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MECHANISTIC STUDIES ON BOVINE BRAIN HEXOKINASE

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Mechanistic studies on bovine brain hexokinase

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GENERAL INTRODUCTION

The first observations leading to the discovery of hexokinase (ATP: D-hexose-6-phosphotransferase, E.C. 2.7.1.1.) were made by Otto Meyerhof (1927, 1932). He found that the normally slow production of lactic acid from hexose in rabbit muscle could be accelerated by the addition of a protein fraction from a yeast autolysate. He called this protein hexokinase because it initiated the oxidation of hexoses. In later studies, Meyerhof found that ATP was necessary for activity and he suggested the reaction,

$$2 \text{ Hexose} + \text{ATP} \overset{Mg^{+2}}{\rightleftharpoons} 2 \text{ Hexose 6-phosphate} + \text{AMP}, \qquad (1)$$

was responsible for hexose phosphorylization. However, Colowick and Kalcker (1941, 1943) found that only the terminal phosphate of ATP was transferred in the hexokinase reaction and they suggested that Meyerhof's muscle preparation probably contained myokinase activity that would transfer a phosphoryl group from one ADP to another forming ATP and AMP. This pair of reactions would yield the stoichiometry suggested by Meyerhof. Hexokinase alone catalyzes the following reaction:

$$MgATP^{2-} + \text{D-Hexose} \rightleftharpoons \text{D-Hexose 6-phosphate}^{2-} + MgADP^{1-} + H^{+} \qquad (2)$$

Hexokinase has been found in yeast (McDonald, 1955); bacteria

(Crane, 1962); locust muscle (Kerly and Leaback, 1957a); intestine, kidney, and heart (Cori, 1945); brain (Sols and Crane, 1954; Crane and Sols, 1954; Kerly and Leaback, 1957b; Schwartz and Basford, 1967); liver and skeletal muscle (Crane and Sols, 1955); and other tissues (Long, 1952; Hoare and Kerly, 1954). Enzymes of this type appear to be ubiquitous in nature and probably all cells capable of metabolizing hexoses contain at least one such enzyme (Crane, 1962). The enzymes show little specificity for hexose substrate(s), catalyzing the phosphorylation of glucose, fructose, mannose, and 2-deoxyglucose, but show little activity with nucleotides other than ATP.

Of the hexokinases isolated, the yeast and mammalian isozymes are the most extensively studied, the yeast enzyme with regard to its kinetic and physical properties, and the mammalian enzymes largely with respect to their regulatory properties. Three isozymes of hexokinase have been isolated from yeast cells and designated A, B, and C. A is composed of identical alpha subunits while B and C are composed of beta subunits (Rustum et al., 1971). Isozymes B and C are conformationally rather than structurally different and C is converted into isozyme B under conditions of high ionic strength (Ramel et al., 1971). The molecular weight of the dimer of each isozyme is approximately 100,000.

The kinetic mechanism of yeast hexokinase has been rigorously investigated, in part due to the new kinetic techniques developed in this time period. Initially, the yeast enzyme was reported to form an enzyme-phosphoryl intermediate (Agren and Engstrom, 1956) or an enzyme-

glucosyl intermediate (Gamble and Najjar, 1954; Najjar and McCoy, 1958). These results suggested that the hexokinase reaction proceeded via a ping-pong mechanism. However, Trayser and Colowick (1961) concluded that neither enzyme intermediate was formed since, by their observations, only negligible amounts of carbohydrate and phosphorus were associated with the enzyme. The mechanism of yeast hexokinase was subsequently demonstrated to be of the random type by the techniques of substrate analog competitive inhibition (Fromm and Zewe, 1962b; Zewe et al., 1964), product inhibition (Fromm and Zewe, 1962b; Zewe et al., 1964; Fromm, 1969), isotope exchange (Fromm et al., 1964), and alternate substrates and products (Fromm and Zewe, 1962b; Fromm, 1964). A more complete development of the kinetic studies of yeast hexokinase is found in the recent review by Purich et al. (1973).

Few studies have been carried out on the mode of yeast hexokinase regulation. Unlike mammalian hexokinases, the yeast isozyme has a high dissociation constant for glucose 6-phosphate (Hammes and Kochavi, 1962) and does not have a great affinity for ADP. Kosow and Rose (1971) have suggested that P_i, citrate, malate, 3-P-glycerate, and ribonucleoside 5'-triphosphates can function as hexokinase activators below pH 7.0. The activation is held to be the result of the existence of two conformational states of the enzyme and accounts for both the negative cooperativity and the burst phenomena seen with the enzyme at neutral pH (Shill and Neet, 1971; Shill and Neet, 1974; Peters and Neet, 1977). These conclusions are questionable, however, in light of a recent report

by Womack and Colowick (1979). These authors show that the apparent negative-cooperativity and burst seen in the hexokinase reaction at pH 7.0 are caused by the presence of aluminum ions in commercial preparations of ATP. Citrate and other ions which activate the enzyme act by complexation of the aluminum.

It was not recognized until 1964 that mammalian hexokinase activity exists as multiple electrophoretically distinct species. Gonzalez et al. (1964) reported that hexokinase isozymes were present in rat liver and shortly thereafter Katzen et al. (1965) also obtained evidence for the presence of isozymic forms in human cell cultures and rat liver. In 1965, Katzen and Schimke (1965) were able to isolate four isozymes of hexokinase from different mammalian tissues. The isozymes were designated type I, II, III, and IV in order of their increasing mobility on starch gel electrophoresis. Types I and II are both low K_m types (K_m with respect to glucose is 10^{-5} M and 10^{-4} M for type I and II, respectively). Type III also has a low K_m for glucose ($\sim 10^{-6}$ M) but exhibits substrate inhibition at higher concentrations of this hexose. These three isozymes represent the bulk of hexokinase found in mammalian cells. Each appears to be a monomer with a molecular weight of about 96,000 to 99,000 daltons as determined by ultracentrifugation (Grossbard and Schimke, 1966). Grossbard and Schimke (1966) also found that the three isozymes had different thermal and proteolytic susceptibilities. Type I is most stable both to thermal and proteolytic attack while type II is most susceptible. All three hexokinase types are stabilized from

tryptic inactivation by saturating levels of glucose, but the proteolytic treatment leads to new, electrophoretically distinct forms. Immunologically, hexokinase I appears distinct from both hexokinase II (anti-hexokinase II does not inhibit the enzymatic activity of hexokinase I (Creighton et al., 1972)) and hexokinase III (the two isozymes show complete absence of immunological crossreactivity (Neumann et al., 1974; Neumann and Pfleiderer, 1974)). Neumann and Pfleiderer (1974) have suggested that their immunological data indicate that the low K_m hexokinases and glucokinase from mammalian tissues are all products of different genes and in this sense are true isozymes. Hexokinase type IV, called glucokinase, is a low glucose affinity isozyme (K_m for glucose $\sim 10^{-2}$ M) found only in the liver. Glucokinase is a monomer as are the other three isozymes, but has a molecular weight of only 47,000 and it can be cleanly separated from types I-III on Sephadex G-100 columns (Pilkis, 1972). Glucokinase from rat liver is reported to have no immunological cross-reactivity with the low K_m hexokinases and is a distinct protein (Pilkis et al., 1968).

The four isozymes of hexokinase found in mammalian tissue have assumed trivial names based upon their tissue locations. Isozyme I is referred to as brain hexokinase because of its abundance in this tissue and because of the low levels of the other isozymes in the brain. Hexokinase I is found in almost all body tissue and is present in high concentration in erythrocytes, kidney, liver, and most organs (Katzen, 1967). The type II isozyme, found plentifully in muscle, fat pads, and several

organs, is referred to as muscle hexokinase, while isozyme IV, found predominantly in the liver is usually called glucokinase (Katzen, 1967). The type III isozyme is found in several tissues (liver, spleen, adrenal, intestines, and kidneys) but not in particularly high activity. The work reported in this dissertation was performed with hexokinase I, brain hexokinase, and unless stated otherwise the information presented here deals with this isozyme.

Recently, there has been some question as to whether or not hexokinase I, as identified from electrophoretic patterns, from various tissues represents a single isozyme. The suggestion has been made that tissues thought to contain hexokinase I exclusively may not also have other isozymes present. In brain tissue, hexokinase activity is found partitioned between the mitochondria and the cytosol in a ratio of approximately 4 to 1 (Crane and Sols, 1953; Johnson, 1960; Bachelard, 1967). Kellogg et al. (1974) showed that this ratio was not fixed, but varied 2 to 3 fold in developing rat brains. The authors, feeling that this ratio would be constant if it were caused by a partitioning of a single enzyme, suggested that distinct cytoplasmic and mitochondrial forms of hexokinase were present in the brain. However, the discovery of a protein which binds hexokinase I to the mitochondria (Felgner et al., 1979) adds a third variable which can change during development so that the partitioning cannot be thought of as a simple bound-soluble equilibrium. A comparison of the kinetic parameters of cytosolic and mitochondrial brain hexokinase showed that the cytosolic enzyme had significantly

higher dissociation constants for the inhibitors N-acetylglucosamine and AMP, perhaps suggesting different isozymes or post-translational modifications of the brain isozyme (Thompson and Bachelard, 1977).

Erythrocytes, thought to contain exclusively hexokinase I, have also been shown to contain hexokinase I subtypes. In the case of erythrocytes, 4 subtypes, designated Ia, Ib, Ic, and Id, were isolated from human red blood cells (Rijksen et al., 1981). The subtypes are named on the basis of their electrophoretic mobilities and all migrate faster than hexokinase II, the muscle isozyme. Kinetically, the subtypes are similar in their K_m's for glucose and ATP and all show marked inhibition by glucose-6-P. All of these parameters are consistent with those of hexokinase I, but the enzymes show variability in the ability of phosphate to reverse sugar-phosphate inhibition, the third marked feature of brain hexokinase activity. The Ib subtype has great phosphate sensitivity, the Ic and Id types intermediate sensitivity and the Ia subtype is relatively insensitive to P_i modulation of glucose-6-P inhibition. When the erythrocytes were fractionated according to age and the distribution of isozymes reexamined, Rijksen et al. (1981) found that hexokinase Ib predominated in young cells. As the cells aged, the concentration of subtypes Ic, Id, and Ia increased as the concentration of hexokinase Ib decreased. Since mature erythrocytes have no protein synthesis, and because the total activity in the cells did not increase, the appearance of the additional subtypes must be due to post-translational modification of the primary enzyme, probably hexokinase I.

Vowles and Easterby (1979) separated two isozymes of hexokinase I from pig heart, one that would bind mitochondria and a second that would not, while Felgner and Wilson (1976) have shown the same phenomena in rat brain. Additional studies on reticulocytes (Stocchi et al., 1981) and erythrocytes (Gahr, 1980) have shown two types of hexokinase present, again one type phosphate sensitive and the second showing less modulation by phosphate. In each study, no attempt had been made to determine whether ot not the subtypes displayed structural heterogeneity, but at this time the isozymes appear to be post-translational modifications of brain hexokinase.

While these studies have all dealt with multiple subtypes of hexokinase I in the same tissue, a recent report by Kurokawa et al. (1979) has indicated the possibility of tissue dependent variation in hexokinase I isozymes. The authors found that isolated mitochondria from rat liver was devoid of bound hexokinase I. To test whether this phenomenon was due to differences in the enzyme or in the mitochondria, Kurokawa et al. conducted a series of experiments comparing the properties of hexokinase I and of mitochondria from brain and liver. They found that hexokinase I from liver did not bind to mitochondria isolated from either liver or brain, while hexokinase I solubilized from rat brain mitochondria would bind to either brain or liver mitochondrial membranes. Although this report is not conclusive and the characteristics of the enzymes were not compared, the data may indicate separate types of hexokinase I are present in the two tissues.

Because of its function in the control of energy metabolism, hexokinase I is the most extensively studied of the mammalian isozymes. The brain, virtually totally dependent upon blood-borne glucose to supply its energy requirements, uses approximately 25% of the glucose and 20% of the oxygen consumed by the body, although it represents only 2-3% of the adult body weight (Bachelard, 1970; Balazs, 1970; Sokoloff, 1977). Erythrocytes, lacking mitochondria, depend primarily on anaerobic glycolysis to supply their energy needs. In both these tissues, the slow utilization of glucose-6-phosphate by the hexose monophosphate shunt (Bachelard and McIlwain, 1969; Winick, 1970) or in glycogen storage (Bachelard and McIlwain, 1969; McIlwain, 1966) make the hexokinase reaction the de facto first committed step of glycolysis. The regulation of hexokinase I controls glycolysis in both brain and erythrocytes (Lowry et al, 1964; Lowry and Passonneau, 1964; Rapoport, 1968). Brain hexokinase's importance in regulation has led to its extensive investigation by many authors.

Various kinetic mechanisms have been proposed for the brain enzyme during the course of its study. Early initial rate studies performed on the particulate bovine brain enzyme (Fromm and Zewe, 1962a) and on the solubilized brain isozyme from rat muscle (Hanson and Fromm, 1965) and from bovine brain (Copley and Fromm, 1967) yielded Lineweaver-Burk (1934) type profiles which were consistent with a ping-pong mechanism and the authors suggested that this was tentative evidence for the formation of a phosphoryl-enzyme or glucosyl-enzyme intermediate. These data were at variance with those for both

the muscle isozyme (Grossbard and Schimke, 1966; Hanson and Fromm, 1967) and yeast hexokinase (Hammes and Kochavi, 1962; Fromm and Zewe, 1962b), each of which showed initial rate kinetics consistent only with a sequential mechanism. This discrepancy was resolved when Fromm and Ning (1968) reexamined the initial rate of the enzyme using D-fructose in place of D-glucose as the sugar substrate. When this substrate was used, the Lineweaver-Burk profiles displayed convergent patterns of lines, indicative of a sequential mechanism. The rate equation for a two substrate sequential mechanism is of the form:

$$\frac{V_m}{V} = 1 + \frac{K_a}{(A)} + \frac{K_b}{(B)} + \frac{K_{ia}K_b}{(A)(B)} \qquad (3)$$

The ping-pong mechanism has a similar equation but does not contain the final term, $\left(\frac{K_{ia}K_b}{(A)(B)}\right)$. Apparently, when glucose is used as the sugar substrate in the brain hexokinase reaction the last term of the rate equation is negligible compared to the others and the kinetic mechanism appears ping-pong. These results have since been obtained with the solubilized rat brain enzyme (Purich and Fromm, 1971), but recent studies on human erythrocyte hexokinase (Gerber et al., 1974) and on hexokinase I from pig brain (Shone and Fromm, 1980) and from pig heart (Vowles and Easterby, 1979) have shown convergent patterns consistent with a sequential mechanism in their initial rate profiles. Presently there is agreement in the literature that the hexokinases operate with a sequential mechanism.

Further elucidation of the kinetic mechanism of brain hexokinase was attempted through the use of product inhibition studies. The first report of the effect of product on the hexokinase reaction was in 1951 when Weil-Malherbe and Bone (1951) reported that glucose-6-P strongly inhibited a crude preparation of rat brain hexokinase in a noncompetitive manner with respect to both glucose and ATP. The authors concluded that glucose-6-P combined with the enzyme at a site different from those which react with either hexose or ATP. Soon after, Crane and Sols (1954), using a purified particulate enzyme, confirmed Weil-Malherbe's and Bone's earlier results in that they showed glucose-6-P was a noncompetitive inhibitor with respect to glucose. In addition, they cataloged the effects of a number of other sugar phosphates. Crane and Sols (1954) took their results to indicate that glucose-6-P occupied a separate regulatory site on the enzyme surface remote from the active site. In 1962, Fromm and Zewe (1962a) found that although glucose-6-P was a noncompetitive inhibitor of brain hexokinase with respect to glucose, the sugar-phosphate inhibited the enzyme in a competitive manner with respect to MgATP. The authors suggested that these results were consistent with glucose-6-P binding to the enzyme primarily at the γ-phosphate subsite within the ATP locus on the enzyme, and that it was not necessary to postulate a second site. The competitive nature of glucose-6-P inhibition of brain hexokinase with respect to ATP has since been confirmed by other authors (Grossbard and Schimke, 1966; Uyeda and Racker, 1965a; Copley and Fromm, 1967; Gerber et al., 1974; Rijksen and Staal, 1977).

If glucose-6-P is binding at the ATP site on the enzyme, this data eliminated the possibility that brain hexokinase operates with a ordered sequential mechanism with glucose binding first. However, the other possible ordered mechanism, that with MgATP adding first and glucose-6-P released last, was not eliminated.

The study of the effect of the other product, ADP, on the enzyme did not resolve the kinetic mechanism but rather displayed noncompetitive inhibition patterns with respect to both substrates (Fromm and Zewe, 1962a; Grossbard and Schimke, 1966; Kosow and Rose, 1968; Hanson and Fromm, 1965; Copley and Fromm, 1967; Purich and Fromm, 1971). This noncompetitive inhibition is noteworthy because most kinases displaying sequential kinetics are inhibited by ADP in a competitive manner with respect to ATP.

In 1969, Ning et al. (1969) tried to distinguish between two possible mechanisms of ADP inhibition of hexokinase which could account for the noncompetitive nature of the inhibition. One mechanism placed the inhibitor across both the sugar and ATP loci within the binding site on the enzyme. This position would prevent the product from inhibiting the enzyme competitively with respect to either substrate. The second mechanism placed ADP at an allosteric site on hexokinase remote from the active site. The first mechanism was excluded by an experiment where first glucose and secondly ATP was held at saturating levels while the other substrate was varied in the presence of ADP. In both cases, the inhibition remained noncompetitive. It was reasoned that if ADP could

bind at either site, saturation of the enzyme with one substrate would make the inhibition competitive. If ADP bound across both substrate binding sites, saturation of the enzyme with one substrate should abolish the inhibition. Additionally, ADP showed multiple binding to the enzyme possibly indicating binding to a regulatory site as well as the active site.

The kinetic mechanism of brain hexokinase was finally resolved by the use of substrate analogs as linear competitive inhibitors for the substrates of the hexokinase reaction (Fromm, 1975; Fromm, 1980). Using N-acetylglucosamine, ATP^{4-}, and β,γ-5'-adenylyl methylene diphosphate as substrate analogs, Ning et al. (1969) found kinetic patterns for the bovine brain enzyme that were fully consistent with a random ordering of substrates. Similar results have been obtained for the ascites tumor (Kosow and Rose, 1968) and rat brain (Purich and Fromm, 1971) hexokinase.

Additional evidence supporting random addition of substrate to hexokinase I comes from binding studies (Ellison et al., 1975), isotope trapping (Fromm and Solheim, 1977), and enzyme protection studies (Redkar and Kenkare, 1972; Redkar and Kenkare, 1975; Wilson, 1978; Wilson, 1979). All of these, when taken together, indicate that each substrate interacts with the enzyme in the absence of other factors.

Along with the study of the kinetic mechanism of brain hexokinase came the elucidation of the regulatory properties displayed by the enzyme. The potent inhibition of the enzyme by glucose-6-P has already

been alluded to. Another aspect of brain hexokinase regulation is the ability for inorganic phosphate to reverse this inhibition. This latter property, first observed in tumor cells by Teidemann and Born (1959), has also been observed in whole cells and cell homogenates (Rose, 1965), reconstructed glycolytic systems (Uyeda and Racker, 1965b), and in isolated hexokinase (Purich and Fromm, 1971; Rijksen and Staal, 1977; Shone and Fromm, 1980). Besides the effects of sugar-phosphate and inorganic orthophosphate, the activity of the enzyme may be regulated by the level of nucleotides present (Ning et al., 1969; Purich and Fromm, 1971; Rijksen and Staal, 1977). Along with inhibition by ADP, brain hexokinase is inhibited by both guanosine di- and triphosphates in a noncompetitive manner with respect to either substrate. At higher concentrations pyrimidine nucleotides proved competitive inhibition of ATP binding (Ning et al., 1969; Purich and Fromm, 1971).

While the regulation of brain hexokinase by glucose-6-P and P_i has been established for some time, the mechanism of action of these effectors is a source of controversy. Most workers in this field believe that glucose-6-P regulates hexokinase activity by binding to an allosteric site on the enzyme. This mechanism was first proposed by Weil-Malherbe and Bone (1951) after they observed that glucose-6-P was a noncompetitive inhibitor (with respect to glucose) of hexokinase activity. Crane and Sols (1954) surveyed the effects of 25 sugar phosphates on hexokinase and found that very few were effective inhibitors of the enzyme. Most notable in their findings was that the 6-phosphates of good substrates

such as mannose, 2-deoxyglucose, and fructose did not inhibit the enzyme. Crane and Sols concluded that glucose-6-P bound at a site specific for this sugar-phosphate remote from the active site. The support for this idea comes from the unusual nature of the inhibition and from the difference in specificity between the active site for hexose and the inhibitory site for hexose-phosphate. In addition, the formation of a tightly binding E·glucose·glucose-6-P abortive complex (Ellison et al., 1975; Wilson, 1978) has been taken by some investigators as further evidence for an allosteric site on brain hexokinase. Much support for this model of brain hexokinase regulation has been expressed in the literature (Colowick, 1973; Rose et al., 1974; Wilson, 1980) with the latest coming in a recent communication by Lazo et al. (1980). Here the authors present evidence for multiple binding of glucose-6-P to brain hexokinase and they conclude that the sugar phosphate is binding to a high-affinity regulatory site and a low-affinity active site in the enzyme. However, several technical problems encountered by the authors during their investigations, plus the type of information yielded by static binding experiments, make the authors' observations inconclusive.

The second model of brain hexokinase regulation was first proposed in 1962 when Fromm and Zewe (1962a) observed that glucose-6-P was a linear-competitive inhibitor of brain hexokinase with respect to MgATP. These authors felt that the effects of glucose-6-P on hexokinase could best be explained by placing the sugar-phosphate at the γ-phosphate subsite within the ATP binding locus on the enzyme. The displacement of

the sugar phosphate from the glucose binding domain upon phosphorylation by ATP would allow addition of a second glucose molecule to the active site, and account for the formation of the E·glucose·glucose-6-P ternary abortive complex. Support for this model is drawn from studies on glucose-6-P inhibition which show that only one inhibitor molecule binds per enzyme monomer, even at high (450 μM to 1000 μM) levels of glucose-6-P (Grossbard and Schimke, 1966; Ellison et al., 1975). Additional evidence comes from studies which indicate that mannose-6-P (Casazza and Fromm, 1976) and fructose-6-P (Rijksen and Staal, 1977) inhibit brain hexokinase with qualitatively the same mechanism as glucose-6-P, but with a lower affinity for the enzyme. This observation is contrary to the findings of Crane and Sols (1954) and weaken their arguments for proposing a second site for glucose-6-P action.

A third type of regulation which has been proposed for brain hexokinase involves the partitioning of the enzyme between soluble and particulate states. This hypothesis arises from the observation that hexokinase I is partitioned, in the brain, between soluble and mitochondrial bound pools of activity (Crane and Sols, 1953; Johnson, 1960; Rose and Warms, 1967; Craven and Basford, 1969; Felgner et al., 1979) and that metabolites in the cell such as glucose-6-P and ATP promote release of hexokinase from the mitochondria while phosphate tends to retard the enzyme's release (Rose and Warms, 1967). Wilson (1968) proposed that the distribution of hexokinase activity was regulated by these metabolites, and decreases in their cellular concentrations, as during increased rates

of glycolysis, displaced the soluble-particulate equilibrium toward increased levels of the particulate form. Since the particulate form of the enzyme displays greater affinity for ATP, and is less inhibited by glucose-6-P (Wilson, 1968; Tuttle and Wilson, 1970), the increased proportion of particulate enzyme would allow maintenance of the higher rate of glycolysis.

In an attempt to determine whether or not changes in the glucose-6-P concentration in the cell can affect the distribution of hexokinase between the soluble and particulate state, Purich and Fromm (1971) evaluated *in vitro* the glucose-6-P potential for solubilization in the presence of "physiological" levels of enzyme, substrates, products, and effectors. The authors found that neither phosphate nor glucose-6-P was able to change the distribution of the enzyme even when present in excess of the reported "physiological" concentrations. More recent *in vivo* studies performed on chick (Knull *et al.*, 1973) and mouse (Bachelard, 1976) have indicated that the distribution of hexokinase between soluble and bound forms does change in the brain during conditions of ischemia, but there is still no evidence that the partitioning of hexokinase changes under normal cellular conditions.

While the kinetic mechanism and regulation of brain hexokinase have been investigated extensively, the chemistry of the active site of this enzyme has received attention only in the last few years. Most of the work in this area has pointed to the presence of thiol groups at or near the active site. Several thiols are available for modification

by 5,5'-dithiobis(2-nitrobenzoic acid) (Redkar and Kenkare, 1972, 1975) while inactivation of the enzyme by tetranitromethane has indicated that the rapid loss of 2 cysteinyl residues accounts for the loss of activity (Subbarao and Kenkare, 1977a). In an attempt to define the location of the reactive thiols, Subbarao and Kenkare (1977b) treated the enzyme with the ATP analog 6-mercapto-9-β-D-ribofuranosylpurine 5'-triphosphate. This reagent showed saturation kinetics in its inactivation of brain hexokinase and, as it functions as a weak substrate (K_m = 0.25 mM), binding probably occurs at the active site. Both glucose and ATP protected the enzyme against inactivation.

A more recent study (Swarup and Kenkare, 1980) has used the glucose analog, N-(bromoacetyl)-D-glucosamine as an active site probe for brain hexokinase. In this study, the modification of one thiol led to inactivation of the enzyme. The reagent displayed both saturation and pseudo-first-order kinetics with the enzyme. In addition, the analog was a competitive inhibitor of glucose binding. The enzyme was protected from inactivation by glucose and glucose-6-P, and, to a lesser extent, ATP. These studies provide good evidence that one or more essential thiols are located around the active site of the enzyme; however, whether or not they are involved in catalysis of the hexokinase reaction or function in maintaining the active conformation of the enzyme is not yet clear.

Few additional studies on the chemical nature of the hexokinase reaction have been attempted; however, one observation

deserves mention here. This involves the inactivation of brain and muscle hexokinase by xylose and ATP (Lazo and Sols, 1979). This reaction seems to be an irreversible autophosphorylation of the enzyme that leads to inactivation. While the reaction has not been well-characterized in mammalian isozymes, the similar reaction that occurs to the yeast isozyme (DelaFuente, 1970) is mediated through the phosphorylation of a serine residue, presumably at the active site of the enzyme (Menezes and Puddles, 1976).

The work presented in this dissertation addresses two aspects of the brain hexokinase problem. The first section attempts to probe the active site of the enzyme by the use of pH kinetics while the second section describes a procedure for the removal of aluminum and other metal ions from commercial preparations of ATP. This treatment of ATP is necessary because of the formation of an AlATP complex which inhibits brain hexokinase at neutral pH. The final section of this dissertation probes the mode of regulation of hexokinase by glucose-6-P. By studying the reverse reaction (Glucose-6-P + ADP $\longrightarrow$ Glucose + ATP), an attempt is made to determine the location of glucose-6-P binding on the enzyme. This dissertation presents research done by myself while under the direction of Dr. H. J. Fromm, and published under our joint authorship (Solheim and Fromm, 1980a, 1980b, 1981).

SECTION I. pH KINETIC STUDIES OF BOVINE BRAIN HEXOKINASE

pH Kinetic Studies of Bovine Brain Hexokinase

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ABSTRACT

The variation of kinetic parameters with pH was examined for bovine brain hexokinase with glucose and MgATP as substrates. The $-\log(V_1)$ and $-\log(V_1/Km)$ profiles for both substrates were examined and seen to decrease below pH 6.5. All profiles asymptotically approached slopes of -1, indicating that the loss of activity in each instance was due to the protonation of a single group on the enzyme. Analysis of the data indicated that two ionizable groups were involved in the reaction. One functions in the binding of ATP and in catalysis, while the other participates in the binding of glucose. The $-\log(V_1)$ profiles both showed a "hump" attributed to a loss of activity in the pH region of 7.5 to 5.5. Addition of aluminum ions to the reaction mixture increased the magnitude of the hump, but the inhibition was abolished by the addition of citrate. Kinetic studies carried out at pH 7 indicated that aluminum was a competitive inhibitor with respect to ATP, and noncompetitive with respect to glucose. However, secondary plots of the kinetic data were nonlinear, concave downward, indicating that the inhibition is not of a simple type. Possible explanations for this phenomenon are presented.

INTRODUCTION

Brain hexokinase (hexokinase I) now is recognized as a primary control point of glycolysis in the brain. Because of its metabolic importance, much time and study have been devoted in attempts to acquire a thorough understanding of the kinetic mechanism of hexokinase (Purich et al., 1973). However, it has only been within the last few years that attention has been directed to the chemistry of this enzyme's active site. Recent studies have shown that sulfhydryl groups are required for activity in both yeast (Jones et al., 1975; Otieno et al., 1975) and mammalian (Redkar and Kenkare, 1972; Chou and Wilson, 1974; Subbarao and Kenkare, 1977a) hexokinase and there is some evidence to indicate that these groups are involved in the binding of substrate (Subbarao and Kenkare, 1977b). Carboxyl groups have been reported to be involved in the action of yeast hexokinase (Pho et al., 1974), and from x-ray crystallographic data, Anderson et al. (1978) have observed what seems to be an interaction between an aspartyl residue and the 4- and 6-hydroxyl groups of the sugar substrate. There is evidence for the involvement of histidyl residues in the active site of the wheat germ enzyme (Higgins and Easterby, 1974), but this residue has been found not to be involved in either the binding of substrate or catalysis of yeast hexokinase (Grouselle et al., 1973).

In this study, we have made use of pH kinetics to gain information about the groups responsible for binding and catalysis of substrate

molecules by bovine brain hexokinase. We report evidence for a single ionizable group involved in the catalysis of the reaction and in the binding of ATP, and a second residue that functions in the binding of the carbohydrate substrate. In addition, we offer observations on an anomalous loss of activity below pH 8 similar to the decrease reported in a recent paper by Viola and Cleland on the pH profiles of yeast hexokinase (Viola and Cleland, 1978). Colowick has suggested that this inhibition may be caused by the trivalent metal ion Al^{3+}.[1] Our results indicate that this ion may indeed be responsible for the pH dependent activity loss. Kinetic evidence indicates that this inhibition is nonlinear competitive with ATP and may be due to the formation of a strongly inhibitory aluminum-ATP complex, as well as interactions of the metal with the enzyme alone.

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[1] Dr. S. P. Colowick, Professor of Microbiology, Vanderbilt University, Nashville, Tennessee, personal communication.

EXPERIMENTAL PROCEDURE

Materials

Glucose 6-phosphate dehydrogenase was purchased from Boehringer Mannheim. Buffers used in the experiments were from Calbiochem. ATP and $NADP^+$ were products of Sigma, and glucose was from Pfhanstiehl. Distilled-deionized water was used in the preparation of all reagents. All other chemicals were of the highest purity available commercially.

Methods

Bovine brain hexokinase was prepared by the method of Redkar and Kenkare (1972) and had a specific activity of 62 units/mg. A unit is defined as 1 μmole of glucose-6-P produced per minute at 28° in a total reaction volume of 3 ml under standard assay conditions. The standard assay contained 10 mM glucose, 5 mM ATP, 50 mM TRIS -Cl (pH 7.6), 0.33 mM NADP, 7 mM $MgCl_2$, and 1.7 units of glucose-6-P dehydrogenase in a total volume of 3.0 ml.

The purified enzyme was stored as previously described (Redkar and Kenkare, 1972) and was desalted before each experiment on a Bio-Gel P-10 column, which had been equilibrated with 20 mM HEPES, pH 7.6, containing 5 mM β-mercaptoethanol. The enzyme was found to be stable for at least 4 h after desalting. For initial rate studies, the enzyme was diluted into 1 mg/ml of bovine serum albumin.

Kinetic assays were carried out in 1.0-ml volumes in 1-cm cuvettes

by using a Cary 118C spectrophotometer with a water-jacketed cell compartment. All assays were carried out at 28° unless otherwise indicated. Assays were initiated by the addition of 0.003 to 0.02 units of hexokinase.

The buffers used for the profile were acetate, 2-(N-morpholino)-ethanesulfonate (MES), piperazine-N,N'-bis(2-ethanesulfonate) (PIPES), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES), 2-(cyclohexylamino)ethanesulfonate (CHES), and 3-(cyclohexylamino)propanesulfonate (CAPS). Each buffer was used within 1 pH unit of its pK value. The buffers had no effects upon activity when the enzyme was assayed in their presence at pH 7.6. Buffer concentrations were maintained at 10 mM in each experiment and adjusted to an ionic strength equivalent to 10 mM salt by addition of KCl.

Reaction rates were determined by measuring the conversion of $NADP^+$ to NADPH in the glucose 6-phosphate dehydrogenase-hexokinase coupled assay. The coupling enzyme was dialyzed before use to remove ammonium sulfate. To verify that the rates measured were not limited by the coupling enzyme, various concentrations of hexokinase were added to assays containing a constant amount of glucose 6-phosphate dehydrogenase at the different pH values used. The plot of velocity *vs.* hexokinase was linear. The amount of couple varied from 0.75 unit/assay at pHs above 7.0 to 8.0 units/assay at pH 4.5. Each assay mixture contained 10 mM buffer, 0.4 mM $NADP^+$, and 2.5 mM Mg^+ in excess of the concentration of ATP. In assays where ATP was varied, glucose

was held at saturating levels (2.2 mM). When glucose was the varied substrate, $MgATP^{2-}$ was saturating (10 mM). When this method is used, breaks in the $-\log(V_1)$ profile will indicate groups ionizing in the ternary complex, and breaks in the $-\log(V_1/K_m)$ profiles will indicate groups ionizing from the EA complex where A is the saturating substrate. Other conditions were as stated in the figure legends.

For the pH studies in the presence of dimethylsulfoxide (DMSO), only cationic buffers were used. At pH values below 5.5, MES buffer (pK 6.5 at 20°) was used in a concentration so as to maintain at least 1.2 mM of the buffer in the neutral form.

Atomic absorption analysis of ATP for aluminum was performed by the Ames Laboratory of the United States Department of Energy, Ames, Iowa.

Contaminating aluminum was removed from ATP by extraction with 8-hydroxyquinoline and chloroform.[2]

Initial rate data were analyzed and fit to specific models by the weighted least-squares method, assuming equal variance of velocities by a computer program written in the OMNITAB language (Siano et al., 1975). The value of α was equal to zero.

The data from the pH experiments were plotted according to the procedure of Dixon (1953), and were fitted by an unweighted nonlinear least squares regression to Eq. 1, where f is $V_{1(app)}$ or $V_{1(app)}/K_{S(app)}$

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[2]Section II.

at the pH studied,

$$\log(f) = \log(C) - \log\left[1 + \frac{(H^+)^n}{(K_a)^n}\right] \qquad (1)$$

and C is V_1 or V_1/K_s. n represents the number of groups involved in a dissociation and was varied to obtain the best fit to the experimental data. K_a is the apparent dissociation constant. For ease in computing, Eq. 1 was rearranged by combining the log terms on the right side of the equation and multiplying the term within the log function by the quantity K_a/K_a (Eq. 2).

$$\log(f) = \log\left[\frac{(K_a)^n(C)}{(K_a)^n + (H^+)^n}\right] \qquad (2)$$

RESULTS

Studies of the variation of kinetic parameters with pH may provide information on the functional enzyme groups associated with binding and catalysis. Because information of this type is lacking in the case of brain hexokinase, experiments were undertaken in an attempt to gain insight into the mode of substrate interaction with this enzyme.

Figures 1a and 1b show Dixon plots for the pH-dependent variation of the maximal velocity (V_1) and maximal velocity/K_m (V_1/K_{ATP}) for varied ATP at a constant glucose concentration. As the pH was lowered below 6, a limiting slope of -1 was obtained, indicating that the protonation of one group on the enzyme was responsible for the loss of activity. Computer fitting of the data yielded pK's of 5.66 $\pm$ 0.10 for the $-\log(V_1)$ profile and 5.20 $\pm$ 0.11 for the $-\log(V_1/K_{ATP})$ profile. No decrease in velocity was seen at basic pH values.

Between the pH values of 7.5 and 5.5, there is a decrease in activity which appears as a "hump" in the $-\log(V_1)$ profile (curve a of Fig. 1). This loss of velocity is similar to that seen by Viola and Cleland (1978) in their study of the pH kinetics of yeast hexokinase. They found that citrate activated the enzyme in this pH region and speclated that this phenomenon may be related to the citrate activation of yeast hexokinase below pH 7 reported by Kosow and Rose (1971). In the fitting of the data, the values constituting the hump were omitted from the data file. When the experiments were repeated with aluminum-free ATP, profiles superimposable on the theoretical curves generated

in Fig. 1 were obtained. In accord with the profiles in Fig. 1, the data gave the best fit to Eq. 2 when one group was assumed to dissociate.

The pH profiles generated with glucose as the varied substrate are shown in Fig. 2. Curve a of this graph shows the $-\log(V_1)$ profile at 28°. As was the case with varied ATP, as the pH was lowered, the slope of the line asymptotically approached a slope of -1 within the pH range studied. Computer fitting of the data gave a pK of 5.85 $\pm$ 0.05. In the pH range from 7.5 to 5.5, a loss in activity resulting in a hump was again observed. These values were omitted from the computer fit. Curve c of Fig. 2 displays the $-\log(V_1/K_{glucose})$ profile at 28°. The curve was fitted assuming one group was involved in the protonation. The computer determined value of the pK is 5.47 $\pm$ 0.14. These experiments were also repeated with aluminum-free ATP and showed no significant variation from the theoretical curves shown in Fig. 2. The best fit was obtained when n = 1.

To investigate the identity of the groups being protonated, the variation of pK with temperature was determined for each of the profiles. For the $-\log(V_1)$ profile with the varied ATP, the determined pK's were 5.96 $\pm$ 0.06 at 20°, 5.66 $\pm$ 0.10 at 28°, and 5.57 $\pm$ 0.08 at 35°. The value for ΔH_{ion}, as estimated from the slope of the van't Hoff plot, is 10 $\pm$ 2 kcal/mol. The values of the pK's in the $-\log(V_1/K_{ATP})$ profiles when ATP concentration was varied were 5.34 $\pm$ 0.11, 5.20 $\pm$ 0.12, and 5.06 $\pm$ 0.09 at 20°, 28°, and 35°, respectively. The ΔH_{ion} value for this group is 8 $\pm$ 2 kcal/mol. Both of these values (8 and 10 kcal/mol)

are much too large to account for the energy required for a simple carboxyl ionization, but they are consistent with the involvement of a histidyl residue ($\Delta H_{ion} \simeq 7$ kcal/mol).

The pK's at the various temperatures for the $-\log(V_1)$ profile with varied glucose concentrations are 5.96 ± 0.10 at 20°, 5.85 ± 0.05 at 28°, and 5.71 ± 0.05 at 35°. As expected, the pK's decreased with increasing temperature. The ΔH_{ion} value, 7 ± 2 kcal/mol, again indicates the possible involvement of a histidyl residue in the catalytic process.

Curves b, c, and d of Fig. 2 display the $-\log(V_1/K_{glucose})$ profiles at 35°, 28°, and 20°, respectively. The determined pK's at these three temperatures are 5.45 ± 0.15, 5.47 ± 0.14, and 5.53 ± 0.07 at 35°, 28°, and 20°, respectively. The value for ΔH_{ion}, at only 2 ± 2 kcal/mol, is much less than that required for the ionization of a histidyl residue, and is only a little higher than the ± 1.5 kcal/mol required to ionize a simple carboxyl group. This is clearly a different group from that involved in catalysis or in the binding of ATP.

Additional evidence on the nature of the ionizing groups was obtained by observing the change in kinetic parameters with respect to pH in the presence of organic solvent. Dimethylsulfoxide (DMSO) was added to the reaction mixture to give a final concentration of 25% (vol/vol), and the pH profiles were generated for both varied ATP and glucose. The buffers used in these experiments were of the cationic type. Since dissociation of a proton from this type of buffer does not increase the net quantity of charged species in solution, lowering

the dielectric constant of the system by inclusion of an organic solvent will not effect the pK of the buffer. If buffers of the neutral acid type were used, the shift in the equilibrium of the system toward uncharged species caused by the lowering of the dielectric constant would produce an apparent shift in the pK of the buffer toward more basic pH values. This same effect is reflected by the ionizable groups within the enzyme. If the experiment is done in a cationic buffer, the pK of a histidyl group on the enzyme will not be changed by inclusion of an organic solvent in the system, while the pK of a neutral acid group will be raised. Similarly, if the experiment is performed in a neutral acid buffer, the pK of histidyl residues will become more acidic and the pK of neutral acid groups will not vary (Findlay et al., 1962; Inagami and Sturtevant, 1960).

Table I shows the results of the DMSO experiments. While the pK's for the kinetic parameters did not vary with varied ATP or with the $\log(V_1)$ profile with varied glucose concentration, the pK for the $\log(V_1/K_{glucose})$ profile shifted about 0.3 pH units toward the basic end of the pH range. Although this change is somewhat small, it tends to support the view that this group is a carboxyl. The data for the other kinetic parameters are in harmony with the ionizing group(s) being one or more histidyl residues.

In 1971, Kosow and Rose (1971) reported the activation of yeast hexokinase by citrate and other ions at pH values below 7. This activation was reduced if reagents were passed through a Chelex column

before use. More recently, Viola and Cleland (1978) have shown that a pH dependent loss of activity by this same enzyme can be restored by the inclusion of citrate in the reaction mixture. Colowick has indicated that this inhibition may be due to aluminum ions present with the ATP in the reaction mixture and can be reversed by citrate, EDTA, and phosphate.[1]

Atomic absorption analysis of the ATP used in these experiments (Product No. A-2383, lot No. 96C-7170, by Sigma) indicated that aluminum was indeed present in this preparation at a concentration of 190 ppm. This is equivalent to a concentration of 1.08 μM aluminum in a 0.25 mM solution of ATP. Our first attempt at demonstrating aluminum inhibition with hexokinase indicated that approximately 15 M aluminum was required to produce 50% inhibition of the enzyme, much more than present in ATP. However, when we preincubated the aluminum with the stock ATP solution for 30 min., we observed significant inhibition of the enzyme with micromolar concentrations of aluminum.

Figure 3 shows the results of an experiment in which the added aluminum was preincubated with 2.5 mM MgATP before addition of the ATP to the reaction mixture. Curve a of Fig. 3 indicates that at pH 7, and when preincubated with ATP, aluminum inhibits brain hexokinase at micromolar concentrations. If one takes into account the aluminum already

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[1]Dr. S. P. Colowick, Professor of Microbiology, Vanderbilt University, Nashville, Tennessee, personal communication.

contained in the ATP (190 ppm), the concentrations expressed on the abscissa are all increased by 1 μM, and the inhibition line is offset one unit to the right (broken line). The intercept of the broken line (the apparent $1/V_{max}$ of the line) is close to that for the velocity of hexokinase at pH 8 (line d). The concentration of aluminum required to cause 50% inhibition of the enzyme is then approximately 2.5 μM. Curve b of this figure demonstrates the effect of aluminum on hexokinase when 1 mM citrate has been included in the system. At the concentrations investigated, aluminum had no inhibitory effect. Curves c and d of Fig. 3 indicate the effects of aluminum and citrate on the enzyme at pH 8. In curve d, all conditions except pH are identical with those in curve a. Curve c indicates that the assay system contains 1 mM citrate. Curves c and d show that at pH 8, aluminum is unable to inhibit the enzyme, and citrate does not activate it.

To determine the effect of aluminum over the range of the pH profile, the effect of pH on the kinetic parameters of brain hexokinase was redetermined in the presence of added aluminum, and in the presence of added aluminum and citrate. Figure 4 shows the results of this experiment. Curve a of this figure indicates the effect of increasing the aluminum content of the ATP to 400 ppm. The added aluminum was preincubated for 30 min. with 50 mM ATP before addition of the nucleotide to the reaction mixture. A comparison of this profile to curve a of Fig. 1 shows that doubling the aluminum contamination of the ATP causes a 0.3 log unit change in the profile in the pH range between 7.5 and

5.0 and makes estimation of the pK almost impossibe.

Curve b of Fig. 4 was generated using conditions identical to those in curve a except for the inclusion of 1 mM citrate in the assay. All evidence of the hump has been removed, and the typical profile of the Dixon plot is observed. Computer fitting of these data gave a pK of 5.71 $\pm$ 0.10, which agrees well with the value initially determined in the absence of citrate, 5.66 $\pm$ 0.10 (Fig. 1, curve a).

In an effort to determine the mode of action of aluminum on hexokinase, we attempted to reverse the aluminum inhibition by the addition of Mg^{2+} to the reaction mixture (Fig. 5). The Mg^{2+} concentration was varied from 1 to 10 mM in the absence and presence of added aluminum. The added Mg^{2+} did not have the ability to lessen the aluminum inhibition.

Figures 6 and 7 show double reciprocal plots of velocity *vs.* varied ATP and glucose concentrations, respectively, at different, fixed levels of $Al(NO_3)_3$. The data show that aluminum is a competitive inhibitor of ATP and a noncompetitive inhibitor of glucose. The secondary plots (see insert of Fig. 6) for slopes in the 1/v *vs.* 1/ATP plots and for slopes and intercepts in the 1/v *vs.* 1/glucose plots (data not shown) *vs.* $Al(NO_3)_3$, are nonlinear, concave downward.

DISCUSSION

The results of studies of pH changes on enzymatic activity are often difficult to interpret. To draw valid conclusions about the identity of ionizing groups involved in the enzymatic process, several types of data must be compared. To this end, three types of observations were compiled in this study, the pK's of the ionizing groups affecting activity, the changes in these pK's with changing temperature, and the affect of organic solvent on the pK values.

The Dixon plots generated from the data gave relatively simple profiles with limiting slopes approaching -1 on the acid side of the pH scale. No variation in activity was observed at basic pH values. Computer analysis of the data gave the best fit to the theoretical curve when the ionization of one group was assumed to be responsible for the loss of activity in each profile. These groups can be broken down into two types, cationic groups with large ΔH_{ion} values, and a neutral acid group with little dependence upon temperature for ionization. Table II gives a summary of the results obtained in these investigations.

Three of the profiles, those for varied ATP concentrations and the $-\log(V_1)$ profile for varied glucose concentration, were caused by a cationic group, and a neutral acid residue was responsible for the change in parameters on the $-\log(V_1/K_{glucose})$ plot. These results can most simply be interpreted in terms of two ionizing groups on the enzyme. The first is involved in the binding of MgATP to the enzyme

and the breakdown of the ternary complex (E·Glc·MgATP) to products. This residue has a pK around 5.7 in the ternary complex and 5.2 in the free enzyme. The high ΔH_{ion} value, and the observation that organic solvent does not shift the pK to more basic pH values, lends credence to the suggestion that a histidyl residue acts in this capacity. The 0.5 pH unit variation between the pK's could be caused by conformational changes that occur in the enzyme upon binding of the substrate. Wilson (1978) has demonstrated such changes in conformation by studies of the rate of inactivation of rat brain hexokinase by chymotrypsin and glutaraldehyde in the presence and absence of substrate, and McDonald et al. (1979) and Pickover et al. (1979) have shown similar changes in conformation with yeast hexokinase and phosphoglycerate kinase and have speculated that this is a general phenomenon in kinase reactions. Alternatively, the variation in pK could indicate that separate histidyl residues are involved in the binding of ATP and the catalysis of the reaction. One or both of these residues may be remote from the active site of the enzyme and function by maintaining the active conformation of the enzyme.

The second ionizable group implicated by these studies participates in the binding of glucose to the enzyme. This group appears, by its low ΔH_{ion} value, to be of the neutral acid type, and this conclusion is reinforced by the shift of its pK 0.3 unit towards the basic end of the pH scale in the presence of organic solvent. This residue is most likely aspartate or glutamate and may function by hydrogen bonding

with a hydroxyl group of the sugar substrate. Although the pK for this group is approximately 1.5 pH units higher than that of free aspartate or free glutamate, hydrophobic residues in close proximity could easily perturb its pK by this much.

The effect of aluminum ions on brain hexokinase is difficult to explain and is complicated by the characteristics of aluminum in solution. Evidence shows that, at both acid and basic pH values, aluminum forms polymeric complexes with water and hydroxides (Akitt et al., 1972; Mesmer and Baes, 1971). This could explain why aluminum only inhibited the enzyme in the pH range of 7.5 to 5.5. Outside of this range the aluminum may assume a polymeric form that is not inhibitory. The inclusion of 1 mM citrate in the reaction reversed the aluminum inhibition, presumably by complexing the aluminum ions. A comparison of the pK values determined in the absence and presence of 1 mM citrate (5.66 ± 0.10 and 5.71 ± 0.10) shows that the aluminum inhibition did not affect the analysis of the data as long as the values between pH 7.5 and 5.5 were omitted from the computer fit.

The site of action of aluminum is not clear. The metal could form an inhibitory complex with ATP (a strong complex since Mg^{2+} was not able to reverse the inhibition), or it could bind directly to the enzyme. Since the inhibition by aluminum is potentiated when it is preincubated with ATP, an AlATP complex is likely to be the inhibitory species. This complex must have a high affinity for the enzyme, however, because it is present at approximately 0.5% of the concentration of ATP. The

high affinity of hexokinase for CrATP (Donenberg and Cleland, 1975) suggests that this is likely.

The kinetic experiments presented in Figs. 6 and 7 lend credence to an $AlATP^{1-}$ complex being responsible for the inhibition of hexokinase as it is a competitive inhibitor of ATP and a noncompetitive inhibitor of glucose. The nonlinear secondary plot, however, indicates that this is not inhibition of a simple type.

Brain hexokinase is thought to operate by using a rapid equilibrium random mechanism (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974). The generalized equation for competitive inhibition in this mechanism is expressed in Eq. 3, where A in this case represents ATP, B is glucose, and I is the inhibitor, in this case aluminum.

$$\frac{1}{V} = \frac{1}{V_1}\left[\frac{K_a}{A}\left(1+\frac{I^n}{K_i}\right) + \frac{K_b}{B} + \frac{K_{ia}K_b}{AB}\left(1+\frac{I^n}{K_{ii}}\right)\right] + \frac{1}{V_1} \qquad (3)$$

In linear competitive inhibition the exponent of I is one, and the replots are linear. If the inhibitor binds multiply to the enzyme, the exponent, n, becomes 2 or higher, yielding replots that are concave upward. If the stoichiometry of inhibitor binding is less than one, n will be less than one, and the secondary plots will be nonlinear concave downward as is seen in this case. The kinetic data presented in Figs. 6 and 7 were fitted to Eq. 3 by the OMNITAB program (Siano et al., 1975) with a variety of n values, and the exponent on the inhibitor term was found to be around 0.75.

This effect could be explained in several different ways. One could be that Al^{3+} ions form a complex with more than one enzyme molecule. If, for example, two molecules of enzyme complex with one Al^{3+}, an $I^{\frac{1}{2}}$ term would be introduced into the rate equation. More complicated expressions would arise if the complex formed with a different stoichiometry and could yield exponents of I varying from 0 to 1. If such a complex is formed, it may be possible to identify it by determining the molecular weight of brain hexokinase in the presence of Al^{3+}. A large change in the weight of the enzyme would indicate complex formation and might allow a determination of the binding stoichiometry.

A second possibility could be that the inhibitor molecule undergoes a concentration-dependent reaction that modifies it into a noninhibiting form. This is most easily visualized by imagining that the inhibitor undergoes a dimerization reaction (Eq. 4). If this happens,

$$2I \rightleftharpoons I_2 \tag{4}$$

$$\bar{I}_o = \bar{I} + 2\bar{I}_2 \tag{5}$$

$$I = \frac{-K + (K^2 + 8K\, I_o)^{\frac{1}{2}}}{4} \tag{6}$$

the added inhibitor (I_o) will be partitioned between I and I_2 (Eq. 5), and the concentration of I would be expressed as in Eq. 6 where K is the dissociation constant of the dimer. If I is inhibiting and I_2 is not, the effect of the dimerization would be to reduce the apparent inhibition caused by an added amount of inhibitor. The formation of such complexes of aluminum is well-documented. Akitt et al. (1972)

have shown that when dissolved in water, $AlCl_3 \cdot 6H_2O$ partitions between the two forms $Al(H_2O)_3^{3+}$ and $Al_2(OH)_2(H_2O)_8^{4+}$. As the pH of the solution is raised, higher order complexes of aluminum form, such as $Al_{13}O_4(OH)_{24}(H_2O)_{12}^{7+}$ and $Al_8(OH)_{20}(H_2O)_x^{4+}$ (Akitt et al., 1972). Other investigators have found species such as $Al_2(OH)_2^{4+}$ and $Al_3(OH)_4^{5+}$ present in solution at acidic pH and more complex forms such as $Al_{14}(OH)_{34}^{8+}$ in alkaline solution (Mesmer and Baes, 1971).

The data presented here support the idea that aluminum is responsible for inhibition of hexokinase below a pH of approximately 8.0. The inhibition is potent and can be reversed by citrate. Above pH 8.0, no inhibition by $Al(NO_3)_3$ is observed, but this could be due to the characteristics of aluminum ions to form polymers at basic pH values.

A paper by Womack and Colowick (1979) has appeared since preparation of the current study for publication. Their findings suggest that aluminum is a proton-dependent inhibitor of yeast hexokinase, and acts by complexation of ATP to form an inhibiting species. They also show that, while aluminum will inhibit rat brain hexokinase, it has little effect on rat muscle hexokinase or rabbit phosphofructokinase, and it was not an inhibitor of yeast glucokinase from Saccharomyces cerevisiae.

REFERENCES

Akitt, J. W., Greenwood, N. N., Khandelwal, B. L., and Lester, G. D. (1972) J. Chem. Soc., Dalton Trans., 604-610.

Anderson, C. M., Stenkamp, R. E., and Steitz, T. A. (1978) J. Mol. Biol. 123, 15-33.

Bachelard, H. S., Clark, A. G., and Thompson, M. F. (1971) Biochem. J. 123, 707-715.

Chou, A. C., and Wilson, J. E. (1974) Arch. Biochem. Biophys. 163, 191-199.

Dixon, M. (1953) Biochem. J. 55, 161-170.

Donenberg, K. D., and Cleland, W. W. (1975) Biochemistry 14, 28-39.

Findlay, D., Mathias, A. P., and Rabin, B. R. (1962) Biochem. J. 85, 139-144.

Gerber, G., Preissler, H., Heinrich, R., and Rapoport, S. M. (1974) Eur. J. Biochem. 45, 39-52.

Grouselle, M., Thiam, A. A., and Puddles, J. (1973) Eur. J. Biochem. 39, 431-441.

Higgins, T. J. C., and Easterby, J. S. (1974) Eur. J. Biochem. 45, 147-160.

Inagami, T., and Sturtevant, J. M. (1960) Biochim. Biophys. Acta 38, 64-79.

Jones, J. G., Otieno, S., Barnard, E. A., and Bhargava, A. K. (1975) Biochemistry 14, 2396-2403.

Kosow, D. P., and Rose, I. A. (1971) J. Biol. Chem. 246, 2618-2625.

McDonald, R. C., Steitz, T. A., and Engelman, D. M. (1979) Biochemistry 18, 338-342.

Mesmer, R. E., and Baes, C. F., Jr. (1971) Inorg. Chem. 10, 2290-2296.

Ning, J., Purich, D. L., and Fromm, H. J. (1969) J. Biol. Chem. 244, 3840-3846.

Otieno, S., Bhargava, A. K., Barnard, E. A., and Ramel, A. H. (1975) Biochemistry 14, 2403-2410.

Pho, O. B., Rouston, C. R., Desvages, G., Pardel, L. A., and Thour, N. K. (1974) FEBS Lett. 45, 114-117.

Pickover, C. A., McKay, D. B., Engelman, D. M., and Steitz, T. A. (1979) J. Biol. Chem. 254, 11323-11329.

Purich, D. L., Fromm, H. J., and Rudolph, F. B. (1973) Adv. Enzymol. Relat. Areas Mol. Biol. 39, 249-326.

Redkar, V. C., and Kenkare, U. W. (1972) J. Biol. Chem. 247, 7576-7584.

Siano, D. B., Zyskind, J. W., and Fromm, H. J. (1975) Arch. Biochem. Biophys. 170, 587-600.

Subbarao, B., and Kenkare, U. W. (1977a) Arch. Biochem. Biophys. 181, 8-18.

Subbarao, B., and Kenkare, U. W. (1977b) Arch. Biochem. Biophys. 181, 19-25.

Viola, R. E., and Cleland, W. W. (1978) Biochemistry 17, 4111-4117.

Wilson, J. E. (1978) Arch. Biochem. Biophys. 185, 88-99.

Womack, F. C., and Colowick, S. P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5080-5084.

Table I

Effects of DMSO on the pK_a of Hexokinase

Computer determined pK's for varied ATP and varied glucose in the absence and presence of DMSO. In the ATP varied experiment, glucose was held at 2.2 mM and $MgATP^{2-}$ was varied from 0.25 mM to 2.0 mM. When glucose was varied (from 0.02 mM to 0.1 mM) $MgATP^{2-}$ was held at 10.0 mM. DMSO was added to a final concentration of 25% (vol/vol). All assays were carried out at 28°.

Varied Substrate	Kinetic Parameter	pK in the absence of DMSO	pK in the presence of DMSO
$MgATP^{2-}$	V_1	5.66 ± 0.10	5.71 ± 0.06
	V_1/K_{ATP}	5.20 ± 0.12	5.18 ± 0.08
Glucose	V_1	5.85 ± 0.05	5.81 ± 0.09
	$V_1/K_{glucose}$	5.47 ± 0.15	5.76 ± 0.12

Table II

pK's of Catalytically Important Groups in Brain Hexokinase

Varied Substrate	Kinetic Parameter	pKa	ΔH_{ion}
$MgATP^{2-}$	V_1	5.66 ± 0.10	10 kcal/mol
	V_1/K_{ATP}	5.20 ± 0.12	8 kcal/mol
Glucose	V_1	5.85 ± 0.05	7 kcal/mol
	$V_1/K_{glucose}$	5.47 ± 0.14	2 kcal/mol

Figure 1. Dixon profiles generated with varied MgATP.

(a) Plot of $-\log(V_1)$ *vs.* pH with 2.2 mM glucose and $MgATP^{2-}$ varied from 0.25 mM to 2.0 mM. Individual points represent computer fits of initial rate data. The pK, determined by computer fit to Eq. 2, is 5.66 ± 0.10.

(b) Plot of $-\log(V_1/Km)$ *vs.* pH. The conditions are the same as in 1a. The pK is 5.20 ± 0.11.

Experiments were carried out at 28°. Other conditions are as stated in the experimental section. The curves through the data are the computer-determined fits, assuming one ionizing group with the pK's given.

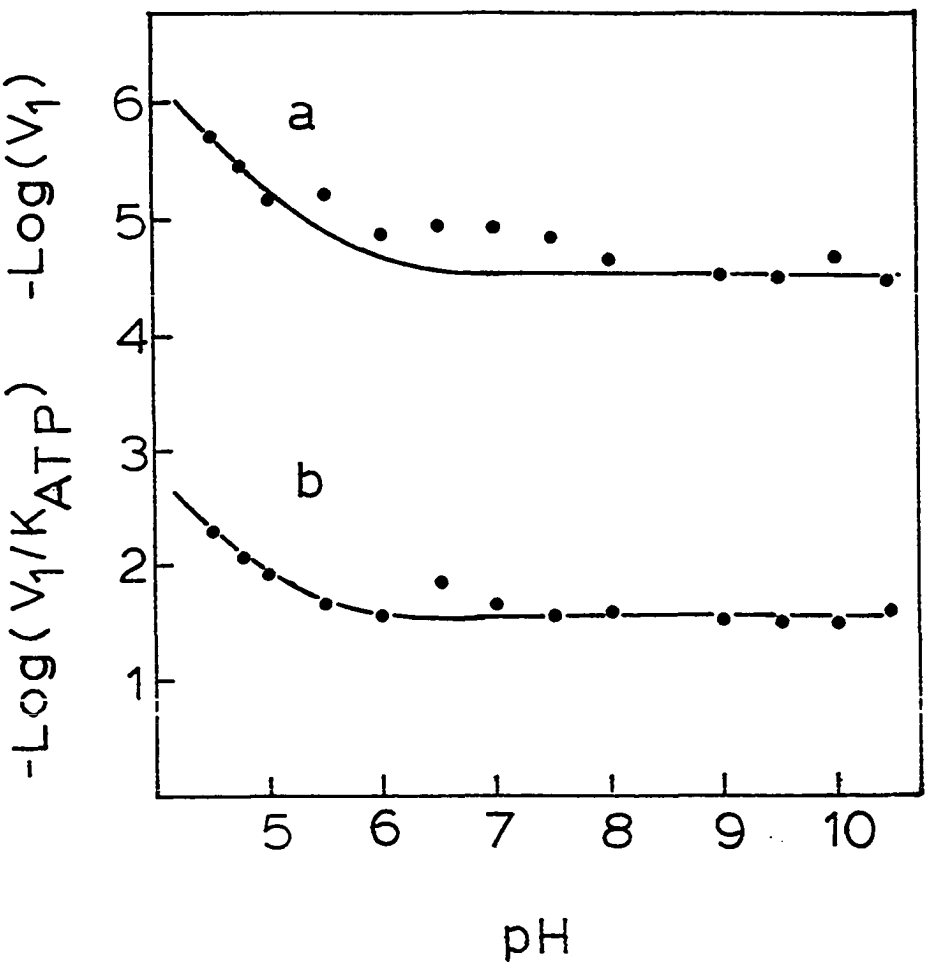

-Log(V1)
-Log(V1/KATP)
a
b
6
5
4
3
2
1
5
6
7
8
9
10
pH

Figure 2. Dixon profiles generated with varied glucose.

(a) Plot of $-\log(V_1)$ vs. pH. $MgATP^{2-}$ held constant at 10.0 mM, glucose is varied from 0.02 mM to 0.1 mM. Individual points represent computer fits of initial rate data. A computer fit of the data to Eq. 2 yielded a pK of 5.85 $\pm$ 0.05.

(b) Plot of $-\log(V_1/Km)$ vs. pH. The conditions are the same as in Fig. 2, line a. The profile was taken at 35°.

(c and d) Plots of $-\log(V_1/Km)$ vs. pH. The conditions are as above. Line c was determined at 28° and line d at 20°.

Other conditions are as stated in the experimental section. The curves through the data were determined by nonlinear fitting to Eq. 2 assuming one group was responsible for the activity loss.

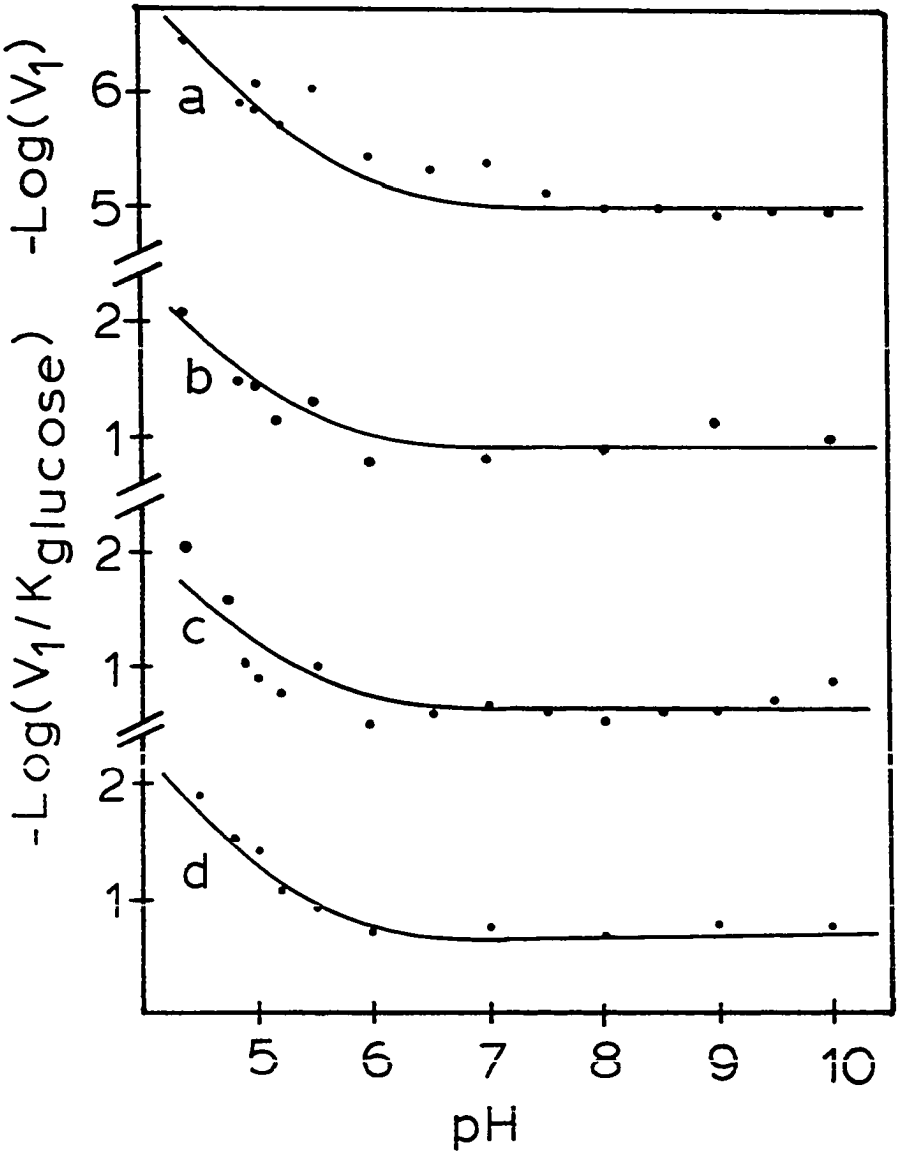

-Log(V1)
-Log(V1/Kglucose)
a
b
c
d
6
5
2
1
2
1
2
1
5
6
7
8
9
10
pH

Figure 3. Plot of 1/velocity (V) *vs.* aluminum at pH 7 and 8. Glucose was held at 2.2 mM and $MgATP^{2-}$ at 0.25 mM. The buffer at both pH values was 10 mM HEPES. A ten-fold concentration of aluminum was preincubated with 2.5 mM ATP for 30 min. before 0.1 ml of this solution was added to complete the 1.0-ml assay mixture. The aluminum concentrations on the abscissa are the concentration of aluminum added in the assay.

(a) 1/V *vs.* [aluminum] at pH 7. The broken line indicates the inhibition if the aluminum present in the ATP is included in calculating the final concentration of aluminum in solution.

(b) 1/V *vs.* [aluminum] at pH 7. The conditions are as in line a but 1 mM citrate was included in the assay.

(c) 1/V *vs.* [aluminum] at pH 8 plus 1 mM citrate.

(d) 1/V *vs.* [aluminum] at pH 8 minus citrate.

1/V is expressed in $(molar/min)^{-1}$.

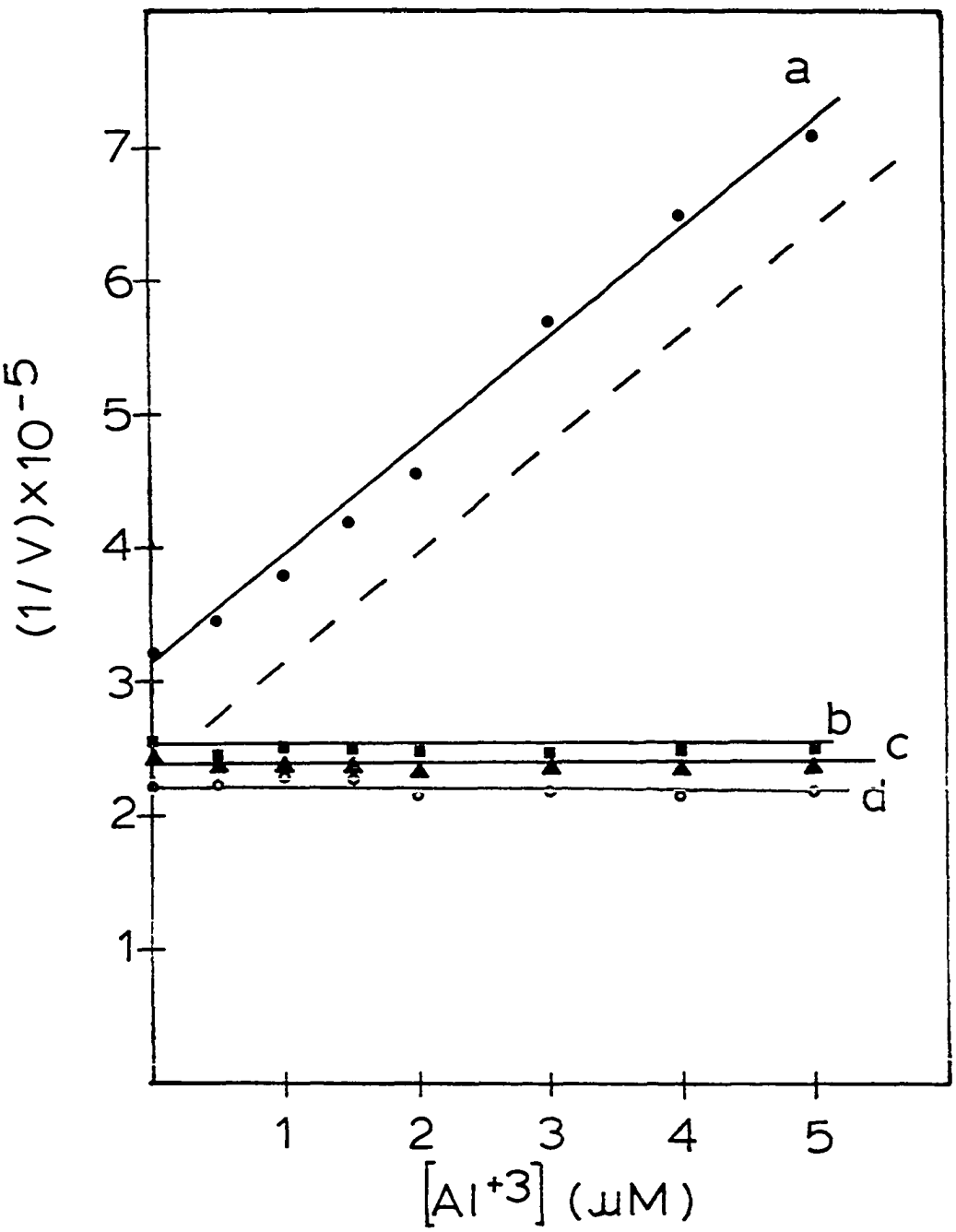

a
b
c
d
7
6
5
4
3
2
1
(1/V)x10-5
1
2
3
4
5
[Al+3] (μM)

Figure 4. Dixon profiles generated in the presence of added aluminum and citrate.

(a) Plot of $-\log(V_1)$ *vs.* pH. The conditions are as in Fig. 1. Glucose was held at 2.2 mM and ATP was varied from 0.25 mM to 2.0 mM. Aluminum nitrate was added to the ATP to increase the contamination to 400 ppm and was preincubated with 50 mM ATP before addition to the assay mix.

(b) Plot of $-\log(V_1)$ *vs.* pH. The conditions were identical to line a, except that 1 mM citrate was included in the assay.

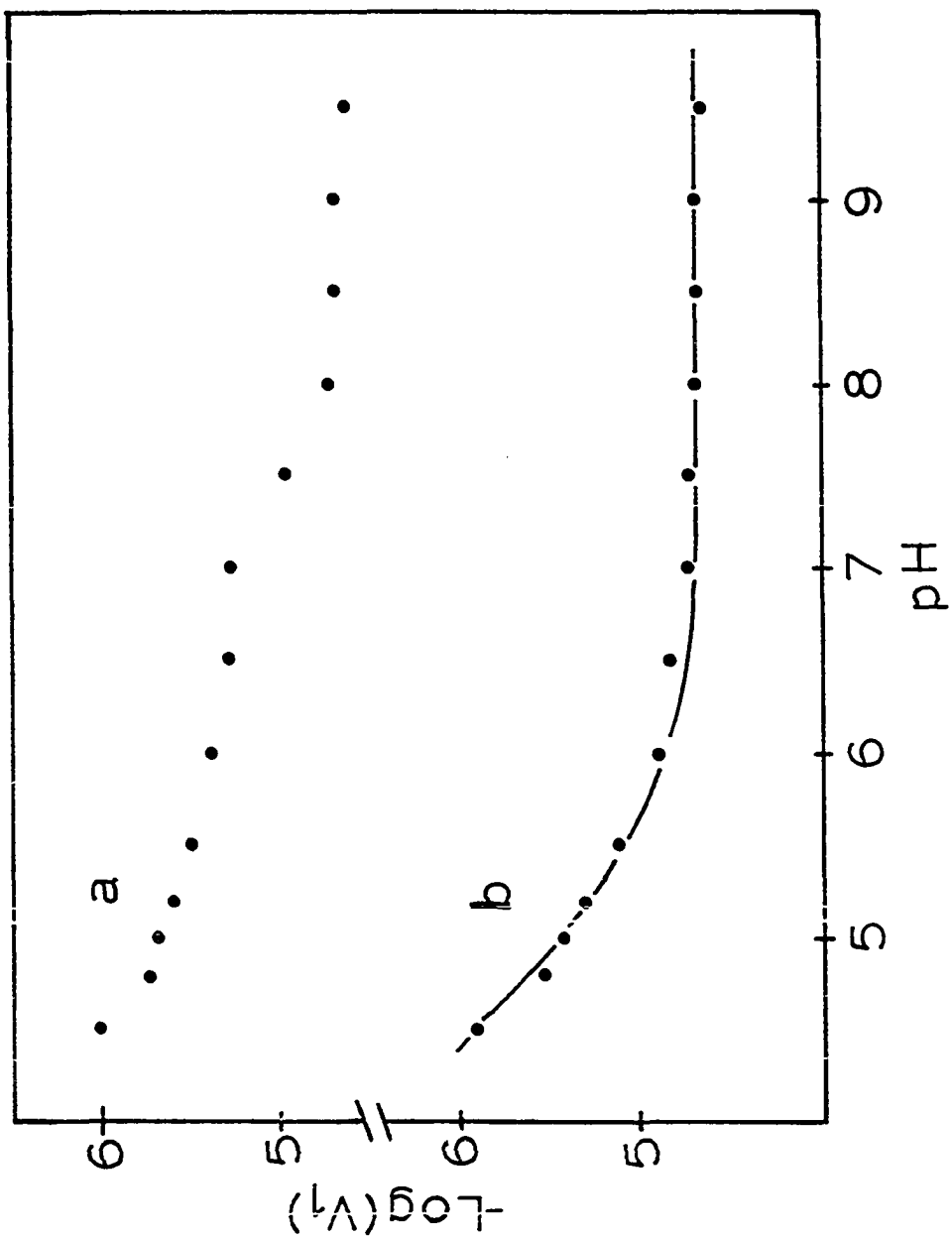

-Log(V1)
6
5
9
5
a
b
5
6
7
8
9
pH

Figure 5. Profile showing the effect of increasing Mg^{2+} on the inhibition caused by 15 μM Al^{3+}. Assays contained 2.2 mM glucose, 2 mM $MgATP^{2-}$, and were done at pH 7 in 10 mM HEPES buffer. Magnesium was varied from 1.0 to 10.0 mM in excess of the concentration of ATP. (▲) 15 μM aluminum nitrate added, (●) no added aluminum.

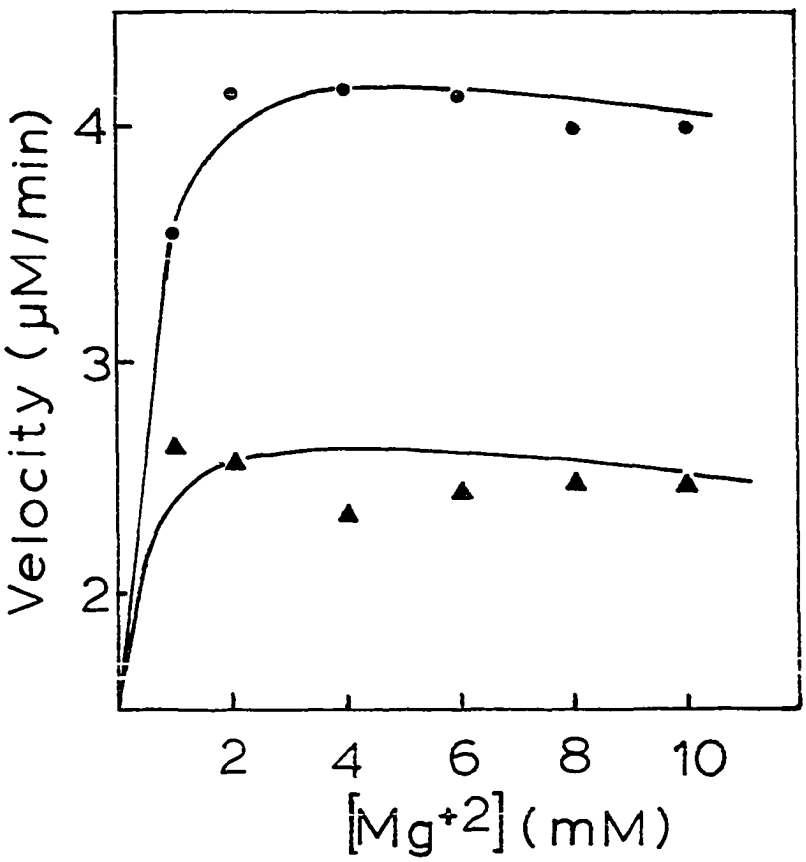

Velocity (μM/min)
4
3
2
2
4
6
8
10
[Mg+2] (mM)

Figure 6. Plot of the reciprocal of the initial velocity vs. the reciprocal of the molar concentration of $MgATP^{2-}$ in the absence (●) and presence of 10.0 μM (■) and 20.0 μM (▲) $Al(NO_3)_3$. Glucose was held at 0.03 mM and $MgATP^{2-}$ was varied from 0.2 to 1.0 mM. The assays were done at 28° in 10 mM HEPES buffer, pH 7.0. 1/ATP is in $(molar)^{-1}$ and 1/V is expressed in $(molar/min)^{-1}$.

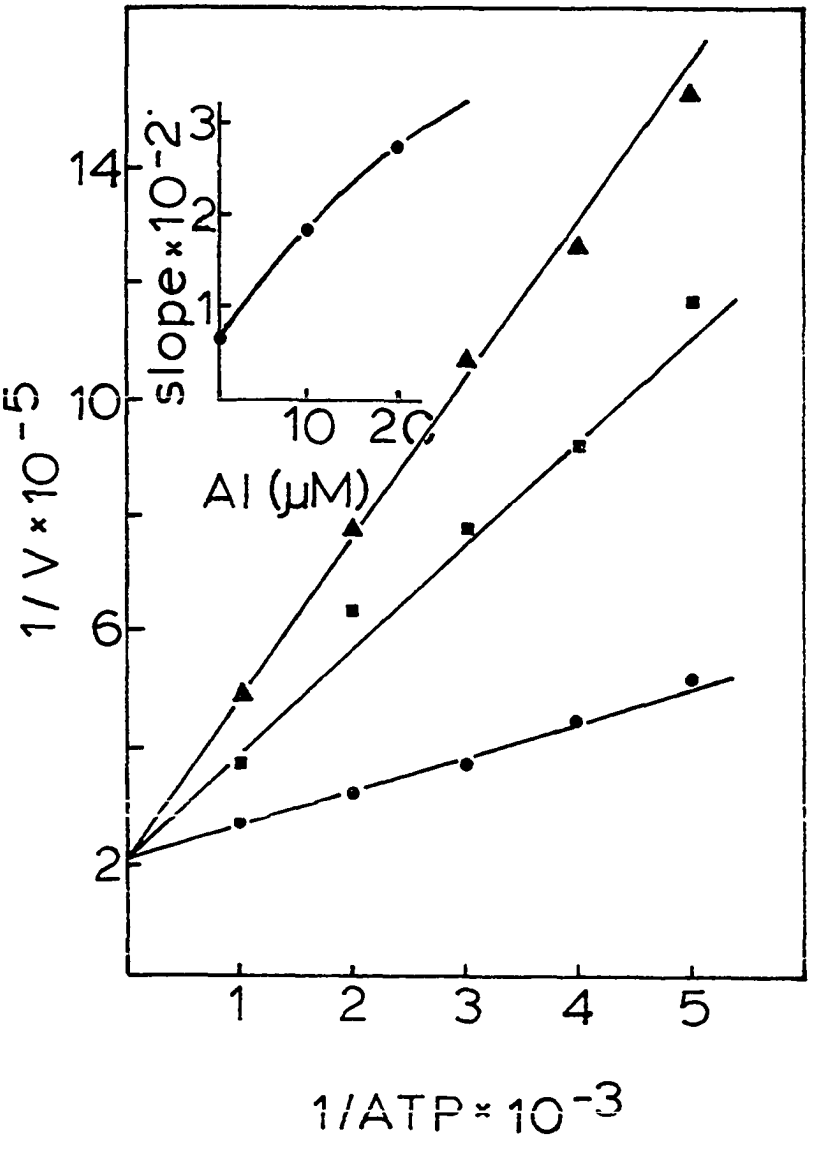
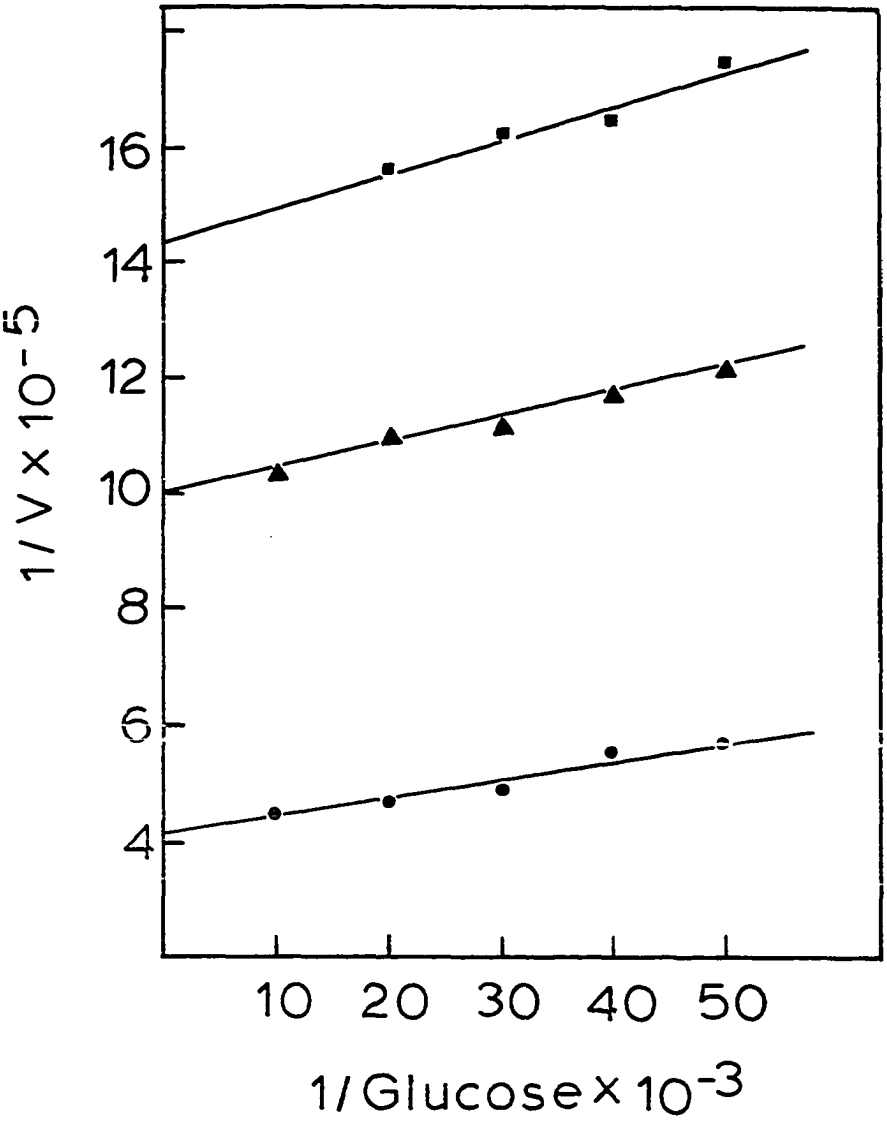

14
10
6
2
1/V × 10⁻⁵
1
2
3
4
5
1/ATP × 10⁻³
slope × 10⁻²
3
2
1
10
20
Al (μM)

Figure 7. Plot of the reciprocal of the initial velocity vs. the reciprocal of the molar concentration of glucose in the absence (●) and presence of 15 μM (▲) and 30 μM (■) $Al(NO_3)_3$. $MgATP^{2-}$ was held at 0.31 mM while glucose was varied from 0.02 mM to 0.10 mM. Assays were carried out at 28° in 10 mM HEPES buffer, ph 7.0. 1/V is expressed in $(molar/min)^{-1}$ and 1/Glucose is in $(molar)^{-1}$.


16
14
12
10
8
6
4
1/V x 10⁻⁵
10 20 30 40 50
1/Glucose x 10⁻³

SECTION II. A SIMPLE METHOD FOR REMOVING ALUMINUM FROM ADENOSINE-5'-TRIPHOSPHATE

A Simple Method for Removing Aluminum from Adenosine-5'-triphosphate

by

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Short Title: Purification of ATP

SUMMARY

Commercial preparations of ATP are contaminated with aluminum, a potent inhibitor of both brain and yeast hexokinase at neutral pH (Womack, F. C., and Colowick, S. P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5080-5084 and Solheim, L. P., and Fromm, H. J. (1980) Biochemistry 19, 6074-6080). In an effort to remove this ion, we have developed an extraction procedure that eliminates more than 99% of the contaminant with no loss of nuceotide. The method involves repeated extractions of the nucleotide solution with 8-hydroxyquinoline in chloroform, followed by one extraction at higher pH, that removes the final 10% of the aluminum contaminant that seems to be sequestered in a slowly dissociating complex and is unavailable to the 8-hydroxyquinoline at low pH.

INTRODUCTION

It is now clear that aluminum has a profound influence on the enzyme hexokinase (Womack and Colowick, 1979; Solheim and Fromm, 1980). The observations that yeast hexokinase is activated by ethylenediaminetetraacetate (EDTA) and citrate (Kosow and Rose, 1971) and that the enzyme exhibits hysteresis (Shill and Neet, 1974), are most likely a manifestation of inhibition by aluminum (Morrison, 1980).

We recently undertook kinetic experiments with brain hexokinase in which initial rates were monitored as a function of pH to gain some insight into the nature of those acid-base groups associated with the catalytic and substrate binding processes (Solheim and Fromm, 1980). The results that we obtained were qualitatively similar to those described by Viola and Cleland (1978) with yeast hexokinase. As did these investigators, we also found that the anomalous V_{max} and V_{max}/K_m versus pH profiles could be "normalized" by addition of either EDTA or citrate (Solheim and Fromm, 1980). Analysis of our commercial ATP samples indicated that they contained 191 ppm (g/g) aluminum (Solheim and Fromm, 1980). We found that this low level of the contaminating metal was, in fact, enough to cause those strange effects with hexokinase reported in the literature (Kosow and Rose, 1971; Shill and Neet, 1974; Viola and Cleland, 1978).

To investigate the role of aluminum in the hexokinase reaction, it was necessary for us to eliminate the metal from our commercial samples

of ATP. We have developed a very simple and innocuous extraction procedure for the removal of aluminum from ATP that takes advantage of the very high stability constant of aluminum-8-hydroxyquinoline and its solubilty in chloroform. This report details the procedure.

MATERIALS AND METHODS

$NADP^+$ was a product of Sigma. Glucose 6-phosphate dehydrogenase was purchased from Boehringer Mannheim, and glucose was from Pfhanstiehl. 8-hydroxyquinoline was of reagent grade and was recrystallized 3 times from water before use. ATP samples were from Sigma. ATP product A2382, lots 119c-7600 and 116c-7110, and product A3377, lot 125c-7320, were used in these experiments. Distilled water, passed through two ion exchange columns, was used in the making of all reagents. Brain hexokinase was prepared by the method of Redkar and Kenkare (1972) and had a specific activity of 62 units/mg.

ATP concentrations were determined spectrophotometrically by a coupled assay using hexokinase and glucose 6-phosphate dehydrogenase (Fromm and Zewe, 1972). ADP was assayed with lactate dehydrogenase and pyruvate kinase (Wellner et al., 1966). All measurements were made by using a Cary 118C spectrophotometer with a water-jacketed cell compartment.

Analysis of ATP samples for aluminum was performed by atomic adsorption spectroscopy at the Analytical Service Division of the Ames Laboratory of the United States Department of Energy, Ames, Iowa.

The extraction procedure was typically performed on 1 g. of ATP. The sample was dissolved in 10 ml of water, and the pH of the solution was adjusted to between 8 and 10 with 1 N sodium hydroxide. 10 ml of 0.1 M 8-hydroxyquinoline in chloroform were added to the ATP solution,

and the two were mixed either by a magnetic stirrer or by a vortex shaker for 5 min. After mixing, the layers were allowed to separate, and the chloroform phase was removed and viewed under ultraviolet light for fluorescence. This procedure was repeated until the fluorescence of the chloroform phase was significantly diminished. This usually occurred within 7 extractions. On the next extraction, while the solutions were mixed, the pH of the mixture was raised with 1 N sodium hydroxide until the solution took on a green color, indicating a partitioning of the 8-hydroxyquinoline between the water and chloroform layers. After being shaken for 30 sec., the pH of the mixture was lowered with 1 N HCl until the green color disappeared. The ATP solution was then extracted twice more with 8-hydroxyquinoline, followed by two extractions with chloroform to remove traces of the chelating reagent from the water phase. The ATP solution was then concentrated by lyophilization or used directly.

RESULTS AND DISCUSSION

8-hydroxyquinoline is known to react with over 50 metals, generally those that form hydroxy- and amino-complexes. Most of these complexes are soluble in chloroform and show absorbances above 375 nm. Furthermore, complexes of aluminum, gallium, and indium fluoresce in the chloroform phase. Because very small quantities of metal cause strong fluorescence, this property has been used for the detection of these metals in solution (Stary, 1964). The high affinity of 8-hydroxyquinoline for metal ions and its ease of extraction from solution made the reagent an obvious choice to remove the aluminum contaminant associated with commercial preparations of ATP. This contaminant is believed to cause the anomalous kinetics associated with yeast and brain hexokinase at neutral pH (Womack and Colowick, 1979; Solheim and Fromm, 1980; Morrison, 1980).

The kinetic data for both yeast and brain hexokinase indicate that aluminum is not inhibitory above pH 8 (Womack and Colowick, 1979; Solheim and Fromm, 1980). Aluminum also is known to form polynuclear complexes at basic pH. These observations led us to believe that there was a fundamental difference between the interaction of aluminum and ATP at neutral and basic pH values, and we decided to attempt to remove the aluminum contaminant from the nucleotide at these higher pH values.

Table I shows our success at purifying 3 different samples of ATP obtained from Sigma. We found that there was a large variance in aluminum content between different products (determinations 1 and 3) and

between different lot numbers of the same product (determinations 3 and 5). Initial attempts to remove the aluminum contaminant by repeated extraction with 8-hydroxyquinoline in chloroform indicated that from 80% to 90% of the ion was removed in 3 or 4 extractions (determinations 2, 4, and 6), but doubling the number of extractions did not remove significantly more contaminant (determination 7). Furthermore, we observed a discoloration of the water phase of the mixture, which increased with repeated extraction of the nucleotide solution. The discoloration was weakly fluorescent under ultraviolet light and was unextractable with chloroform.

We believe that this discoloration and weak fluorescence represent the remaining 10% of the aluminum in the sample, either still combined with ATP in a slowly dissociating complex or in some other way rendered inaccessible to the 8-hydroxyquinoline. We attempted to remove the remaining aluminum by two different procedures, both of which were successful. First, we incubated the nucleotide solution overnight at room temperature to allow any slow dissociation to equilibrate before extraction. This procedure, shown in determination 8, allowed extraction of 98% of the contaminating aluminum. Second, if the remaining aluminum was complexed with ATP, then a shifting of pH toward the basic end of the pH scale could speed the breakdown of the complex. Furthermore, at increased pH, 8-hydroxyquinoline becomes ionic and soluble in the water phase of the mixture. To achieve this end, we increased the pH of the solution to approximately 12 with sodium hydroxide during an

extraction. The water phase of the solution became green as the solubility of 8-hydroxyquinoline increased. After 30 sec. of mixing, the pH was lowered to between 8 and 10 with HCl, and the mixing continued for 5 min. When the phases separated, we observed that the discoloration of the water layer had abated and that it was no longer fluorescent under ultraviolet light. Determinations 9, 10, and 11 show the results of this procedure with various numbers of extractions performed before raising the pH. In each instance, 99% or more of the contaminant was removed.

Quantitation of the yield of the extraction procedure indicated that no ADP was generated during the procedure. Yield, in terms of initial ATP, ranged from 89% to 95%, depending upon how carefully the water and chloroform phases were separated between extractions. Losses in product are due entirely to manipulation of the sample.

The method itself is quick and easy to perform. Several samples can be treated in a few hours, and gram quantities of nucleotide can be handled in a single sample. Besides aluminum, other contaminating metal ions are likely to be removed from solution inasmuch as 8-hydroxyquinoline has a strong affinity for many other metals under these conditions (Stary, 1964).

To demonstrate the effect of the purified ATP preparation on the activity of brain hexokinase, we generated the pH profile with commercial ATP and with ATP that had been freed of aluminum by the extraction procedure. Fig. 1 shows the Dixon profile (Dixon, 1953) of this exper-

iment. When commercial ATP is used in the generation of the profile, an anomalous hump is present in the pH region 7.5 to 5.5. Because this is in the region of the pK for hexokinase (Solheim and Fromm, 1980), estimation of pK is uncertain. A similar finding was made with yeast hexokinase by Viola and Cleland (1978). When the purified ATP preparation is used to generate the same profile, all points lie close to the theoretical curve, and the normal profile of the Dixon plot is observed.

The advantages of this procedure are many. It is simple, rapid, and avoids harsh conditions. It quantitatively removes aluminum from the sample and leads to little loss of the nucleotide. Besides aluminum, this procedure should be useful in removing other metals from nucleotide preparations.

REFERENCES

Dixon, M. (1953) Biochem. J. 55, 161-170.

Fromm, H. J., and Zewe, V. (1972) J. Biol. Chem. 237, 1661-1667.

Kosow, D. P., and Rose, I. A. (1971) J. Biol. Chem. 246, 2618-2625.

Morrison, J. F. (1980) Fed. Proc. 39, abs. 1329.

Redkar, V. C., and Kenkare, U. W. (1972) J. Biol. Chem. 247, 7576-7584.

Shill, J. P., and Neet, K. E. (1974) Biochemistry 13, 3864-3871.

Solheim, L. P., and Fromm, H. J. (1980) Biochemistry 19, 6074-6080.

Stary, J. (1964) The Solvent Extraction of Metal Chelates, pp. 80-92. Pergamon Press, Oxford.

Viola, R. E., and Cleland, W. W. (1978) Biochemistry 17, 4111-4117.

Wellner, V. C., Zoukis, M., and Meister, A. (1966) Biochemistry 5, 3509-3514.

Womack, F. C., and Colowick, S. P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5080-5084.

Table I

Effect of 8-Hydroxyquinoline Extraction on

Aluminum-contaminated ATP

The concentration of aluminum in ATP was determined by atomic adsorption spectroscopy before and after extraction with 8-hydroxyquinoline in chloroform. Each sample consisted of 1 g. of ATP dissolved in 10 ml of water. After extraction, each sample was concentrated by lyphilization before analysis.

Deter.	ATP Sample	No. of extract.	Aluminum[a] (μg/ml)	Fraction removed
1.	lot 125c-7320	0	11.06	0.0
2.	"	3	1.18	0.893
3.	lot 119c-7600	0	4.62	0.0
4.	"	3	0.52	0.887
5.	lot 116c-7110	0	26.30	0.0
6.	"	4	4.66	0.823
7.	"	8	3.52	0.866
8.	"	8[b]	0.37	0.986
9.	"	11[c]	<0.05	>0.998
10.	"	9[d]	<0.06	>0.998
11.	"	7[d]	0.22	0.991

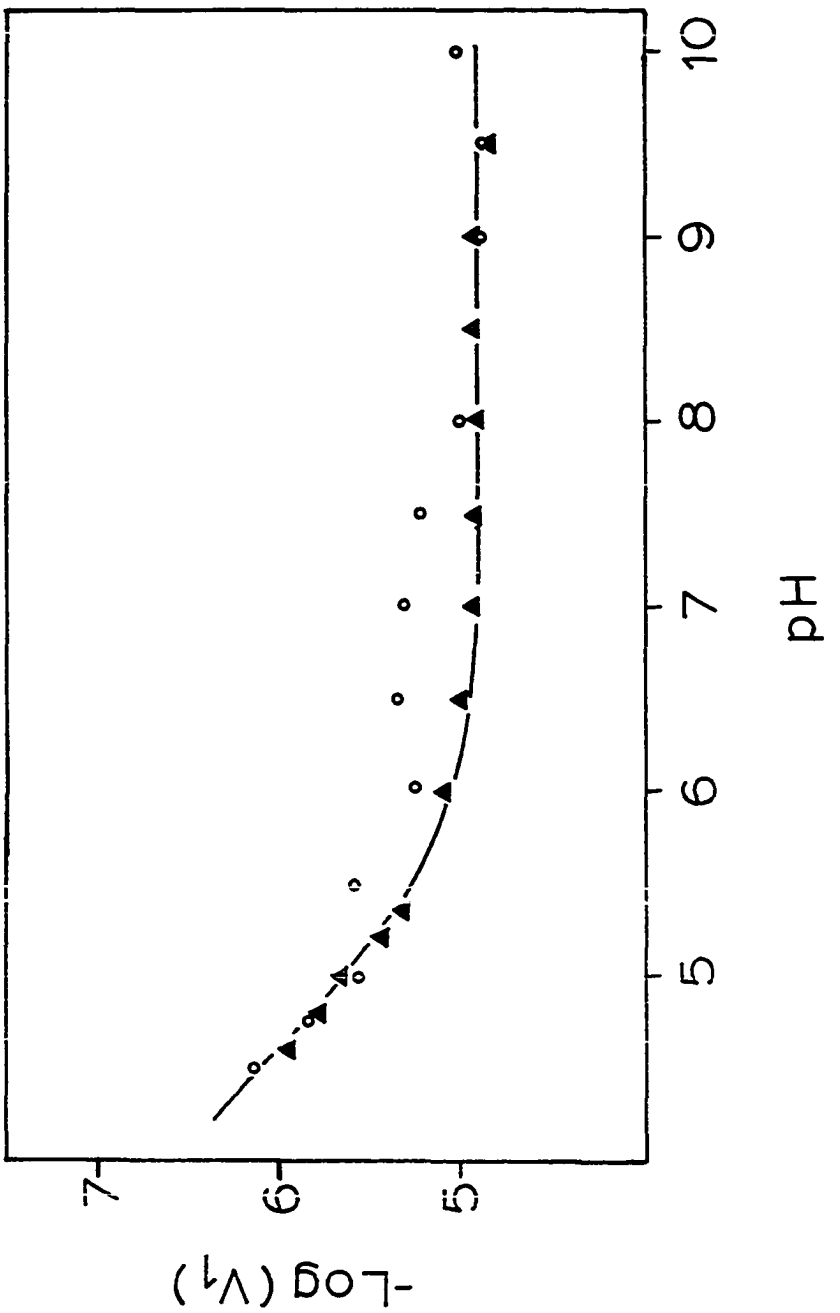
[a]Values reported represent the concentration of aluminum contained in a 0.1 M solution of nucleotide. Standard errors of the values are ± 5% with a maximum accuracy of ± 0.1 μg.

[b]The solution was equilibrated at room temperature for 16 h before extraction.

[c]On the 8th extraction the pH was raised and after 30 sec. of mixing was again lowered. The solution was extracted 3 additional times following this step. (See the methods section for greater detail.)

[d]The nucleotide solution was extracted 2 times after the high pH step.

Figure 1. Plot of $-\log(V_1)$ vs. pH with 2.2 mM glucose and commercial (●) and aluminum-free (▲) $MgATP^{2-}$ varied from 0.2 mM to 1.0 mM. The lines represent computer fits of the initial rate data as described elsewhere (Solheim and Fromm, 1980). Experiments were carried out at 28°. Buffer concentrations were maintained at 10 mM in each experiment and were used within one pH unit of their pK. These curves are normalized to 0.005 unit of brain hexokinase used per assay. Other experimental details are available in the literature (Solheim and Fromm, 1980).


-Log (V1)
7
6
5
pH
5
6
7
8
9
10

SECTION III. KINETIC EVIDENCE THAT THE HIGH AFFINITY GLUCOSE 6-PHOSPHATE SITE ON HEXOKINASE I IS THE ACTIVE SITE

Kinetic Evidence that the High-affinity Glucose 6-Phosphate Site on Hexokinase I is the Active Site

By

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Short Title: Glucose 6-Phosphate binds at the active site of Hexokinase I.

Key Words: Brain hexokinase, hexokinase I, glucose-6-P inhibition.

Correspondence to: H. J. Fromm, Dept. Biochem. Biophys., Iowa State U., Ames, Iowa 50011. Tel # (515)294-4971.

ABSTRACT

Two mechanisms have been suggested to account for the regulation of brain hexokinase by glucose 6-phosphate. One mechanism places glucose-6-P at an allosteric site, remote from the active site, while the second describes glucose-6-P as a simple product inhibitor of the enzyme, binding at the γ-phosphate subsite within the ATP locus of the active site. To resolve this question, we have undertaken a study of the back reaction of hexokinase I. Our data indicate that glucose-6-P displays classical Michaelis-Menten kinetics with brain hexokinase. This finding is consistent only with the high-affinity glucose-6-P site on the enzyme being the catalytic site. The dissociation constant estimated from the initial rate experiments is approximately 25 μM, a value that agrees well with the inhibition constant for glucose-6-P in the forward direction. These findings are consistent with an earlier model (Ellison et al., 1975) which maintains that glucose-6-P inhibition of brain hexokinase is a manifestation of product inhibition.

In a recent paper, Lazo et al. (1980) reported data obtained from binding studies with rat brain hexokinase at an elevated (250 μM) level of glucose-6-P. These authors believe that their results indicate multiple binding of glucose-6-P to the enzyme and interpret the data in terms of a high-affinity allosteric site and a low-affinity catalytic site. Our results are at variance with this interpretation, and are consistent only with the high affinity site for glucose-6-P on brain hexokinase being the active site.

INTRODUCTION

It is generally accepted today that hexokinase I, brain hexokinase is the pacemaker of glycolysis in brain tissue (Lowry and Passonneau, 1964) and the erythrocyte (Rapoport, 1968). The observation by Tiedemann and Born (1959), that P_i (inorganic phosphate) increases the activity of glucose-6-P-inhibited brain hexokinase, provides an attractive model for coordinated glycolytic regulation in tissues that do not rely heavily on glycogen metabolism. However, an understanding of the molecular mechanism of brain hexokinase regulation requires knowledge of the nature of glucose-6-P binding to the enzyme. Most workers in this field believe that the regulatory ligand binds to an allosteric site in the enzyme. We, on the other hand, have maintained that the effect of glucose-6-P on hexokinase can best be explained by assuming that the sugar phosphate acts as a normal product inhibitor of the enzyme, binding tightly within the ATP domain at the active site (Ellison et al., 1975). If this ligand also binds at an allosteric site, binding is very weak (Ellison et al., 1975).

To further elucidate the role of sugar phosphate in the hexokinase I reaction, we have undertaken initial-rate studies of the back reaction of the enzyme. Our results indicate that the reverse reaction of brain hexokinase displays classical Michaelis-Menten kinetics. Glucose-6-P binds to the active site of the enzyme with high affinity (K_{ia} = 25 μM) and shows substrate inhibition of the enzyme beginning at 60 μM.

Recently, Lazo et al. (1980) reported multiple binding of glucose-6-P to brain hexokinase. These findings led them to conclude that there are two binding sites for glucose-6-P per molecule of enzyme, one with a high affinity for ligand and the other with a much lower affinity. They interpret their results, as well as other data in the literature, to mean that the high-affinity binding of glucose-6-P occurs at an allosteric locus on hexokinase, whereas weak binding takes place at the catalytic site.

In 1976, Sols (1976) reported that glucose-6-P is a classical substrate inhibitor of the reverse reaction of an animal hexokinase. Until the recent report by Lazo et al., we took this to be the final proof that glucose-6-P was in fact binding at the active site of hexokinase I. To resolve this question we undertook the current study and our findings strongly imply that the high-affinity binding site for glucose-6-P is the active site.

EXPERIMENTAL PROCEDURES

Materials

Glucose oxidase (210 units/mg) and horseradish peroxidase (250 units/mg) were purchased from Boehringer Mannheim. Mutarotase was from Sigma, as was ADP, glucose-6-P, 4-aminoantipyrine, and PIPES buffer (piperazine-N,N'-bis(2-ethanesulfonate)). Glucose dehydrogenase was kindly supplied by Dr. R. Ramaley of the Univ. Nebr. Med. School. Distilled-deionized water was used in the preparation of all reagents. All other chemicals were of the highest purity available commercially.

Methods

Bovine brain hexokinase was purified by the method of Redkar and Kenkare (1972) and had a specific activity of 62 units/mg. A unit of activity is defined as 1 μmole of glucose 6-phosphate per minute at 28° in a total reaction volume of 3 ml under standard assay conditions. The standard assay contained 10 mM glucose, 5 mM ATP, 50 mM TRIS-Cl (pH 8), 0.33 mM NADP, 7 mM $MgCl_2$, and 1.7 units of glucose 6-phosphate dehydrogenase in a total volume of 3 ml.

The purified enzyme was stored as previously described (Redkar and Kenkare, 1972) and was desalted before each experiment on a Bio-Gel P2 column, which had been equilibrated with 100 mM PIPES pH 6.0. The enzyme was found to be stable for at least 12 h after desalting. For initial-rate studies, the enzyme was diluted into 1 mg/ml of bovine serum albumin

in 100 mM PIPES, pH 6.0.

Aluminum, contaminating the commercial preparations of ADP and ATP, was removed by the procedure of Solheim and Fromm (1980) with 8-hydroxyquinoline in chloroform. The coupling enzymes were dialyzed against 5 mM NaCl in 10 mM PIPES buffer, pH 6.0, overnight before used to remove ammonium sulfate which activated the reverse reaction.

Reaction rates for the reverse reaction were determined in a Cary 118c spectrophotometer by measuring the conversion of phenol and 4-aminoantipyrine to quinoneimine in the glucose oxidase-peroxidase coupled assay (Gierow and Jergil, 1980). To verify that the rates measured were not limited by the coupling enzymes, various concentrations of hexokinase were added to assays containing constant amounts of mutarotase, glucose oxidase, and peroxidase. Hexokinase concentrations used in the initial-rate experiments were from well within the linear portion of the velocity *vs.* enzyme profile. The typical assay contained 4 mM ADP, 10 mM $MgCl_2$, 10 mM phenol, and 0.4 mM 4-aminoantipyrine in 100 mM PIPES buffer pH 6.0. Glucose-6-P was varied from 3 μM to 450 μM; 10 units of glucose oxidase, 5 units of peroxidase, and 20 units of mutarotase were used in each assay. The reaction was initiated with brain hexokinase (0.06-0.01 units). To verify that changes in absorbance were due only to the reverse reaction catalyzed by hexokinase, two blanks were run. One contained the total assay mixture without hexokinase; the other contained hexokinase, but ADP and glucose-6-P were omitted. In each case, no change in absorbance was observed.

Initial-rate data were analyzed and fit to specific models by the weighted least-squares method, assuming equal variance of velocities, by a computer program written in the OMNITAB language (Siano et al., 1975). The value of α was set equal to zero. Data where glucose-6-P inhibition was obvious were eliminated from the data set.

RESULTS

In order to gain additional insight into the kinetic mechanism and mode of glucose-6-P binding in the brain hexokinase reaction, initial-rate studies were undertaken for the reverse reaction. The data, plotted in the double reciprocal form, are shown in Figs. 1 and 2. The following conclusions can readily be drawn from these results: the kinetic mechanism is sequential[1], the kinetics are Michaelian, and an evaluation of the Michaelis and dissociation constants can be made for the substrates. The kinetic mechanism of brain hexokinase is rapid-equilibrium random (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974). Since the reverse reaction is extremely slow, relative to the forward reaction, with conversion of the central complex the rate limiting step, binding of the substrates in the reverse reaction will be rapid-equilibrium as well (Fromm, 1975).

The kinetic parameters for the substrates are: 28.5 $\pm$ 5.0 μM, 14.8 $\pm$ 5.1 μM, 1.66 $\pm$ 0.53 mM, and 0.86 $\pm$ 0.18 mM for $K_{iglucose\text{-}6\text{-}P}$, $K_{glucose\text{-}6\text{-}P}$, K_{iADP}, and K_{ADP}, respectively. The two-fold discrepancy between the $K_{iglucose\text{-}6\text{-}P}$ value reported for this experiment and from experiments of the forward reaction (Copley and Fromm, 1967) is probably due to the fact that the latter experiments were carried out at pH 7.6.

---

[1]The nomenclature is that of Cleland (1963).

Recent studies of the reverse reaction from Sols' laboratory (1976) suggest that the brain hexokinase reaction is markedly inhibited at glucose-6-P levels exceeding approximately 0.1 mM. On the other hand, aside from the obvious excess substrate inhibition, there was no hint of sigmoidicity from his kinetic data. Because the experimental conditions used in these studies were not available in the literature, we undertook the experiment illustrated in Fig. 3. These data confirm the findings of Sols (1976) and suggest that the back hexokinase reaction follows Michaelis-Menten kinetics.

DISCUSSION

Lazo et al. (1980) have recently reported data from binding experiments that they interpret to mean that two sites exist on hexokinase for glucose-6-P, one a high-affinity allosteric inhibitory site, and the other a low-affinity site. It will be useful in the development of this discussion to place this report in historical perspective. In 1951, Weil-Malherbe and Bone (1951) published results indicating the noncompetitive nature of glucose-6-P inhibition of rat brain hexokinase. From their results, they concluded that glucose-6-P binds at a site other than the site or sites that bind either glucose or ATP, but that, when occupied, prevented catalysis. In their 1954 paper, Crane and Sols (1954) expanded upon this concept by requiring that the site of glucose-6-P inhibition be remote from the active site and that the sugar phosphate dissociate from and recombine with the enzyme to cause inhibition. This proposal has come to be regarded as the allosteric model of brain hexokinase regulation and is based in part on the observation that good substrates such as D-mannose, D-fructose, and 2-deoxy-D-glucose yield phosphorylated products, which either do not inhibit hexokinase or inhibit very poorly. Thus, it was suggested (Crane and Sols, 1954) that glucose-6-P and other sugar phosphates cannot bind to the same locus on the enzyme. Fromm and Zewe (1962) showed in 1962 that, although glucose-6-P inhibition relative to glucose was noncompetitive, the sugar phosphate affected the enzyme as a classical product inhibitor

with regard to ATP. These authors indicated that it was unnecessary to postulate a second site in the enzyme; rather, they suggested that the phosphate of glucose-6-P interacts primarily with the γ-phosphate subsite of the ATP pocket on brain hexokinase. Other investigators have obtained similar product inhibition data with hexokinase I (Rapoport, 1968; Copely and Fromm, 1967; Grossbard and Schimke, 1966; Uyeda and Racker, 1965). Replots of double-reciprocal data shown in a report by Ellison et al. (1975) and which we have drawn from the data of Grossbard and Schimke (1966), indicate that glucose-6-P is a linear competitive (relative to ATP) or linear noncompetitive (relative to glucose) inhibitor of hexokinase I, even at high concentrations of the sugar phosphate (1000 μM and 450 μM, respectively). These data indicate that only one inhibitor molecule binds per enzyme monomer. Additional work by Purich and Fromm (1971 and 1972a) has shown that glucose-6-P is a potent inhibitor of brain hexokinase even in the presence of high concentrations of nucleotides, and finally, recent work by Casazza and Fromm (1976) has shown that, contrary to the suggestion of Crane and Sols (1954), mannose-6-P inhibits brain hexokinase in an identical fashion with that of glucose-6-P, but with a lower affinity for the enzyme. This result has been confirmed in the recent report by Lazo et al. (1980) in which their binding studies indicate that mannose-6-P, as well as the sugar phosphates of other alternative substrates, was able to compete with glucose-6-P for the high-affinity binding site on brain hexokinase.

One of the seeming anomalies in the proposal of Fromm and his co-

workers comes from their suggestion originally alluded to from kinetic studies (Rapoport, 1968; Copley and Fromm, 1967; Weil-Malherbe and Bone, 1951; Crane and Sols, 1954; Fromm and Zewe, 1962; Grossbard and Schimke, 1966; Uyeda and Racker, 1965) and subsequently confirmed by binding experiments (Ellison et al., 1975; Lazo et al., 1980; Ellison et al, 1974), that an abortive complex of enzyme-glucose-glucose-6-P forms at the enzyme's active site. The model of Crane and Sols (1954), and now espoused by Wilson (Lazo et al., 1980), would place glucose at the active site and glucose-6-P at an allosteric site. Quite clearly the sugar phosphate must bind to the active site and Lazo et al. (1980) in their recent study have provided for two glucose-6-P binding sites. Our view (Ellison et al., 1975) is that in the forward reaction, after glucose-6-P is formed, the phosphate moiety is anchored to the γ-phosphate subsite of the ATP locus on the enzyme. The glucose portion of the glucose-6-P next binds to a new position on hexokinase where the binding determinants differ somewhat from the binding determinants at the glucose subsite (Ellison et al., 1975). This explains the different specificities of the two subsites and permits formation of the abortive ternary complex. The rationale for the different specificities of the sugars and sugar phosphates are presented elsewhere (Casazza and Fromm, 1976). Abortive complex formation leads to tighter binding of glucose-6-P (Ellison et al., 1975; Lazo et al., 1980) as a consequence of the law of mass action.

In addition to the binding experiment at low glucose-6-P concentra-

tion, referred to above, Lazo et al. (1980) also conducted an experiment at high glucose-6-P concentration, which led the authors to conclude that brain hexokinase had two binding sites for glucose-6-P, a high-affinity allosteric site and the low-affinity catalytic site. However, there are several problems associated with this report which led us to question the reliability of the data of Lazo et al. (1980). These are as follows: 1. The authors admit that because of technical difficulties with their assay, Scatchard plots could not be constructed, and they rely upon binding experiments performed at one concentration (250 μM) of glucose-6-P to support their conclusions. 2. Although their data (Table I) indicate that 1 mM 2-deoxyglucose-6-P is able to reduce by half the binding of glucose-6-P to the high-affinity site at 5.4 μM glucose-6-P, the authors state that "2-deoxyglucose-6-P is a poor ligand for the inhibitory site and thus would be ill-suited to compete with glucose-6-P at this site." Furthermore, because 2-deoxyglucose-6-P is a substrate for hexokinase (Sols, 1976), it must bind to both sites. This analog, at a concentration of 10 mM, however, can only displace glucose-6-P from 1 site (Table II (Lazo et al., 1980)), presumably the allosteric site (see Table I (Lazo et al., 1980)). Yet, Lazo et al. (1980) go on to state that "weak binding of 2-deoxyglucose-6-P at the inhibitory site is also consistent with the failure of 2-deoxyglucose-6-P to inhibit brain hexokinase when the reverse reaction is studied". The inhibition referred to is relative to glucose-6-P inhibition (Fig.3 and (Sols, 1976)) and is low-affinity excess substrate inhibition.

In this context, the data of Table I (Lazo et al., 1980) show that 1,5-anhydroglucitol-6-P is effective in removing glucose-6-P from the allosteric site, as is 2-deoxyglucose-6-P. Lazo et al. (1980) state "in combination, 2-deoxyglucose-6-P and 1,5-anhydroglucitol-6-P virtually abolish binding of glucose-6-P". It is not at all clear how two ligands that compete for the allosteric site (Table I (Lazo et al., 1980)) can effectively remove glucose-6-P from both sites. 3. In order for the results of Fig. 3 or of Sols' data (Sols, 1976) to be consistent with the Lazo-Sols-Wilson model (Lazo et al., 1980), a third type of binding site, weaker in affinity than either the postulated allo or active sites, must be proposed, i.e., the inhibitory effect at this site is manifested only at very high levels of glucose-6-P. 4. Most troubling is the result obtained when the binding of glucose-6-P to hexokinase I is measured in the presence of glucose. At the concentration of glucose used in the experiment (20 mM), the binding site for glucose is saturated, i.e., the K_D for glucose is approximately 40 μM. This precludes binding of glucose-6-P at the glucose binding site of the enzyme, and the ratio of binding of glucose-6-P per molecule of hexokinase cannot be greater than 1. The value determined by Lazo et al. (1980) is 1.34 ± 0.26 in 6 determinations. Because this value cannot be greater than unity, one must conclude that the excess in the value is due to the technical difficulties alluded to in the manuscript. If one now compares this value to that determined in the absence of additions (1.55 ± 0.39 in 17 determinations) with a simple t test, one finds that these

values are not significantly different at the 20% level. Although this limitation is acknowledged by Lazo et al.(1980), they suggest that the glucose analog N-acetylglucosamine (10 mM) "produced a substantial decrease in glucose-6-P binding". Yet we found that there is no statistical difference between the values for 10 mM glucose and 10 mM N-acetylglucosamine. These results lead one to question whether any of the binding data at high levels of glucose-6-P provide meaningful results. In an attempt to explain why glucose is an ineffective inhibitor of glucose-6-P binding to the active site, Lazo et al. (1980) evoke the concept of synergistic interactions between the two hexose binding sites. They suggest that although glucose enhances binding of glucose-6-P to the regulatory site, it may be that binding of the sugar phosphate to the catalytic site may have an adverse effect on binding of glucose-6-P to the regulatory site. 5. Finally, binding data do not provide any information about the progress of an enzymatic reaction and thus cannot tell what effect a ligand has on the course of the catalytic reaction.

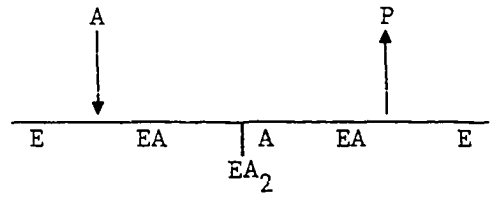
Because of the problems involved in interpreting the work of Lazo et al. (1980), and generally because of the inability of binding data to provide information dealing with enzyme catalysis, we have undertaken the study of the back reaction of bovine brain hexokinase to determine the characteristics of the glucose-6-P binding site (Figs. 1 and 2). Fig. 3 illustrates the velocity dependence of the reverse reaction of glucose-6-P at a fixed concentration of ADP. These data describe

classical substrate inhibition and show beyond doubt that high-affinity binding by the sugar-phosphate occurs at the active site of hexokinase I. The apparent K_m, estimated from this profile, is approximately 20 μM. This value is in good agreement with the K_i ($\sim$12 μM) for glucose-6-P inhibition of hexokinase I in the forward direction (Rapoport, 1968) and with the values determined for the reverse reaction in Figs. 1 and 2. As a confirmation of this profile, the experiment described by Fig. 3 was repeated with glucose dehydrogenase as the coupling enzyme. The profile was qualitatively identical with Fig. 3 and yields an apparent K_m of 20 to 30 μM. It is of some interest that these results are qualitatively identical with findings published by Sols in 1976 (Sols, 1976). Taken at face value these results seem to exclude glucose-6-P binding at a high-affinity allosteric site. It is important to note that the dissociation constant determined for glucose-6-P is similar whether it is a product inhibitor (forward reaction) or a substrate (reverse reaction).

The Lazo-Sols-Wilson model (Lazo et al., 1980) of hexokinase regulation by glucose-6-P in fact requires 3 sugar phosphate binding sites. At low levels of ligand, in the presence of ADP, binding would occur at the high-affinity allosteric site and no reaction would occur. As the level of glucose-6-P is increased, glucose-6-P would bind to the catalytic site and leave the allosteric site (Lazo et al., 1980). In order to explain the substrate inhibition at still higher levels of glucose-6-P (Fig. 3 and (Sols, 1976)), one would have to postulate

either a lower-affinity inhibitory allosteric site for ligand or a mechanism for rebinding of glucose-6-P at the high-affinity allosteric site. In addition to all of this, the model requires synergistic interactions between the ligands at the allosteric and catalytic sites (Lazo et al., 1980). This is a highly complex and unprecedented proposal when compared to our model which can account for the effects of glucose-6-P using classical assumptions of product and substrate inhibition (Ellison et al., 1975). Although it is difficult to prove that the latter model is correct, it is clear, at least in our opinion, that the Lazo-Sols-Wilson model (Lazo et al., 1980) is incorrect. We arrive at this conclusion based not only on the inconsistencies alluded to regarding the findings of Lazo et al. (1980), but also based on the type of kinetic response one could predict for brain hexokinase when the reverse reaction is studied.

The mechanism for substrate inhibition (for simplicity represented as a one substrate system) is shown in Scheme I



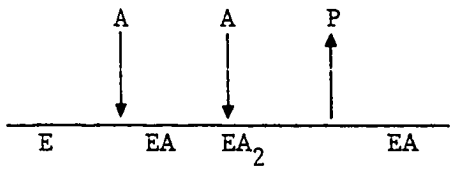
Scheme I

and has a rate equation of the type described by Eq. 1,

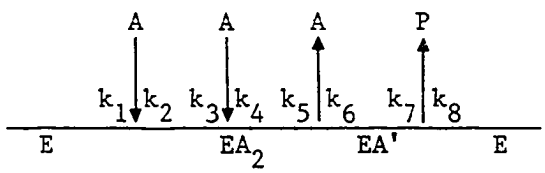
$$v = \frac{V_1}{1 + \frac{K_a}{A} + \frac{A}{K_i}} \qquad (1)$$

where K_a is the Michaelis constant for A, and K_i is the dissociation constant for the EA_2 complex. The results of Fig.3 and the data of Sols (1976) are in harmony with Eq. 1 and, thus, with the mechanism in Scheme I. It is of interest to note that multiple binding of glucose-6-P is not seen in the forward reaction when studies of glucose-6-P inhibition of brain hexokinase are undertaken even at elevated levels of the inhibitor (Ellison et al., 1975; Grossbard and Schimke, 1966). This is not an unusual occurrence, inasmuch as enzymes often show substrate inhibition when studied from the nonphysiological direction. Because hexokinase I is believed to operate with a rapid equilibrium random mechanism (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974), this inhibition is most likely due to the addition of glucose-6-P to the E·ADP·G-6-P ternary complex (Fromm, 1975; Cleland, 1979). Ironically, the incidence of the substrate inhibition lends support to Lazo et al.'s (1980) contention that there are two binding sites for glucose-6-P on hexokinase I, but it cannot be stressed too strongly that the evidence supports binding with high affinity at the active site of the enzyme, with only weak binding at a second site.

The mechanism proposed by Lazo et al. (1980) is actually a manifestation of substrate activation by glucose-6-P when looking at the back reaction of hexokinase. If we assume for simplicity that we are dealing with a one-substrate system, the Lazo-Sols-Wilson model (Lazo et al., 1980) can be described by either of two mechanisms shown in Schemes II and III.



Scheme II



Scheme III

The mechanism in Scheme II is an example of classical substrate activation and leads to a rate equation of the type shown in Eq. 2.

$$v = \frac{V_1}{1 + \frac{K_i}{A} + \frac{K_{ia}}{A^2} \cdot K_i} \tag{2}$$

where K_{ia} is the dissociation constant for EA and K_i is the dissociation constant for the EA_2 complex. The rate expression for the mechanism for Scheme III, assuming the steady-state assumption, is of the form

$$v = \frac{V_1}{1 + \frac{A}{K_i} + \frac{K_{ii}}{A} + \frac{K_{a'}}{A^2}} \tag{3}$$

where $V_1 = \frac{k_3 k_5 k_7 E_0}{k_3 k_5 + k_4 k_6 + k_3 k_7}$, $K_i = \frac{k_3 k_6}{k_3 k_5 + k_4 k_6 + k_3 k_7}$,

$$K_{ii} = \frac{k_1k_4k_7 + k_2k_4k_6 + k_5k_7(k_1 + k_3)}{k_1(k_3k_5 + k_4k_6 + k_3k_7)}$$, and

$$K_{a'} = \frac{k_7k_2(k_4 + k_5)}{k_1(k_3k_5 + k_4k_6 + k_3k_7)}$$

and arises from the model of Lazo et al. (1980). In this model, glucose-6-P binds first to the high-affinity allosteric site and then to the low-affinity active site. Furthermore, when binding occurs at the catalytic site, the sugar phosphate dissociates from the allosteric site. This model predicts that a plot of velocity vs. A will be sigmoidal at low substrate concentrations, reach a maximum, and approach zero as a limit. The initial, sigmoidal portion of this type of profile is illustrated in Fig. 4. It is of interest to note that in the model described by Scheme III, it is not necessary to suggest an additional site for glucose-6-P. The data of Figs. 1-3 and results from Sols' laboratory (Sols, 1976) argue against sigmoidicity at low substrate levels of glucose-6-P. One could suggest, of course, that the sigmoidicity exists, but is too subtle to be discerned. The suggestion must be viewed, however, in context of what is known regarding the mechanism of inhibition of brain hexokinase by glucose-6-P and the fact that the $K_{iglucose\text{-}6\text{-}P}$ is about 20 μM.

The data of Figs. 1 and 2 permit calculation of the kinetic parameters for the reverse hexokinase reaction, and provide us with a fuller understanding of how brain hexokinase is coordinately regulated by its products. We proposed some time ago (Purich and Fromm, 1972b) that the

kinetic mechanism may play a fundamental role in enzyme regulation. Purich and Fromm (1971, 1972a) reported that hexokinase is inhibited about 65% by intercellular levels of ADP and other nucleotides and approximately 75% by intracellular levels of glucose-6-P. On the other hand, these effectors exhibit synergism when present together and produce approximately 95% inhibition of the brain hexokinase reaction. These data were obtained in the absence of phosphate which modulates the flux through the hexokinase reaction by deinhibiting the glucose-6-P inhibited enzyme (Ellison et al., 1975). To explain these findings, we used the rate equation for the rapid-equilibrium random Bi Bi mechanism and the kinetic parameters obtained from this and another report (Ellison et al., 1974).

$$v = \frac{\left[(Glc)(ATP) - \frac{(Glc\text{-}6\text{-}P)(ADP)}{K_{eq}}\right] V_1}{K_{ATP}(Glc) + K_{Gl}(ATP)(Glc) + \frac{(V_1/V_2)}{K_{eq}}\left[K_{Glc\text{-}6\text{-}P}(ADP) + K_{ADP}(Glc\text{-}6\text{-}P) + (ADP)(Glc\text{-}6\text{-}P)\right]} . \quad (4)$$

$v = 6.4 \times 10^{-3} V_1$ with both products present, and $21.1 \times 10^{-3} V_1$ and 13.1×10^{-3} with only ADP or glucose-6-P present alone, respectively. These calculations indicate that both products aid to inhibit the hexokinase reaction so as to dampen and thus control the potentially rapid flux through the hexokinase reaction when the level of phosphate increases

in the brain cell in order to increase glycolysis (Purich and Fromm, 1971; Purich and Fromm, 1972a).

REFERENCES

Bachelard, H. S., Clark, A. G., and Thompson, M. F. (1971) Biochem. J. 123, 707-715.

Casazza, J. P., and Fromm, H. J. (1976) Arch. Biochem. Biophys. 177, 480-487.

Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 104-137.

Cleland, W. W. (1979) Methods Enzymol. 63, 500-513.

Copley, M., and Fromm, H. J. (1967) Biochemistry 6, 3503-3509.

Crane, R. K., and Sols, A. (1954) J. Biol. Chem. 210, 597-606.

Ellison, W. R., Lueck, J. D., and Fromm, H. J. (1974) Biochem. Biophys. Res. Commun. 57, 1214-1220.

Ellison, W. R., Lueck, J. D., and Fromm, H. J. (1975) J. Biol. Chem. 250, 1864-1871.

Fromm, H. J., and Zewe, V. (1962) J. Biol. Chem. 237, 1661-1667.

Fromm, H. J. (1975) Initial Rate Enzyme Kinetics, Springer-Verlag, Berlin/Heidelberg/New York.

Gerber, G., Preissler, H., Heinrich, R., and Rapoport, S. M. (1974) Eur. J. Biochem. 45, 39-52.

Gierow, P., and Jergil, B. (1980) Anal. Biochem. 101, 305-309.

Grossbard, L., and Schimke, R. T. (1966) J. Biol. Chem. 241, 3546-3560.

Lazo, P. A., Sols, A., and Wilson, J. E. (1980) J. Biol. Chem. 255, 7548-7551.

Lowry, O. H., and Passonneau, J. V. (1964) J. Biol. Chem. 239, 31-42.

Ning, J., Purich, D. L., and Fromm, H. J. (1969) J. Biol. Chem. 244, 3840-3846.

Purich, D. L., and Fromm, H. J. (1971) J. Biol. Chem. 246, 3456-3463.

Purich, D. L., and Fromm, H. J. (1972a) J. Biol. Chem. 247, 5660.

Purich, D. L., and Fromm, H. J. (1972b) Current Topics in Cellular Regulation, Horecker, B. L., and Stadtman, E. R., eds., Vol. 6, 131-167.

Rapoport, S. M. (1968) Essays in Biochemistry, Camphett, P. N., and Greville, G. O., eds., Vol. 4, pp. 69-103, Academic Press, New York.

Redkar, V. D., and Kenkare, V. W. (1972) J. Biol. Chem. 247, 7576-7584.

Siano, D. B., Zyskind, J. W., and Fromm, H. J. (1975) Arch. Biochem. Biophys. 170, 587-600.

Solheim, L. P., and Fromm, H. J. (1980) Anal. Biochem. 109, 266-269.

Sols, A. (1976) Reflections on Biochemistry, Kornberg, A., Horecker, B. L., Cornudella, L., and Oro, J., eds., pp. 199-206, Pergamon, New York.

Tiedemann, H., and Born, J. (1959) Z. Naturforsch. 14b, 477-478.

Uyeda, K., and Racker, E. (1965) J. Biol. Chem. 240, 4682-4688.

Weil-Malherbe, H., and Bone, A. D. (1951) Biochem. J. 49, 339-347.

Wilson, J. E. (1978) Arch. Biochem. Biophys. 185, 88-99.

Figure 1. Plot of the reciprocal of initial velocity (V) versus the reciprocal of the molar concentration of glucose 6-phosphate at 1.0 mM (●), 0.50 mM (▲), 0.33 mM (▼), 0.25 mM (■), and 0.20 mM (+) $MgADP^-$. Glucose-6-P was varied from 12.5 μM to 66.7 μM. Other experimental details are described under "Experimental Procedures". 1/V is expressed in $(molar/min)^{-1}$ and 1/Glc-6-P is $(molar)^{-1}$.

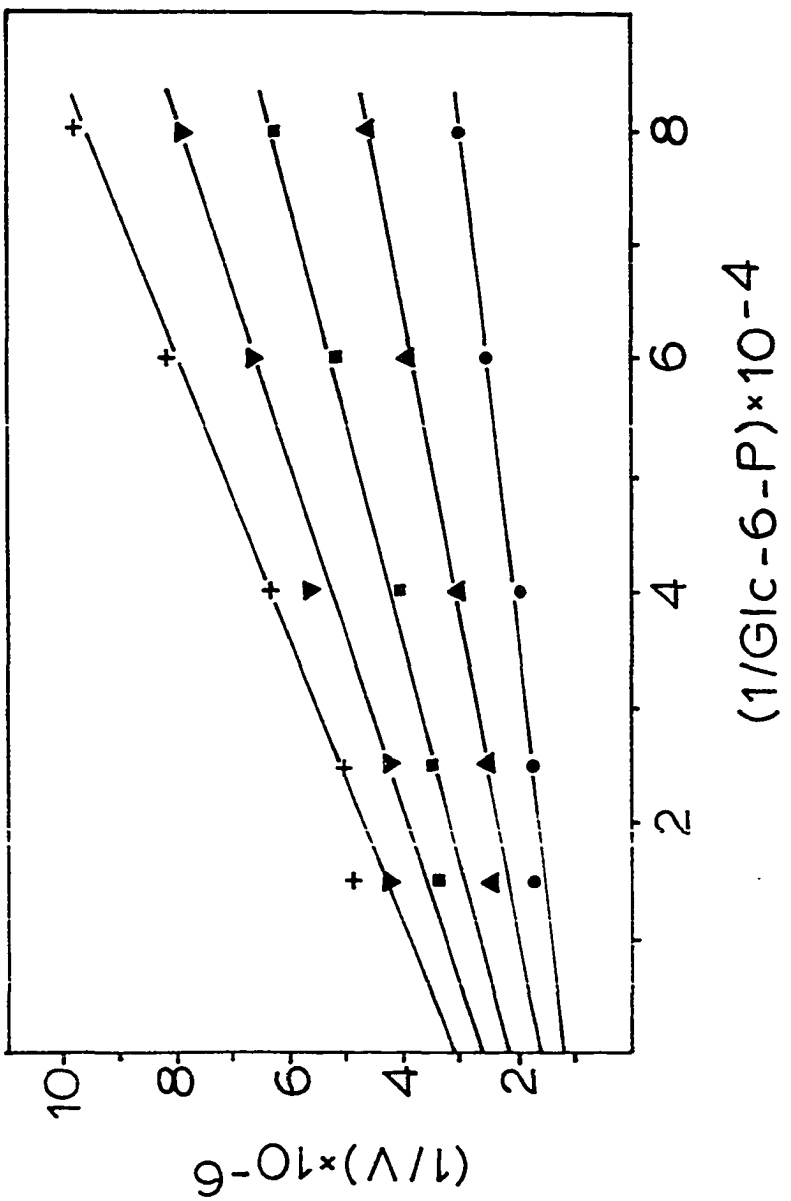

(1/V)×10-6
10
8
6
4
2
2
4
6
8
(1/Glc-6-P)×10-4

Figure 2. Plot of the reciprocal of initial velocity (V) *versus* the reciprocal of the molar concentration of $MgADP^-$ at 40 μM (●), 25.0 μM (▲), 16.7 μM (■), and 12.5 μM (+) glucose-6-P. $MgADP^-$ was varied from 0.20 mM to 1.0 mM. Other experimental details are described under "Experimental Procedures". 1/MgATP is expressed in $(molar)^{-1}$ and 1/V is in $(molar/min)^{-1}$.

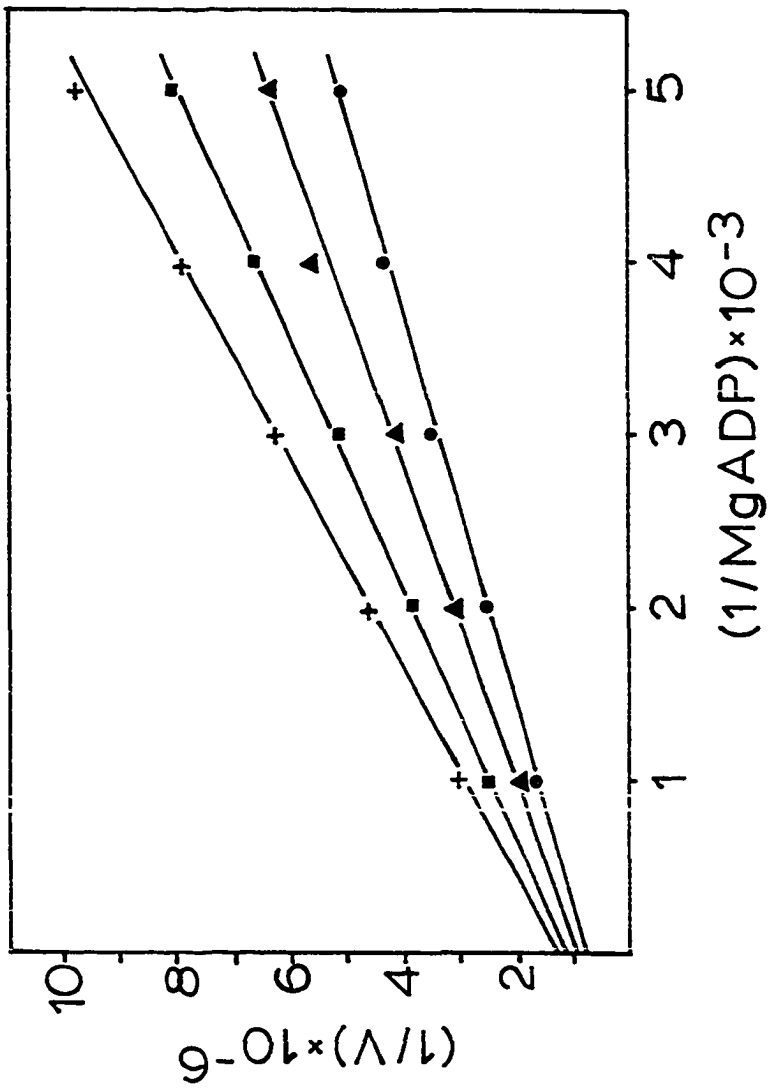

(1/V)×10⁻⁶
10
8
6
4
2
1
2
3
4
5
(1/MgADP)×10⁻³

Figure 3. Plot of initial velocity versus glucose-6-P concentration in the presence of 4 mM ADP. Assays were performed as outlined in the "Experimental Procedures". Each assay contained 0.06 I.U. of bovine brain hexokinase, as assayed in the forward direction. Velocity is expressed in molar/min.

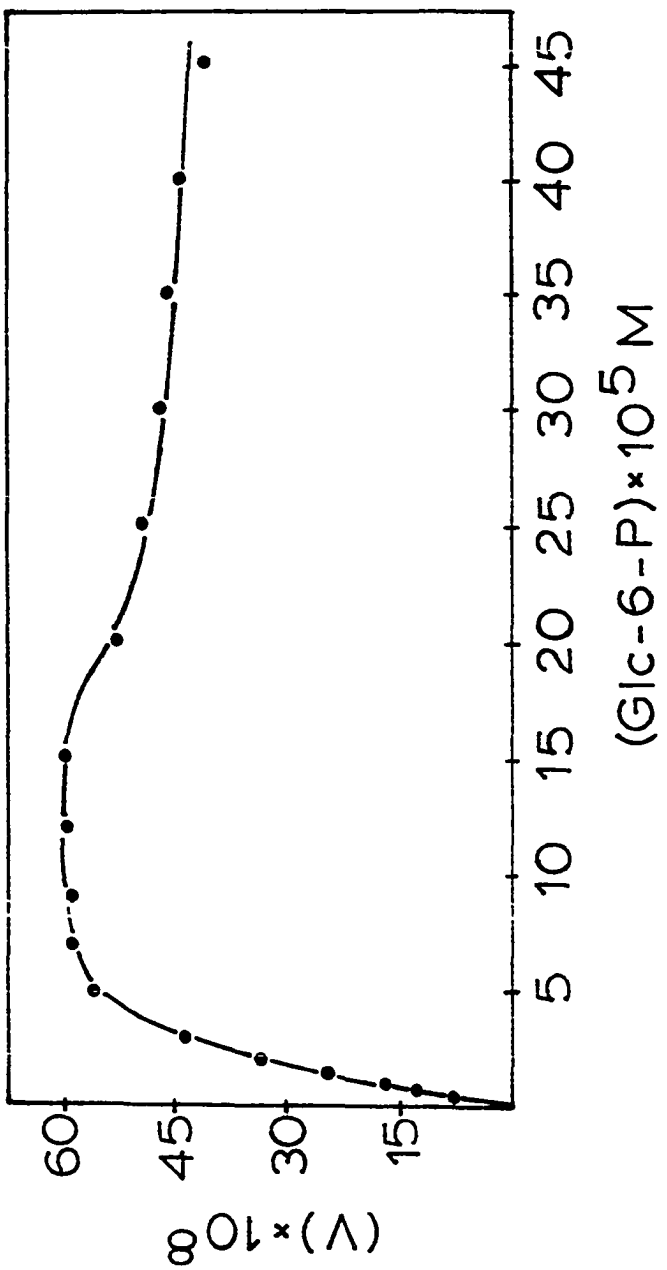
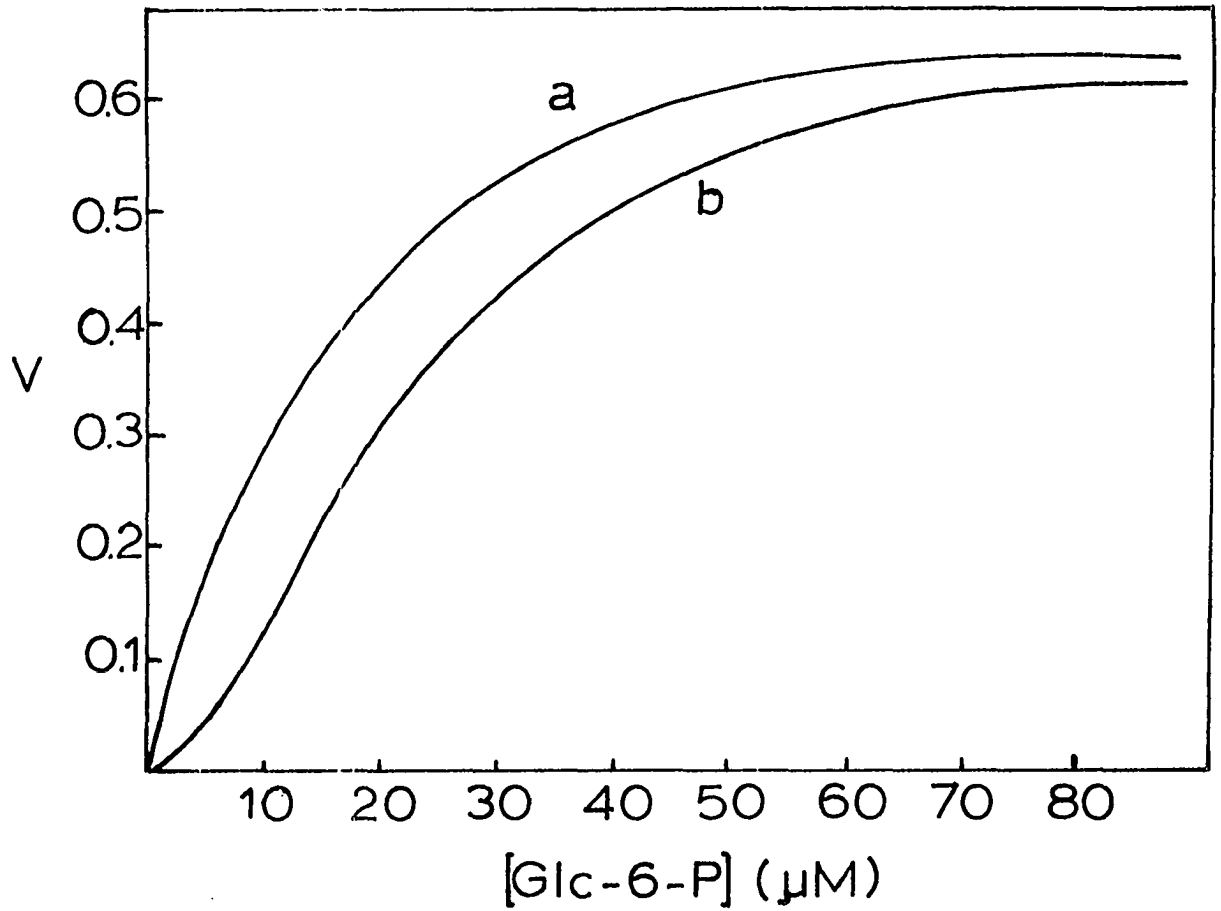

(V)×10⁸
60
45
30
15
5
10
15
20
25
30
35
40
45
(Glc-6-P)×10⁵ M

Figure 4. Theoretical plot of initial velocity *versus* glucose-6-P concentration.

(a) generated from Eq. 1 where K_i = 300 μM, K_a = 25 μM, and V_1 = 1.

(b) generated from Eq. 3 where K_i = 300 μM, K_{ii} = 25 μM, $K_{a'}$ = 400 μM^2, and V_1 = 1. K_{ii} and $K_{a'}$ in Eq. 3 are analogous to K_i and $K_{ia} \cdot K_i$, respectively, in Eq. 2.


0.6
0.5
0.4
0.3
0.2
0.1
V
a
b
10
20
30
40
50
60
70
80
[Glc-6-P] (μM)

SUMMARY AND DISCUSSION

Investigations into the kinetic mechanism and regulation of hexokinase I have been progressing for the last 25 years. During this period, a comprehensive model for the action of the brain isozyme has been developed. It is now established that the kinetic mechanism of brain hexokinase is rapid equilibrium random (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974) and with the exception of a disagreement on the mode of action of glucose-6-P inhibition, the regulation of hexokinase I by glucose-6-P and P_i is accepted and recognized as the primary control point of energy metabolism in nonglycogen using tissues. In the present studies, we have attempted to gain information about the groups involved in binding and catalysis of substrates in the hexokinase reaction and to resolve the question of the site of action of glucose-6-P on the enzyme by a study of the reverse reaction kinetics.

The variation of kinetic parameters with pH was examined for bovine brain hexokinase using glucose and MgATP as substrates. The Dixon plots generated from the data yielded profiles with limiting slopes approaching -1 on the acid side of the pH scale. No variation in activity was observed at basic pH values. From a comparison of 3 types of data, the pK's of ionizing groups affecting enzyme activity, changes in these pK's with temperature, and the effect of organic solvent on the pK values, tenative assignments of residues have been made for the detected ionizations. Breakdown of the ternary complex (E·Glc·MgATP) to product appears

to be facilitated by histidine residue as does binding of MgATP to the E·Glc complex. A neutral acid group, possibly aspartate or glutamate, appears to be involved in the binding of glucose to the E·MgATP complex. Whether these residues are directly interacting with the substrates in the reaction or are important in maintaining the active configuration of the enzyme, cannot be determined by this technique.

During the course of these investigations, an anomalous inhibition of the enzyme was observed around neutral pH. This inhibition was traced to an aluminum contamination of commercial preparations of ATP. The observed inhibition could be reversed by the addition of citrate to the reaction mixture but citrate had no effect on the enzyme other than relief of the aluminum inhibition. Kinetic data indicated that aluminum inhibited the enzyme in a competitive manner with respect to ATP and is probably acting in the form of an AlATP complex.

In order to determine the effect that the aluminum inhibition had on estimation of the pK's, we developed a method for removing aluminum from ATP. The method, which removes greater than 99% of the aluminum from the nucleotide, involves repeated extraction of the nucleotide solution with 8-hydroxyquinoline in chloroform at basic pH. Analysis of several lots of ATP indicated that the quantity of contamination varied greatly and that the extraction procedure removed virtually all of the contaminant. Regeneration of the pH profiles using the purified ATP preparations yielded Dixon profiles with no significant variation from the original data.

In an attempt to resolve the question of the site of action of glucose-6-P on the enzyme, a study of the reverse reaction of brain hexokinase was undertaken. The initial rate studies using glucose-6-P and ADP as substrate indicated that hexokinase I displayed normal Michaelian kinetics. Glucose-6-P bound to the enzyme with high affinity ($K_{ia} \sim 25$ μM) and displayed inhibition only at elevated concentrations of sugar phosphate. These data are only consistent with the high-affinity site for glucose-6-P on brain hexokinase being the active site.

To further elucidate both the chemical mechanism and the role of glucose-6-P in the regulation of hexokinase I additional experiments are being undertaken. These studies take two forms: i) kinetic studies on the reverse hexokinase reaction to determine the effect of inorganic orthophosphate on the reaction, and ii) chemical inactivation of the enzyme using active site directed protein modification reagents.

Since phosphate relieves inhibition by glucose-6-P when the enzyme is assayed in the forward direction, the effect of phosphate on the reverse reaction should provide addition evidence on the site of action of glucose-6-P. These data, plus additional experiments determining the effects of products, ATP, and hexose, on the reverse reaction, will allow the construction of a more complete description of the hexokinase mechanism.

The use of analogs for ATP such as 5'-fluorosulfonylbenzoyladenosine (Colman et al., 1977) and 8[m-(m-fluorosulfonylbenzamido)benzylthio] adenine (Baker and Kozma, 1968) will allow direct probing of the active

site of the enzyme. These reagents, unlike that used by Subbarao and Kenkare (1977b) place the reactive portion of the molecule in the area of the phosphates. Modification of the enzyme in this location should provide information on both catalysis and regulation of the enzyme. A survey of protection from inactivation provided by substrates and products may allow one to determine common loci of interaction on the enzyme, specifically in this instance whether or not glucose-6-P and ATP are interacting at a common site. Furthermore, isolation of peptides and residues modified by the reagent can allow identification of residues in and about the enzyme's active site.

Because of its unique position as a control point of glycolysis, hexokinase I has been extensively studied to delineate its kinetic and regulatory mechanism. The present studies address two of the remaining questions about mode of action of this important enzyme. Evidence for glucose-6-P acting within the ATP binding site has been presented indicating that the sugar phosphate is a simple product inhibitor of the hexokinase reaction. In addition, data derived from the effect of changing pH upon the kinetic parameters indicate two types of residues important in hexokinase activity. These residues are histidine, acting in the binding of ATP and in the breakdown of the ternary complex, and an acid residue acting in the binding of glucose to the E·ATP complex.

ADDITIONAL LITERATURE CITED

Agren, G., and Engstrom, L. (1956) Acta Chem. Scand. 10, 489-490.

Bachelard, H. S. (1967) Biochem. J. 104, 286-292.

Bachelard, H. S. (1970) Handbook of Neurochemistry, Lajtha, A., ed., Vol. 4, pp. 1-12, Plenum, New York.

Bachelard, H. S. (1976) Biochemistry and Neurological Disease, Davison, A. N., ed., pp. 228-277, Blackwell, London.

Bachelard, H. S., Clark, A. G., and Thompson, M. F. (1971) Biochem. J. 123, 707-715.

Bachelard, H. S., and McIlwain, H. (1969) Comprehensive Biochemistry, Florkin, M., and Stotz, E. H., eds., Vol. 17, pp. 197-218, Elsevier, New York.

Baker, B. R., and Kozma, J. A. (1968) J. Med. Chem. 11, 652-655.

Balazs, R. (1970) Handbook of Neurochemistry, Lajtha, A., ed., Vol. 1, pp. 1-36, Plenum, New York.

Casazza, J. P., and Fromm, H. J. (1976) Arch. Biochem. Biophys. 177, 480-487.

Colman, R. F., Pal, P. K., and Wyatt, J. L. (1977) Methods in Enzymology, Colowick, S. P., and Kaplan, N. O., eds., Vol. XLVI, pp. 240-249, Academic Press, New York.

Colowick, S. P. (1973) The Enzymes, Boyer, P. D., ed., Vol. 9, pp. 1-48, Academic Press, New York.

Colowick, S. P., and Kalcker, H. M. (1941) J. Biol. Chem. 137, 789-790.

Colowick, S. P., and Kalcker, H. M. (1943) J. Biol. Chem. 148, 117-126.

Copley, M., and Fromm, H. J. (1967) Biochemistry 6, 3503-3509.

Cori, C. F. (1945) Harvey Lecture Series, 41, 253-272.

Crane, R. K. (1962) The Enzymes, Boyer, P. D., Lardy, H., and Myrback, K., eds., Vol.6, pp. 47-66, Academic Press, New York.

Crane, R. K., and Sols, A. J. (1953) J. Biol. Chem. 203, 273-292.

Crane, R. K., and Sols, A. J. (1954) J. Biol. Chem. 210, 597-606.

Crane, R. K., and Sols, A. (1955) Methods in Enzymology, Colowick, S. P., and Kaplan, N. O., eds., Vol. I, pp. 277-286, Academic Press, New York.

Craven, P. A., and Basford, R. E. (1969) Biochemistry 8, 3520-3525.

Creighton, S. J., McClure, A. M., Watrous, B. J., and Hansen, R. J. (1972) Comp. Biochem. Physiol. 42B, 509-516.

DelaFuente, G. (1970) Eur. J. Biochem. 16, 240-243.

Ellison, W. R., Lueck, J. D., and Fromm, H. J. (1975) J. Biol. Chem. 250, 1864-1871.

Felgner, P. L., and Wilson, J. E. (1976) Biochem. Biophys. Res. Commun. 68, 592-597.

Felgner, P. L., Messer, J. L., and Wilson, J. E. (1979) J. Biol. Chem. 245, 4946-4949.

Fromm, H. J. (1964) Biochim. Biophys. Acta 81, 413-417.

Fromm, H. J. (1969) Eur. J. Biochem. 7, 385-392.

Fromm, H. J. (1975) Initial Rate Enzyme Kinetics, pp. 83-120, Springer-Verlag, Berlin/Heidelberg/New York.

Fromm, H. J. (1980) Methods in Enzymology, Colowick, S. P., and Kaplan, N. O., eds., Vol. 63, pp. 467-486, Academic Press, New York.

Fromm, H. J., and Ning, J. (1968) Biochem. Biophys. Res. Commun. 32, 672-677.

Fromm, H. J., and Solheim, L. P. (1977) 174th American Chemical Society Meeting, Abstract 6 (Biochemical Section).

Fromm, H. J., and Zewe, V. (1962a) J. Biol. Chem. 237, 1661-1667.

Fromm, H. J., and Zewe, V. (1962b) J. Biol. Chem. 237, 3027-3032.

Fromm, H. J., Silverstine, E., and Boyer, P. D. (1964) J. Biol. Chem. 239, 3645-3652.

Gahr, M. (1980) Hoppe-Seyler's Z. Physiol Chem. 361, 829-837.

Gamble, J. L., and Najjar, V. A. (1954) Science 120, 1023-1024.

Gerber, G., Preissler, H., Heinrich, R., and Rapoport, S. M. (1974) Eur. J. Biochem. 45, 39-52.

Gonzalez, C., Ureta, T., Sanchez, H., and Niemeyer, H. (1964) Biochem. Biophys. Res. Commun. 16, 347-352.

Grossbard, L., and Schimke, R. T. (1966) J. Biol. Chem. 241, 3546-3560.

Hammes, G. G., and Kochavi, D. (1962) J. Amer. Chem. Soc. 84, 2069-2073.

Hanson, T. L., and Fromm, H. J. (1965) J. Biol. Chem. 240, 4133-4139.

Hanson, T. L., and Fromm, H. J. (1967) J. Biol. Chem. 242, 501-508.

Hoare, D. S., and Kerly, M. (1954) Biochem. J. 58, 38-44.

Johnson, M. K. (1960) Biochem. J. 77, 610-618.

Katzen, H. M. (1967) Advan. Enzyme Regulation 5, 335-356.

Katzen, H. M., and Schimke, R. T. (1965) Proc. Natl. Acad. Sci. U.S.A. 54, 1218-1225.

Katzen, H. M., Soderman, D. D., and Nitowsky, H. M. (1965) Biochem. Biophys. Res. Commun. 19, 377-382.

Kellogg, E. W., Knull, H. R., and Wilson, J. E. (1974) J. Neurochem. 22, 461-463.

Kerly, M., and Leaback, D. H. (1957a) Biochem. J. 67, 245-250.

Kerly, M., and Leaback, D. H. (1957b) Biochem. J. 67, 250-252.

Knull, H. R., Taylor, W. F., and Wells, W. W. (1973) J. Biol. Chem. 248, 5414-5417.

Kosow, D. P., and Rose, I. A. (1968) J. Biol. Chem. 243, 3623-3630.

Kosow, D. P., and Rose, I. A. (1971) J. Biol. Chem. 246, 2618-2625.

Kurokawa, M., Kimura, J., Tukuoka, S., and Ishibashi, S. (1979) Brain Research 175, 169-173.

Lazo, P. A., and Sols, A. (1979) FEBS Lett. 98, 88-90.

Lazo, P. A., Sols, A., and Wilson, J. E. (1980) J. Biol. Chem. 255, 7548-7551.

Lineweaver, H., and Burk, D. (1934) J. Amer. Chem. Soc. 56, 658-666.

Long, O. (1952) Biochem. J. 50, 407-415.

Lowry, O. H., and Passonneau, J. V. (1964) J. Biol. Chem. 239, 31-42.

Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W. (1964) J. Biol. Chem. 239, 18-30.

McDonald, M. R. (1955) Methods in Enzymology, Colowick, S. P., and Kaplan, N. O., eds., Vol. 1, pp. 269-276, Academic Press, New York.

McIlwain, H. (1966) Biochemistry and the Central Nervous System, 3rd ed., pp. 36, 62, 98, Little and Brown, Boston.

Menezes, L. C., and Puddles, J. (1976) Eur. J. Biochem. 65, 41-47.

Meyerhof, O. (1927) Biochem. Z. 183, 176-215.

Meyerhof, O. (1932) Biochem. Z. 246, 249-284.

Najjar, V. A., and McCoy, E. E. (1958) Fed. Proc. 17, 1141-1144.

Neumann, S., and Pfleiderer, G. (1974) Biochim. Biophys. Acta 334, 343-353.

Neumann, S., Falkenberg, F., and Pfleiderer, G. (1974) Biochim. Biophys. Acta 334, 328-342.

Ning, J., Purich, D. L., and Fromm, H. J. (1969) J. Biol. Chem. 244, 3840-3846.

Peters, B. A., and Neet, K. E. (1977) J. Biol. Chem. 252, 5345-5349.

Pilkis, S. J. (1972) Arch. Biochem. Biophys. 149, 349-360.

Pilkis, S. J., Hansen, R. J., and Krahl, M. E. (1968) Comp. Biochem. Physiol. 25, 903-912.

Purich, D. L., and Fromm, H. J. (1971) J. Biol. Chem. 246, 3456-3463.

Purich, D. L., Fromm, H. J., and Rudolph, F. B. (1973) Advances in Enzymology, Meister, A., ed., Vol. 39, pp. 250-326, Wiley, New York.

Ramel, A. H., Rustum, Y. M., Jones, J. G., and Barnard, E. A. (1971) Biochemistry 10, 3499-3508.

Rapoport, S. M. (1968) Essays in Biochemistry, Campbell, P. N., and Greville, G. D., eds., Vol. 4, pp. 69-103, Academic Press, New York.

Redkar, V. D., and Kenkare, U. W. (1972) J. Biol. Chem. 247, 7576-7584.

Redkar, V. D., and Kenkare, U. W. (1975) Biochemistry 14, 4704-4712.

Rijksen, G., Jansen, G., Kraaijenhagen, R. J., VanDerVlist, M. J. M., Vlug, A. M. C., and Staal, G. E. J. (1981) Biochim. Biophys. Acta 659, 292-301.

Rijksen, G., and Staal, G. E. J. (1977) Biochim. Biophys. Acta. 485, 75-86.

Rose, I. A. (1965) Control of Energy Metabolism, Chance, B., Estabrook, R. W., and Williamson, J. R., eds., pp. 177-185, Academic Press, New York.

Rose, I. A., and Warms, J. V. B. (1967) J. Biol. Chem. 242, 1635-1645.

Rose, I. A., Warms, J. V. B., and Kosow, D. P. (1974) Arch. Biochem. Biophys. 164, 729-735.

Rustum, Y. M., Massaro, E. J., and Barnard, E. A. (1971) Biochemistry 10, 3509-3516.

Schwartz, G. P., and Basford, R. E. (1967) Biochemistry 6, 1070-1078.

Shill, J. P., and Neet, K. E. (1971) Biochem. J. 123, 283-285.

Shill, J. P., and Neet, K. E. (1974) Biochemistry 13, 3864-3871.

Shone, C. C., and Fromm, H. J. (1980) Comp. Biochem. Physiol. 67B, 697-699.

Sokoloff, L. (1977) Nutrition and the Brain, Wurtman, R. J., and Wartman, J. J., eds., Vol. 1, pp. 87-139, Raven, New York.

Solheim, L. P., and Fromm, H. J. (1980a) Biochemistry 19, 6074-6080.

Solheim, L. P., and Fromm, H. J. (1980b) Anal. Biochem. 109, 266-269.

Solheim, L. P., and Fromm, H. J. (1981) Arch. Biochem. Biophys. 211, 92-99.

Sols, A., and Crane, R. K. (1954) J. Biol. Chem. 210, 581-595.

Stocchi, V., Magnani, M., Canestrari, F., Dacha, M., and Forraini, G. (1981) J. Biol. Chem. 256, 7856-7862.

Subbarao, B., and Kenkare, U. W. (1977a) Arch. Biochem. Biophys. 181, 8-18.

Subbarao, B., and Kenkare, U. W. (1977b) Arch. Biochem. Biophys. 181, 19-25.

Swarup, G., and Kenkare, U. W. (1980) Biochemistry 19, 4058-4064.

Thompson, M. F., and Bachelard, H. S. (1977) Biochem. J. 161, 593-598.

Tiedemann, H., and Born, J. (1959) Z. Naturforch. 14b, 477-478.

Trayser, K. A., and Colowick, S. P. (1961) Arch. Biochem. Biophys. 94, 161-176.

Tuttle, J. P., and Wilson, J. E. (1970) Biochim. Biophys. Acta 212, 185-188.

Uyeda, K., and Racker, E. (1965a) J. Biol. Chem. 240, 4682-4688.

Uyeda, K., and Racker, E. (1965b) J. Biol. Chem. 240, 4689-4693.

Vowles, D. T., and Easterby, J. S. (1979) Biochim. Biophys. Acta 566, 283-295.

Weil-Malherbe, H., and Bone, A. D. (1951) J. Biol. Chem. 49, 339-347.

Wilson, J. E. (1968) J. Biol. Chem. 243, 3640-3647.

Wilson, J. E. (1978) Arch. Biochem. Biophys. 185, 88-99.

Wilson, J. E. (1979) Arch. Biochem. Biophys. 196, 79-87.

Wilson, J. E. (1980) Current Topics in Cellular Regulation, Horecker, B. L., and Stadtman, E. R., eds., Vol. 16, pp. 2-40, Academic Press, New York.

Winick, M. (1970) Fed. Proc. 29, 1510-1515.

Womack, F. C., and Colowick, S. P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5080-5084.

Zewe, V., Fromm, H. J., and Fabiano, R. (1964) J. Biol. Chem. 239, 1625-1634.

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