Activation of Brain Hexokinase by Magnesium Ions and by Magnesium Ion–Adenosine Triphosphate Complex

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1. An alternative explanation for the kinetic data obtained by Bachelard (1971) for the brain hexokinase reaction is presented. 2. Apparently sigmoidal saturation curves for MgATP^{2-} based upon Bachelard's (1971) studies can be corrected to hyperbolic curves by use of a stability constant for MgATP^{2-} complex formation. 3. A number of other effects related to the concentration-dependent stability of the MgATP^{2-} complex and to the presence of the inhibitory free uncomplexed ATP^{4-} concentration are also explained in terms of a non-allosteric role for either Mg^{2+} or MgATP^{2-} fully consistent with a number of previous reports on this enzyme. 4. A brief discussion of the validity of Hill plots in studies of multisubstrate co-operative enzymes is presented. 5. A simple model is presented that demonstrates how enzymes obeying Michaelis–Menten kinetics can demonstrate sigmoidal velocity responses if the true substrate of the reaction is the metal–substrate complex.

In a recent report Bachelard (1971) described the allosteric activation of brain hexokinase by Mg^{2+} and MgATP^{2-} . He found that substrate-saturation curves of brain hexokinase for MgATP^{2-} were sigmoidal at subsaturating concentrations of glucose when the $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratio was unity. On the other hand, he observed that these saturation curves were strictly hyperbolic in the presence of excess of Mg^{2+} (i.e., $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}} = 5$). Hill plots of these data indicated that the number of binding sites for MgATP^{2-} varied from 1.05 to 1.8 depending upon the Mg^{2+} concentration. In addition, the sigmoidicity and deviation from Michaelis–Menten kinetics at a $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratio of 1.0 became less pronounced with increasing glucose concentration. Finally, he found that although substrate-saturation curves for glucose were hyperbolic when the $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratio was unity, reciprocal plots were slightly non-linear. Bachelard interpreted these results to reflect homotropic co-operative binding of MgATP^{2-} to brain hexokinase and postulated that Mg^{2+} acts as a topologically distinct activator site at low $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratios, but acts as an inhibitor whenever this ratio exceeds five.

Examination of Bachelard's (1971) data has led us to an alternative interpretation fully in agreement with his experimental results but in harmony with previous studies in which no evidence for such complex binding of MgATP^{2-} was obtained (Copley & Fromm, 1967; Ning *et al.*, 1969; Purich & Fromm, 1971). Our interpretation, which does not require postulating co-operative MgATP^{2-} binding or the presence of a distinct activator site for Mg^{2+} , considers the presence of large quantities of ATP^{4-} that

may arise whenever the $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratio is near unity. Further, it is also possible to rationalize the glucose effects on the sigmoidicity and deviation from Michaelis–Menten kinetics at low Mg^{2+} concentrations. Changes in the Hill coefficient for MgATP^{2-} binding at different $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratios are explained, and a discussion of the applicability of the Hill equation in velocity studies of multisubstrate co-operative enzyme systems is presented. These studies serve to suggest that there is no allosteric involvement of Mg^{2+} or MgATP^{2-} in the brain hexokinase reaction, but that the concentration of free uncomplexed Mg^{2+} in cerebral tissue may play an important role in the regulation of the phosphorylation of glucose by adjusting the concentration of MgATP^{2-} and ATP^{4-} .

Results

Co-operative effects of MgATP^{2-}

Bachelard (1971) recently explored the possibility that Mg^{2+} is involved in the hexokinase reaction in a fashion distinct from its role as MgATP^{2-} by examining the initial reaction velocity dependence on $[\text{MgATP}^{2-}]$ at $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratios of 1.0 and 5.0. Whereas the velocity dependence in the presence of excess of Mg^{2+} appeared to be strictly hyperbolic, slightly sigmoidal responses were observed at the lower Mg^{2+} concentrations. Because all known nucleoside di- and tri-phosphate-dependent phosphotransferase reactions require a bivalent metal ion to combine with the nucleotide to form the active substrate (Cohn, 1968), we became interested in

Fig. 1. $MgATP^{2-}$ saturation curves for brain hexokinase at a $Mg^{2+}_{total}/ATP_{total}$ ratio of 1:1

Glucose concentrations were: $\circ$, 0.1 mM; $\bullet$, 0.05 mM; $\square$, 0.035 mM; and $\blacksquare$, 0.025 mM. The velocity measurements are those of Bachelard (1971) and the $MgATP^{2-}$ concentrations on the abscissa are corrected based upon a stability constant of $73000 M^{-1}$, as described in the text. Corrections were made on photographic enlargements of the original data presented by Bachelard (1971) in Fig. 1(b).

determining the percentage of ATP_{total} that is chelated with Mg^{2+} under the conditions used by Bachelard (1971). When the $Mg^{2+}_{total}/ATP_{total}$ ratio was 1.0, the percentage of ATP_{total} present in solution as $MgATP^{2-}$ varied with the extent of dilution. For instance, the concentration of Mg^{2+}_{total} and ATP_{total} varied from 0.05 mM to 0.8 mM in the experiment presented in Fig. 1(b) of Bachelard's (1971) report. If one assumes that the stability constant for the

$MgATP^{2-}$ complex in 0.15 M-triethanolamine-HCl buffer (pH 7.6) is $73000 M^{-1}$ (O'Sullivan & Perrin, 1964; Bachelard, 1971), then the concentration of $MgATP^{2-}$ will vary from 0.03 mM to 0.71 mM. Thus considerable correction is necessary for the abscissa values presented in Fig. 1(b) of Bachelard's (1971) report. Since the correction is more substantial at the lower end of this concentration range, it appeared to us that such effects might well explain the sigmoidal curvature that he observed. When such corrections were made on photographic enlargements of Fig. 1(b) of Bachelard's (1971) paper, the rather normal hyperbolic saturation curves shown in Fig. 1 were observed. For the sake of clarity, only the corrected curves are presented.

Additional corrections are also possible for the data presented by Bachelard (1971). For example, at pH 7.6 approximately 18.3% of ATP_{total} is present as the protonated species, $HATP^{3-}$, if one assumes that $HATP^{3-}$ has a pK_a of 6.95 (Smith & Alberty, 1956). The stability constant for the $MgHATP^{1-}$ complex is approximately $31 M^{-1}$ (Smith & Alberty, 1956), and it is quite unlikely that very much of the $HATP^{3-}$ is present as its Mg^{2+} ion complex. Further, ATP^{4-} is a potent inhibitor of yeast and brain hexokinases (Hammes & Kochavi, 1962a,b; Ning *et al.*, 1969; Bachelard & Goldfarb, 1969; Purich & Fromm, 1971). Corrections for $HATP^{3-}$ and ATP^{4-} were not made as the deviations from hyperbolic velocity responses observed by Bachelard (1971) appear to be accountable in terms of the correction made in Fig. 1 alone.

The possibility that $HATP^{3-}$ inhibition can lead to residual sigmoidicity remaining after the above correction of Bachelard's (1971) data seems remote. As shown by Dalziel (1963) the presence of a competitive inhibitor as a constant fraction of the true substrate will still generate hyperbolic velocity versus [substrate] plots. At pH 7.6 we have observed that, over a wide range of glucose saturation, velocity versus [ATP] plots remain hyperbolic (Ning *et al.*, 1969). These measurements were made at 1.0 mM-free uncomplexed Mg^{2+} , and the concentration and effects of ATP^{4-} are quite small. The observation of hyperbolic responses under these conditions suggests that $HATP^{3-}$ is either a competitive inhibitor relative to $MgATP^{2-}$ or not an inhibitor at all. If $HATP^{3-}$ acts as a competitive inhibitor, then the following rate expression describes the velocity of the hexokinase reaction at limiting $[Mg^{2+}]$ in the presence of ATP^{4-} and $HATP^{3-}$:

$$\frac{V_{max}}{v} = 1 + \frac{K_4}{[MgATP^{2-}]} \left(1 + \frac{[ATP^{4-}_{free}]}{K_{ATP^{4-}_{free}}} + \frac{[HATP^{3-}]}{K_{HATP^{3-}}} \right) + \frac{K_3}{[Glucose]} + \frac{K_1 K_3}{[Glucose] [MgATP^{2-}]} \left(1 + \frac{[ATP^{4-}_{free}]}{K_{ATP^{4-}_{free}}} + \frac{[HATP^{3-}]}{K_{HATP^{3-}}} \right)$$

Since at any pH the $\text{HATP}^{3-}_{\text{total}}/\text{ATP}^{4-}_{\text{total}}$ ratio is equal to some constant (k), then $[\text{HATP}^{3-}] = k[\text{MgATP}^{2-}] + k[\text{ATP}^{4-}_{\text{free}}]$. Substitution of this equality into the above rate expression yields upon rearrangement:

$$\frac{V_{\text{max.}}}{v} = 1 + \frac{kK_4}{K_{\text{HATP}^{3-}}} + \frac{K_4}{[\text{MgATP}^{2-}]} \left(1 + \frac{[\text{ATP}^{4-}_{\text{free}}]}{K_{\text{ATP}^{4-}_{\text{free}}}} + \frac{[\text{ATP}^{4-}_{\text{free}}]}{K_{\text{HATP}^{3-}}} \right) + \frac{K_3}{[\text{Glucose}]} \left(1 + \frac{kK_1}{K_{\text{HATP}^{3-}}} \right) + \frac{K_1K_3}{[\text{Glucose}][\text{MgATP}^{2-}]} \cdot \left(1 + \frac{[\text{ATP}^{4-}_{\text{free}}]}{K_{\text{ATP}^{4-}_{\text{free}}}} + \frac{[\text{ATP}^{4-}_{\text{free}}]}{K_{\text{HATP}^{3-}}} \right)$$

Although this expression appears complex, one should note that it predicts strictly hyperbolic velocity versus $[\text{MgATP}^{2-}]$ plots. The absence of quadratic terms in $[\text{MgATP}^{2-}]$ precludes any higher-order dependence of velocity upon $[\text{MgATP}^{2-}]$.

Bachelard (1971) attempted to illustrate more clearly the departure from Michaelis-Menten kinetics by comparing plots of $[\text{MgATP}^{2-}]/v$ versus $[\text{MgATP}^{2-}]$ at several Mg^{2+} concentrations. Again, in the presence of low $[\text{Mg}^{2+}]$ he found marked curvature in these plots. If one corrects these data as described in the legend to Fig. 1, the curvature of such plots is far less pronounced. It should be noted

that correction of Fig. 2(b) of Bachelard's (1971) paper requires large changes in the values plotted on both the ordinate and the abscissa. The corrected plot is shown in Fig. 2 of the present paper.

Effect of glucose on MgATP^{2-} binding

In his studies of the effects of Mg^{2+} and MgATP^{2-} on the kinetic properties of brain hexokinase, Bachelard observed non-linear velocity versus $[\text{MgATP}^{2-}]$ plots became less pronounced at elevated glucose concentrations and was apparently absent at saturating concentrations of glucose (Bachelard, 1971; Bachelard & Goldfarb, 1969). In a companion experiment, Bachelard (1971) observed that plots of $[\text{glucose}]/v$ versus $[\text{glucose}]$ were linear in the presence of excess of Mg^{2+} , but slightly curved when the $\text{Mg}^{2+}_{\text{total}}/\text{ATP}_{\text{total}}$ ratio was 1.0. Whereas Bachelard (1971) interprets these results to suggest that alterations in the conformation of the MgATP^{2-} -binding sites may affect the conformation of the glucose-binding site, a much simpler explanation can be offered in terms of the kinetic reaction mechanism of brain hexokinase action.

The kinetic mechanism appears to be of the sequential random type for rat and bovine brain hexokinase, and it is generally assumed that all substrates bind rapidly relative to the interconversion of the productive ternary complex. These conclusions were made on the basis of initial-rate studies in the absence and the presence of competitive inhibitors and product-inhibition studies (Fromm & Ning, 1968; Ning *et al.*, 1969; Purich & Fromm, 1971), and have been independently confirmed by the use of similar approaches (Bachelard *et al.*, 1971). In addition, we have found that the uncomplexed form of ATP acts as a linear competitive inhibitor with respect to MgATP^{2-} for these mammalian enzymes (Ning *et al.*, 1969; Purich & Fromm, 1971), whereas studies from Bachelard's laboratory appear to indicate 'mixed' inhibition (Bachelard & Goldfarb, 1969). The latter observation can be better understood if one considers the concentrations of ATP^{4-} present in the experiments of Bachelard and Goldfarb (1969) (assuming for the purpose of this analysis that all the uncomplexed ATP is ATP^{4-} , although under the conditions used 20% of it would exist as HATP^{3-}). They observed relatively small intercept changes in Lineweaver-Burk-type plots (Lineweaver & Burk, 1934) at the higher concentrations of ATP^{4-} which correspond to concentrations that are from 11.3- to

Fig. 2. Plot of $[\text{MgATP}^{2-}]/v$ versus $[\text{MgATP}^{2-}]$ for brain hexokinase at a $\text{Mg}^{2+}_{\text{total}}/\text{ATP}_{\text{total}}$ ratio of 1:1

Glucose concentrations were: ○, 0.1 mM; ●, 0.05 mM; □, 0.035 mM; and ■, 0.025 mM. The velocity measurements are those of Bachelard (1971) and have been corrected on the ordinate and abscissa by multiplication of a factor representing the fraction of $\text{ATP}_{\text{total}}$ as MgATP^{2-} , calculated as described in the legend to Fig. 1. Corrections were made on photographic enlargements of Fig. 2(b) of Bachelard's (1971) reported data.

Scheme 1.

17.5-fold higher than the dissociation constant for this inhibitor (Bachelard & Goldfarb, 1969). At such high concentrations of the free uncomplexed ATP, it is possible that these small intercept changes could result from a weak non-specific binding not related to the mode of binding at lower inhibitor concentrations where their results appear to suggest competitive inhibition. If we assume that ATP^{4-} is indeed a competitive inhibition of the brain enzyme, then Scheme 1 outlines the various interactions of substrates and ATP^{4-} with this phosphotransferase. The rate expression for the above kinetic mechanism can be expressed in the following form:

$$v = \frac{V_{\max.}}{1 + \frac{K_4}{A} \left[1 + \frac{I}{K_{11}} \right] + \frac{K_3}{B} + \frac{K_1 K_3}{AB} \left[1 + \frac{I}{K_1} \right]} \quad (1)$$

where v , $V_{\max.}$, A , B and I represent initial-reaction velocity, maximal velocity, $[\text{MgATP}^{2-}]$, $[\text{glucose}]$, and $[\text{ATP}^{4-}]$, respectively. The various K 's are dissociation constants defining the equilibria described in Scheme 1. If $K_1 \neq K_{11}$ or $K_1 \neq K_4$, increasing the glucose concentration will make the AB term in the denominator smaller and thereby lessen the inhibition by ATP^{4-} . At the $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratio of 1, there is a relatively high concentration of uncomplexed ATP^{4-} at the most dilute $[\text{ATP}_{\text{total}}]$, and the amount becomes less significant as the concentration of $\text{ATP}_{\text{total}}$ increases. These considerations therefore explain why one would observe an interdependence between the binding of MgATP^{2-} and glucose at the lower Mg^{2+} concentration. In a similar manner, one can understand why $[\text{glucose}]/v$ versus $[\text{glucose}]$ plots are slightly curved at sub-optimal amounts of the bivalent cation. Although we believe that ATP^{4-} inhibition is competitive, it may be important to note that similar arguments could also be made if the inhibition were 'mixed'. The rate equation for ATP^{4-} acting as a 'mixed' inhibitor would contain an AB term in its denominator as in eqn. 1. Elevated concentrations of glucose would again be expected to decrease the ATP^{4-} inhibition in the manner described for the effect of glucose on a competitive inhibitor of MgATP^{2-} .

Discussion

This report attempts to re-interpret Bachelard's (1971) data in terms of previous kinetic studies in which no evidence was obtained for an allosteric role for either Mg^{2+} or MgATP^{2-} , and is similar to a report (Blair, 1969) which re-evaluated the role of Mg^{2+} and ATP^{4-} in the pyruvate carboxylase reaction (Keech & Barritt, 1967). Our major contention is that Bachelard's (1971) kinetic data must be corrected to reflect the concentration-dependent stability of the MgATP^{2-} complex whenever $[\text{Mg}_{\text{total}}^{2+}] = [\text{ATP}_{\text{total}}]$ and their respective concentrations are sufficiently low. If such corrections are made, the velocity dependence upon the concentration of MgATP^{2-} becomes strictly hyperbolic. In addition, corrections of this sort and also for ATP^{4-} inhibition appear to reconcile the non-hyperbolic glucose-saturation curves with all previous studies, in which strictly Michaelis-Menten isotherms were observed. Although these corrections serve to clarify the action of Mg^{2+} and MgATP^{2-} in the brain hexokinase reaction, it may be of value to briefly summarize several additional arguments against an allosteric action by these compounds.

On the basis of Hill plots, Bachelard (1971) has stated that the number of binding sites for MgATP^{2-} on brain hexokinase is 1.8 at a $\text{Mg}_{\text{total}}^{2+}/\text{ATP}_{\text{total}}$ ratio of unity, and 1.0 in the presence of excess of Mg^{2+} . Here again, corrections for the large percentage of total ATP that exists as the uncomplexed ATP^{4-} and for the inhibitory action of this species suggest that the Hill coefficient at high and low $[\text{Mg}^{2+}]$ is near unity (i.e., $n < 1.25$). In this regard, we should also like to point out some possible limitations in the use of Hill plots in kinetic studies of multi-substrate co-operative enzyme systems. The original formulation of the Hill plot was in terms of ligand binding to a protein assuming that the concentrations of all protein-ligand complexes having less than n molecules of bound ligand are negligibly small (Hill, 1913). This was later put in terms of velocity on the assumption that $v/V_{\max.}$, the fractional attainment of maximal initial velocity, for a one-substrate enzymic reaction is strictly proportional to $\bar{Y}$, the fractional

saturation of substrate-binding sites (Atkinson *et al.*, 1965). Unfortunately, this equation can be rather indiscriminately applied to studies of multisubstrate co-operative enzymes where the above proportionality may or may not hold depending upon the concentration of the other substrate(s) involved in the reaction. For example, by analogy with the rapid-equilibrium random mechanism described earlier, the dependence of $v/V_{\max}$ on the concentration of substrate A is also related to the concentration of substrate B, as indicated by the following relationship:

$$\bar{Y} = \frac{(EA) + (EAB)}{E_0} = \frac{v(1 + K_3/B)}{V_{\max}} \quad (2)$$

where the different enzyme forms and kinetic terms are as defined earlier, and E_0 is the total enzyme concentration. Clearly, $\bar{Y} = v/V_{\max}$ only when substrate B is saturating, and the apparent values for the maximal velocity, obtained by the usual extrapolation of Lineweaver-Burk plots, cannot be used in place of the true $V_{\max}$. Finally, it is not altogether clear what n truly means; Atkinson (1966) has stated that treatments considering the slope to be either an 'interaction factor' or an indication of the number of binding sites may be equally in error without further analysis. These considerations point to an obvious need for the cautious application of the Hill equation in kinetic studies of multisubstrate co-operative enzyme reactions.

One additional point should be made regarding the differences in the observed maximal velocity at $Mg^{2+}_{\text{total}}/ATP_{\text{total}}$ ratios of 1.0 and 5.0 in Bachelard's (1971) study of the velocity dependence of the hexokinase reaction upon $[MgATP^{2-}]$. From Fig. 4 of his paper, it seems clear that the excess of Mg^{2+} inhibits the enzyme and may account for the lower maximal velocities at $Mg^{2+}_{\text{total}}/ATP_{\text{total}}$ ratio of 5.0.

It should be noted that whereas it is possible to rationalize all of Bachelard's (1971) data in terms of normal Michaelis-Menten kinetics, it is not possible to make similar arguments regarding a recent study suggesting allosteric interactions in the yeast hexokinase reaction (Kosow & Rose, 1971). In the latter study, velocities were determined at pH 6.9, where the kinetics with respect to ATP are complicated by the presence of ATP^{4-} , $MgATP^{2-}$, $HATP^{3-}$, and $MgHATP^{-}$. In addition, the stability constant for the $MgATP^{2-}$ complex is only $15000 M^{-1}$ in the buffer used in those studies (D. L. Purich & H. J. Fromm, unpublished work), and the buffer, itself, binds appreciable amounts of the Mg^{2+}_{total} (Good *et al.*, 1966). It should be pointed out, however, that our studies of the forward and reverse reaction kinetics of yeast hexokinase at pH 6.5 in another buffer showed no deviation from normal kinetics in either initial-rate studies or isotope-exchange at equilibrium studies (Fromm *et al.*, 1964).

With nucleoside 5'-triphosphate-dependent transphosphorylases, we have found that the free uncomplexed Mg^{2+} concentration must be maintained at a fixed value in each reaction mixture. This procedure ensures that a constant fraction of the total nucleotide is in the form of the Mg^{2+} -nucleotide complex. Whereas this free Mg^{2+} concentration is frequently around 1 mM (Copley & Fromm, 1967; Rudolph & Fromm, 1969), the free $[Mg^{2+}]$ optimum depends upon the enzyme under study and, to a large extent, upon the concentration, pH, and type of buffer used in the rate experiments. It is therefore advantageous to evaluate the optimum free Mg^{2+} concentration experimentally at the extremes of the concentration ranges of substrates to be studied. It is generally insufficient to maintain the $Mg^{2+}_{\text{total}}/ATP_{\text{total}}$ ratio at 2.0, as has been done in many rate studies. For such cases, the percentage of ATP^{4-}_{total} existing in solution as $MgATP^{2-}$ will vary with the extent of dilution. If, for example, the $Mg^{2+}_{\text{total}}/ATP_{\text{total}}$ ratio is 2.0 and the stability constant for this complex is taken to be $73000 M^{-1}$ (O'Sullivan & Perrin, 1964), then the ATP_{total} concentration must exceed 0.25 mM or else the free uncomplexed ATP^{4-} will exceed 5% of the total ATP concentration. The potential seriousness of such an approach can be illustrated by a kinetic study of rabbit muscle phosphofructokinase (Uyeda, 1970). In that study, the ratio of total Mg^{2+} to total ITP was maintained at 2.0, and the total ITP was varied in the concentration range from 12 to 200 μM . If one assumes that the stability constant for the $MgITP^{2-}$ complex is $20000 M^{-1}$ for the Mg^{2+} -nucleotide complex in tris buffer (O'Sullivan & Perrin, 1964), simple calculation shows that the percentage of total ITP as $MgITP^{2-}$ varied from approximately 29 to 83%. This variation becomes especially important if the free uncomplexed nucleotide is inhibitory. However, it is also inadvisable to utilize higher $Mg^{2+}_{\text{total}}/ATP_{\text{total}}$ ratios as the free uncomplexed Mg^{2+} is often inhibitory at higher concentrations. The above statements also pertain to studies of nucleoside 5'-diphosphate-dependent transphosphorylases, but the free Mg^{2+} concentration must be maintained at a higher constant and non-inhibitory concentration.

Finally, we should note here that it is not our intent to suggest that the kinetic studies of Bachelard (1971) describing the sigmoidal dependence of reaction velocity upon [total ATP] may not be of functional importance. Indeed, in cases where the true substrate of an enzymic reaction is the metal-substrate complex, functionally important non-hyperbolic velocity responses to changes in the total substrate concentration may occur. In addition, if the uncomplexed substrate acts as a competitive inhibitor relative to the metal-substrate complex, the deviation from normal hyperbolic responses may be more pronounced. This can be illustrated in the case of a simple one-substrate

Fig. 3. Plot of $v/V_{\max}$ versus the millimolar concentration of total substrate for a model one-substrate enzyme

The concentrations of S_{free} and the metal-S complex were estimated assuming a stability constant of 10000M^{-1} . The K_m and K_i for metal-S complex and S_{free} , respectively, were assumed to be 0.5mM . The ratio $v/V_{\max}$ was calculated from the rate expression for a simple one-substrate enzyme obeying Michaelis-Menten kinetics. Curve A (∇) represents the case where total $[\text{substrate}] = [\text{metal-S complex}]$; curve B ($\circ$) is where the velocity is strictly a function of the metal-S complex concentration, as determined by using the stability constant; curve C (Δ) is where velocity is dependent upon metal-S complex concentration as in curve B, but also accounts for competitive inhibition by S_{free} relative to the metal-S complex. The total metal ion to total substrate ratio was maintained at 1.0. Other details are described in the text.

model enzyme system for which it is assumed that the active substrate species is the 1:1 complex of substrate and metal ion. For example, the theoretical velocity-response curves presented in Fig. 3 show how the normal hyperbolic dependence (curve A) can give sigmoidal substrate-saturation curves (curves B and C). For curve A, the stability constant for the complex is assumed to be sufficiently great that $[S_{\text{total}}]$ is equivalent to the concentration of the metal-substrate complex. Alternatively, one may assume that the stability constant is lower but that there is a sufficient concentration of free metal ion to force the substrate into combination with the metal ion. The responses in curves B and C were computed assuming

a stability constant of 10000M^{-1} . For curve B it is assumed that the free uncomplexed substrate is non-inhibitory, whereas for curve C it is assumed that free substrate acts as a competitive inhibitor. The non-linear responses of hexokinase reaction velocities to total ATP concentration may represent such an example for a two-substrate enzymic reaction. These responses may be of functional importance since it is likely that the non-particulate Mg^{2+} concentration in cerebral cortical tissue is approximately 1.5 to 3.5mM (Bachelard & Goldfarb, 1969), slightly lower than the ATP concentration in this tissue. Whereas one might expect that the amount of Mg^{2+} will remain constant, it is important to recognize that the amount of Mg^{2+} associated with the ATP pool is the important factor. Clearly, this will change with changes in the ATP concentration, and the other Mg^{2+} -metabolite complexes within the cell will act as the source of this bivalent metal ion. Moreover, there is growing evidence suggesting that the free uncomplexed Mg^{2+} concentration in many tissues is below 0.4mM (Rose, 1968; England *et al.*, 1967; Peck & Ray, 1971). These results suggest that appreciable fractions of the total ATP pool within the cell may be present as the inhibitory free uncomplexed ATP^{4-} . The effect of suboptimal Mg^{2+} concentrations and the presence of free ATP^{4-} on the responses of several glycolytic phosphotransferases to the adenylate energy charge has been recently evaluated (Purich & Fromm, 1972). These experiments would suggest that the above factors may play metabolically important roles.

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