The Kinetics and Regulation of Rat Brain Hexokinase*

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SUMMARY

Initial rate kinetic studies of soluble rat brain hexokinase indicate that the kinetic mechanism is random Bi Bi. These studies also indicate that the ADP interaction with enzyme cannot be ascribed merely to action at a product site and suggest the presence of a separate inhibitory nucleotide-binding site. Rates of glucose phosphorylation were evaluated in the presence of a number of metabolites at or near their intracellular levels to determine their regulatory potential. Inhibition from ADP, GTP, and UTP was insensitive to fluctuations in the P_i level; whereas, glucose-6-P inhibition was partially relieved by P_i. Studies of the enzyme cumulatively inhibited by the above nucleotides and glucose-6-P indicate that the reaction rate was markedly depressed to a level corresponding to only 3 to 5% of the uninhibited rate. Under these conditions the rate responds to changes in glucose concentration and only large changes in P_i level. The soluble-particulate distribution of the brain enzyme was found to be relatively insensitive to changes in metabolite levels.

Recent reports from this laboratory (1, 2) have detailed the kinetic properties of bovine brain hexokinase (ATP:hexose 6-phosphotransferase, EC 2.7.1.1). These studies have shown that (a) the kinetic properties are most reasonably rationalized in terms of a rapid equilibrium random Bi Bi mechanism, (b) the enzyme catalyzes the reverse reaction at elevated levels of Mg2+, (c) pyrimidine nucleotides bind only at the ATP site, and (d) ADP inhibition arises from binding at an inhibitory nucleotide site that is kinetically distinct from the substrate-binding sites. These conclusions were drawn from the results of an extensive reinvestigation of the bovine enzyme which, like the rat enzyme (4), had previously been considered to have a "ping-pong" kinetic mechanism. The inhibitory nature of ADP prompted our suggestion that there may be a regulatory site on the enzyme that permits hexokinase to be sensitively responsive to changes in the nucleotide level.

Lowry and Passonneau (5) have argued that the only true control points in brain glycolysis are at the hexokinase and phosphofructokinase steps. Since brain hexokinase is present in cerebral cortical tissue in amounts far exceeding the amount necessary to produce the 20 to 30 μmoles of glucose-6-P required by 1 g of tissue each hour (6), the enzyme must be markedly inhibited and yet capable of accelerated rates for brief periods of time. This implies that hexokinase must be under the sensitive control of a number of factors.

It appeared advantageous to reinvestigate the properties of rat brain hexokinase to determine whether the kinetic observations with the bovine enzyme are a general feature of mammalian 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 metabolites upon hexokinase could now be evaluated. The effects of these levels of metabolites on the soluble-particulate distribution of hexokinase were also of interest.

The results of the present report suggest that the rat enzyme is kinetically very similar to the bovine brain enzyme. The substrates appear to add in a random fashion, and ADP inhibition is again attributable to binding at a site that is topologically distinct from the active site. Rat brain hexokinase differs from the bovine system in that both pyrimidine and purine nucleotides appear to bind at the inhibitory site. The studies with the hexokinase effectors at their approximate intracellular levels indicate that both ADP and glucose-6-P are required to account for the substantially depressed rates in vivo. Finally, studies presented here indicate that the distribution of hexokinase does not change appreciably with glucose-6-P when the other metabolites are present.

EXPERIMENTAL PROCEDURE

Materials—Mannitol, D-glucose, and D-fructose were obtained from Fannstiel Laboratories, Waukegan, Illinois. D-Glucose-6-P, P-enolpyruvate, DPNH, glucose-6-P dehydrogenase, rabbit muscle lactate dehydrogenase, pyruvate kinase, and yeast hexokinase were products of Calbiochem. ATP, GTP, UTP, ADP, GDP, UTP, AMP, GMP, UMP, and TPN+ were purchased from Sigma. Analytical grade Dowex 1-X2 (200 to 400 mesh) was obtained from Bio-Rad Laboratories, Richmond, California. Uniformly labeled D-glucose-14C (specific radioactivity 240 μCi per mmole) was the product of Schwarz BioResearch. Ion low water, obtained from passage of distilled water through a Rohm and Haas MB-3 resin bed, was used to prepare all reagents. Rat brain hexokinase I was purified through the first ammonium sulfate step of a procedure reported elsewhere (7),
and after dialysis, was rechromatographed on a DEAE-cellulose column equilibrated with 0.01 M potassium phosphate, 0.005 M 2-mercaptoethanol, 0.01 M glucose, 0.005 M EDTA (pH 7.0). The enzyme was eluted with the same buffer containing 0.15 M KCl. The enzyme, which was stable for months in neutral 3.0 M ammonium sulfate-0.01 M glucose, was dialyzed against 0.05 M Tris-HCl (pH 7.6) immediately prior to use. Male Wistar strain rats were obtained from Schettle Biologicals, St. Paul, Minnesota.

Methods—The substrates and products of the hexokinase reaction were enzymatically assayed as described elsewhere (8, 9). Nucleotide solutions were prepared as previously described (2). Unless otherwise stated, the reactions were carried out in 0.05 M Tris-HCl (pH 7.6).

Initial velocity measurements were made in a Cary model 15 recording spectrophotometer (0 to 0.1 slide wire), maintained at 28° by circulating water from a temperature-controlled bath through thermospacers. 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 μM TPN⁺ and a substantial excess of glucose-6-P dehydrogenase. When fructose was employed as the sugar substrate, excess phosphoglucomutase was added to the above solution. Initial rates of ADP production were evaluated by use of a coupled enzyme system containing, in addition to the other components, 10 mM potassium chloride, 0.5 mM P-enolpyruvate, 50 μM DPNH, and an excess of lactate dehydrogenase and pyruvate kinase (10). 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 as described earlier (11). Velocities in all experiments reported here are expressed as molarity of product formed per minute.

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%, respectively, by volume, respectively (12, 13). We further assume that the tissue is 80% water and that the metabolites are uniformly distributed throughout the cell. This latter assumption requires 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 (14). Analytical values for ATP, AMP, and glucose-6-P (15) 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. Values for GTP (16) and UTP (16, 17) were corrected to 1.5 and 1.3 mM, respectively. Similarly, the analytical level of Pi was corrected to 9.2 mM (6). Finally, the intracellular concentration of "free" nonparticulate Mg²⁺ was taken to be 1.5 mM (18).

In those experiments where the free Mg²⁺ concentration was adjusted to 1.0 mM, calculation of the proper Mg:ATP ratio used values of 20,000 M⁻¹ for the stability constant of MgATP²⁻ (19), and 2,000 and 100 M⁻¹ for MgADP⁻³ and MgAMP (20).

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When HEPES buffer was utilized, similar adjustments were made assuming 100,000 M⁻¹ for MgATP²⁻ (21).

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 (22). 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 homoge-
nizer 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 × 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 × 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.

RESULTS

Initial Velocity Measurements—In earlier studies of rat brain hexokinase glucose was utilized as the sugar substrate (4). Lineweaver-Burk plots (23) of 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 that the kinetic mechanism of rat brain hexokinase was ping-pong. Recently, studies of bovine brain hexokinase, however, indicated that the mechanism was sequential with fructose as the sugar substrate (1). The findings of Fig. 1, A and B, indicate that this is also the case for the rat enzyme. To exclude the possibility that there are separate sites for each sugar the experiment described in Fig. 2 was carried out. It is clear that fructose acts as a competitive inhibitor of glucose when the reaction is monitored with glucose-6-P dehydrogenase. These data are in conflict with a ping-pong mechanism, but are in harmony with the family of kinetic mechanisms involving a sequential interaction of enzyme and substrates prior to release of any product. The rate equation for a sequential mechanism has the following general form.

\[
\frac{V_m}{v} = 1 + \frac{K_A}{(A)} + \frac{K_B}{(B)} + \frac{K_{AB}}{(A)(B)}
\]

where \(V_m\), \(v\), \(A\), \(K_A\), \(B\), \(K_B\), and \(K_{AB}\) represent maximal velocity, initial reaction velocity, Substrate A, Michaelis constant for A, Substrate B, Michaelis constant for B, and a complex constant, as first defined by Alberty (24). The values for \(K_{fructose}\) and \(K_{ATP}\) determined from the data above are 2.4 and 0.51 mM, respectively. It is obvious from the form of Equation 1 that if the \(K_{AB}/(A)(B)\) term is small relative to the other terms in the rate equation, the apparent kinetic expression would yield Lineweaver-Burk plots in agreement with the ping-pong mechanism.

Fig. 2. Plot of reciprocal of initial velocity (v) against reciprocal of 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 (△), 1.00 mM (○), and 3.00 mM (□); v was determined as a function of glucose concentration which was varied in the range 0.11 to 1.0 mM. Other experimental details are given under ‘‘Experimental Procedure.’’

Fig. 3. A, 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 (△), 0.4 mM (○), and 0.8 mM (□). B, 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 (○), 0.4 mM (△), and 0.8 mM (□). Other experimental details are described under ‘‘Experimental Procedure.’’
FIG. 4. A, 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 (□). B, 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 (□). All reactions were carried out in 0.020 M HEPES (pH 7.6). Other experimental details are given under "Experimental Procedure."

Fig. 5. A, 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 × $K_{\text{glucose}}$) and ATP was varied in the concentration range from 0.256 to 2.3 mM. ADP concentrations were none (O), 3.0 mM (▲), 6.0 mM (●), and 9.0 mM (▽). B, 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 × $K_{\text{ATP}}$) and glucose was varied in the concentration range from 0.1 to 0.9 mM. ADP concentrations were none (▽), 2.5 mM (O), 5.0 mM (△), and 7.5 mM (●). Other experimental details are described under "Experimental Procedure."
Fig. 6. Plot of reciprocal of initial velocity (v) against 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 (○), 15.0 mM (△), and 30.0 mM (□). Other experimental details are described under “Experimental Procedure.”

These data are taken as evidence for the interaction of ADP with a site on the enzyme that is distinct from the substrate-binding sites. Studies with the bovine brain enzyme indicated that the random kinetic mechanism can be modified to provide for these interactions with ADP (2). It is also interesting to note that, in contrast with the bovine brain enzyme, the results of Fig. 6 indicate that pyrimidine nucleotides are not competitive inhibitors of ATP.

**Effect of the Hexokinase Modulators on Soluble-Particulate Distribution of 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 (26, 27). The elegant studies of Rose and Warms (29) on the release, re-binding, and location of mitochondrial hexokinases suggest that a number of metabolites and inorganic ions affect the partitioning of the soluble and particulate hexokinase activities. They found that glucose-6-P, ATP, ADP, and AMP can release the enzyme from the mitochondria. In addition, their work indicated that P1 could prevent the release of the enzyme by glucose-6-P, and that the extent of re-binding of the enzyme to the mitochondrial fraction is sensitive to Mg++ levels. In similar studies with rat brain mitochondria, Wilson (28) 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.

We felt that it was necessary to define the distribution of hexokinase activity under the conditions that would be utilized to evaluate the effects of various compounds on the kinetic properties of the soluble and particulate forms. Table I lists the effects of various incubation conditions on this distribution. Results of the first experiment indicate that glucose-6-P does not appreciably alter the distribution if the other substrates, products, and effectors are present at their estimated intracellular levels. There is little effect even at glucose-6-P concentrations that far exceed the level thought to exist in brain tissue (15). From the second experiment we find that P1 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 (27). 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 P1 level, but the effect is not completely abolished at 10 mM P1; 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 I, 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.

\[
M + E = ME
\]

(2)

\[
ME + S = ES + M
\]

(3)

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 (28) has shown that glucose 6 P is a competitive inhibitor of ATP in the solubilization of hexokinase. This is in agreement with the
FIG. 7. A, plot of initial reaction velocity (v) against molar concentration of P<sub>i</sub>. Glucose (including 100,000 cpm of <sup>14</sup>C-glucose), ATP, Mg, 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 the production of <sup>14</sup>C-glucose-6-P 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, and 1.3 mM UTP ( ), and 0.15 mM glucose-6-P, 0.75 mM ADP, 1.5 mM GTP, and 1.3 mM UTP ( ). Reactions were carried out in 0.5-ml reaction mixtures and were initiated by the addition of particulate brain hexokinase. The enzyme concentrations and incubation times were adjusted to yield initial rates and were then corrected to constant enzyme activity. Suitable controls were prepared in which water was used in place of enzyme and processed in an identical manner. B, plot of initial velocity ( ) against molar concentration of P<sub>i</sub> in the presence of soluble brain hexokinase. The conditions used were identical with those presented above. Other experimental details are described under “Experimental Procedure.”

observation that glucose-6-P is also a competitive inhibitor with respect to ATP (2, 7). The ability of glucose-6-P, ATP, ADP, and AMP to solubilize hexokinase activity follows quite well their respective K<sub>M</sub> and K<sub>I</sub> values for hexokinase. For these reasons we feel that it is attractive to consider that some change in the affinity of the mitochondria for hexokinase attends the binding of these ligands to the enzyme.  

1 Another mechanism for the solubilization of hexokinase from the mitochondria is expressed by the following equilibria: ME → M + E; E + S = ES, where ME, M, E, S, and ES are defined as the text. The sum of these equilibria would imply that the percentage of hexokinase solubilized is dependent upon mitochondrial concentration which is in variance with our observations. Such a mechanism also appears to be in disagreement with the observations of Wilson (28) described in the text.

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<sub>i</sub> by utilizing a reconstructed segment of glycolysis (29). 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<sub>i</sub> (30, 31). 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 Fig. 7, A and B, permit the following preliminary conclusions. P<sub>i</sub> 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<sub>i</sub> 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 mM glucose. Finally, it is apparent that the stimulation caused by P<sub>i</sub> is similar for both forms of the enzyme, suggesting that there is little functional difference between the soluble and particulate enzymes.

The findings presented in Fig. 8 show that the nucleotide inhibition by ADP, GTP, and UTP remains substantial at higher levels of Mg<sup>2+</sup>. These data further suggest that the inhibition does not arise solely from the binding of Mg<sup>2+</sup> to these nucleotides.
DISCUSSION

The results of the present report serve to support our recent studies on the kinetic mechanism of brain hexokinase, and, in addition, serve to place several earlier investigations of hexokinase regulation into better perspective.

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 mechanism is ordered with glucose as the obligatory first substrate has been ruled out on the basis of the following evidence. The product of the first substrate in an ordered mechanism should be a competitive inhibitor with respect to that substrate (32). Several laboratories, however, have indicated that glucose-6-P is a competitive inhibitor of ATP and a mixed inhibitor of glucose (4, 7, 10), and that ADP is a mixed inhibitor of both substrates (4, 10). Furthermore, the use of alternative substrates has also served to exclude this possibility. If the kinetic mechanism were ordered with glucose adding first, an alternative substrate of glucose would appear to act as a competitive inhibitor of glucose and inhibition relative to ATP would be nonlinear (i.e. concave upward) (11). We have reported in the past that mannose does act like a competitive inhibitor of glucose but it is a mixed inhibitor of MgATP when the velocity was measured as a function of glucose-6-P production (4). The ordered mechanism above has also been eliminated from consideration by the use of the competitive inhibition studies presented in this report.

The possibility that ATP is the leading substrate exists only for the case where its product is considered to be glucose-6-P and not ADP. The random mechanism is the only kinetic mechanism that appears to reconcile all the kinetic findings on the brain enzyme.

The present studies indicate that the existence of an inhibitory nucleotide site is also a common feature of the rat and bovine brain systems (2). The experiments on ADP inhibition at saturating substrate levels have given qualitatively identical results for the rat and bovine enzymes. Although Kosow and Rose (33) have taken exception to our suggestion that there is a special site for nucleotides in addition to the product site, our evidence showing that more than 1 molecule of ADP interacts with the enzyme cannot be rationalized in terms of a single binding site (2). It was the complex nature of this inhibition that has focused our attention upon the regulatory role of this inhibitory site. The concentration ranges of substrates, products, inhibitors, and Mg++ that are normally employed in studies of kinetic mechanism, are not representative of the conditions present within the cell. Our re-evaluation of the rates of glucose phosphorylation under simulated intracellular conditions has served to confirm that ADP and other nucleotides are quite effective in substantially depressing the reaction rate. Under the experimental conditions employed, the data suggest that both glucose-6-P and several nucleotides act to regulate hexokinase. The inhibition caused by glucose-6-P was found to be somewhat sensitive to variation in the Pi level with greatest sensitivity in the concentration region corresponding to the intracellular level of Pi as judged by available analytical data. This activation may arise either from direct displacement of the sugar-P from the enzyme or from an indirect process involving action at another site. It is of interest to note that, although both ATP and glucose 6-P appear to bind to the same locus on hexokinase, Pi has no effect on the Michaelis constant for ATP.

In fact, the Michaelis constants for glucose and ATP were identical in the absence and presence of 15 mM Pi. Nevertheless, our experiments indicate that under simulated intracellular conditions both products are quite effective inhibitors. These data also support our earlier suggestion that action at the inhibitory site for nucleotides may serve to dampen the response of glucose-6-P inhibition to Pi (2).

The potent inhibition by the intracellular levels of GTP, UTP, and ADP should not be attributed merely to a trivial mechanism only involving competition with ATP. On the contrary, our data suggest that at higher levels of Mg++ the inhibition is even more appreciable. This would be anticipated if the metal-nucleotide complexes bind more tightly to the inhibitory site than do the free nucleotides. Although it is true that these compounds will bind a significant amount of Mg++, the amount of divalent cation associated with each species will remain quite constant as long as the total Mg++ concentration does not exceed the total nucleotide concentration. This is apparent from consideration of the stability constants for these complexes. Therefore, any error in the value of "free" non-particulate intracellular Mg++ level will not be of consequence so long as this value does not exceed 7 mM. From the range of values for this metal ion in brain tissue (18), this possibility appears to be remote.

It appears that hexokinase regulation results from the interplay of several factors. 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. The transport of glucose into the tissue has been shown to be a saturable carrier-mediated form of active transport (34, 35) and may prove to be an important control point responding to changes in metabolite levels. Bachelard has suggested that the rate of glucose permeation may even, at times, be the rate-limiting step in the utilization of glucose (36). The studies of Uveda and Raeker (29) have indicated that glucose-6-P serves as an effective regulator and is also sensitive to changes in Pi. It seems clear that inhibition of the phosphofructokinase reaction will lead to higher levels of glucose-6-P and will further potentiate this effect. 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 altered by Pi. We further feel that our studies indicate that the partitioning of hexokinase activity into soluble and particulate 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 glucose-6-P concentration, and that both ATP and glucose-6-P act to solubilize the enzyme. This view is also supported by recent studies on the association of brain hexokinase with mitochondria (37). Although it is possible that some other, yet unknown, factors may also affect brain hexokinase, it is now attractive to consider that regulation of glucose utilization arises chiefly from changes in glucose permeation, changes in the levels of glucose-6-P and Pi, and variation in the size and composition of the free nucleotide pool.

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Corrections

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In PURICH, DANIEL L., AND HERBERT J. FROMM. The Kinetics and Regulation of Rat Brain Hexokinase

Page 3461, the legend to Fig. 7 is incomplete; the entire, correct legend should read:

**Corrected Legend to Figure 7**

Fig. 7. A, plot of the initial reaction velocity (v) versus molar concentration of Pi. Glucose (including 100,000 cpm of [14C]-glucose), ATP, Mg²⁺, 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 the production of [14C]glucose-6-P 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 (●). Reactions were carried out in 0.5-ml reaction mixtures and were initiated by the addition of particulate brain hexokinase. The enzyme concentrations and incubation times were adjusted to yield initial rates and were then corrected to constant enzyme concentration. Suitable controls were prepared in which water was used in place of enzyme and processed in an identical manner. B, plot of initial velocity (v) versus molar concentration of Pi in the presence of soluble brain hexokinase. Reaction conditions were identical with those described above, and velocities were determined in the absence of any additional components (▼), and in the presence of 0.15 mM glucose-6-P (●), 0.75 mM ADP, 1.5 mM GTP, and 1.3 mM UTP (■), and 0.15 mM glucose-6-P, 0.75 mM ADP, 1.5 mM GTP, and 1.3 mM UTP (▲). Other experimental conditions are described under “Experimental Procedure.”

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