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Kinetic and regulatory properties
of rat brain hexokinase

by

Daniel Lee Purich

A Dissertation Submitted to the
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INTRODUCTION

Interest in enzymes catalyzing glucose phosphorylation has been considerable ever since 1927 when Meyerhof obtained an enzyme from yeast extracts that enabled old muscle juice to regain its ability to ferment sugars. The Meyerhof enzyme, termed hexokinase (ATP:D-hexose-6 phosphotransferase, EC 2.7.1.1), was found to be identical to the yeast enzyme, heterophosphatase, which von Euler and Adler (1935) and Lutwak-Mann and Mann (1935) also found to be capable of catalyzing the transfer of the terminal phosphoryl group of ATP to a number of hexoses according to the following reaction:

\[
\text{D-Hexose} + \text{MgATP}^{2-} \rightleftharpoons \text{D-Hexose-6-P}^{2-} + \text{MgADP}^{1-} + H^+ 
\]

Although the enzyme is apparently ubiquitous in nature, the yeast and mammalian enzymes are probably the best characterized. The yeast enzyme has been studied in extenso with regard to its kinetic and physical properties. In fact, a number of new kinetic techniques for evaluating multisubstrate kinetic mechanisms with regard to the order of substrate addition and product removal were used during their development to study the yeast enzyme. These include the use of product inhibitors (Fromm and Zewe, 1962b; Zewe, Fromm, and Fabiano, 1964; Fromm, 1968), substrate analogs as competitive inhibitors (Fromm and Zewe, 1962b; Zewe, Fromm, and Fabiano, 1964), isotope exchange at equilibrium (Fromm, Silverstein,
and Boyer, 1964), and alternative substrates and products (Fromm and Zewe, 1962b; Fromm, 1964). In addition, there has been a lasting interest in the ATP-phosphohydrolase activity of the yeast enzyme (Kaji and Colowick, 1965; Trayser and Colowick, 1961; Rudolph and Fromm, 1971). On the other hand, the mammalian hexokinases have been examined largely with interest in their regulatory properties. As early as 1945, Utter, Wood, and Reiner reported that centrifugation of rat brain homogenates resulted in substantial loss of hexokinase activity, and this finding may have been the basis for the suggestion of Meyerhof and Geliazkowa (1947) that the enzyme exists primarily in a bound form in marked contrast with the other soluble glycolytic enzymes. In addition, there has been great interest in the manner in which mammalian hexokinase activity is coordinated with phosphofructokinase to provide for effective control of glycolysis.

Over the years since Meyerhof's discovery of the hexokinase reaction, there have been numerous studies that have contributed to our present understanding of the mode of action and regulation of the hexokinases. A complete discussion of these investigations of yeast and mammalian hexokinases is clearly beyond the scope of this introduction which will be restricted to the mammalian brain enzyme. For a more extensive treatment of this topic, the reader is
referred to a forthcoming review by Rudolph, Purich, and Fromm (1973).

Early investigations of glucose-6-P inhibition of brain hexokinase suggested that this sugar-P acted as a specific potent noncompetitive product inhibitor relative to the sugar substrate. This conclusion was alluded to when it was observed that high concentrations of glucose did not reverse the inhibition of glucose-6-P. Weil-Malherbe and Bone (1951) concluded from such studies that glucose and glucose-6-P occupy separate sites on the brain enzyme, and Crane and Sols (1953) came to a similar conclusion. Later studies by Crane and Sols (1954) showed that low levels (~100 μM) brought about potent inhibition of brain hexokinase whereas other possible reaction products such as 2-deoxyglucose-6-P, mannose-6-P, and fructose-6-P were considerably less effective. They also found that sorbose-1-P and 1,5-anhydroglucitol-6-P were also excellent inhibitors. It was this specificity of product inhibition and their observation of noncompetitive inhibition relative to glucose that led Crane and Sols (1954) to conclude that brain hexokinase possesses a glucose-6-P specific allosteric site.

In 1954, Racker proposed that the potent glucose-6-P inhibition of mammalian hexokinases might play an important role in the Pasteur effect which refers to the acceleration of the glycolytic rate when respiration is prevented by
anaerobiosis. Subsequent studies by the Racker group established that the maximal *in vivo* hexokinase activity was consistently lower than the optimal activity of any other glycolytic enzyme in ascites tumor cells, HeLa cells, chicken leukocytes, and mouse brain, and that only with hexokinase was a considerable portion of any glycolytic enzymatic activity associated with particulate fractions (Wu and Racker, 1959a,b; Racker, 1965). Lowry and Passonneau (1964) concluded on the basis of a similar analysis and additional experiments on the effects of anoxia on the intracellular content of key glycolytic intermediates that the hexokinase and phosphofructokinase reactions were the important regulatory steps in brain glycolysis.

Racker has also provided several studies that illustrate the functional significance of differences between the mammalian and yeast enzyme which is only weakly inhibited by glucose-6-P (Fromm and Zewe, 1962b). The early experiments of Gatt and Racker (1959) with reconstructed glycolytic systems were performed using highly purified enzymes in proportion to their measured optimal enzymic activities. All of these enzymes were purified from rabbit muscle with the exception of yeast hexokinase which was the only highly purified and soluble hexokinase available. In the presence of mitochondria and the reconstructed glycolytic system, competition for common cofactors such as $P_i$ and ADP was
observed. In the presence of limiting concentrations of $P_i$ and ADP, respiration was inhibited whenever the glycolytic enzymes predominated, and the rate of lactate production was inhibited whenever the mitochondria were present. Although these phenomena resembled the well-known Pasteur effect (1876) and Crabtree effect (1929) observed in intact cells, the inhibition of lactate production in the reconstructed systems was accompanied by an accumulation of a rather large amount of fructose-1,6-diP which is not characteristic of these effects as observed in vivo. A re-examination of similar reconstructed systems by Uyeda and Racker (1965a,b) in the presence of ascites tumor hexokinase demonstrated that the source of hexokinase activity greatly affected the behavior of the entire reconstructed pathway. The Pasteur and Crabtree effects could again be demonstrated, but, more significantly, there was no fructose-1,6-diP accumulation or change in the glucose-6-P level even at greatly different rates of the overall reaction sequence. Thus, use of mammalian hexokinase in place of the yeast enzyme brought the properties of the reconstructed pathway into qualitative agreement with events occurring in vivo. Moreover, the observation that the glucose-6-P level did not change appreciably suggested that the rates of the hexokinase and phosphofructokinase reactions are rigorously coordinated. Uyeda and Racker (1965a,b) suggested that $P_i$ played a major role by countering
the glucose-6-P inhibition of tumor hexokinase. Such an observation would be in agreement with the observation of Rose and O'Connell (1964) that glucose-6-P inhibition of red cell hexokinase is diminished in the presence of orthophosphate.

In 1962, initial rate studies of the particulate calf brain enzyme were carried out in order to define the kinetic reaction mechanism (Fromm and Zewe, 1962b). These investigators found that Lineweaver-Burk type plots of the initial reaction velocity dependence on one substrate at several constant levels of the other substrate were parallel, suggesting that a covalent enzyme-substrate intermediary was formed in the course of the reaction. In addition, their studies of the glucose-6-P inhibition indicated that the sugar-P product was a linear competitive inhibitor relative to ATP and a noncompetitive inhibitor with respect to glucose. That glucose-6-P and ATP compete for the same locus on the enzyme was clear evidence against an allosteric role for glucose-6-P as proposed earlier by Weil-Malherbe and Bone (1951) and Crane and Sols (1954). The competitive nature of the glucose-6-P with respect to ATP was rationalized in terms of a common binding site on the enzyme for the 6-phospho and γ-phosphoryl groups on glucose-6-P and ATP, respectively.

Fromm and Zewe (1962b) also found that ADP inhibition relative to either substrate was noncompetitive, and suggested that
this type of inhibition could result from ADP binding at the
ADP site on the phosphoryl-enzyme and the ATP site on the
free uncomplexed enzyme. Similar results were observed by
Hanson and Fromm (1965) for the soluble brain isozyme iso­
lated from rat skeletal muscle and by Copley and Fromm (1967)
for the Triton X-100 solubilized bovine brain enzyme. In
contrast, Hanson and Fromm (1967) found that the rat skeletal
muscle isozyme had kinetic properties similar to the yeast
enzyme, for which a rapid equilibrium random mechanism appar­
ently pertains on the basis of numerous kinetic experiments
(Fromm and Zewe, 1962b; Fromm, Silverstein, and Boyer, 1964;
Rudolph and Fromm, 1971). More recent studies of the kinetic
mechanism of the bovine brain enzyme have shown that (a) the
kinetic properties of the bovine brain enzyme are most
reasonably rationalized in terms of a rapid equilibrium
random mechanism, (b) the enzyme catalyzes the reverse reac­
tion at elevated levels of Mg$^{2+}$ at pH 6.5 where the apparent
equilibrium constant for the hexokinase reaction is only 490,
(c) pyrimidine nucleotides can only bind at the ATP site, and
(d) ADP inhibition arises from binding at an inhibitory
nucleotide site that is distinct from the active site on the
basis of kinetic criteria (Ning, Purich, and Fromm, 1969).

It appeared advantageous to reinvestigate the properties
of rat brain hexokinase to determine whether the kinetic
observations with the bovine enzyme represent general features
of mammalian brain hexokinases. Furthermore, since the analytical concentrations of the various hexokinase effectors in rat brain are available in the literature, it was felt that the direct effects of these compounds on soluble and particulate brain hexokinase could be evaluated. Moreover, the effects of these metabolites on the \textit{in vitro} soluble-particulate distribution of hexokinase activity could be measured. The role of Mg$^{2+}$ and MgATP$^{2-}$ in the hexokinase reaction was also studied. Finally, the response of brain hexokinase to the adenylate energy charge was evaluated under simulated intracellular conditions.
EXPERIMENTAL PROCEDURE

Mannitol, D-glucose, and D-fructose were obtained from Pfanstiehl Laboratories, Waukegan, Illinois. D-Glucose-6-P dehydrogenase, DPNH, glucose-6-P, PEP, rabbit skeletal muscle lactate dehydrogenase (specific activity, 750-1500 units/mg), rabbit skeletal muscle pyruvate kinase (specific activity, 178 units/mg), and yeast hexokinase (200 units/mg) were products of Calbiochem. ATP, GTP, UTP, ADP, GDP, UDP, AMP, GMP, UMP, TPN\(^+\) and phosphoglucoisomerase were purchased from Sigma Chemical Company. Analytical grade Dowex 1-X2 (200-400 mesh) was obtained from Bio-Rad Laboratories, Richmond, California. Uniformly labeled D-glucose\(^{14}\)C (specific radioactivity, 240 mCi/mmole) was the product of Schwartz Bio-Research. Ion-low water, obtained from passage of distilled water through a Rohm and Haas MB-3 resin bed, was used to prepare all reagents.

Male Wistar strain rats (200-250 g), obtained from Schettle Biologicals, St. Paul, Minnesota, were allowed free access to Kent animal feed pellets and water. The animals were maintained on this diet for no more than 2 weeks before they were sacrificed. On occasion, male rats were also obtained from the rat colony maintained at the Department of Food and Nutrition, Iowa State University.
**Standard Hexokinase Assay**  
Determination of hexokinase activity during the purification was made by observing the absorbancy increase at 340 nm accompanying conversion of TPN⁺ to TPNH. The routine assay mixture contained in a total volume of 2.0 ml, 50 mM Tris-HCl (pH 7.4), 25 mM D-glucose, 0.5 mM TPN⁺, 3.7 mM ATP, 7.4 mM MgCl₂, 5 mM 2-mercaptoethanol, and approximately 1 international unit of glucose-6-P dehydrogenase. The reactions were followed at 28°C in a Cary model 15 recording spectrophotometer with a thermostatted cell housing. One unit of activity is defined as the amount of enzyme required to catalyze the reduction of 1μ mole of TPN⁺ per min.

**Protein Determination**  
The protein concentration was determined by use of the Biuret method (Gornall et al., 1949) with bovine serum albumin as a standard. Specific activity is expressed as units of enzyme activity per mg of protein.

**TPN⁺ Assay**  
TPN⁺ concentration was assayed spectrophotometrically on the basis of its molar extinction coefficient, 18.0 x 10³ M⁻¹ cm⁻¹, at pH 7.0 and 260 nm. Stock solutions were stored at -15° when not in use.

**DPNH Assay**  
DPNH concentration was assayed spectrophotometrically on the basis of its molar extinction coefficient, 6.2 x 10³ M⁻¹ cm⁻¹, at pH 8 and 340 nm. Stock
solutions were prepared immediately prior to use by dissolving the sodium salt of this nucleotide directly into buffer.

**Nucleotide Concentration and Purity** Concentrations used in this study were determined from their characteristic ultraviolet absorption (P-L Laboratories Circular OR-24). The purity of the nucleotides was determined by chromatography on DEAE-cellulose ion-exchange paper essentially as described by Morrison (1968). The developing solvent was prepared by a 1:5 dilution (v/v) of a 0.6 M ammonium formate-0.005 M EDTA solution which was adjusted to pH 3.1 by the addition of concentrated formic acid. Only those nucleotides that appeared homogeneous under ultraviolet illumination were used.

**Glucose Assay** Glucose concentration was enzymatically determined using commercial yeast hexokinase and glucose-6-P dehydrogenase. Reaction mixtures (3.0 ml final volume) contained 0.5 mM ATP, 1.5 mM MgSO\textsubscript{4}, 0.05 M Tris-HCl buffer (pH 7.6), 0.3 mM TPN\textsuperscript{+}, and a substantial excess of yeast hexokinase and glucose-6-P dehydrogenase. The assay also contained from 0.1 to 0.2 mM D-glucose or water which served as the absorbance blank. Optical density measurements were made at 340 nm in a Beckman DU spectrophotometer fitted with a Gilford model 220 optical density converter.

The purity of \textsuperscript{14}C-D-glucose was confirmed by ascending
paper chromatograph on Whatman 3M paper using 70% n-propanol. Dried chromatograms were subjected to radioautography, and it was found that more than 98% of the radioactivity was concentrated in a single spot. The location corresponded to an approximate $R_f$ of 0.5.

**Fructose Assay** The concentration of stock fructose solutions was estimated as with glucose but in the presence of rabbit phosphoglucoisomerase. Stock solutions were stored at $-15^\circ$ when not in use.

**Preparation of Brain Hexokinase** A modification of the procedure of Grossbard and Schimke (1966) was utilized. Rats were killed by a blow to the head, decapitated, and the entire brain was removed and placed on ice. Brain (27 g) was homogenized in a hand operated Potter-Elvehjem homogenizer fitted with a teflon pestle in an equal volume of ice-cold 0.01 M potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, 5 mM disodium EDTA, and 10 mM glucose. (This buffer is hereafter referred to as Standard Buffer). The homogenate was centrifuged at 30,000 $x$ g for 30 min in a Sorvall refrigerated centrifuge, and the supernatant extract (~25 ml) was decanted and retained. The tissue was then extracted with 15 ml Standard Buffer, re-centrifuged as above, the supernatant fluids were combined, and dialyzed against 2000 ml of Standard Buffer in a continuous flow
rocking dialyzer.

The dialysate (45 ml) was then applied to a Whatman DE-52 DEAE-cellulose anion-exchange column, 1.0 x 13.0 cm, that was packed under approximately 1 pound pressure of nitrogen. The column was washed with Standard Buffer (150 ml), and then developed with a 250 ml linear gradient from 0 to 0.6 M KCl in Standard Buffer. Type I hexokinase, the first hexokinase activity to issue from the column, was collected and dialyzed for approximately 4 hours against 2000 ml Standard Buffer. It was reapplied to a 1 x 4 cm DEAE-cellulose column which had been equilibrated with Standard Buffer. The enzyme was eluted with the same buffer containing 0.15 M KCl, and solid glucose was added to a concentration of 0.1 M. The eluate was concentrated by dialysis against dry Sephadex G-25 (coarse) and purified through the first ammonium sulfate fractionation as described by Grossbard and Schimke (1966).

The enzyme (specific activity, 0.5 units/mg), which was stable for months in neutral 3.0 M ammonium sulfate-0.01 M glucose, was exhaustively dialyzed against 0.05 M Tris-HCl (pH 7.6) immediately prior to use. It was also shown to be free of glucose-6-phosphatase, ATPase, and myokinase.

**Magnesium Ion Levels** The concentration of free uncomplexed magnesium ion (as MgSO$_4$) was rigorously controlled in all experiments. Calculation of the proper Mg$^{2+}$:ATP$^{4-}$
ratio in Tris-HCl (pH 7.6) was based upon a value of 20,000 M\(^{-1}\) for the MgATP\(^{2-}\) complex (O'Sullivan and Perrin, 1964). Similar calculations were made for MgADP\(^{1-}\) and MgAMP assuming stability constants of 2,000 and 100 M\(^{-1}\), respectively (Bock, 1960). When HEPES buffer was utilized, adjustments were made assuming a value of 100,000 M\(^{-1}\) for the MgATP\(^{2-}\) complex (Rudolph and Fromm, 1969).

**Kinetic Measurements**

Initial velocity measurements were made in a Cary model 15 spectrophotometer maintained at 28°. All reactions were initiated by the addition of the brain enzyme. Initial rates of glucose-6-P production were assayed with a coupled enzyme system containing, in addition to the other components, 60 \(\mu\)M TPN\(^+\), Tris-HCl (pH 7.6), and a substantial excess of glucose-6-P dehydrogenase. When fructose was employed as the sugar substrate, excess phosphoglucoisomerase was added to the above solution. Initial rates of ADP production were evaluated by use of a coupled enzyme system containing, in addition to other components, 10 mM potassium chloride, 0.5 mM P-enolpyruvate, 50 \(\mu\)M DPNH, and an excess of lactate dehydrogenase and pyruvate kinase (Copley and Fromm, 1967). In those experiments where both glucose-6-P and the nucleoside 5'-diphosphates were present as inhibitors, the initial rate of conversion of radioactive glucose to radioactive glucose-6-P was measured. The reactions were stopped by boiling for 1 min. Samples were assayed at various
time intervals to ensure linearity with time, and blanks were analyzed at each time and each glucose concentration. Ten milliliters of 40 mM glucose were added to the boiled reaction mixture, and 10 ml of the sample were washed onto an AG1-X2-C1- column (2 x 4 cm) which had been exhaustively washed with water and then equilibrated with 40 mM glucose. The column was then washed with 50 ml of 40 mM glucose to remove the $^{14}$C-glucose present. Finally, 50 ml of H$_2$O were passed through the column, and the glucose 6-phosphate was removed with 0.1 N HCl. Ten milliliters of the acid effluent were collected in a volumetric flask, and 2 ml of this solution were placed in 15 ml of the naphthalene-dioxane fluid and counted. A Packard Tri-Carb liquid scintillation spectrometer was used for all counting. Unless otherwise stated, velocities in all experiments are expressed as molarity of product formed per min.

**Estimated Intracellular Metabolite Levels**

Estimation of the intracellular metabolite concentrations requires conversion of analytical data (usually expressed in terms of micromoles per g of fresh tissue) into units of molarity. Corrections were made for the volumes of blood, cerebrospinal fluid, and extracellular space. These values were taken to be 3, 9, and 20%, by volume, respectively (Bachelard and McIlwain, 1969; Bachelard, 1969; Bachelard, 1970). We further assume that the tissue is 80% water and that the metabolites are uniformly distributed throughout the cell. This latter
assumption requires that metabolite concentration gradients are reasonably small, if present, and that the mitochondrial adenylate pool does not constitute a large fraction of the total adenylate pool. This is justified in part by the observation that rat brain mitochondria have lower concentrations of adenylates than other mitochondria (Clark and Nicklas, 1970). Analytical values for ATP, AMP, and glucose-6-P (Lowry et al., 1964) were corrected to 4.4, 0.4, and 0.15 mM, respectively. The ADP level was calculated to be 0.75 mM based upon an analytical concentration of 0.5 μmole per g of tissue (Lowry, 1969). Values for GTP (Tarr et al., 1962) and UTP (Tarr et al., 1962; Schmitz et al., 1954) were corrected to 1.5 and 1.3 mM, respectively. Similarly, the analytical level of $P_i$ was corrected to 9.2 mM (McIlwain, 1966). Finally, the intracellular concentration of "free" nonparticulate Mg$^{2+}$ was taken to be 1.5 mM (Bachelard and Goldfarb, 1969).

**Determination of Soluble-Particulate Distribution of Hexokinase Activity** Each study on the soluble-particulate distribution of hexokinase used freshly prepared mitochondria from a single rat brain (1.9 to 2.0 g). The procedures outlined here are similar to those used to study the distribution of hexokinase activity in ascites tumor cells and bovine cerebral cortical tissue (Rose and Warms, 1967). The animal was killed by suffocation in a nitrogen atmosphere and its brain immediately removed intact. The whole rat brain was
homogenized in 10 ml of 0.25 M mannitol in a hand-operated Potter-Elvehjem homogenizer. The homogenate was freed of cellular debris by centrifugation at 800 x g for 25 min, and the supernatant solution was collected and centrifuged at 15,000 x g for 1 hour. The mitochondrial pellet was washed three times by suspension in mannitol solution with the homogenizer and centrifugation as above. Finally, the mitochondria were evenly suspended in 10 ml of mannitol solution in a similar manner. This material, when incubated at 36° for 30 min in the presence of 25 mM Tris-HCl (pH 7.6), had no detectable hexokinase activity in the supernatant fluid obtained after centrifugation at 15,000 x g for 1 hour.

Aliquots of the mitochondrial suspension were incubated at 36° for 30 min in the presence of the various compounds listed in the text. Next, the 3.0-ml samples were cooled in an ice bath and centrifuged at 15,000 x g for 1 hour. The supernatant solution was carefully decanted and then the walls and pellet were gently washed with 7.0 ml of mannitol solution which was removed and combined with the previous decantant. The pellet was transferred to a homogenizer with 10 ml of mannitol solution and evenly suspended. A 0.025-ml aliquot was assayed for hexokinase activity, and the activity is reported as the percentage of hexokinase remaining associated with the mitochondrial pellet after treatment. No correction was necessary for time-dependent changes in turbidity.
Correction of Rate Data to Account for the Concentration-Dependent Stability of MgATP$^{2-}$

To estimate the true MgATP$^{2-}$ complex concentration over a range of ATP$^{4-}$ and Mg$^{2+}$ total concentrations, the following quadratic equation was solved for the real root:

\[
(MA) = \frac{(1 + KM + KA) \pm \sqrt{(1 + KM + KA)^2 - 4K^2M_A}}{2K}
\]

$MA$, $K$, $M_A$, and $A$ represent the MgATP$^{2-}$ concentration, the stability constant for the MgATP$^{2-}$ complex, total magnesium ion concentration, and total ATP$^{4-}$ concentration. $K$ was taken to be 73,000 M$^{-1}$ for MgATP$^{2-}$ in 0.15 M triethanolamine-HCl, pH 7.6 (O'Sullivan and Perrin, 1964; Bachelard, 1971b). Corrections of the MgATP$^{2-}$ concentration in the initial rate experiments of Bachelard (1971b) to account for the MgATP$^{2-}$ stability were made on photographic enlargements of published figures.
RESULTS AND DISCUSSION

Initial Velocity Measurements and Kinetic Reaction Mechanism

In earlier studies of rat brain hexokinase, glucose was utilized as the sugar substrate (Hanson and Fromm, 1965). Lineweaver-Burk type plots of their initial rate data yielded parallel lines when either substrate was varied at different constant levels of the second substrate. These results were taken as evidence for the participation of an enzyme-glucosyl or enzyme-phosphoryl intermediary [i.e. the kinetic mechanism was Ping-Pong]. Scheme I depicts the model that appeared to be most reasonable in view of the above initial rate data. In this model, E, A, EA, E', P, B, E'B,

\[
\begin{align*}
E + A & \xrightleftharpoons[k_2]{k_1} EA & \xrightarrow[k_4]{k_3} E' + P \\
E' + B & \xrightleftharpoons[k_6]{k_5} E'B & \xrightarrow[k_8]{k_7} E + Q
\end{align*}
\]

Scheme I

and Q are taken to represent free enzyme, substrate A, enzyme substrate A Michaelis complex, the covalent enzyme-substrate intermediary, product P, substrate B, covalent enzyme-substrate substrate B Michaelis complex, and product Q. The rate expression for this mechanism in Dalziel (1957) form is:
\[
\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{(A)} + \frac{\phi_2}{(B)}
\]  

(1)

where the \( \phi \) values are collections of rate constants as first defined by Dalziel (1957), and \( E_0 \) and \( v \) are total enzyme concentration and initial reaction velocity, respectively.

Similar parallel-line reciprocal plots have been observed with particulate and solubilized calf brain hexokinase (Fromm and Zewe, 1962a; Copley and Fromm, 1967). Recently, studies of bovine brain hexokinase, however, indicated that the mechanism appeared sequential when fructose was utilized as the sugar substrate (Fromm and Ning, 1968). Their findings were consistent with either of the general two substrate mechanisms shown in Scheme 2.

A. Ordered Ternary Complex Mechanism
Figure 1. Plot of the reciprocal of the initial reaction velocity (v) versus the reciprocal of the molar concentration of ATP. Fructose concentrations were held constant at 1.11 mM (◇), 1.43 mM (○), 2.00 mM (□), 3.33 mM (△), and 10.0 mM (▲); v was determined as a function of ATP concentration in the range from 0.28 to 2.5 mM. Other experimental details are described under "Experimental Procedure."
Figure 2. Plot of the reciprocal of the initial reaction velocity (v) versus the reciprocal of the molar concentration of fructose.

ATP concentrations were held constant at 0.28 mM (▲), 0.36 mM (■), 0.50 mM (○), 0.83 mM (△), and 2.5 mM (□); v was determined as a function of fructose concentration in the range from 1.11 to 10.0 mM. Other experimental details are described under "Experimental Procedure."
B. Rapid Equilibrium Random Mechanism

Scheme II

It was originally shown by Alberty (1953) that the rate expression for a sequential mechanism will contain an \((A)(B)\) term. When expressed in Dalziel form, the general rate equation for sequential kinetic mechanisms is:

\[
\frac{E_0}{v} = 1 + \frac{\theta_1}{(A)} + \frac{\theta_2}{(B)} + \frac{\theta_{12}}{(A)(B)}
\]  

(2)

It is clear that plots of \(1/v\) versus \(1/A\) or \(1/B\) at several constant levels of \(B\) or \(A\), respectively, will result in both slope and intercept changes (i.e., the lines converge). When similar studies were carried out with rat brain hexokinase, the data presented in Figs. 1 and 2 were obtained. The data suggest that the kinetic mechanism with fructose as the sugar substrate is sequential, and that the \(\theta_{12}/(A)(B)\) apparently is not appreciable with glucose as the sugar substrate.

To discount the unlikely possibility that rat brain hexokinase possessed separate sugar binding sites and catalyzed
two independent phosphorylation reactions, one sequential (with fructose) and the other Ping-Pong (with glucose), further studies were carried out. It was found that the alternative substrate, D-fructose, competed with D-glucose for the same site on the brain enzyme. This was established by the results of the experiment presented in Fig. 3. The initial rate of glucose-6-P production was measured by using glucose-6-P dehydrogenase which is specific for D-glucose-6-P and will not utilize fructose-6-P.

Studies of AMP inhibition of rat (Hanson and Fromm, 1965) and bovine brain hexokinase (Fromm and Zewe, 1962a; Copley and Fromm, 1967) indicated that AMP was a noncompetitive inhibitor relative to either substrate. These data were compatible with the Ping-Pong mechanism if it was assumed that AMP could bind to the two enzyme forms, E and E' (i.e., AMP binds at the ATP and ADP sites on the enzyme. Such behavior is, for example, observed with the Escherichia coli acetate kinase for which there is both chemical (Anthony and Spector, 1970) and kinetic (Purich and Fromm, 1972a) evidence for the participation of a phosphoryl-enzyme intermediary. On the other hand, the behavior of AMP in the brain hexokinase reaction is not consistent with sequential pathways of enzyme and substrate interactions. Ning, Purich, and Fromm (1969), however, demonstrated that one could reconcile the AMP data with a rapid equilibrium random mechanism if one postulates
Figure 3. Plot of the reciprocal of initial reaction velocity ($v$) versus the reciprocal of the molar concentration of glucose in the absence and presence of fructose. ATP concentration was maintained at 0.5 mM, and fructose concentrations were held constant at none ($\nabla$), 1.0 mM (O), and 3.0 mM (□); $v$ was determined as a function of glucose concentration which was varied in the range from 0.11 mM to 1.0 mM. Other experimental details are described under "Experimental Procedure."
the existence of an additional nucleotide site topologically distinct from the ATP substrate site. The following experiments were carried out to demonstrate that a similar mechanism and nucleotide site pertains in the rat brain hexokinase reaction.

The use of competitive inhibitors of reaction substrates permits segregation of possible sequential mechanisms (Fromm, 1964; Zewe, Fromm, and Fabiano, 1964; Zewe and Fromm, 1965). The idea involved when using competitive inhibitors is that one observes unique inhibition patterns for random and ordered mechanisms as is illustrated in Table 1.

| Table 1. Inhibition Patterns of Competitive Substrate Inhibitors for Several Bisubstrate Reactions |
|---------------------------------|---------------------------------|
| Competitive Inhibitor for substrate | Inhibition relative to other substrate |
| Ordered Ternary Complex         | A                                | Mixed                        |
| Theorell-Chance (Binary Complex) | B                                | Uncompetitive                |
| Rapid Equilibrium Random        | A                                | Mixed                        |
|                                 | B                                | Uncompetitive                |
|                                 | A                                | Noncompetitive               |
|                                 | B                                | Noncompetitive               |

One may therefore use competitive inhibitor studies to "rule out" mechanisms whose theoretical inhibition patterns fail to conform to the experimentally observed mode of inhibition.
The findings presented in Figs. 4 and 5 indicate that N-acetyl glucosamine behaves as a linear competitive inhibitor relative to glucose and as a noncompetitive inhibitor with respect to MgATP$^{2-}$. ATP$^{4-}$ behaves as a linear competitive inhibitor relative to MgATP$^{2-}$ but ATP$^{4-}$ inhibition with respect to glucose is noncompetitive (See Figs. 6 and 7).

Equation 2 for the random mechanism in the absence of inhibitors can be rewritten in the following form:

\[
V = \frac{V_m}{1 + \frac{K_A}{(A)} + \frac{K_B}{(B)} + \frac{K_B K_D}{(A)(B)}}
\]  

(3)

The K's are dissociation constants for substrates from the several enzyme forms for mechanism A shown in Scheme II.

If A in Equation 3 is taken to represent ATP and B is the sugar substrate, then for a competitive inhibitor of A such as ATP$^{4-}$, the inhibitor (I) may react with the enzyme forms as follows.

\[
\begin{align*}
E + I &= EI, K_1 \\
EI + B &= EIB, K_2 \\
EB + I &= EIB, K_3
\end{align*}
\]

The following rate expression (Equation 4) indicates that competitive inhibitors of the nucleotide substrate, would be expected to act as mixed inhibitors of the sugar substrate as shown in Fig. 7.
Figure 4. Plot of reciprocal of initial velocity (v) against reciprocal of molar concentration of glucose in the absence and presence of N-acetylglucosamine. ATP was held constant at 0.5 mM and glucose was varied in the concentration range from 0.11 to 1.0 mM. N-Acetylglucosamine concentration was none (Δ), 0.4 mM (O), and 0.8 mM (V). Other experimental details are described under "Experimental Procedure."
Figure 5. Plot of reciprocal of initial velocity (v) against reciprocal of molar concentration of ATP in the absence and presence of N-acetylglucosamine.

Glucose concentration was held at 0.2 mM and ATP varied in the concentration range of 0.228 to 2.50 mM. N-Acetylglucosamine concentration was none (O), 0.4 mM (Δ), and 0.8 mM (□). Other experimental details are described under "Experimental Procedure."
Figure 6. Plot of reciprocal of initial velocity (v) against reciprocal of molar concentration of MgATP$^{2-}$ in the absence and presence of ATP$^{4-}$.

Glucose was held constant at 0.2 mM and MgATP$^{2-}$ was varied in the concentration range from 0.22 to 2.0 mM. ATP$^{4-}$ concentration was maintained at 0.5 mM (O) and 1.5 mM (□). Other experimental details are given under "Experimental Procedure."
Figure 7. Plot of reciprocal of initial velocity (v) against reciprocal of molar concentration of glucose in the absence and presence of ATP$^4^-$.

MgATP$^2^-$ was held constant at 0.5 mM and glucose was varied in the concentration range 0.11 to 1.0 mM. ATP$^4^-$ concentrations were 0.5 mM (O) and 1.5 mM (C). All reactions were carried out in 0.020 M HEPES (pH 7.6). Other experimental details are given under "Experimental Procedure."
N-Acetylglucosamine is also expected to function as a mixed inhibitor of ATP in a pathway involving random interaction of enzyme and substrate. The reaction of N-acetylglucosamine (I) with the enzyme is: 

\[ E + I = EI, \quad K_1; \quad EI + A = EIA, \quad K_2; \quad EA + I = EIA, \quad K_3. \]

In the case of the random mechanism the following inhibited rate expression is obtained.

\[
v = \frac{V_m}{1 + \frac{K_A}{A} \left[ 1 + \frac{I}{K_3} \right] + \frac{K_B}{B} \left[ \frac{K_B K_D}{(A)(B)} \left[ 1 + \frac{I}{K_1} \right] \right]}
\]

It is clear that the results depicted in Figs. 4 to 7 are consistent with Equations 4 and 5, and it can be shown that these results are at variance with ordered sequential mechanisms (See Table 1).

**ADP Inhibition of Rat Brain Hexokinase**

Product inhibition studies are often a useful adjunct to initial velocity and competitive inhibition experiments. Although use of competitive inhibitors offers the most direct approach toward segregating two substrate sequential kinetic mechanisms, product inhibition studies can provide important supplemental information. Moreover, product inhibition represents one of the most fundamental and generally available
control phenomena known, and some possible roles for kinetic reaction mechanism-dependent product effects in cellular regulation have been recently discussed (Purich and Fromm, 1972b). While a number of studies have focused on the potential regulatory role of glucose-6-P inhibition of mammalian hexokinases (Crane and Sols, 1954; Rose and O'Connell, 1964), surprisingly little attention has been given to the role of ADP inhibition. In 1969, Ning, Purich and Fromm presented evidence for an allosteric inhibition of solubilized bovine brain hexokinase. Unlike glucose-6-P, which is a linear competitive inhibitor with respect to MgATP (Fromm and Zewe, 1962a; Copley and Fromm, 1967), these investigators observed that a plot of $1/v$ versus ADP was nonlinear. Moreover, they observed that the noncompetitive nature of the ADP inhibition with respect to either substrate could not be rationalized with simultaneous binding at both substrate sites. In the light of these observations, it was of interest to assess the effect of ADP on the soluble rat enzyme. Like the bovine brain enzyme, Hanson and Fromm (1965) found that ADP acted as a noncompetitive inhibitor relative to each substrate.

The following experiments were carried out to exclude the possibility that the inhibitor bound simultaneously at the glucose and ATP loci on the enzyme resulting in an apparent noncompetitive inhibition relative to each substrate.
In Fig. 8 are presented the findings of an ADP inhibition experiment in which the glucose site was more than 98% saturated with glucose. The noncompetitive nature of the inhibition is quite apparent. It was reasoned that, had ADP bound to the glucose site in earlier experiments carried out in the neighborhood of the Michaelis Constant for glucose, 98% saturation of the site with glucose would have eliminated (if binding occurred exclusively at the sugar adsorption site) or the inhibition would have appeared competitive with respect to ATP. A similar experiment, presented in Fig. 9, was undertaken in which the enzyme was 90% saturated with ATP (i.e., ATP was maintained at a concentration ten-fold in excess of the Michaelis constant for ATP), and glucose was varied in the absence and presence of ADP. Again, inhibition relative to the sugar substrate was noncompetitive, suggesting that although ADP may bind at the ATP site, it also binds at a separate site topologically distinct from the binding sites for ATP and glucose. These results are essentially identical with corresponding studies of bovine brain hexokinase (Ning, Purich, and Fromm, 1969). No attempt was made, however, to demonstrate that two ADP molecules bind simultaneously. On the contrary, it appears that over the ADP concentration range indicated (0 - 9 mM) the ADP inhibition is linear noncompetitive. It is of interest to note that $1/v$ versus ADP plots of the ADP inhibition of the bovine
Figure 8. Plot of reciprocal of initial velocity (v) against reciprocal of molar concentration of ATP in the absence and presence of ADP

Glucose concentration was held constant at 9.0 mM (approximately 50 x $K_{glucose}$) and ATP was varied in the concentration range from 0.256 to 2.3 mM. ADP concentrations were none (O), 3.0 mM (V), 6.0 mM (●), and 9.0 mM (●). Other experimental details are described under "Experimental Procedure."
Figure 9. Plot of reciprocal of initial velocity (v) against reciprocal of molar concentration of glucose in the absence and presence of ADP.

ATP concentration was held constant at 5.0 mM (approximately 10 x $K_{ATP}$) and glucose was varied in the concentration range from 0.1 to 0.9 mM. ADP concentrations were none (▽), 2.5 mM (○), 5.0 mM (△), and 7.5 mM (●). Other experimental details are described under "Experimental Procedure."
brain enzyme are also linear in this range (Ning, Purich, and Fromm, 1969).

**Pyrimidine Nucleotide Inhibition of Rat Brain Hexokinase**

Studies of bovine brain hexokinase indicated that purine nucleotides, except ATP, bound at the allosteric inhibitory nucleotide site and possibly at the ATP site as well (Ning, Purich, and Fromm, 1969). On the other hand, pyrimidine nucleotides bound only at the ATP site as indicated by their action as linear competitive inhibitors relative to ATP. It was of interest to determine if this specificity was a general property of the brain isozyme, and similar inhibition studies were undertaken with the soluble rat brain hexokinase. The data shown in Figs. 10 and 11 indicate, however, that UMP and UTP inhibition relative to ATP is noncompetitive. It thus appears that the allosteric site of the rat brain enzyme is not as specific.

**Effect of Hexokinase Modulators on the Soluble-Particulate Distribution of Brain Hexokinase**

Early studies on the mammalian hexokinases established that a large portion of the enzymatic activity is associated with the mitochondrial fraction of the cell (Utter, Wood, and Reiner, 1945; Meyerhof and Gelliazkowa, 1947; Crane and Sols, 1953). The elegant studies of Rose and Warms (1967) on the release, rebinding, and location of mitochondrial hexokinases suggest that a
Figure 10. Plot of reciprocal of initial velocity (v) versus reciprocal of molar concentration of ATP in the absence and presence of UMP.

Glucose concentration was maintained at 0.18 mM and ATP was varied in the concentration range 0.255 to 2.30 mM. UMP concentrations are none (O), 15.0 mM (▽), and 30.0 mM (□). Other experimental details are described under "Experimental Procedure."
Figure 11. Plot of the reciprocal of initial reaction velocity \((v)\) versus the reciprocal of the molar concentration of ATP in the absence and presence of UTP.

Glucose concentration was maintained at 0.15 mM, and ATP was varied from 0.22 mM to 2.00 mM. UTP concentrations were none (\(\times\)), 1.6 mM (\(\bullet\)) and 3.2 mM (\(\blacksquare\)). Other experimental details are described under "Experimental Procedure."
number of metabolites and inorganic ions affect the partitioning of hexokinase into soluble and particulate activity pools. They found that glucose-6-P, ATP, ADP, and AMP can release the enzyme from the mitochondria. In addition, their experiments indicated that Pi could prevent the release of the enzyme by glucose-6-P, and that the extent of rebinding of the enzyme to the mitochondria is affected by Mg2+. In similar studies with rat brain mitochondria, Wilson (1968) observed that ATP, ADP, and AMP can solubilize the enzyme in the presence of Pi, whereas the solubilization by glucose-6-P is substantial only in the absence of Pi.

It was of interest to evaluate the ability of Pi and glucose-6-P to alter the soluble-particulate distribution in the presence of other hexokinase substrates and effectors. Furthermore, since particulate hexokinase preparations incubated in the presence of such metabolites will redistribute into soluble and particulate activity pools, it was of interest to establish how this distribution was altered in those experiments on the initial rate kinetics of soluble and particulate hexokinase in the presence of intracellular levels of substrate, product, and effector. Table 2 summarizes the effects of such metabolites on this distribution. Results of the first experiment indicate that increases in glucose-6-P concentration do not appreciably alter the distribution provided the other hexokinase substrates and
Table 2. Soluble-Particulate Distribution of Brain Hexokinase Activity

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Percentage bound</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>50 mM Tris-HCl (pH 7.6)</td>
<td>97</td>
</tr>
<tr>
<td>Solution A&lt;sup&gt;a&lt;/sup&gt; + no glucose-6-P</td>
<td>75</td>
</tr>
<tr>
<td>Solution A + 0.1 mM glucose-6-P</td>
<td>66</td>
</tr>
<tr>
<td>Solution A + 0.2 mM glucose-6-P</td>
<td>68</td>
</tr>
<tr>
<td>Solution A + 0.4 mM glucose-6-P</td>
<td>68</td>
</tr>
<tr>
<td>Solution A + 0.8 mM glucose-6-P</td>
<td>68</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>50 mM Tris-HCl (pH 7.6)</td>
<td>98</td>
</tr>
<tr>
<td>Solution B&lt;sup&gt;b&lt;/sup&gt; + 10 mM Pi</td>
<td>84</td>
</tr>
<tr>
<td>Solution B + 8 mM Pi</td>
<td>82</td>
</tr>
<tr>
<td>Solution B + 6 mM Pi</td>
<td>79</td>
</tr>
<tr>
<td>Solution B + 4 mM Pi</td>
<td>82</td>
</tr>
<tr>
<td>Solution B + 2 mM Pi</td>
<td>77</td>
</tr>
<tr>
<td>Solution B</td>
<td>76</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
</tr>
<tr>
<td>50 mM Tris-HCl (pH 7.6)</td>
<td>100</td>
</tr>
<tr>
<td>0.1 mM glucose-6-P + 10 mM Pi</td>
<td>64</td>
</tr>
<tr>
<td>0.1 mM glucose-6-P + 8 mM Pi</td>
<td>65</td>
</tr>
<tr>
<td>0.1 mM glucose-6-P + 6 mM Pi</td>
<td>69</td>
</tr>
<tr>
<td>0.1 mM glucose-6-P + 4 mM Pi</td>
<td>66</td>
</tr>
<tr>
<td>0.1 mM glucose-6-P + 2 mM Pi</td>
<td>64</td>
</tr>
<tr>
<td>0.1 mM glucose-6-P + 0 mM Pi</td>
<td>50</td>
</tr>
<tr>
<td>10 mM Pi</td>
<td>81</td>
</tr>
</tbody>
</table>

<sup>a</sup>Solution A contained 50 mM Tris-HCl, 4.4 mM ATP, 1.5 mM Mg<sup>2+</sup>, 0.75 mM ADP, 1.5 mM GTP, 1.3 mM UTP, and 9 mM Pi.

<sup>b</sup>Solution B contained 50 mM Tris-HCl, 4.4 mM ATP, 1.5 mM Mg<sup>2+</sup>, 0.75 mM ADP, 1.5 mM GTP, 1.3 mM UTP, and 0.15 mM glucose-6-P. Other experimental details are given under "Experimental Procedure."
effectors are present at their estimated intracellular levels. There is little effect even at glucose-6-P concentrations that far exceed what Lowry et al. (1964) found to exist in brain tissue. From the second experiment we find that $P_i$ does not alter the distribution. These data indicate that roughly 80% of the enzymatic activity remains associated with the mitochondrial fraction at 'physiological' effector levels. This is in good agreement with the observed in vivo distribution (Johnson, 1960). The findings of the third experiment show that glucose-6-P can release slightly more than one-half of the hexokinase activity in the absence of the other metabolites used above. The glucose-6-P effect does appear to depend upon $P_i$ level, but the effect is not completely abolished as 10 mM $P_i$ can also solubilize some activity.

It should be made clear that these data appear to be independent of mitochondrial concentration. In a companion experiment to those presented in Table 1, the percentage of bound enzyme was not changed when the mitochondrial concentration was varied over an 8-fold range. This can be rationalized by the following equilibria.

\[ M + E = ME \]  
\[ ME + S = ES + M \]

where $M$, $ME$, $E$, $S$, and $ES$ represent the concentrations of mitochondria, mitochondrial-enzyme complex, enzyme, substrate
and enzyme-substrate complex. The sum of these equilibria has no M or ME terms indicating that the solubilization process is independent of these factors. The assumption made here is that the substrate or effector mediates the solubilization by binding to the enzyme and not the mitochondria. Wilson (1968) has shown that glucose-6-P is a competitive inhibitor of ATP in the solubilization of hexokinase. This is in agreement with the observation that glucose-6-P is also a competitive inhibitor with respect to ATP (Fromm and Zewe, 1962a; Copley and Fromm, 1967). The ability of glucose-6-P, ATP, ADP, and AMP to solubilize brain hexokinase parallels their respective Michaelis constants and inhibition constants. For these reasons it is attractive to consider that some change in the stability of the mitochondria-enzyme complex attends the binding of ligands to the enzyme.

Initial Rate Kinetics of Soluble and Particulate Hexokinase in Presence of Intracellular Levels of Substrates, Products, and Effectors

Earlier studies on the regulation of hexokinase investigated the regulatory roles of glucose-6-P, ADP, and P_i by utilizing a reconstructed segment of glycolysis (Uyeda and Racker, 1965a, b). In those studies the rate of formation of triose-P from glucose was measured by the oxidation of DPNH in the presence of triose-P isomerase and
α-glycerol-P dehydrogenase. This segment of glycolysis includes the phosphofructokinase step which is known to be regulated by ATP, ADP, and $P_i$ (Lowry, 1965; Uyeda and Racker, 1965b). Furthermore, this segment does not permit studies at high glucose-6-P levels. The following experiments were carried out to further explore the direct effects of some glycolytic intermediates upon the hexokinase reaction. To our knowledge, this is the first attempt to evaluate these effects at approximate intracellular levels of these metabolites.

The results presented in Figs. 12 and 13 permit the following preliminary conclusions. $P_i$ can partially relieve the inhibition caused by glucose-6-P in the concentration range used. Inhibition by ADP, GTP, and UTP is not sensitive to changes in the $P_i$ level. When the velocities of the reaction were estimated in the presence of each nucleotide, ADP, GTP, and UTP inhibited the rates by 26, 37, and 35%, respectively. In the presence of these nucleotides and glucose-6-P the rates of the hexokinase reaction are potently inhibited. Although these data were obtained at a glucose concentration of 1 µM, similar depressions in the initial velocity were seen at 50 and 300 µM glucose. Finally, it is apparent that the stimulation caused by $P_i$ is similar for both forms of the enzyme, suggesting that there is little functional difference between the soluble and particulate
Figure 12. Plot of initial velocity ($v$) versus molar concentration of $P_i$

Glucose (including $10^5$ cpm $^{14}$C-glucose), ATP, Mg$^{2+}$, and Tris-HCl (pH 7.6) were maintained at 1 μM, 4.35 mM, 1.5 mM, and 50 mM, respectively. Velocities were measured by $^{14}$C-glucose-6-P produced in the absence of any additional components (▲), in the presence of 0.15 mM glucose-6-P (■), 0.75 mM ADP, 1.5 mM GTP, 1.3 mM UTP (▼), and 0.15 mM glucose-6-P, 0.75 mM ADP, 1.5 mM GTP, and 1.3 mM UTP (●). Reaction mixtures (0.5-ml) were initiated with addition of particulate brain hexokinase which was adjusted to give initial rates and later corrected to constant enzyme concentration.
Figure 13. Plot of initial velocity ($v$) versus molar concentration of $P_i$ for soluble rat brain hexokinase.

Reaction conditions were identical with those described in the legend to Figure 12, and velocities were determined in the absence of any components (▼), in the presence of glucose-C-P (●), ADP, GTP, and UTP (■), and glucose-6-P, ADP, GTP, and UTP (▲).
Figure 14. Plot of initial velocity (v) against Mg$^{2+}$ (as MgSO$_4$)

Each reaction mixture contained 50 μM glucose, 4.4 mM ATP, 0.1 mM TPN$^+$, 50 mM Tris-HCl (pH 7.6), and a variable amount of MgSO$_4$. Velocities were measured in the absence (○) and presence of 0.75 mM ADP, 1.5 mM GTP, and 1.3 mM UTP (■). Other experimental details are described under "Experimental Procedure."
enzymes.

The findings presented in Fig. 14 show that the nucleotide inhibition by ADP, GTP, and UTP remains substantial at higher levels of Mg\(^{2+}\). These data further suggest that the inhibition does not arise solely from the binding of Mg\(^{2+}\) to these nucleotides.

**Effect of Mg\(^{2+}\) and MgATP\(^{2-}\) on Brain Hexokinase**

In a recent report Bachelard described the allosteric activation of brain hexokinase by Mg\(^{2+}\) and MgATP\(^{2-}\) (Bachelard, 1971b). He found that substrate-saturation curves of brain hexokinase for MgATP\(^{2-}\) were sigmoidal at subsaturating levels of glucose when the Mg\(^{2+}\)\_total/ATP\_total ratio was unity. On the other hand, he observed that these saturation curves were strictly hyperbolic in the presence of excess magnesium ion (i.e., Mg\(^{2+}\)\_total/ATP\_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 magnesium ion concentration. In addition, the sigmoidicity and deviation from Michaelis-Menten kinetics at a Mg\(^{2+}\)\_total/ATP\_total of 1.0 became less pronounced with increasing glucose concentration. Finally, he found that although substrate-saturation curves for glucose were hyperbolic when Mg\(^{2+}\)\_total/ATP\_total was unity, reciprocal plots were slightly nonlinear. Bachelard interpreted these results to reflect homotropic cooperative binding of MgATP\(^{2-}\) to brain hexokinase and postulated that
Mg$^{2+}$ acts at a topologically distinct activator site at low $\frac{Mg_{\text{total}}}{ATP_{\text{total}}}$ ratios but acts as an inhibitor whenever this ratio exceeds 5.

Examination of Bachelard's data suggests that there is an alternative interpretation fully in agreement with his experimental data but in harmony with previous studies in which no evidence for such complex binding of MgATP$^{2-}$ was obtained (Copley and Fromm, 1967; Ning, Purich, and Fromm, 1969; Purich and Fromm, 1971). This reinterpretation, which does not require postulating cooperative MgATP$^{2-}$ binding or the presence of a distinct activator site for Mg$^{2+}$, considers the presence of large quantities of ATP$^{4-}$ that arise whenever $\frac{Mg_{\text{total}}}{ATP_{\text{total}}}$ is near unity. Furthermore, it is also possible to rationalize the glucose effects on the sigmoidicity and deviation from Michaelis-Menten kinetics at low magnesium ion concentrations. Changes in the Hill coefficient for MgATP$^{2-}$ binding at different $\frac{Mg_{\text{total}}}{ATP_{\text{total}}}$ ratios are explained, and a discussion of the applicability of the Hill equation in velocity studies of multisubstrate cooperative 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 level 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$^{4-}$. 
Bachelard 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 upon MgATP\(^{2-}\) at Mg\(^{2+}\)\_total/ATP\_total ratios of 1.0 and 5.0. While the velocity dependence in the presence of excess Mg\(^{2+}\) appeared to be strictly hyperbolic, sigmoidal responses were observed at the lower Mg\(^{2+}\) levels. In view of the fact that all known nucleoside di- and tri-phosphate dependent phosphotransferase reactions require a divalent metal ion to combine with the nucleotide to form the active substrate (Cohn, 1969), it became of interest to determine the percentage of ATP\_total chelated with magnesium ion under the conditions used by Bachelard. 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. 1b of Bachelard's report. If one assumes that the stability constant for the MgATP\(^{2-}\) complex in 0.15 M triethanolamine-HCl buffer (pH 7.6) is 73,000 M\(^{-1}\) (O'Sullivan and Perrin, 1964; Bachelard, 1971b), 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. 1b of Bachelard's report. Since the correction is more substantial at the lower end of this concentration range, it
appeared that such effects might well explain the sigmoidal curvature that he observed. When such corrections were made on photographic enlargements of Fig. 1b of Bachelard's paper, the rather normally hyperbolic saturation curves shown in Fig. 15 were observed. For the sake of clarity, only the corrected curves are presented.

Additional corrections also were possible for the data presented by Bachelard. For example, at pH 7.6 approximately 18.3% of ATP$^{\text{total}}$ is present as the protonated species, HATP$^{3-}$, if one assumes that HATP$^{3-}$ has a $pK_a$ of 6.95 (Smith and Alberty, 1956). The stability constant for the MgHATP$^{1-}$ complex is approximately 31 M$^{-1}$ (Smith and Alberty, 1956), and it is quite unlikely that very much of the HATP$^{3-}$ is present as its magnesium ion complex. Furthermore, ATP$^{4-}$ is a potent inhibitor of yeast and brain hexokinases (Rudolph and Fromm, 1971; Ning, Purich, and Fromm, 1969; Bachelard and Goldfarb, 1969; Purich and Fromm, 1971). Corrections for HATP$^{3-}$ and ATP$^{4-}$ were not made as the deviations from hyperbolic velocity responses observed by Bachelard seem fully accountable in terms of the correction made in Fig. 15 alone.

Bachelard 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 magnesium ion levels. Again, in the presence of low Mg$^{2+}$ he found marked curvature in these plots. If one corrects these data as
Figure 15. Plot of velocity (v) versus the [MgATP] for brain hexokinase at a Mg$^{2+}$/ATP ratio of 1.0
Glucose concentrations were 0.1 mM (O), 0.05 mM (●), 0.035 mM (□), and 0.025 mM (■). The velocity measurements are those of Bachelard (1971b) and the MgATP$^{2-}$ concentrations on the abscissa were corrected for the concentration dependence of the MgATP$^{2-}$ complex. Corrections were made directly upon photographic enlargements of Bachelard's data as described under "Experimental Procedure."
described in the legend to Fig. 15 the curvature of such plots is far less pronounced as shown in Fig. 16. It should be noted that corrections of Fig. 2b of Bachelard's report requires large changes in the values plotted on both the ordinate and the abscissa. The uncorrected and corrected plots are shown in Fig. 16. Although the curves remain somewhat nonlinear, one must note that no corrections were made for ATP inhibition.

In his studies of the effects of Mg and MgATP on the kinetic properties of brain hexokinase, Bachelard observed that nonlinear velocity versus MgATP plots became less pronounced at elevated glucose concentrations and was apparently absent at saturating levels of glucose (Bachelard, 1971b; Bachelard and Goldfarb, 1969). In a companion experiment, Bachelard observed that plots of [glucose]/v versus [glucose] were linear in the presence of excess Mg, but slightly curved when the ratio of Mg to ATP was 1.0 (Bachelard, 1971b). While Bachelard interprets these results to suggest that alterations in the conformation of the MgATP-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.

As stated earlier, the kinetic mechanism appears to be of the sequential random type for rat and bovine brain
Figure 16. Plots of \([\text{MgATP}^{2-}] / v\) versus \([\text{MgATP}^{2-}]\) for brain hexokinase at a \(\text{Mg}^{2+}/\text{ATP}_{\text{total}}\) ratio of 1.0. \(\text{MgATP}^{2-}\) was varied in the range from 0.05 mM to 0.6 mM, and the glucose concentration was maintained at 0.1 mM (O), 0.05 mM (●), 0.035 mM (□), and 0.025 mM (■). Reaction rates are expressed as \(\text{OD}_{340}/\text{min}\). Plot A is the uncorrected data of Bachelard (1971b), and Plot B is corrected for the concentration dependence of the \(\text{MgATP}^{2-}\) stability as described under "Experimental Procedure."
hexokinase, and it is generally assumed that all substrates bind rapidly relative to the interconversion of the productive ternary complex. This has been independently confirmed by the use of approaches similar to those outlined on pages 26 and 27 (Bachelard, Clark, and Thompson, 1971). In addition, the uncomplexed form of ATP acts as a linear competitive inhibitor with respect to MgATP\(^2^-\) for these mammalian enzymes (Ning, Purich, and Fromm, 1969; Purich and Fromm, 1971), whereas studies from Bachelard's laboratory appear to indicate 'mixed' inhibition (Bachelard and Goldfarb, 1969). The latter observation can be better understood if one considers the levels of ATP\(^4^-\) used in the experiments of Bachelard and Goldfarb. They observed relatively small intercept changes in Lineweaver-Burk type plots (Lineweaver and Burk, 1934) at the higher levels of ATP\(^4^-\) which correspond to levels that are from 11.3 to 17.5-fold higher than the dissociation constant for this inhibitor (Bachelard and Goldfarb, 1969). At such high levels of the free uncomplexed ATP, it is possible that these small intercept changes could result from a weak nonspecific binding not related to the mode of binding at lower inhibitor concentrations where their data appear to suggest competitive inhibition. If we grant that ATP\(^4^-\) is indeed a competitive inhibitor of the brain enzyme, then the following scheme outlines the various interactions of substrates and ATP\(^4^-\)
with this phosphotransferase:

\[ \text{E-ATP}^{2-} \xrightarrow{K_1} \text{E-MgATP}^{2-} \xrightarrow{K_3} \text{glucose} \xrightarrow{K_4} \text{products} \]

\[ \text{E-glucose} \]

\[ \text{E-glucose-ATP}^{4-} \]

The rate expression for the above kinetic mechanism can be expressed in the following form:

\[
v = \frac{V_m}{1 + \frac{K_4}{A} \left[1 + \frac{I}{K_{ii}}\right] + \frac{K_3}{B} + \frac{K_1K_3}{AB} \left[1 + \frac{I}{K_i}\right]} \tag{8}
\]

where \( v, V_m, A, B, \) and \( I \) represent initial reaction velocity, maximal velocity, \( \text{MgATP}^{2-} \), glucose, and \( \text{ATP}^{4-} \), respectively. The various \( K \)'s are dissociation constants defining the equilibria described in the scheme. If \( K_i \neq K_{ii} \) 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 \( \text{ATP}^{4-} \). At the \( \text{Mg}^{2+}/\text{ATP}_{\text{total}} \) ratio of 1, there is a relatively high level of uncomplexed \( \text{ATP}^{4-} \) 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$^{2+}$ level. In a similar manner, one can understand why $\frac{[\text{glucose}]}{v}$ versus $[\text{glucose}]$ plots are slightly curved at suboptimal amounts of the divalent 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'.

On the basis of Hill (1913) plots, Bachelard has stated that the number of binding sites for MgATP$^{2-}$ on brain hexokinase is 1.8 at a Mg$^{2+}_{\text{total}}$/ATP$^{4-}_{\text{total}}$ ratio of unity, and 1.0 in the presence of excess magnesium ion. 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 Mg$^{2+}$ is near unity (i.e., $n < 1.25$).

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

Responses of Brain Hexokinase to the Adenylate Energy Charge The concept that biosynthetic and biodegradative
reactions are sensitively responsive to changes in the adenylate energy charge of the cell has rapidly gained favor in recent years. The original proposal of Atkinson and Walton (1967) suggested that energy charge be formally defined as one-half the number of anhydride-bound phosphates per adenosine moiety (i.e., energy charge = [(ATP) + ½(ADP)]/[(AMP) + (ADP) + (ATP)]). Atkinson has argued that the adenylate energy charge represents an appropriate input parameter in the control of individual enzymes and metabolic sequences that utilize or regenerate ATP (Atkinson, 1968). The energy charge model assumes that enzymes have evolved to respond to the over-all state of the adenine nucleotide pool rather than to concentration changes of any single species within it, so that a clearer understanding of the in vivo control of enzymes and metabolism will be obtained by studying this over-all response, rather than the response to AMP, ADP, or ATP, separately. At present, the response of a number of diverse enzyme systems in vitro has been evaluated (Klungsøyr, Hagemen, Fall, and Atkinson, 1968; Shen, Fall, Walton, and Atkinson, 1968; Atkinson and Fall, 1967; Shen and Atkinson, 1970). Basically, these studies have monitored the response of initial reaction velocity to alteration in the energy charge, assuming that the distribution of the adenylates is governed by the mass action ratio of the adenylate kinase reaction. Only one study has attempted to
consider the importance of evaluating these responses under conditions that reasonably reflect intracellular conditions (Villar-Palasi and Wei, 1970).

Studies from several laboratories have indicated that the mammalian hexokinases are quite sensitive to inhibition by glucose-6-P and ADP (Crane and Sols, 1953; Weil-Malherbe and Bone, 1951). Recent studies from our laboratory suggest that ADP and several other nucleotides can severely inhibit rat brain hexokinase under simulated intracellular conditions (Purich and Fromm, 1971). Rapoport et al. (Rapoport, Hinterberger, and Hofmann, 1961) first proposed that hexokinase represented the pacemaker in red cell glycolysis. More recent studies have confirmed this proposal and have described the role of glucose-6-P in this process (Rose and O'Connell, 1964). We felt that it would be useful to test the responses of these enzymes under conditions that approximate the intracellular level of substrates, Mg$^{2+}$, and adenylates. Considering the potent inhibition of hexokinases by ATP$^4-$ described earlier, it appeared that magnesium ion levels might play an important role in enzyme responses to energy charge. The data presented in Fig. 17 indicate that a wide spectrum of responses can be obtained depending on the Mg$^{2+}$ level. It is clear that the responses observed at intracellular levels of magnesium ion do not resemble the generalized response of an ATP-utilizing system as described
by Atkinson (1968).

Since it is likely that, for nearly all enzymic reactions under normal physiological conditions, there are finite levels of all reaction substrates and products, it was felt that the inhibitory effects of the nonadenylate reaction product should thus be considered in evaluations of the sensitivity of brain hexokinase to alteration in the energy charge. Product effects can be especially critical for kinetically reversible enzymes since the net reverse reaction can also take place over the range of energy charge used. The ease with which one can accurately experimentally evaluate energy charge responses in the presence of all substrates and products, however, depends largely upon the physical and kinetic properties of the enzyme catalyzed reaction under study. Ideally, one could monitor a spectral change due to the net conversion of substrate to product as with the pyridine-linked dehydrogenases. In the absence of such optical changes during the reaction, one may encounter some experimental difficulties in measuring such responses. For example, in experiments measuring the activity of yeast hexokinase (which is readily reversible) in the presence of all reaction substrates and products, one must take care to measure the rate of the net phosphorylation of glucose as opposed to the rate of a particular exchange reaction. To make this distinction, one may simultaneously measure both
Figure 17. Plot of initial reaction velocity (expressed as \( \Delta \text{OD}_{340} \text{ min}^{-1} \)) catalyzed by soluble brain hexokinase versus energy charge.

Each reaction mixture contained a total of 5.5 mM adenylates as AMP, ADP, and ATP in sufficient quantity to satisfy the desired energy charge and an adenylate kinase mass action ratio of 0.8, 50 mM Tris-HCl (pH 7.6), 20 mM potassium chloride, 50 \( \mu \)M glucose, 60 \( \mu \)M TPN\(^+\), excess glucose-6-P dehydrogenase, and 1.5 mM Mg\(^{2+}\) (\(\bullet\)), 5.5 mM Mg\(^{2+}\) (\(\bigtriangleup\)), and 25 mM Mg\(^{2+}\) (\(\bigtriangledown\)). Other experimental details are presented under "Experimental Procedure."
the rate of incorporation of $^{14}$C-glucose into the glucose-6-P pool and the rate of incorporation of $^{32}$P-glucose-6-P into the ATP pool. The difference between these rates defines the rate and direction of the net reaction. This procedure would be necessary for any enzymic reaction for which the $V_f/V_r$ ratio is relatively low. One may obviate this difficulty by using an enzyme which is essentially irreversible such as the brain hexokinase for which the $V_f/V_r$ ratio is approximately 13,200 at pH 7.6 (Rudolph and Fromm, 1971). For this latter hexokinase, except at thermodynamic equilibrium, the rate of the reverse reaction is negligibly small in comparison to the forward reaction rate, and the net reaction is measurable to well within experimental error by the rate of $^{14}$C-glucose incorporation into the glucose-6-P pool. The findings presented in Fig. 18 indicate that glucose-6-P at its approximate intracellular level (Lowry and Passonneau, 1964) alters the energy charge response of soluble rat brain hexokinase under approximate in vivo levels of glucose and Mg$^{2+}$. The response in the absence of glucose-6-P measured by the radioactive assays used in these studies is similar to the response previously measured by coupling to glucose-6-P dehydrogenase. These data also suggest that the enzyme is relatively insensitive to the energy charge in the absence of reaction product and becomes even less sensitive in its presence.
Although one might reasonably argue that the adenylates are directly concerned with most energy utilizing and producing reactions, one must also consider the approximately 40% of the total cellular nucleoside-5'-triphosphates are not adenylates in such widely different sources as rat brain tissue (Lowry et al., 1964) or Escherichia coli (Lowry et al., 1971). These compounds are also important in view of their potential action as competitive inhibitors or weak alternative substrates of many phosphotransferase catalyzed reactions. It was of interest to investigate the effect of approximately intracellular levels of such nonadenine nucleotides on the response of rat brain hexokinase to the adenylate energy charge. The findings presented in Fig. 19 suggest that the presence of these nucleotides serves to further suppress the noticeably insensitive response of this enzyme to changes in the adenylate charge. This observation suggests that, if increases in the adenylate energy charge are accompanied by increases in the energy charge of the nonadenylates through the nucleoside diphosphate kinase reaction (Thompson and Atkinson, 1971), then some ATP-utilizing reactions will become more inhibited by the nonadenine nucleoside di- and tri-phosphates as the charge increase. Such action would not be consistent with the adenylate charge hypothesis which predicts acceleration of ATP-utilizing reactions at high energy charge.
Figure 18. Plot of the initial reaction velocity (v) versus the energy charge in the absence (▲) and presence (▼) of 0.15 mM glucose-6-P.

Each reaction mixture also contained 50 mM Tris-HCl (pH 7.5), 40 μM glucose (including approximately 100,000 cpm of 14C-glucose), 5.5 mM adenine nucleotides, and 2.5 mM MgCl2. The adenylate distribution was determined as described in the legend to Fig. 17. Reactions were initiated by the addition of soluble rat brain hexokinase and were incubated for a period of 20-60 min, over which period the reaction rate was constant. Samples were quenched and processed as described under "Experimental Procedure."
Figure 19. Plot of the initial velocity (v) of the rat brain hexokinase reaction versus the energy charge in the absence (○) and presence (▲) of GTP and UTP.

Each reaction mixture contained 50 mM Tris-HCl (pH 7.5), 40 μM glucose (including 108,000 cpm of \(^{14}\)C-glucose), 5.5 mM adenine nucleotides, 2.5 mM MgCl\(_2\), 1.5 mM GTP, and 1.3 mM UTP. Other experimental details are described in the legend to Fig. 18 and under "Experimental Procedure."
GENERAL DISCUSSION

The results of the kinetic experiments on soluble rat brain hexokinase described herein support in general the recent kinetic studies of bovine brain hexokinase by Ning, Purich, and Fromm (1969). These investigators had concluded that solubilized bovine brain hexokinase has a random Bi Bi kinetic mechanism in which all steps equilibrated rapidly relative to the interconversion of the enzyme MgATP$_2$-glucose and enzyme MgADP$_1$-glucose-6-P ternary Michaelis complexes. They also found that the enzyme was allosterically inhibited by the reaction product, ADP, and other purine nucleoside-5'-mono-, di-, and tri-phosphates. The studies of Ning, Purich, and Fromm (1969) were carried out on an enzyme which during the course of purification and solubilization from the outer mitochondrial membrane was subjected to the possibly desensitizing action of deoxycholate, Triton X-100, and a-chymotrypsin (Schwartz and Basford, 1967). The present studies, however, have employed a hexokinase preparation from the soluble rat brain fraction (Grossbard and Schimke, 1966), thereby obviating the use of such agents. This enzyme appears to be identical to the Triton X-100 solubilized rat brain hexokinase based upon starch block electrophoresis (Wilson, 1967). In addition to the use of a soluble hexokinase, the present studies differ from previous studies (Copley and Fromm, 1967; Ning, Purich, and Fromm, 1969) which did not
attempt to evaluate the regulatory potential of a number of modulators that have been postulated to have specific effects on brain hexokinases or general effects on transphosphorylases.

Although the methodology and limitations of the experimental approaches used in these studies have been discussed in some detail, it may be of value to further qualify some of the conclusions drawn in these studies. Moreover, it may be of particular value to outline the manner in which rat brain hexokinase may be regulated in vivo based upon these in vitro experiments.

There is now ample evidence supporting the contention that the kinetic mechanism of brain hexokinase involves a random interaction of enzyme and substrates. The possibility that the kinetic mechanism is ordered with glucose acting as the obligatory first substrate has been ruled out on the basis of the following evidence. The product which corresponds to the first substrate in an ordered mechanism should act as a competitive inhibitor with respect to that substrate (Alberty, 1958). Several laboratories, however, have indicated that glucose-6-P is a competitive inhibitor of ATP and a mixed inhibitor of glucose (Grossbard and Schimke, 1966; Copley and Fromm, 1967), and that ADP is a mixed inhibitor of both substrates (Hanson and Fromm, 1965; Grossbard and Schimke, 1966; Copley and Fromm, 1967; Ning, Purich, and
Fromm, 1969). Furthermore, the use of alternative substrates has also served to exclude this possibility. If the kinetic reaction mechanism were ordered with glucose adding first, an alternative substrate of glucose would appear to act like a competitive inhibitor of glucose, and inhibition relative to ATP would be nonlinear concave upward when plotted as velocity$^{-1}$ versus ATP$^{-1}$ (Fromm, 1964; Rudolph and Fromm, 1971). Past reports have demonstrated that mannose does, indeed, act like a competitive inhibitor of glucose, but it is a mixed inhibitor of MgATP$^2-$ when the initial reaction velocity was measured as a function of glucose-6-P production which is monitored by use of the specific yeast glucose-6-P dehydrogenase (Hanson and Fromm, 1965; Copley and Fromm, 1967; Ning, Purich, and Fromm, 1969). The ordered mechanism described above has also been eliminated from consideration by the use of competitive inhibition studies presented in this report. Finally, the possibility that ATP is the leading substrate in an ordered ternary complex mechanism exists only for the case where its product is considered to glucose-6-P and not ADP. Considering that yeast hexokinase operates with a random kinetic mechanism (Fromm and Zewe, 1962a; Fromm, 1968; Rudolph and Fromm, 1971), this possibility appears remote. The random mechanism therefore appears to be the only likely kinetic mechanism that reconciles all the kinetic findings of the mammalian brain enzyme.
The present studies indicate that the existence of a distinct allosteric inhibitory nucleotide site may be a common feature of the rat and bovine brain enzymes (Ning, Purich, and Fromm, 1969; Purich and Fromm, 1971). The experiments on ADP inhibition at saturating levels of either substrate have given qualitatively identical results for the soluble cytoplasmic rat brain enzyme and the Triton X-100 solubilized bovine brain hexokinase. These enzymes do differ, however, with respect to the apparent nucleotide specificity of the inhibitory site. For the brain enzyme, only purine nucleotides bind at the allosteric site, as indicated by the observation that pyrimidine nucleotides act as linear competitive inhibitors relative to ATP (Ning, Purich, and Fromm, 1969). On the other hand, both pyrimidine and purine nucleotides appear to bind at the allosteric site on the rat brain enzyme. It should be noted that no attempt has been made to carry out equilibrium binding studies of the ADP interaction with the brain enzyme. The amount of purified hexokinase that can be obtained precludes use of binding studies at present. For example, with the method of Grossbard and Schimke (1966), one would require approximately 500 rat brains (average weight, 1.6 g) to obtain 3 mg of hexokinase with a final specific activity of 80 units/mg. Assuming a dimer molecular weight of $10^5$ g (Grossbard and Schimke, 1966), this amount would represent less than $6 \times 10^{-8}$ moles of hexokinase active or allosteric sites. Moreover,
reliable equilibrium binding data would be difficult to obtain in view of the fact that the ADP binding constant is less than $10^3 \text{ M}^{-1}$ for the enzyme ADP complex as estimated from inhibition studies (Grossbard and Schimke, 1966; Hanson and Fromm, 1965; Purich and Fromm, 1971).

An alternative explanation of the noncompetitive nature of the ADP inhibition of ascites tumor hexokinase has been presented by Kosow and Rose (1970). They suggest that the interconversion of the ternary complexes in the random mechanism may not be rate limiting and that glucose-6-P release may, in fact, be the slowest step in the process. Kosow and Rose (1970), therefore, favor the possibility that the effect of ADP on the maximal velocity (i.e., the intercept effects in Lineweaver-Burk type plots of ADP inhibition data) must result from ADP interacting at the ADP product site on the enzyme-glucose-6-P binary complex. Their proposal is based, in part, upon the fact that the $\text{ADP} \rightleftharpoons \text{ATP}$ equilibrium exchange rate exceeds the $\text{glucose} \rightleftharpoons \text{glucose-6-P}$ equilibrium exchange rate in the yeast hexokinase reaction (Fromm, Silverstein, and Boyer, 1964). The analogous experiments have not been carried out for mammalian hexokinases since the ratio of the maximal velocities of the forward and reverse reactions is very large (Purich and Fromm, 1972b). Their proposal does not, however, account for the higher order inhibition of brain hexokinase by ADP and cannot apply to the bovine brain enzyme (Ning, Purich,
and Fromm, 1969). It is also of interest to note that Coffee and Hu (1972) have found that our analysis (Ning, Purich, and Fromm, 1969; Purich and Fromm, 1971) does fully account for the ADP inhibition of a pseudomonad gluconokinase. They found that plots of \((1/\text{velocity})^2\) versus ADP were linear, suggesting that ADP bound at two sites on the enzyme.

It was the complex nature of the ADP inhibition of the mammalian brain hexokinases that focused our attention upon the regulatory role of nucleotide inhibition. Although the kinetic studies on the order of substrate addition and ADP inhibition suggested a regulatory role for nucleotide inhibition, the levels of substrates, products, metal ions, and hexokinase effectors used in such studies, are not representative of the conditions thought to exist within the cell (Lowry et al., 1964; McIlwain, 1966). This is especially true for the concentration of free uncomplexed magnesium ion which is generally maintained at a fixed optimal level in kinetic studies but is variable in the cell. The best current estimates of the magnesium ion concentration in rat brain place the normal intracellular level in the range of 1.5 mM to 3.5 mM (Bachelard and Goldfarb, 1969), considerably below optimal. Re-evaluation of the rates of glucose phosphorylation under simulated intracellular conditions has served to demonstrate that glucose-6-P, \(P_i\), ADP, and other nucleotides may regulate brain hexokinase. Inhibition caused by glucose-
6-P was found to be somewhat sensitive to changes in the $P_i$ level with greatest sensitivity in the concentration range corresponding to the intracellular $P_i$ content (Lowry et al., 1964; McIlwain, 1966). ADP inhibition was found to be $P_i$-insensitive over the $P_i$ range examined. Under conditions corresponding to the content of glucose-6-P, $P_i$, ADP, GTP, and UTP, the cumulative inhibition of brain hexokinase amounted to 95-97%, and there was little difference between the soluble and particulate responses to $P_i$. This inhibition corresponds rather favorably with estimates of the in vivo inhibition of hexokinase (Lowry and Passonneau, 1964).

The $P_i$ stimulation of glucose-6-P-inhibited brain hexokinase may arise from a number of mechanisms. Since $P_i$ has no apparent effect on the velocity of the uninhibited reaction rate, $P_i$ stimulation of glucose-6-P-inhibited brain hexokinase is a deinhibition rather than an activation. In this regard, $P_i$ deinhibition is analogous to the AMP, ADP or 3',5'-cyclic AMP mediated deinhibition of ATP-inhibited rabbit skeletal muscle phosphofructokinase (Mansour, 1972). It is of interest to note that, although both ATP and glucose-6-P appear to bind at the same locus on the enzyme (Fromm and Zewe, 1962a; Copley and Fromm, 1967; Grossbard and Schimke, 1966), $P_i$ has no effect on the Michaelis constant for ATP. In fact, the Michaelis constants for glucose and ATP were identical to within experimental error in the absence and presence of
15 mM P$_i$ (Purich and Fromm, 1971). Nevertheless, our experiments indicate that under simulated intracellular conditions both products are quite effective as inhibitors with glucose-6-P inhibition depending inversely upon the cellular P$_i$ content.

The potent inhibition by intracellular levels of GTP, UTP, and ADP should not be attributed merely to a trivial mechanism involving competition with ATP$^4^-$ for the available Mg$^{2+}$. On the contrary, our data suggest that at higher levels of Mg$^{2+}$ the inhibition is even more appreciable. This would be anticipated if the metal-nucleotide complexes bound more tightly to the inhibitory nucleotide site than do the free nucleotides. Although it is certain that these compounds will bind a significant amount of the magnesium ion, the amount of this divalent cation associated with each metal-nucleotide species will remain quite constant as long as the total Mg$^{2+}$ concentration does not exceed the total nucleotide concentration and the ratio of total nucleotide remains constant. This is apparent from considerations of the stability constants for these complexes. Therefore, any error in the value of available nonparticulate intracellular magnesium ion will not be of major consequence so long as the value does not exceed 7 mM. From the range of values estimated for this metal ion in brain tissue (Bachelard and Goldfarb, 1969), this possibility appears to be remote.
The nature of the ADP inhibition and the fact that hexokinase can be considered as the first committed step in brain glycolysis had suggested that brain hexokinase may be influenced strongly by the adenylate energy charge. The energy charge is considered by proponents of the concept to be of central importance in the regulation of all reactions utilizing or re-supplying ATP (Atkinson, 1968; Atkinson and Walton, 1967). The studies described in this report indicate that rat brain hexokinase does not respond as predicted by the energy charge model. A number of factors were found to influence the response of phosphotransferases to the adenylate energy charge. The responses of human erythrocyte hexokinase and pyruvate kinase, rabbit skeletal muscle pyruvate kinase and phosphofructokinase, and yeast hexokinase all appear to be markedly affected by a number of experimentally adjustable factors. These include: a) the free uncomplexed magnesium ion concentration, b) total adenylate concentration, c) pH, d) the adenylate kinase mass action ratio, e) the non-adenine nucleotide substrate and product levels, f) the size and composition of the nonadenine nucleotide pool, and g) the kinetic reaction mechanism of the enzymic reaction under study (Purich and Fromm, 1972d). The studies with rat brain hexokinase served as the basis for the first theoretical and practical quantitative appraisal of the adenylate energy charge model (Purich and Fromm, 1972d). The results of these
studies suggest that the use of the energy charge index,
\[
\frac{(ATP) + \frac{1}{2}(ADP)}{(ATP) + (ADP) + (AMP)},
\]
to describe the basic control of energy metabolism is very incomplete, and it remains to be convincingly demonstrated that this parameter plays an important \textit{in vivo} role.

It now appears that hexokinase regulation results from the interplay of several factors, and that, in brain, it is coordinated with the phosphofructokinase reaction to provide for effective glycolytic control. A schematic model for this coordination is shown in Fig. 20. The kinetics of glucose transport in cerebral cortical tissue argue strongly that the intracellular level of glucose is really quite low and that nearly all of the transported glucose is promptly phosphorylated (Bachelard, 1971a). The transport of glucose into the tissue has been shown to be a saturable carrier-mediated form of active transport (Fishman, 1964; Crone, 1965) and may prove to be an important control point responding to changes in metabolite and hormonal levels. Bachelard has suggested that the rate of glucose permeation may, at times, even be the rate limiting step in the utilization of glucose (Bachelard, 1970). The studies of Uyeda and Racker (1965b) and those presented here suggest that glucose-6-P serves as an effective regulator and is also sensitive to changes in the $P_i$ level. It seems clear that, since glycogen storage in brain is quite low (Bachelard and McIlwain, 1969), in the
Figure 20. Schematic representation of the coordinated control of the reactions catalyzed by brain hexokinase and phosphofructokinase.
absence of $P_i$ deinhibition of hexokinase a rise in the $P_i$ level would affect the rate of the phosphofructokinase reaction both positively (by lowering the $K_m$ for fructose-6-P) and negatively (by lowering the intracellular level of glucose-6-P and fructose-6-P). The $P_i$ deinhibition of glucose-6-P-inhibited brain hexokinase appears to permit the rate of glycolysis to increase by permitting the rapid phosphorylation of glucose at moderate levels of glucose-6-P and fructose-6-P. Our present studies point to a multivalent inhibition of hexokinase involving glucose-6-P, ADP, GTP, UTP and possibly other nucleotides as well. These studies have also indicated that the binding of these nucleotides to the inhibitor site is not $P_i$-sensitive, suggesting that changes in $P_i$ alone cannot lead to marked increases in the hexokinase rate. Further, it is felt that the partitioning of hexokinase activity into soluble and particulate activity pools does not represent a dynamic regulatory process. This appears evident from the observations that the distribution is not markedly affected by even large changes in the glucose-6-P concentration, and that both ATP and glucose-6-P act to solubilize the enzyme. This view is also supported by the recent studies of Vallejo et al. (1970) on the association of bovine brain hexokinase with mitochondria. Although it is possible that some, yet unknown, factors may also affect rat brain hexokinase, it is now attractive to consider that the regulation
of glucose utilization arises chiefly from changes in glucose permeation, changes in the levels of glucose-6-P and $P_i$, and changes in the size and composition of the free cytoplasmic nucleotide pool.

Finally, it may be of value to discuss briefly some of the possible criticisms of the various kinetic approaches used here to delineate the regulation of rat brain hexokinase. The following partial listing of the assumptions used throughout this study may serve as a basis for this discussion. We have generally assumed that the kinetic properties of hexokinases studies in glass apply in the cell. The hexokinase reaction has been considered as an isolated element, rather than as a coordinated part of the glycolytic pathway in which it participates. It is also assumed that the substrate concentrations are much greater than the levels of the enzymes acting upon them. Initial rate responses are measured throughout, and no attempt has been made to simulate the simultaneous variation of more than one parameter at a time. In addition, it is tacitly assumed that the simulations of intracellular conditions reflect the performance of rat brain hexokinase in the cell. All of these assumptions point toward our current ignorance of the actual status of in vivo chemical events, but a brief discussion of these factors may be of considerable value.

Undoubtedly, the kinetic mechanism of any enzyme deter-
mined in vitro can only serve as a rough guide to the behavior of enzymes in cells. In fact, there is a real need for evaluating enzyme kinetic mechanisms and parameters under conditions thought to prevail in vivo. While it is difficult to presently study the effect of numerous metabolites on particular enzymes which are present at high concentrations in the cell, it is becoming possible for biochemists to study the kinetic properties of concentrated enzyme solutions. Srere (1967) was among the first to point out the need for studying enzymic catalysis and regulation at levels of enzymes corresponding to their intracellular concentrations. Only recently were the kinetic parameters of several enzymes evaluated at such high concentrations (Barwell and Hess, 1970; Hemphill, Zielke, and Suelter, 1971). The major difficulty has been the high rates of enzymic catalysis which require the use of fast reaction equipment, usually of the stopped-flow type. Alternatively, one may study the reaction by conventional means by use of an alternative substrate which is acted upon only feebly. This latter approach suffers the disadvantage that the enzyme may not behave on the weak alternative substrate in a manner analogous to its action on the true substrate. Furthermore, the assumption that the total substrate concentration is much greater than the enzyme concentration may not necessarily hold, and corrections for the fraction of total substrate present as the enzyme-substrate
Michaelis complex must be employed (Rhoads and Garfinkel, 1971). To the best of our knowledge, complete kinetic studies, designed to elucidate an enzyme's kinetic reaction mechanism and evaluate the true rather than apparent Michaelis constants, are not available except for several single-substrate systems at levels of 1 μM enzyme or less. Purich and Fromm (1972c) recently suggested that the technique of isotope exchange at equilibrium may provide a more convenient inexpensive means for the probing of kinetic properties of highly concentrated enzyme solutions. Because enzyme catalyzed reactions often proceed much more slowly at equilibrium than in the absence of the reaction products, it is feasible to use very high concentrations of enzyme in isotope exchange at equilibrium studies. For example, they found that with yeast hexokinase one can use as high as 0.1 mg enzyme/ml of assay solution and short incubation periods (0.5 to 1 min) to characterize its properties. They also presented a table, based upon published equilibrium exchange kinetic data, that suggests that many reactions can be studied at concentrations as high as 50 mg/ml depending upon their unique kinetic parameters.

As mentioned earlier, the assumption that the substrate levels in cells are much greater than the levels of enzymes acting upon them may not be valid in a number of cases. Sols and Marco (1970) have listed a number of metabolites,
including DPNH, fructose-1,6-P₂, glyceraldehyde-3-P, and AMP, whose free concentrations in the cell are probably quite low. They have presented a systematic explanation for observed discrepancies between the total analytical intracellular concentration and the free unbound metabolite levels in terms of the in vivo levels of enzymes, themselves capable of strongly binding these metabolites. In the simulations presented in this report we have assumed that the enzyme binds a negligible portion of the substrate. While this assumption is valid in vitro and in vivo for glucose-6-P, the adenine nucleotides, GTP, UTP, AND Pi, this assumption may not apply for glucose at normal intracellular levels of hexokinase and glucose. The extent to which this limitation affects the results of our studies remains to be determined.

Although we have considered brain hexokinase to be an isolated element and not as a functionally coordinated part of the glycolytic pathway, the results should be applicable to the coupled processes. This is probably true because for any pathway there are steady-state levels of substrates and products, and the responses simulated here can be thought of in terms of anticipated responses to changes in the steady-state level of one or more substrates and products.
BIBLIOGRAPHY


Copley, M., and Fromm, H. J. (1967), Biochemistry, 6, 3503.


Lutwak-Mann, C., and Mann, T. (1935), Biochem. Z., 281, 140.
Meyerhof, O. (1927), Biochem. Z., 183, 176.
Meyerhof, O., and Geliazkowa, N. (1947), Arch. of Biochem., 12, 405.


Schwartz, G. P., and Basford, R. E. (1967), Biochemistry, 6, 1070.


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