixture could exert such an appreciable influence on the reaction kinetics as to deserve discussion

Reaction velocity was measured in the absence of K^+ and in the presence of NH_4^+ and SO_4^{2-} at an approximate concentration of 1 mM These ions are known to act as stimulators of phosphofructokinase[5,6,9,10,13,17,22,31–34] In addition, NH_4^+ was found to modify the ATP-induced inhibition of the enzymes from rat liver[6], sheep brain[9] and parasite[11] Recent reports on the enzyme from rabbit muscle, however, showed that the degree of ATP inhibition and modification of inhibition by metabolites were not affected by the presence of NH_4^+ (refs. 5, 17) Since a concentration of these ions is little influenced by a change in pH or ATP concentration, it is likely that the kinetic properties of phosphofructokinase observed here are not dependent upon these ions.

A role of Mg^{2+} in the ATP-induced inhibition of phosphofructokinase has also been discussed by several investigators[3,4,7,9,10,17,20,21,34] ATP is more inhibitory when free than when combined with Mg^{2+} (refs. 9, 20). On the other hand, excess of Mg^{2+} also causes an inhibition of the enzyme reaction[7,9,15] and prevents the crystallization observed in the presence of ATP[17] MgATP was found to be more inhibitory in regard to tumor phosphofructokinase than free ATP under certain conditions[7] In the present study concentration of total Mg^{2+} was maintained at 3 mM, a concentration not inhibitory to muscle enzyme[5,10], in an attempt to avoid an excess of Mg^{2+} which could occur at higher ATP concentrations when the ratio of Mg^{2+} to ATP was kept constant (also see ref 34) As a result, the relative concentration of one of the molecular species of ATP to another changed greatly when the concentration of ATP was altered, MgATP was predominant at pH's below 7 6 because the molecular concentrations of ATP employed were lower than those of Mg^{2+}, whereas a larger part of added ATP remained free when ATP was present far in excess of Mg^{2+} Kinetic data presented

here, therefore, represent a combined effect of various species of ATP, though a marked modification of kinetic parameters depending on a change in pH was observed when MgATP was predominant and the concentration of Mg^{2+} was sufficient (Fig. 4) Little information has been obtained so far on the specificity of molecular species of ATP as substrate of phosphofructokinase Whether the inhibitor sites of phosphofructokinase are occupied predominantly by one or another of the ion species of ATP also remains as a subject for further investigation

The kinetic parameter n, usually referred to as an index of kinetic order of reaction or interaction (cooperativity) coefficient, is a function of the number of interacting substrate-binding sites per enzyme molecule and of the strength of interaction[37] As is evident in deriving Eqn 2, n is equal to the number of the binding sites when a sufficiently high cooperativity is assumed for ATP binding. In the present study, as many as 12 ATP sites were obtained at pH 7 3, no such high estimate has been suggested by the kinetic study for the number of binding sites for a single ligand Recent findings on purified or crystallized rabbit muscle phosphofructokinase by PAETKAU AND LARDY[34] and LING, MARCUS AND LARDY[38], as well as PARMEGGIANI[17] and KEMP AND KREBS[18], clearly showed that phosphofructokinase undergoes reversible association and dissociation depending on the concentration of the enzyme protein, pH, addition of ATP or kind of buffer. According to physical studies, 3 molecules of ATP are bound to the minimal binding unit having a molecular weight of 90 000[18], which can aggregate in the solution[17] A value of 360 000 calculated by PAETKAU AND LARDY[34] for the molecular weight of the smallest molecule of the active enzymes shows that their enzyme consists of four binding units and might possibly possess 12 binding sites for ATP. Owing to differences in experimental conditions, no decisive information is available for the polymerization state of the enzyme in the present study However, it is highly probable that phosphofructokinase exists in Tris buffer in monomer–dimer–tetramer system as suggested by LING, MARCUS AND LARDY[38]. If an interaction between ATP sites is assumed to be not very strong, the actual number of the binding sites might be larger than n estimated in the present study. Such a possibility may not be excluded because the molecular weight of 1300 000 was calculated for the most slowly sedimenting species of rabbit muscle enzyme[34,38], indicating a much higher degree of aggregation of the protein molecule

Values similar to n in the present study have been proposed for the combined number of various kinds of binding sites of sheep brain[9] and rabbit muscle[21] phosphofructokinase Based on the physical studies[18] as well as the present kinetic finding that binding of ATP at the inhibitor sites is alternated by excess of Fru-6-*P*, it may be possible to postulate that some binding sites are common to several ligands and that the number of the ATP inhibitor sites estimated here has something to do with the combined number of all the binding sites for substrates and modifiers

MANSOUR[39], using phosphofructokinase from mammalian heart, reported that active enzyme was reversibly converted to inactive enzyme during incubation at acid pH. The inactivation of the enzyme, which took place after 10-min incubation of the concentrated enzyme solution at pH levels below 6.5 at 37°, was found to be accompanied by a change in sedimentation behavior suggesting that dissociation of the enzyme protein into subunits had occurred Though the experimental conditions employed by MANSOUR are more drastic than the conditions of enzyme assay, it is possible that his finding is somewhat related to the pH dependence of kinetic parameters observed

here. Two forms of yeast phosphofructokinase with different sensitivity to ATP inhibition were also identified by VINUELA *et al*[25] The requirement of the specific enzyme along with NaF and 3′, 5′-AMP for their interconversion appears to make it implausible that a similar phenomenon takes place during incubation of phosphofructokinase in the present study Instead, the ionizable group(s) on the enzyme protein may play a significant role in the association-dissociation state of muscle phosphofructokinase as suggested by the symmetrical form of the *n*–pH curve (Fig 4) Kinetic behavior of phosphofructokinase was not studied at pH levels below 6 8, because it was impossible to maintain an incubation medium at constant acid pH's with the aid of Tris–HCl buffer Though replacement of HCl by maleic acid lowered the pH range of Tris buffer, the addition of maleic acid was found, even at higher pH's, to impair enzyme activity This finding is in agreement with the previous report by PASSONNEAU AND LOWRY[22] that dicarboxylic acid was inhibitory to phosphofructokinase

As mentioned above, it is possible to assume that n in Eqn. 2 corresponds to cooperativity of binding of ATP at the inhibitor sites rather than actual multiplicity of the inhibitor sites When cooperativity is taken into consideration in deriving the rate equation, Eqn 4 should be replaced by Eqn 5,

$$v = \frac{V}{1 + n\frac{s}{K_i} + \frac{n(n-1)}{2}\alpha^{1+2}\left(\frac{s}{K_i}\right)^2 + \cdots \alpha^{1+2+\cdots n}\left(\frac{s}{K_i}\right)^n} \qquad (5)$$

where α represents cooperativity (interaction) factor of ATP binding at the inhibitor sites (The term $(1 + Ka/s)$ is omitted) Fitting of kinetic data to Eqn 5 with the aid of a computer, assuming $n = 12$, resulted in the following approximate values for α at pH's from 6 8 to 9.6 pH 6 8, 4, pH 7.0, 10; pH 7 3, > 50, pH 7 6, 10, pH 8 0, 4, pH 8.3, 4, pH 8.8, 4, pH 9 3, 4; pH 9 6, 4 Though the degree of fitting to this equation is inferior to that to Eqn. 2, the trend of alteration of α in response to a pH change is consistent with the change of n in Eqn 2 listed in Table III.

It would appear that these kinetic properties of phosphofructokinase play a significant role in the control mechanism of cellular glycolytic systems Owing to the striking pH dependence of the affinity of ATP to the inhibitor sites, an extremely minute change of the intracellular pH in the presence of Fru-6-*P* and ATP at normal concentrations could result in an appreciable alteration of the rate of phosphofructokinase reaction On the other hand, the high cooperativity observed around the possible cellular pH as to binding of ATP at the inhibitor sites makes it possible that a small change of intracellular ATP concentrations results in a marked modification of phosphofructokinase activity Since the intracellular concentration of ATP and hydrogen ions is considered to be dependent upon the activity of oxidative phosphorylation of mitochondria, it is tempting to speculate that these peculiar kinetic properties of phosphofructokinase are closely related to the mitochondrial inhibition of glycolysis, the Pasteur effect This speculation might be favored by our unpublished preliminary observations that phosphofructokinase from rat erythrocytes, which are lacking in mitochondria, exhibits kinetic patterns rather independently of the pH in the incubation medium Recently, TRIVEDI AND DANFORTH[27], on the basis of their kinetic studies of frog muscle enzyme, also proposed that the shifts in intracellular pH are significant in the regulation of phosphofructokinase activity.

REFERENCES

1 J V PASSONNEAU AND O H LOWRY, *Biochem Biophys Res Commun*, 7 (1962) 10
2 E VINUELA, M L SALAS AND A SOLS, *Biochem. Biophys Res Commun*, 12 (1963) 140
3 T E MANSOUR, *J Biol Chem*, 238 (1963) 2285
4 D E ATKINSON AND G M WALTON, *J Biol Chem*, 240 (1965) 757
5 K UYEDA AND E RACKER, *J Biol Chem*, 240 (1965) 4682
6 A H UNDERWOOD AND E A NEWSHOLME, *Biochem J*, 95 (1965) 868
7 R WU, in B CHANCE, R W ESTABROOK AND J R WILLIAMSON, *Control of Energy Metabolism*, Academic Press, New York, 1965, p 87
8 C L MOORE, A BETZ AND B CHANCE, in B CHANCE, R W ESTABROOK AND J R WILLIAMSON, *Control of Energy Metabolism*, Academic Press, New York, 1965, p 97
9 O H LOWRY AND J V PASSONNEAU, *J Biol Chem*, 241 (1966) 2268
10 C I POGSON AND P J RANDLE, *Biochem J*, 100 (1966) 683
11 E BUEDING AND J FISHER, *Biochem Pharmacol*, 15 (1966) 1197
12 R WU, *J Biol Chem* 241 (1966) 4680
13 R M DENTON AND P J RANDLE, *Biochem J*, 100 (1966) 420
14 C C GRIFFIN, B N HOUCK AND L BRAND, *Biochem Biophys Res Commun*, 27 (1967) 287.
15 T E MANSOUR AND J M MANSOUR, *J Biol Chem*, 237 (1962) 629
16 A RAIMAIAH, J A HATAWAY AND D E ATKINSON, *J Biol Chem*, 239 (1964) 3619
17 A PARMEGGIANI, J H LUFT, D S LOVE AND E G KREBS, *J Biol Chem*, 241 (1966) 4625
18 R G KEMP AND E G KREBS, *Biochemistry*, 6 (1967) 423
19 M F LOU AND J H. KINOSHITA, *Biochim Biophys Acta*, 141 (1967) 547
20 H A LARDY AND R E PARKS, in O H GAEBLER, *Enzymes, Units of Biological Structure and Function*, Academic Press, New York, 1956, p 584
21 D GARFINKEL, *J Biol Chem*, 241 (1966) 286
22 J V PASSONNEAU AND O H LOWRY, *Biochem. Biophys Res Commun*, 13 (1963) 372
23 A PARMEGGIANI AND R H BOWMAN, *Biochem Biophys Res Commun*, 12 (1963) 268
24 P B GARLAND, P J RANDLE AND E A NEWSHOLME, *Nature*, 200 (1963) 109
25 E VINUELA, M L SALAS, M SALAS AND A SOLS, *Biochem Biophys Res Commun*, 15 (1964) 243
26 M L SALAS, E VINUELA, M SALAS AND A SOLS, *Biochem Biophys Res Commun*, 19 (1965) 371
27 B TRIVEDI AND W H DANFORTH, *J Biol Chem*, 241 (1966) 4110
28 M UI, *Biochim Biophys Acta*, 124 (1966) 310
29 S GATT AND E RACKER, *J Biol Chem*, 234 (1959) 1015
30 J F TAYLER, in S P COLOWICK AND N O KAPLAN, *Methods in Enzymology*, Vol 1, Academic Press, New York, 1955, p 310
31 J A MUNTZ AND J HURWITZ, *Arch Biochem Biophys*, 32 (1951) 137
32 J A MUNTZ, *Arch Biochem Biophys*, 42 (1953) 435
33 S C RIZZO, R E ECKEL, *Am J Physiol*, 211 (1966) 429
34 V PAETKAU AND H. A LARDY, *J Biol Chem*, 242 (1967) 2035
35 M V VOLKENSTEIN AND B N. GOLDSTEIN, *Biochim Biophys Acta*, 115 (1966) 471
36 W. W. CLELAND, in F F NORD, *Advances in Enzymology*, Vol 29, Interscience, New York, 1967, p 1
37 D E ATKINSON, J A HATTAWAY AND E C SMITH, *J Biol Chem*, 240 (1965) 2682.
38 K H LING, E MARCUS AND H A LARDY, *J Biol Chem*, 240 (1965) 1893
39 T E MANSOUR, *J Biol Chem*, 240 (1965) 2165
10 G N. WILKINSON, *Biochem J*, 80 (1961) 324