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YIP, Berwin Ping Yuen, 1951-
THE ROLE OF NUCLEOTIDE FOR THE FUNCTION
AND CONFORMATION OF ENZYMES.

Rice University, Ph.D., 1977
Chemistry, biological

Xerox University Microfilms, Ann Arbor, Michigan 48106
RICE UNIVERSITY

THE ROLE OF NUCLEOTIDE FOR
THE FUNCTION AND CONFORMATION OF ENZYMES

by

BERWIN PING YUEN YIP

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Thesis Director's Signature:

Houston, Texas

October 28, 1976
TO MY GRANDPARENTS AND PARENTS
ACKNOWLEDGEMENTS

The author wishes to thank Dr. Frederick Rudolph whose invaluable suggestions and encouragement aided immeasurably the progress of these studies. Also, his thanks to Dr. Rudolph for the preparation of CrATP used in these studies.

Thanks are also due to Dr. Florante Quicocho for kindly providing the tetraiodofluorescein and the pH-stat system used in this investigation, and for his helpful discussions; to Mr. Tim Woodward of the University of Texas Health Science Center Ultracentrifuge Facility for his technical assistance in performing the ultracentrifuge experiments.

The author also wishes to express his sincere appreciation to Cathleen Parris for encouragement and critical reading of the manuscript.

This work has been supported by grants from the Robert A. Welch Foundation and the National Institutes of Health. The author gratefully acknowledges the support of a Robert A. Welch Foundation Predoctoral Fellowship.
TABLE OF CONTENTS

LIST OF ABBREVIATIONS ........................................ v

GENERAL INTRODUCTION ........................................ 1

I.  INTERACTION OF TETRAIODOFUORESCEIN WITH YEAST HEXOKINASE........................................ 3
   A.  INTRODUCTION ........................................ 4
   B.  EXPERIMENTAL PROCEDURE ............................ 10
   C.  RESULTS ........................................... 15
   D.  DISCUSSION ........................................ 30

II. EFFECT OF CRATP ON THE CONFORMATION OF YEAST HEXOKINASE ........................................ 34
   A.  INTRODUCTION ........................................ 35
   B.  EXPERIMENTAL PROCEDURE ............................ 39
   C.  RESULTS ........................................... 42
   D.  DISCUSSION ........................................ 53

III. THE KINETIC MECHANISM OF RAT KIDNEY 
     \gamma-GLUTAMYLGLYCSTEINE SYNTHETASE ................... 56
   A.  INTRODUCTION ........................................ 57
   B.  EXPERIMENTAL PROCEDURE ............................ 64
   C.  RESULTS ........................................... 67
   D.  DISCUSSION ........................................ 103

REFERENCES .................................................... 112
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>$\alpha$-Aminobutyric acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>8-BrATP</td>
<td>8-Bromoadenosine-5'-triphosphate</td>
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<td>CrATP</td>
<td>Chromium-adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>Cr(NH$_3$)$_2$ATP</td>
<td>Chromium-diammonium-adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>DE-52</td>
<td>Microgranular diethylaminoethyl cellulose</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>hepes</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid</td>
</tr>
<tr>
<td>MgADP</td>
<td>Magnesium-adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>MgATP</td>
<td>Magnesium-adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>P$_1$</td>
<td>Inorganic orthophosphate</td>
</tr>
<tr>
<td>pipes</td>
<td>Piperazine-N-N'-bis(2-ethane-sulfonic acid)</td>
</tr>
<tr>
<td>PP$_1$</td>
<td>Inorganic pyrophosphate</td>
</tr>
<tr>
<td>TEA</td>
<td>Tris(hydroxyethyl)-amine</td>
</tr>
<tr>
<td>TIF</td>
<td>2',4',5',7'-tetraiodofluorescein</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Adenosine triphosphate (ATP) was first isolated from acid extracts of muscle by Fiske and Subbarow in 1929. A clear picture of its central role in energy transfer did not emerge until about 1940 when Lipmann proposed that ATP is the agent that links energy yielding and energy requiring functions in the cell.

ATP, as an energy source to drive a reaction, participates in two main types of reactions. The first is a phosphoryl transfer reaction resulting in a phosphorylated product and ADP:

\[ X + ATP \rightarrow X-P + ADP \]

The second is the formation of an amide or ester linkage between two compounds, coupled with hydrolysis of ATP to ADP and P\(_i\) or to AMP and PP\(_i\):

\[ X + Y + ATP \rightarrow X-Y + ADP + P_i \]
\[ \text{or} \quad X + Y + ATP \rightarrow X-Y + AMP + PP_i \]

Reactions of the first type can be visualized as an intermediate step in a series of reactions in which the ultimate product is ADP and P\(_i\). For example, the reaction catalyzed by hexokinase,

\[ \text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP} \]

can be coupled to a series of enzymatic reactions in which the final result is ATP dependent sucrose biosynthesis:

\(\text{a. } \text{Glucose-6-phosphate} \rightarrow \text{Glucose-1-phosphate} \)
\(\text{b. } \text{Glucose-1-phosphate} + \text{Fructose} \rightarrow \text{Sucrose} + P_i \)

Sum: \[ \text{Glucose} + \text{Fructose} + \text{ATP} \rightarrow \text{Sucrose} + \text{ADP} + P_i \]

Thus, the final goal of these two types of reactions is the same:
the thermodynamically favorable process of ATP hydrolysis (to ADP and P$_i$ or to AMP and PP$_i$) is used to drive the unfavorable process of $X + Y \rightarrow X-Y$.

Spectrophotometric titration, reacting enzyme ultracentrifugation, and kinetic analysis were used to study nucleotide binding to yeast hexokinase and $\gamma$-glutamylcysteine synthetase. The results indicate that ATP binding has a strong influence on the structure and catalytic functions of ATP requiring enzymes.
I. INTERACTION OF TETRAIODOFLUORESCEIN WITH YEAST HEXOKINASE

A. INTRODUCTION
B. EXPERIMENTAL PROCEDURE
C. RESULTS
D. DISCUSSION
INTRODUCTION

Yeast hexokinase (ATP: D-hexose b-phosphotransferase, EC 2.7.1.1), first discovered in 1927 by Meyerhof (1927), catalyzes the phosphorylation of hexose as follows:

\[ \text{MgATP} + \text{hexose} \rightarrow \text{MgADP} + \text{hexose-6-phosphate} \]

Yeast hexokinase has a rather broad specificity toward hexoses. Glucose, fructose, and mannose (but not galactose) are phosphorylated (Slein et al., 1950). The specificity is much greater for the phosphoryl donor than for the acceptor (DelaFuente, 1969). By far, the best phosphoryl donor is ATP with a \( K_m \) of 0.2 mM, which can be compared to 2 and 1.2 mM for ITP and GTP, respectively. The \( V_{\text{max}} \)'s for ITP and GTP are only 33% and 0.1% of the \( V_{\text{max}} \) for ATP respectively. Hexokinase requires no protein-bound cofactor and is essentially free of bound phosphate or glucose (Trayser and Colowick, 1961; Lazarus et al., 1966).

When proteolysis is excluded at all steps in the purification procedure, three distinct isoenzyme forms, designated A, B, and C according to the order of their elution from a DEAE-cellulose column, can be obtained (Rustum et al., 1971a). Hexokinase isozyme A has been shown to be composed of identical subunits, designated as alpha. The subunits of which the B and C isozymes are composed are designated as beta (Rustum et al., 1971b). The B and C isozymes are not structural isozymes. Rather, they appear to be conformationally different, as C can be converted to B by high ionic strength (Ramal et al., 1971b). Isozymes A and B also
differ in their hexose specificity. The ratio for enzymatic phosphorylation at pH 8.3, 25°C, in the presence of 27 mM sugar and 3 mM MgATP, of fructose to that of glucose for A is 2.3 and for B is 1.1 (Ramel et al., 1971b). Other isozyme forms were shown to exist in the yeast cell. An alpha-beta hybrid form, termed hexokinase A', was found in fresh yeast cell lysates (Ramel et al., 1971b). This hybrid isozyme was converted into isozymes A and B at low pH (pH = 5.3) (Ramel et al., 1971b). Multiple forms of hexokinase were also detected by polyacrylamide gel electrophoresis in crude extracts obtained from yeast cells of different strains (Kopperchlager and Hofman, 1969). The number of isozymes detected depended on the conditions of cell disruption and varied with the respective yeast strain. The pattern of the relative isozymic activities differed in exponential and stationary growth phases. Although the physiological roles of these different isozymic forms remain unclear, the dependence of the relative proportion of these forms upon stages of growth suggests that each isozyme may perform a specialized function.

Purified native yeast hexokinase isozymes A and B have molecular weights of 102,000 and both isozymes are composed of 2 subunits (Rustum et al., 1971b). The enzyme undergoes changes in the state of polymerization and/or conformation depending on the pH, presence of substrates, and various other factors. With equilibrium sedimentation and sedimentation velocity studies, Derechin et al (1972) have shown that the enzyme tends to be dissociated by alkali (pH 9.0) or by high ionic strength or by a
mixture of glucose and MgADP. However, based on results of reacting enzyme sedimentation studies, Shill et al. (1974) have suggested that the presence of glucose and MgATP causes association of hexokinase at protein levels (below 50 µg/ml) where the free protein is monomeric. The apparent contradiction in these studies has been explained by Shill et al. (1974) as being due to two different conformations of the enzyme, one an equilibrium form and the other a reacting steady state form that is induced by the presence of MgATP and glucose.

X-ray Analysis of Hexokinase

X-ray diffraction techniques were used to study the structure of yeast hexokinase. Three different crystal forms of yeast hexokinase B, designated BI, BII, and BIII, were obtained. The three crystal forms vary in their ability to bind ligands (Anderson et al., 1974; Fletterick et al., 1975). BI crystals do not bind substrate ligands and so structural studies have focused on forms BII and BIII. Results at 7 Å resolution with the crystal form BII have shown that: (1) each crystal unit contains the dimer, (2) the subunits are non-equivalently associated, with molecular screw relationship of 156° rotation and a 14 Å translation, (3) only one nucleotide binds strongly per dimer at an intersubunit site, (4) there is an allosteric interaction between the sugar and nucleotide sites such that bound hexose is required for nucleotide binding at the intersubunit interface and that bound nucleotide promotes hexose binding, and (5) Very unequal binding to the two subunits is observed with some sugars (Anderson et al., 1974).
X-ray diffraction data at 2.7 Å resolution from BIII crystals show that they contain only one monomer per asymmetric unit. Both glucose and AMP bind to this crystal form. Glucose binds in the deep cleft, as was observed previously in the BII crystal of the dimeric enzyme. AMP, however, binds to a site which is different from the major intersubunit ATP binding site observed in the crystalline dimer. It is not known which site is the catalytic site for ATP (Fletterick et al., 1975).

**Kinetic Mechanism of Hexokinase**

The order of binding of substrates to yeast hexokinase has been an area of active research and controversy in recent years. Initial rate (Fromm and Zewe, 1962; Rudolph and Fromm, 1971a, 1971b, 1970), inhibition (Fromm and Zewe, 1962; Rudolph and Fromm, 1971a; DelaFuente and Sols, 1970), and isotope exchange (Fromm et al., 1964; Purich and Fromm, 1972) studies are generally all consistent with the steady state random mechanism as suggested originally by Rudolph and Fromm (1971b) and recently confirmed by Danenberg and Cleland (1975). The experimental data that have been interpreted as evidence for an ordered binding of glucose followed by ATP generally involve binding type experiments. These include equilibrium dialysis (Noat et al., 1969), rapid dialysis (Colowick and Womack, 1969), and difference spectroscopy (Roustan et al., 1974). Glucose binding was observed with a dissociation constant at or above its kinetically determined Michaelis constant while a dissociation constant for MgATP could not usually be determined. The enzyme has an intrinsic ATPase activity suggesting that MgATP can bind
to the free enzyme, but the $K_m$, which is also the $K_d$ for MgATP in the ATPase reactions, is 5 mM (Rudolph and Fromm, 1970, 1971b). The discrepancy between the ATPase $K_m$ value and the $K_m$ for the normal reaction (0.2 mM) has been cited as evidence for an ordered mechanism (Kaji and Colowick, 1965). Conversely, under conditions where the enzyme was maximally dissociated by salt (1.5 M NaCl) the $K_m$ values for both reactions were similar (about 7.3 mM) (Rudolph and Fromm, 1970). It was suggested in that study (Rudolph and Fromm, 1970) that the physical state of hexokinase was a critical determinant for binding of substrates.

**Tetraiodofluorescein as Chromophoric Probe**

One approach to the study of the binding of ligands to protein and the conformational states of a protein is to use a chromophoric reagent that will interact with the protein at a specific site and produce a difference spectrum. Any conformational changes caused by substrate binding can be monitored from the resulting changes in the difference spectrum. The chromophore, $2',4',5',7'$-tetraiodofluorescein (TIF), the structure of which is shown below, has been shown to be an adenosine analogue.

![Tetraiodofluorescein structure](image)
X-ray crystallographic studies have shown that this dye binds to lactate dehydrogenase at a position coincident with that of the adenosine portion of the cofactor NAD (Wasserman and Lentz, 1971). TIF also interacts with aspartate transcarbamylase to cause effector (i.e. ATP) - like activation of the native enzyme at low TIF concentrations (Jacobsberg et al., 1973). It also inhibits aspartate transcarbamylase at high TIF concentrations and inhibits the isolated catalytic subunit at all concentrations of TIF (Jacobsberg et al., 1975). It has also been shown by X-ray crystallography that diiodofluorescein, another fluorescein analogue, binds to yeast hexokinase at the same site as AMP (Fletterick et al., 1975). All of this evidence support that TIF is an adenosine analogue. With both lactate dehydrogenase and aspartate transcarbamylase, the difference absorption spectra obtained proved to be useful as reporters of protein conformational changes resulting from the binding of substrates and effectors.

In the present report, the interaction between TIF and hexokinase was studied in order to gain insight on the nucleotide binding site of the protein.
EXPERIMENTAL PROCEDURE

Materials:
ATP, hepes and glucose were purchased from Sigma. DE-52 was obtained from Whatman and Sephadex G-100 was obtained from Pharmacia. TIF was a product of Eastman Kodak. TIF was purified on a silicic acid column with 3% acetic acid in chloroform as the solvent (Jacobsberg et al., 1975). The purified material exhibited essentially one spot on thin layer chromatography on Silica Gel G plate by visible or fluorescent detection (Plate 1, panels a - d) (solvent systems: acetic acid-ethyl acetate-H₂O, 2:8:1; n-butanol (H₂O saturated)-concentrated NH₄OH, 99:1; ethyl acetate-methanol-H₂O, 60:66:10; acetic acid-chloroform, 3:97). Upon spraying with 2N H₂SO₄ in methanol and incubating at 100°C overnight, only one spot of organic material corresponding to the pink color material before charring, plus the buffer spot, was detected (Plate 1, panels e - h). The identity of the buffer spot was established by running a control with buffer only (Plate 1, panel i). Also, results from preliminary experiments performed with TIF without purification were the same as the results obtained with purified TIF. Other chemicals were of the highest purity available.

Hexokinase was purified from Baker's yeast as described previously (Rustem et al., 1971a), with the exception that phenylmethanesulfonyl fluoride was used to inhibit proteolysis in place of diisopropyl fluorophosphate. The specific activities of A and B isoforms were 240 and 720, respectively (Rustem et al., 1971a).
Plate 1. Thin layer chromatographic analysis of tetraiodo-fluorescein on Silica Gel G plate. The TIF was dissolved in 0.1M hepes, pH 7.0. After a photography was taken of each plate (panels a - d), the plates were sprayed with 2N $\text{H}_2\text{SO}_4$ in methanol and incubated at 100° overnight. A photography of the charred plates was then taken (panels e - h). The only exception is panels d and e, where the data shown are from different plates. Solvent systems are: panels a and f, acetic acid-ethyl acetate-$\text{H}_2\text{O}$, 2:8:1; panels b and g, n-butanol ($\text{H}_2\text{O}$ saturated)-concentrated $\text{NH}_4\text{OH}$, 99:1; panels c and h, ethyl acetate-methanol-$\text{H}_2\text{O}$, 60:66:10; panels d and e, acetic acid-chloroform, 3:97. Panel i is the plate obtained with the buffer only and charred as described above. Solvent system for panel i is ethyl acetate-methanol-$\text{H}_2\text{O}$, 60:66:10.
Both isozymes were homogenous as evidenced by a single species on gel electrophoresis (Davis, 1964) and sedimentation velocity centrifugation (Schachman, 1959).

Crystalline yeast hexokinase was purchased from Boehringer Mannheim. The enzyme was dialyzed extensively against 0.1M hepes, pH 7.0, prior to use.

Methods:

**Kinetic Assays**
Kinetic experiments with TIF were performed by continuous measurement of $H^+$ production (Hammers and Kochavi, 1962) using a Radiometer pH Stat system. CO$_2$-free NaOH was used and the titration was done at 30° under a nitrogen atmosphere, with pH 8.0 as the set end point. The pH stat assay was used in order to avoid potential inhibition of the coupling enzyme (glucose-6-phosphate dehydrogenase) used in normal assays.

**Spectrophotometric Titrations**
Titration experiments were conducted in 0.1M hepes, pH 7.0, using a Cary 118 recording spectrophotometer, thermostated at 30°. Briefly, microliter volumes of the indicated ligand solution were added to both sample and reference cuvettes, with and without the protein, respectively. After thorough mixing, the resulting difference spectrum was recorded.

**Gel Filtration**
The molecular weight of native hexokinase and commercial crystalline hexokinase was determined using molecular sieve chromatography with Sephadex G-100. Cytochrome C
(M.W. 12,500), bovine albumin (M.W. 45,000), and aldolase (M.W. 158,000) were used as protein standards.

Protein Determination

Protein concentrations were determined by absorbance at 280 nm, using the previously determined extinction coefficient of 0.92 for a 1 mg/ml solution (Rustum et al., 1971a).
RESULTS

Tetraiodofluorescein was found to be a competitive inhibitor relative to ATP of the phosphotransferase kinetic reaction of catalytic concentration of hexokinase B, as shown in fig. 1, with a $K_i$ of about 7 $\mu$M. The slope replot of the inhibition study done at higher concentrations of TIF was parabolic, suggesting a second binding effect at high levels of the inhibitor. The inhibition relative to glucose was noncompetitive as shown in fig. 2. In the presence of 0.6 M NaCl, TIF was also a competitive inhibitor relative to ATP with a $K_i$ of about 30 $\mu$M. The apparent inhibition constants reported here were calculated from the equation

$$K_i = \frac{(I) \cdot (S_0)}{S_1 - S_0}$$

where $K_i$ is the apparent inhibition constant, I is the concentration of the inhibitor, $S_0$ is the slope of the uninhibited line from the competitive inhibition plot, and $S_1$ is the slope of the inhibited line on the same graph for a particular (I). Strictly speaking, the above equation applies to linear competitive inhibition. Since at higher concentrations of the inhibitor, the slope replot is parabolic, the apparent inhibition constants calculated from the above equation are actually $K_i$ (slope), which is not the $K_i$ for the dissociation of an EI complex, but rather a more complex function of $K_i$ which varies with (I). However, with low TIF concentrations, the slope replot was approximately linear and the apparent inhibition constants reported can be taken as the dissociation constant.
Fig. 1. Plot of reciprocal of initial reaction velocity (v) versus reciprocal of the concentration of MgATP$^{2-}$ in the absence and presence of TIF. Glucose and MgCl$_2$ were held at 10 mM and 2 mM, respectively. MgATP$^{2-}$ was varied from 0.5 to 0.125 mM. The concentrations of TIF were: none (○); 8.1 x 10$^{-6}$ M (×); 1.6 x 10$^{-5}$ M (○); 2.4 x 10$^{-5}$ M (●). Velocities were expressed as (H$^+$) moles produced / sec. Other details are described under "Experimental Procedure".
Fig. 2. Plot of reciprocal of initial reaction velocity (v) versus reciprocal of the concentration of glucose in the absence and presence of TIF. MgATP$^{2-}$ and MgCl$_2$ were held constant at 0.5 and 2 mM respectively. Glucose was varied from 1 mM to 0.125 mM. The concentrations of TIF were: none (•); 13 M (x); 27 M (o); 40 M (v). Velocity was expressed as (H$^+$) moles produced / sec. Other details were described under "Experimental Procedure".
The binding interactions of TIF with yeast hexokinase were monitored by measuring the difference absorption spectrum. The data of fig. 3 are for the hexokinase B at a concentration of 0.6 mg/ml in 0.1 M hepes, pH 7.0. Under these conditions the enzyme exists as a dimer. The maximum absorbance difference is at 543 nm and the minimum difference occurs at 519 nm. At low TIF concentrations, the $1/\text{OD}_{543}$ vs $1/\text{TIF}$ plot is linear and gives a $K_d$ for TIF with hexokinase B of 11 μM. Analysis of the data with Hill and Scatchard plots (Hill, 1910; Scatchard, 1949) shows that the binding of TIF is independent and apparently at only one site per dimer (100,000 molecular weight). At higher TIF concentrations, the double reciprocal plot becomes non-linear, curving downward at higher TIF concentrations. There is also a shift of $\lambda_{\text{max}}$ and isobestic point toward longer wavelengths, indicating a more complicated interaction. This is consistent with the nonlinear slope replots of the kinetic experiments. However, the possibility that the dye dimerizes at higher concentrations, causing the nonlinearity, cannot be ruled out (Wasserman and Lentz, 1971). Addition of MgATP (2 mM), glucose (20 mM), or MgATP and glucose together at these concentrations did not measurably perturb the difference spectrum. MgATP and glucose in the concentration ranges used in these studies had no effect on the visible absorption spectrum of TIF alone.

To evaluate the binding of TIF to monomeric enzyme, the protein was converted to monomer by addition of 0.6 M NaCl (Easterby and Rosemeyer, 1972; Shill et al., 1974). The dissociation was confirmed by sedimentation velocity experiments. The difference spectrum
Fig. 3. Spectrophotometric titration of yeast hexokinase with TIF. 3 ml of hexokinase B at a concentration of 0.6 mg/ml in 0.1 M Hepes buffer, pH 7.0 were used and microliter amounts of TIF solution were added to both the enzyme and the blank cuvettes to reach the concentration indicated: (a) 0 µM; (b) 3 µM; (c) 6 µM; (d) 9 µM; (e) 12 µM; (f) 15 µM. $\lambda_{max}$ is at 543 nm and $\lambda_{min}$ is at 519 nm.

INSET: Double reciprocal plot of absorbance at 543 nm versus TIF concentration from the difference spectrum shown.
obtained with TIF and hexokinase B in the presence of 0.6 M NaCl is similar to that obtained with the dimeric protein except that a shift of \( \lambda_{\text{min}} \) and \( \lambda_{\text{max}} \) to higher wavelengths occurs. The value of the dissociation constant is about twice that without salt (Table I), consistent with the higher \( K_d \) found in the kinetic experiments. As shown in fig. 4, the addition of MgATP to solutions containing the yeast hexokinase B monomer:TIF complex caused a significant change in the spectral properties of the complex. In addition to a decrease in absorption at 545 nm, presumably due to displacement of TIF, there is also a shift of the difference spectrum toward longer wavelengths. The addition of glucose to the mixture caused a further decrease in absorbance. Glucose by itself did not have any significant effect. However, it was not possible to abolish the spectrum completely.

Since MgATP lowered the absorbance, a difference spectrum obtained by titrating the TIF:monomeric enzyme complex with MgATP allowed the determination of the dissociation constant for MgATP. The changes observed are small, which makes accurate determinations difficult, but a value of 7 mM was estimated for the dissociation constant of MgATP (fig. 5). This is in good agreement with the value of 7.3 mM for the \( K_m \) of ATP in the ATPase reaction in the presence of 1.5 M NaCl (Rudolph and Fromm, 1970).

Difference spectra, similar to those observed with the B isozyme, are obtained with crystalline hexokinase. The commercial sample used in this study, although likely to be partially proteolytically degraded, had a molecular weight of 94,000 on a Sephadex G-100 column
TABLE I

Interaction of Hexokinase with TIF in the Absence and Presence of Salt

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Condition a</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\lambda_{\text{min}}$ (nm)</th>
<th>$K_d$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.6 mg/ml; [TIF]; 1.4 - 10 $\mu$M</td>
<td>543</td>
<td>519</td>
<td>$1.1 \times 10^{-5}$</td>
</tr>
<tr>
<td>B</td>
<td>0.6 mg/ml; 0.6 M NaCl; [TIF]; 1.4 - 10 $\mu$M</td>
<td>545</td>
<td>521</td>
<td>$2.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>Crystalline</td>
<td>0.4 mg/ml; [TIF]; 1.4 - 7 $\mu$M</td>
<td>543</td>
<td>519</td>
<td>$0.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>Crystalline</td>
<td>0.4 mg/ml; 0.6 M NaCl; [TIF]; 1.4 - 7 $\mu$M</td>
<td>543</td>
<td>519</td>
<td>$1.2 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

a At the indicated protein concentration in 0.1 M hepes, pH 7.0
Fig. 4. Effect of the addition of substrates on the difference absorption spectrum of the hexokinase:TIF complex in the presence of salt. The difference spectrum was obtained with hexokinase at 0.6 mg/ml in 0.1 M Hepes, pH 7.0, 0.6 M NaCl and 11.2 M TIF (3 ml total volume) (curve a). Microliter aliquots of MgATP or glucose were added to both the protein and the blank cuvettes to reach the concentration indicated: (b) 1 mM; (c) 2 mM MgATP; (d) 2 mM MgATP, 5 mM glucose.
Fig. 5. Difference spectrum produced by the perturbation of TIF\textsubscript{hexokinase} complex with MgATP after correction of baseline absorbance. Titration experiments were performed in double sector cuvettes. Both sample and reference cuvettes contained 1 ml of TIF\textsubscript{hexokinase} and 1 ml of buffer in the two sectors. To the reference cuvette, microliter aliquots of MgATP were added to the buffer sector and to the sample cuvette, equal amount was added to the TIF\textsubscript{hexokinase} sector. Enzyme concentration was 0.41 mg/ml, TIF concentration was 7 \( \mu \)M and the buffer used was 0.1 M hepes, 0.6 M NaCl, pH 7.0. The MgATP concentrations (mM) reached were: (a) 0.5; (b) 1.0; (c) 1.5; (d) 2.0; (e) 2.5; (f) 3.0; (g) 3.5.

INSET: Determination of dissociation constant by double reciprocal plotting of the magnitude of the difference spectrum at 535 nm versus MgATP concentration.
and thus is predominantly a dimer under the conditions employed.
The dissociation constant for TIF is compared to those for the native B isozyme in Table I. The spectral changes are nearly identical except that the $\lambda_{\text{max}}$ and $\lambda_{\text{min}}$ are the same in both the absence and presence of 0.6 M NaCl. The addition of 0.6 M NaCl is also required to allow ATP perturbation of the spectrum to be observed, similar to the effect observed with native B isozyme. This is consistent with the observation that the crystalline enzyme is predominantly dimeric.
DISCUSSION

The noncompetitive inhibition relative to glucose by TIF is consistent with the results obtained previously for AMP, ATP$^4$ (Rudolph and Fromm, 1971), and CrATP (Danenberg and Cleland, 1975). This is further confirmation of the proposed steady state random mechanism (Rudolph and Fromm, 1971).

The competitive nature of the inhibition by TIF of the kinetic reaction of hexokinase would suggest that the difference absorption spectrum produced by binding of TIF to hexokinase would be due to interaction at or near a nucleotide binding site. The similarity between the spectral changes observed with hexokinase and those observed with lactate dehydrogenase (Wasserman and Lentz, 1971), creatine kinase (Quirocho, 1976) and aspartate transcarbamylase (Jacobsberg et al., 1973, 1975) where the spectra are perturbed by addition of substrates, also supports the idea that TIF is a nucleotide analogue. The shift in spectra observed in the presence of salt can be explained in either of two ways; high ionic strength may cause a conformational change in the protein resulting in a more hydrophobic interaction between TIF and the protein, or the conformational change induced by high salt may open up a different class of nucleotide site. Although there is no evidence to disprove either case, the second situation seems unlikely because the spectral change observed is only a subtle one. However, the shift definitely shows that the interaction between TIF and dimer is different than that between TIF and monomer. The ability of substrates to perturb
the dimeric hexokinase B while a perturbation in the presence of salt would indicate that the nucleotide binding sites are made more accessible in the monomer, allowing a lower dissociation constant. This may explain the difficulty in previous studies of determining nucleotide binding. This result is important because it is known that at concentrations used in kinetic experiments, the enzyme exists as a monomer (Shill et al., 1974). The lower dissociation constant for nucleotide binding to monomer is consistent with the finding that the monomer is at the most only half as active as the dimer (Derechin et al., 1972). In the case of dimer, the free energy of the protein-substrate interaction is used in catalysis, but in the monomer, a higher proportion of the free energy is spent on binding, resulting in a lower dissociation constant (Jencks, 1975).

The inability to completely abolish the difference spectrum of the monomeric protein:TIF complex with nucleotide and the shift in position of the absorption maximum in the presence of ATP suggest that TIF is able to bind to the monomer:ATP complex. A similar result was reported with lactate dehydrogenase (Wasserman and Lentz, 1971). These results might be explained by the binding of additional dye molecule at other sites of the binary enzyme:ATP complex. These sites are made more accessible by the binding of ATP to the enzyme. This proposition is consistent with the nonlinearity of the slope replot of the inhibition experiment and of the double reciprocal plot of the difference spectrum at higher TIF concentrations, which show that TIF at higher concentrations is able to interact with the protein at more than one site. A
similar conclusion was also reached by Eden and Horowitz (1974) from studies on the interaction of 1-anilino-8-naphthale sulfonic acid with hexokinase. There are at least two classes of apolar sites that 1-anilino-8-naphthale can interact with the protein. However, this proposition will have to be tested by X-ray crystallography.

The number of substrate binding sites found per dimer is puzzling. Different methods have been used to study the binding of glucose and, with less success, of ATP to the enzyme. However, only a few studies have been addressed to the problem of the number of substrate binding sites. Conclusions from some of the previous studies are additionally complicated because the enzyme used may have been a mixture of different proteolytically digested forms. A value of one glucose site per monomer has been shown (Noak et al., 1969) and should be the minimum figure because the monomer itself is also active (Shill et al., 1974). However, a value of one site per dimer has also been reported (Danenberg and Cleland, 1975; Trayser and Colowick, 1961). This situation is similar in the case of the nucleotide binding site (Danenberg and Cleland, 1975; Bhargava et al., 1976) and, very recently, a nucleotide activator site has also been indicated (Peter and Shill, 1976). The reason behind this is not understood and may be due to different conformational states of the enzyme under different conditions. X-ray studies with different hexokinase B crystal forms are not able to offer a unique solution at the present time (Anderson et al., 1974; Fletterick et al., 1975).

Recent studies on hysteresis in hexokinase (Shill and Neet, 1974), and other changes in activity with time (Danenberg and Cleland,
1975), are consistent with multiple molecular conformations of the protein. If a binding study is done on the protein in a conformation that does not bind ATP well, the binding order may appear to be sequential. At concentrations of the enzyme used for kinetic investigations, the conformation will likely be different, an effect previously suggested to explain the differences in binding in the ATPase reaction (Rudolph and Fromm, 1970). These results can explain the controversy concerning the mechanism of hexokinase. The binding properties depend on the conformational state of the enzyme, making comparisons between kinetic and binding techniques quite difficult.
II. EFFECT OF CaATP ON THE CONFORMATION OF YEAST HEXOKINASE

A. INTRODUCTION

B. EXPERIMENTAL PROCEDURE

C. RESULTS

D. DISCUSSION
INTRODUCTION

Slow Transient Kinetic Properties of Yeast Hexokinase

In addition to the general properties described in the introduction to Part I, yeast hexokinase exhibits a substrate-induced slow transient during catalysis at pH 6.5 (Shill and Neet, 1974). The reaction velocity curves go from an initial high activity to a lower steady state activity. At pH 6.5, hexokinase is subject to activation by low concentrations of various metabolites and exhibits deviation from Michaelis-Menten kinetics (negative cooperativity) with ATP (Kosow and Rose, 1971). Ainslie et al. (1972) have proposed a slow transition model which may account both for slow transients in reaction progress curves and for cooperative kinetics in a monomeric enzyme with no need for more than a single catalytic site. The regulatory properties of yeast hexokinase have been attributed to a substrate-induced slow conformational change of the enzyme. As discussed in the introduction to Part I, ultracentrifugation studies have shown that the enzyme undergoes changes in the state of polymerization and/or conformation depending on the pH and presence of substrates (Derechin et al., 1972; Shill et al., 1974).

CrATP Inhibition of Yeast Hexokinase

Chromium-ATP, a virtually inert, inner sphere metal-ligand complex, is useful as a dead end inhibitor in kinetic studies, paramagnetic probe in nuclear magnetic resonance and electron paramagnetic studies, and, finally, for isomorphic replacement in X-ray crystallographic studies.
of enzymes. Danenberg and Cleland (1975) have investigated the kinetic mechanism of yeast hexokinase with CrATP. In the hexokinase reaction, CrATP is a competitive inhibitor versus MgATP and a noncompetitive inhibitor versus glucose, with a dissociation constant of 4 - 6 μM in either the presence or absence of glucose (Danenberg and Cleland, 1975). CrATP prepared by the method of DePamphilis and Cleland (1974) contains two β,γ-bidentate and four α,β,γ-tridentate isomers. The four tridentate CrATP isomers correspond to attachment of adenosine to oxygens a, b, c, or d in the following structure (a). For the two bidentate CrATP isomers, adenosine attaches to either oxygen a or b in the following structure (b), and H₂O replaces the oxygen of the α-phosphate group as the coordination ligand.

These possible CrATP isomers have not been separated from each other. However, preliminary evidence indicated that it is the bidentate isomers only that inhibit yeast hexokinase (Brummond and Cleland, 1974). During the investigation of CrATP inhibition of yeast hexokinase, it was observed that the rate of the reaction at pH 7 slows down over the first several minutes in the presence
of CrATP with the inhibition constant of the steady state being lower than that of initial velocity. It was suggested that a slow conformational change of hexokinase occurs when both glucose and CrATP are present, which tightens the binding of CrATP to the enzyme (Danenberg and Cleland, 1975). The obvious similarity between the slow conformational change induced by glucose and CrATP at pH 7 and the slow transient in the reaction curves induced by glucose and MgATP at pH 6.75 suggest that they may be the same phenomenon, especially since CrATP has been shown to be a very poor substrate (Danenberg and Cleland, 1975).

**Reacting Enzyme Sedimentation**

To study the effect of CrATP binding on the structure of hexokinase, reacting enzyme ultracentrifugation was carried out. This elegant technique, which allows positive determination of the reacting forms of an enzyme, was first developed by Cohen and co-workers (Cohen et al., 1967; Cohen and Mire, 1971) and later modified by others (Taylor et al., 1972; Shill et al., 1974). In this method, a very small quantity of the enzyme is layered in the ultracentrifuge cell on top of a complete assay mix for spectrophotometric determination of the enzyme. As the band of enzyme sediments through the assay mix, the progress of the actual reacting form or forms of the enzyme is followed by observing the disappearance of substrates or formation of products by optical means. This technique allows investigation at concentrations of proteins that approach those employed in kinetic studies.
The following part of the report describes the results of ultracentrifugation experiments with hexokinase in the presence of CrATP.
EXPERIMENTAL PROCEDURE

Materials:

The following chemicals were purchased from the indicated sources: glucose, pipes, ATP, TEA, p-nitrophenol (Sigma); D$_2$O (Stohler Isotope Chemicals); glucose-6-phosphate dehydrogenase (Boehringer). CrATP was synthesized as described previously (DePamphilis and Cleland, 1973). The CrATP used has the same spectral characteristics as reported by DePamphilis and Cleland (1973) (absorption maxima in visible region are at 435 and 620 nm; ultraviolet absorption maximum is at 260 nm; A$_{260}$/A$_{435}$ = 770; A$_{435}$/A$_{620}$ = 1; values reported were measured at pH 7.0).

Hexokinase was purified from Baker’s yeast as described in Part I.

Methods:

Protein Determination  Protein concentration was determined by absorbance at 280 nm as described in Part I.

Kinetic Assay  The more sensitive glucose-6-phosphate dehydrogenase coupled enzyme assay was used in the determination of inhibition constant for CrATP. The assay mixture for kinetic studies included 50 mM pipes, pH 7.0, 100 mM TPN, 100 mM MgATP, 10 mM glucose, 5 mM MgCl$_6$ and glucose-6-phosphate dehydrogenase in excess. Inhibition constants were calculated from the relationship for a competitive inhibitor:

$$K_i = \left( \frac{I}{1 + \frac{A}{K_a}} \right) \left( \frac{v_0}{v_i} - 1 \right),$$
where \( I \) is the inhibitor concentration, \( A \) is MgATP concentration (100 \( \mu M \)), \( K_a \) its apparent \( K_m \) (100 \( \mu M \)), \( v_0 \) is the velocity in the absence of inhibitor, and \( v_1 \) is the velocity in the presence of inhibitor. Initial \( K_1 \) was calculated from initial velocity. Final \( K_1 \) was calculated from the rate seen after several minutes when the lower steady-state rate appeared to have become established.

**Reacting Enzyme Sedimentation**

Reacting enzyme sedimentation studies were performed on a Beckman-Spinco Model E analytical centrifuge with a RTIG temperature control unit and photoelectric scanner. The progress of the enzyme band was observed as described by Shill et al. (1974) by the absorbance change of p-nitrophenol as it was titrated by the \( H^+ \) produced in the enzymatic reaction.

The assay mix contained 0.1 mM TEA-chloride, 0.01 mM p-nitrophenol, 2 mM ATP, 20 mM glucose and 8 mM MgCl\(_2\) in 50% D\(_2\)O at pH 6.75.

Hexokinase was diluted in a dilution mix similar to the assay mix with ATP and D\(_2\)O omitted and the pH adjusted to 6.55. For experiments with CrATP, the enzyme was mixed with CrATP (250 \( \mu M \)) and glucose (10 mM) before each ultracentrifugation experiment.

The technical aspects of the centrifugation were described by Shill et al. (1974). Both sectors of the double sector cell were filled with 0.29 ml of the reaction mix and the capillary chamber of the blank sector was filled with 10 \( \mu l \) of the enzyme diluted to the proper concentration with the dilution mix (the enzyme was placed in the blank sector side because the absorbance change is a negative one). 10 \( \mu l \) of dilution mix was placed in the sample capillary chamber as the blank. Centrifugations were conducted at 60,000 rpm and the change in absorbance was followed at 400 nm.
using the photoelectric scanner at 4 or 8 min intervals. The sedimentation coefficients were determined from boundary midpoints as described by Taylor et al. (1972) and Shill et al. (1974), corrected for the density and viscosity of D₂O and deuteration of the protein as described by Shill et al. (1974).

**Sedimentation Velocity** Sedimentation velocity experiments were also performed with the Beckman-Spinco Model E ultracentrifuge, at 20⁰ and 60,000 rpm. The sedimentation coefficient was calculated from schlieren patterns as described previously (Goldberg, 1953).
RESULTS

CrATP Inhibition of A and B Isozymes

Previous studies on the effect of chromium nucleotide have been done with commercially prepared hexokinase that is a mixture of partially degraded isozymes (Danenberg and Cleland, 1975). In the present study, CrATP was a strong inhibitor of both pure native isozymes at pH 7.0 and an increase in inhibition occurs with both. The apparent $K_i$ for the initial and steady state inhibition were 5.4 and 0.25 $\mu$M and 2.2 and 0.1 $\mu$M for isozyme A and B, respectively.

Sedimentation Velocity

The physical effect of CrATP on both isozymes was evaluated by sedimentation velocity experiments (Table II). At a protein concentration of about 1 mg/ml at pH 6.75, the sedimentation coefficients were identical in the presence and absence of CrATP (250 $\mu$M). The sedimentation coefficient was consistent with either isozyme being dimeric under these conditions (Derechin et al., 1972).

Reacting Enzyme Sedimentation

The reacting form of both isozymes was determined with and without preincubation of the enzyme with CrATP and glucose. An example of an experiment with and without CrATP preincubation is shown in figs. 6 and 7, respectively. Consistent with the idea that the monomer and dimer are in rapid equilibrium, no separation of boundaries due to separation of monomer and dimer under centrifugal force was observed. The results of a series of experiments at different enzyme concentrations are shown in figs. 8 and 9. They indicate that preincubation with CrATP caused the monomeric form of hexokinase to predominate at a
TABLE II

Effect of CrATP on the sedimentation value of hexokinase
determined at pH 6.75 at a protein concentration of 1 mg/ml

<table>
<thead>
<tr>
<th>ISOZYME</th>
<th>$S_{20,w}$ (0 CrATP)</th>
<th>$S_{20,w}$ (250 μM CrATP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.6 (3)*</td>
<td>5.75 (4)</td>
</tr>
<tr>
<td>B</td>
<td>5.3 (3)</td>
<td>5.2 (4)</td>
</tr>
</tbody>
</table>

* Number indicates the number of determinations which were averaged.
Fig. 6. Reacting enzyme sedimentation of yeast hexokinase preincubated with CrATP and glucose.

(A) Scanner tracings (change in absorbance at 400 nm versus the distance from the center of rotation, r) at 8 min. intervals, are superimposed upon a common point of reference (the two reference lines). The enzyme loading concentration was 7.5 µg/ml isozyme B, preincubated with 250 µM CrATP and 10 mM glucose. Other details are described under "Experimental Procedure".

(B) Plot of the log of the radius of the boundary mid-point against time (minutes) from the sedimentation data shown in (A).
Fig. 7. Reacting enzyme sedimentation of yeast hexokinase B.

(A) Scanner tracings (change in absorbance at 400 nm versus the distance from the center of rotation, r) at 8 min. intervals, are superimposed upon a common point of reference (the two reference lines). The enzyme loading concentration was 7.5 µg/ml isozyme B. Other conditions of centrifugation are described under "Experimental Procedure".

(B) Plot of the log of the radius of the boundary mid-point against time (minutes) from the sedimentation data shown in (A).
A. Absorbance

- Reference
- Meniscus
- Direction of Sedimentation
- Radius

B. Log r

- \( S_{20, w} = 5.5 \)
- Time (min)
Fig. 8. Reacting enzyme centrifugation of yeast hexokinase B in the presence (●) and absence (○) of preincubation of the enzyme with CrATP and glucose at different enzyme concentrations. Experimental conditions are as described under "Experimental Procedure".
Fig. 9. Reacting enzyme centrifugation of yeast hexokinase A in the presence (●) and absence (○) of preincubation of the enzyme with CrATP and glucose at different enzyme concentrations. Experimental conditions are as described under "Experimental Procedure".
higher protein concentration than the native enzyme. At a higher loading concentrations of both isozymes, the sedimentation value increased above 6 due to overloading of the system. Shill et al. (1974) have studied this system with computer simulation studies. They concluded that at loading concentrations of less than 10 μg/ml for isozyme B, overloading is not significant and the data were an accurate description of the association-dissociation behavior of the enzyme. As shown in fig. 8, when hexokinase B was preincubated with CrATP and glucose, the sedimentation value was lower than the corresponding value for the enzyme in the absence of CrATP at similar protein concentrations. At higher enzyme concentrations, the sedimentation value did increase for the treated enzyme, indicating that association still occurred but CrATP caused a shift in the equilibrium between monomer and dimer. The data for A isozyme, presented in fig. 9, show a similar shift toward monomer in the presence of CrATP. An interesting result with the A isozyme was that even at the relatively high concentration tested, the sedimentation coefficient observed did not reach the dimeric value of 5.5.

As an additional control, two reacting enzyme sedimentation experiments with CrATP (20 μM) present in both the incubation and the reaction mix were performed at 2 different enzyme concentrations. In both cases, even though the enzyme activity was small, boundary midpoints were still obtainable and, throughout centrifugation, the sedimentation value was consistently lower at a given protein concentration.
DISCUSSION

The inhibition constants for the pure isozymes are similar to the values obtained with commercially available partially degraded enzymes. That the increase in inhibition with time occurs with both isozymes indicates a possible common characteristics in nucleotide active centers between them. This is consistent with the observation that the kinetic constant ($K_m$) for MgATP is similar for both forms. However, the specificity toward the sugar substrate varies between the two isozymes (Ramel et al., 1971).

The results of sedimentation studies show that CrATP binding to the enzyme causes the reacting state of the enzyme to undergo a conformational change that favors the monomeric state. This effect is dependent on the protein concentration because at protein concentrations used in sedimentation velocity studies (1 mg/ml), no dissociation effect was observed.

The assay mix in the centrifuge cell did not contain CrATP and a question can be raised on how an enzyme saturated with an inhibitor can exhibit activity. Thus, an alternate explanation that CrATP bound only to the dimeric form of hexokinase and that the activity observed in the ultracentrifuge was only due to the uninhibited monomeric form may be possible. However, this seemed unlikely because association was still observed, even though a higher protein concentration was required. As the enzyme sediments, the concentration of free CrATP decreased, but some tightly bound CrATP would be expected to sediment with the enzyme. Since the time
required for an ultracentrifugation experiment was about 1 hour, MgATP had to be in equilibrium with the CrATP on the enzyme. Thus, the activity observed in the centrifuge could be explained by this equilibrium process, and the conformational change caused by CrATP binding was also maintained, as evidenced by the linear log r versus time plot through the time range observed. That similar results were obtained when CrATP was present in the assay mix also supported the idea that MgATP and CrATP competed for the enzyme during centrifugation. Thus, the sedimentation value observed in the presence of CrATP must be the result of at least two forms under rapid equilibrium.

Since dissociation is taking place in addition to a conformational change upon CrATP binding, it is possible to rationalize the increase in CrATP inhibition observed in kinetic assay by either the conformational change or the resulting change in association state of the enzyme. It is not possible to determine which is the primary cause of the increase in inhibition. However, a conformational change would be consistent with the conclusion obtained by Shill and Neet (1974), on the related phenomenon of slow transition during catalysis.

The results with the A isozyme are less prone to interpretation problems due to enzyme overloading than for the B isozyme. Even above a loading concentration of 20 µg/ml, the sedimentation value does not rise above 5, as contrasted with isozyme B shown previously by Shill et al. (1974) and confirmed in this study. The transitions are clearly dimer to monomer for A and overloading
problems which would make interpretation difficult are unlikely. Shill et al. (1974) have studied both the B and C isozymes and each has different association behavior as compared to the present results for A isozyme. It seems that although the nucleotide binding sites of the isozymes appear to be related, other structural features of the isozymes are quite different.

Very recently, Peters and Neet (1976) have studied the effect of Cr(NH$_3$)$_2$ATP on hexokinase B, at pH 6.5. This analogue activated both the initial and steady state reactions at low concentrations. At higher concentrations it was a competitive inhibitor relative to MgATP. At pH 7.5, Cr(NH$_3$)$_2$ATP behaved similar to CrATP, causing a decrease in activity with time. These results have been interpreted as being indicative of a slow conformational change occurring at both pH values but with different consequences, depending on the charge of the enzyme. The data in this report are not inconsistent with that view, but reacting enzyme sedimentation at various pH values, together with other techniques, would possibly aid in elucidation of the phenomena observed.
III. THE KINETIC MECHANISM OF RAT KIDNEY

\(\gamma\)-GLUTAMYLGLYSTEINE SYNTHETASE

A. INTRODUCTION
B. EXPERIMENTAL PROCEDURE
C. RESULTS
D. DISCUSSION
INTRODUCTION

\( \gamma \)-Glutamylcysteine synthetase (EC 6.3.2.2) catalyzes the following reaction:

\[
\text{L-glutamic acid} + \text{L-cysteine} + \text{ATP} \xrightarrow{\text{M}^{2+}} \text{L-} \gamma \text{-glutamyl-L-cysteine} + \text{ADP} + \text{P}_i
\]

The synthesis of \( \gamma \)-glutamylcysteine, the first of the two steps involved in the enzymatic synthesis of glutathione, was initially studied by Bloch and his colleagues (Bloch, 1949; Johnston and Bloch, 1949, 1951). Current information indicates that glutathione is synthesized from its constituent amino acids in virtually all living cells. It is generally thought that glutathione plays a fundamental biological role. The functions that have been ascribed to glutathione include (a) maintenance of the SH groups of proteins and other molecules; (b) destruction of hydrogen peroxide, other peroxides, and free radicals; (c) catalyst for disulfide exchange reactions; (d) coenzyme for certain enzymes (e.g. glyoxalase); (e) detoxification of foreign compounds (e.g. by the mercapturic acid pathway) (Meister, 1975). In addition, there are evidences indicating extensive formation of glutathionyl-spermidine in Escherichia Coli (Tabor and Tabor, 1975), a function of glutathione and glutathione disulfide in protein synthesis (Zehavi-Willner et al., 1970; Kosower and Kosower, 1973), in oxidative phosphorylation (Painter and Hunter, 1970) and regulation of the hexose monophosphate pathway (Eggleston and Krebs, 1974). It must be emphasized that the evidence for each of these proposed functions is incomplete. Thus, there are gaps in the knowledge of the apparent
roles of glutathione in maintaining the SH groups of proteins and as a catalyst in disulfide exchange reactions. Although glutathione is a specific coenzyme of glyoxalase, the function of this enzyme is not clear. The significance of the interaction of glutathione with foreign compounds and of mercapturic acid formation is not yet known.

Recently, the glutathione biosynthetic pathway was linked to a series of enzymatic reactions in a number of different mammalian cells to form a cycle, called the \( \gamma \)-glutamyl cycle (Orlowski and Meister, 1970). This \( \gamma \)-glutamyl cycle is illustrated in fig. 10. It has been suggested that the \( \gamma \)-glutamyl cycle (and thus, glutathione) functions in the transport of amino acids across cell membranes (Orlowski and Meister, 1970; Meister, 1973). A scheme has been proposed (Meister, 1973) according to which (a) the amino acid to be transported binds noncovalently to a cell membrane site, (b) a group on the membrane bound \( \gamma \)-glutamyl transpeptidase interacts with the \( \gamma \)-glutamyl moiety of intracellular glutathione (or of other \( \gamma \)-glutamyl compounds) to yield a \( \gamma \)-glutamyl enzyme, (c) there is an attack of the amino acid nitrogen atom on the \( \gamma \)-carbon of the \( \gamma \)-glutamyl enzyme to yield \( \gamma \)-glutamyl amino acid, (d) the formation of the \( \gamma \)-glutamyl amino acid is associated with removal of the amino acid from its
Fig. 10. The γ-Glutamyl Cycle: 1. γ-glutamyl transpeptidase, 2. γ-glutamyl cyclotransferase, 3. dipeptidase, 4. 5-oxoprolinase, 5. γ-glutamylcysteine synthetase, 6. glutathione synthetase
membrane binding site and movement of the amino acid moiety into the cell, (e) the amino acid is released from its γ-glutamyl moiety carrier within the cell and (f) the ATP dependent decyclization of 5-oxoproline and the synthesis of glutathione are energy-requiring recovery steps needed for resynthesis of the carrier precursor. This idea of amino acid transport is consistent with several observations (Meister, 1973): (a) γ-glutamyl transpeptidase is membrane bound, (b) the γ-glutamyl cycle is detected in most mammalian cells, (c) in the kidney and intestine, γ-glutamyl transpeptidase is found where active absorption is most active, (d) a small amount of γ-glutamyl amino acid is found in the urine and (e) in vivo administration of L-2-imidazolidone-4-carboxylate, a competitive inhibitor of 5-oxoproline, to mice increases the excretion of amino acids.

However, this proposal can only be considered tentative because the γ-glutamyl cycle fails to explain the following known characteristics of amino acid transport systems (Christensen, 1973; Segal and Thier, 1973): (a) the amino acid transport systems are specific; (b) active transport of amino acid requires Na⁺; (c) the translocation of amino acid through the membrane, the key step in the proposed scheme, is still not understood; (d) the energy requirement of 3 molecules of ATP for each amino acid molecule seems too high, especially since the γ-glutamyl cycle is non-specific with respect to amino acids. However, even though the function of the cycle is still not well understood, the cycle is definitely an important part of metabolism in a large number of mammalian cells and an understanding of the characteristics of the
different enzymes will contribute to the understanding of the cycle.

A highly purified and apparently homogenous preparation of
\( \gamma \)-glutamylcysteine synthetase has been obtained from rat kidney
(Orlowski and Meister, 1971). The molecular weight of the enzyme
was estimated by gel filtration to be 92,000 (Orlowski and Meister,
1971b). The enzyme exhibits maximal activity in the pH range
8.0 - 8.4. The enzyme is rather specific toward L-glutamate but
a number of other amino acids are also active in the synthesis
reaction in place of L-cysteine. L-\( \alpha \)-aminobutyrate is as good
as L-cysteine and is often used as a substitute of L-cysteine
because use of \( \alpha \)-aminobutyrate in the assay prevents complications
associated with spontaneous oxidation of cysteine. The apparent
\( K_m \) values for L-\( \alpha \)-aminobutyrate, L-glutamate, and ATP in the
presence of \( Mg^{2+} \) are 1.25, 1.59 and 0.20 mM respectively (Orlowski
and Meister, 1971a).

\( \gamma \)-Glutamylcysteine synthetase catalyzes several partial
reactions which appear relevant to the mechanism of action of this
enzyme (Orlowski and Meister, 1971b; Richman et al., 1973).

1. Hydrolysis of ATP:
\[ ATP + H_2O \rightarrow ADP + P_i \]

This ATPase activity is inhibited by L-glutamate but is
activated by L-\( \alpha \)-aminobutyrate, such activation being prevented by
L-glutamate or methionine sulfoximine, a glutamate analogue.

2. Pyrophosphate synthesis:
\[ ATP + P_i \rightarrow PP_i + ADP \]

This partial reaction is also inhibited by L-glutamate and
its analogues and is activated by L-\( \alpha \)-aminobutyrate.

3. Formation of 5-oxoproline from D and L-glutamate:
Glutamate + ATP → 5-oxoproline + ADP + P_i

This reaction proceeds at a rate that is only about 0.3% of that of the synthesis reaction. Addition of L-α-aminobutyrate inhibits the formation of 5-oxoproline from L-glutamate.

4. Phosphorylation of L-methionine-S-sulfoximine:

\[
\text{enzyme} + \text{methionine sulfoximine} + \text{ATP} \rightarrow \text{(enzyme-methionine sulfoximine-phosphate)(ADP)}
\]

Methionine sulfoximine inhibits γ-glutamylcysteine synthetase competitively with respect to glutamate (Richman et al., 1973). Incubation of the enzyme with methionine sulfoximine, ATP and magnesium ion leads to inactivation associated with stoichiometric formation of ADP and methionine sulfoximine phosphate. Both of these products are bound tightly to the enzyme.

5. Exchange reaction:

γ-Glutamylcysteine synthetase does not catalyze exchange between inorganic phosphate and ATP or between ADP and ATP in the absence of other substrates. It can catalyze the incorporation of α-aminobutyrate and of glutamate into γ-glutamyl-α-aminobutyrate in the presence of ADP and inorganic phosphate. The rate of incorporation of α-aminobutyrate is about twice the rate observed for glutamate.

The partial reaction of phosphorylation of methionine sulfoximine suggests that γ-glutamyl phosphate is an intermediate in the catalytic reaction. The tentative mechanism that was proposed can be visualized either as Bi Uni Uni Bi Ping Pong if P_i is released before cysteine binds to the enzyme or an ordered sequential if P_i is not released (Meister, 1974). To elucidate the actual
mechanism of this enzyme, kinetic studies were carried out. This report details results of kinetic experiments that are consistent with a substrate binding mechanism that can explain the previous findings on partial reactions catalyzed by this enzyme.
EXPERIMENTAL PROCEDURE

Materials:

ATP, ADP, glutamate, \( \alpha \)-aminobutyrate, NADH, L-methionine-DL-sulfoximine, L-methionine-DL-sulfoxide, \( \alpha \)-methylglutamate, \( \beta \)-aminobutyrate, \( \gamma \)-aminobutyrate, glycine, serine, O-phosphoserine and TEA were obtained from Sigma. 2-amino-1-butanol, 1,2-butanediol, 2-bromobutyric acid, 2-amino-1-propanol and N-propylurea were obtained from Aldrich Chemical Co. All other reagents were of the highest purity available. All solutions used were adjusted to pH 8.2 with NaOH or HCl.

\( \gamma \)-GlutamyEcysteine synthetase was isolated from frozen rat kidneys as described by Orlowski and Meister (1971a) with the following modifications. The final concentration step was done on a 2 ml DEAE cellulose (Whatman DE-52) column that was equilibrated with 0.05 M tris-HCl, pH 8.2, 0.005 M L-glutamate and 0.005 M MgCl\(_2\) at 4\( ^\circ \). The active fractions from the DEAE-cellulose column (step 4 in Orlowski and Meister, 1971a) were pooled and dialyzed overnight against the equilibrating buffer and then applied to the column. After a brief wash with buffer the protein was eluted with 0.2 M NaCl in the equilibrating buffer. Pooled active fractions were dialyzed against 0.05 M tris-HCl, pH 8.2 and stored at 4\( ^\circ \). The enzyme prepared by this procedure from rat kidneys stored frozen had a specific activity of 150 \( \mu \)moles phosphate released/mg protein/hr. This specific activity is somewhat lower than reported in previous preparations but no contaminating enzymes such as glutaminase or ATPase were present in
the final preparation.

Methods:

$\gamma$-Glutamylcysteine synthetase activity was followed during purification by measurement of $P_i$ release as described by Orlowski and Meister (1971a) using the Fiske and Subbarow (1925) method. For the kinetic experiments a modification of the more sensitive Martin and Doty (1949) $P_i$ assay was used. The reaction mixture (2.5 ml) was incubated for a known period of time at 30\(^\circ\)C and the reaction was terminated by addition of 1 ml of cold 10\% trichloroacetic acid. Five ml of a 1:1 mixture of isobutanol and benzene (water saturated) was added, the tube mixed vigorously on a vortex mixer and then 0.4 ml of ammonium molybdate (0.04 M in 4 N sulfuric acid) was added followed by 30 sec. of vigorous mixing. After allowing separation of the phases, three ml of the organic layer was transferred to 1 ml of 95\% ethanol/5\% sulfuric acid. 0.2 ml of SnCl\(_2\) (0.018 M in 1 N sulfuric acid) was then added to reduce the phosphomolybdate complex. After allowing ten minutes for color development, the absorbance was measured at 660 nm.

All the kinetic experiments were carried out in 0.05 M TEA buffer, pH 8.2, with 1 mM free Mg\(^{2+}\) (as magnesium chloride) and the appropriate substrates and inhibitors for each experiment. Any ATP or ADP used was complexed with sufficient magnesium chloride as described (Rudolph and Fromm, 1967). The amount of enzyme used was adjusted so that the maximum extent of reaction obtained was within the linear portion of initial rate previously
determined in a different experiment. The intensity of the blue color was compared to that of an appropriate blank. Initial reaction rate is expressed as μmoles of phosphate released per hour.
RESULTS

Initial rate studies were performed as described by Fromm (1967). Two substrates were varied in a constant ratio at different levels of the third substrate. The results of initial rate studies with $\gamma$-glutamylcysteine synthetase are shown in figs. 11-13. All three plots have lines that intersect to the left of the $1/v$ axis.

Further distinctions can be made among the possible quaternary complex mechanisms by examination of replots of the initial rate data (Rudolph and Fromm, 1967; Clayton and Rudolph, 1976). The replots are shown in fig. 14. Only the slope replot from the $1/\text{MgATP}^2-$ reciprocal plot has a zero intercept. The significance of the initial rate and the replot data will be discussed in the discussion.

To differentiate between the possible mechanisms, various inhibition experiments were carried out. MgADP$^-$ was found to be a competitive inhibitor of MgATP$^2-$ as shown in fig. 15. The data of figs. 16 and 17 indicate that MgADP$^-$ is noncompetitive with respect to both $\alpha$-aminobutyrate and glutamate. The slopes of the $1/$glutamate plot vary only slightly but multiple experiments have consistently shown the effect to be present. To confirm that the inhibition was noncompetitive, NADH was tested as an inhibitor relative to MgATP$^2$-. Jackson (1969) had shown that NADH inhibited $\gamma$-glutamylcysteine synthetase in crude haemolysates and that the inhibition was complex. However, with the purified rat kidney enzyme, NADH is a competitive inhibitor of MgATP$^2-$.
Fig. 11. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of MgATP$^{2-}$. The respective mM concentrations of α-aminobutyrate and glutamate were: 10.7 and 13.3 (●); 5.3 and 6.7 (×); 2.7 and 3.3 (○). The MgATP$^{2-}$ concentration was varied from 0.2 to 2.0 mM. Other experimental details are described under "Experimental Procedure".
Fig. 12. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of glutamate. The respective mM concentrations of α-aminobutyrate and MgATP$^{2-}$ were: 8 and 2 (○); 4 and 1 (x); 2.4 and 0.6 (○); 2 and 0.5 (△). The glutamate concentration was varied from 1.25 to 10 mM. Other experimental conditions are described under "Experimental Procedure".
Fig. 13. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of α-aminobutyrate (αAB). The respective mM concentrations of glutamate and MgATP$^{2-}$ were: 11.2 and 2.8 (●); 8.8 and 2.0 (x); 4.8 and 1.2 (○); 3.2 and 0.8 (▽). The α-aminobutyrate concentration was varied from 2.5 to 20 mM. Other experimental details are described under "Experimental Procedure".
Fig. 14. Replot of the slopes and intercepts with respect to the reciprocal of the concentration of one of the varied substrates: A, B, C; Replot with data from figs. 1, 2, 3, respectively.
Fig. 15. Plot of reciprocal of initial reaction velocity \((v)\) with respect to the reciprocal of the concentration of \(\text{MgATP}^2\) in the presence and absence of \(\text{MgADP}^-\).

\(\alpha\)-Aminobutyrate and glutamate were held constant at 4 and 5 mM respectively and \(\text{MgATP}^2\) was varied from 0.29 to 2.0 mM. \(\text{MgADP}^-\) concentrations were 0 (●); 1 mM (x); 2 mM (○). Other experimental details are described under "Experimental Procedure".
Fig. 16. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of α-aminobutyrate (αAB) in the presence and absence of MgADP⁻. Glutamate and MgATP²⁻ were held at 16 mM and 4 mM respectively and α-aminobutyrate (αAB) was varied from 2.5 to 20 mM. MgADP⁻ concentrations were 0 (●); 0.5 mM (x); 1 mM (○); 1.5 mM (▽). Other experimental details are described under "Experimental Procedure".
Fig. 17. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of glutamate in the presence and absence of MgADP⁻. α-Aminobutyrate and MgATP²⁻ were held constant at 7.3 mM and 1.6 mM respectively. Glutamate was varied from 1.25 to 5.0 mM. MgADP⁻ concentrations were 0 (●), 2 mM (x), 4 mM (○). Other experimental details are described under "Experimental Procedure".
(fig. 18) and clearly noncompetitive relative to both glutamate (fig. 19) and α-aminobutyrate (fig. 20), consistent with the results of the MgADP\(^-\) inhibition studies. GTP and 8-BrATP were also tested but were found to be substrates. 8-BrATP appears to be nearly as good a substrate as ATP itself.

It has been shown previously that L-methionine-DL-sulfoximine is a competitive inhibitor relative to glutamate (Richman et al., 1973). In the present study the competitive behavior was confirmed (fig. 21) and as shown in figs. 22 and 23, methionine sulfoximine is a noncompetitive inhibitor relative to α-aminobutyrate, but uncompetitive with respect to MgATP\(^{2-}\). This uncompetitive pattern has been consistently observed in multiple experiments. To confirm this inhibition pattern, other compounds were tested as a competitive inhibitor relative to glutamate. α-Methyl-glutamate has been reported to be a weak substrate (Orlowski and Meister, 1971a). In the present study, it was found to be a competitive inhibitor with respect to glutamate at concentrations where its activity as a substrate was negligible (fig. 24). The inhibition patterns with respect to the other two substrates were identical to those found with the sulfoximine (figs. 25 and 26).

Attempts to find a competitive inhibitor relative to α-aminobutyrate have thus far been unsuccessful. The following compounds were found not to inhibit the reaction significantly (20%) at the indicated mM concentrations: N-butyric acid (5), 1,2-butanediol (10), 2-bromobutyric acid (10), 2-amino-1-butanol (10), 2-amino-1-propanol (10), glycine (100), serine (5), cysteic acid (5),
Fig. 18. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of MgATP$^{2-}$ in the presence and absence of NADH. α-Aminobutyrate and glutamate were held constant at 6 and 8 mM respectively. MgATP$^{2-}$ was varied from 0.5 mM to 2 mM. NADH concentrations were 0 (o); 1.8 mM (x); 3 mM (o). Other experimental details are described under "Experimental Procedure".
Fig. 19. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of α-aminobutyrate (αAB) in the presence and absence of NADH. Glutamate and MgATP²⁻ were held constant at 8 mM and 2 mM respectively. α-Aminobutyrate (αAB) was varied from 1.25 mM to 5 mM. NADH concentrations were 0 (•); 2 mM (x); 3 mM (o). Other experimental details are described under "Experimental Procedure".
Fig. 20. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of glutamate in the presence and absence of NADH. α-Aminobutyrate and MgATP$^{2-}$ were held constant at 8 mM and 2 mM respectively. Glutamate was varied from 1.25 to 5.0 mM. NADH concentrations were 0 (•), 2 mM (x), 3 mM (○). Other experimental details are described under "Experimental Procedure".
Fig. 21. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of glutamate in the presence and absence of methionine-\textit{RS}-sulfoximine. $\alpha$-Aminobutyrate and MgATP$^{2-}$ were held constant at 8 mM and 2 mM respectively and glutamate was varied from 1.4 mM to 10 mM. Methionine sulfoximine concentrations were 0 (e); 0.5 mM (x); 1 mM (o). Other experimental details are described under "Experimental Procedure".
Fig. 22. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of \(\alpha\)-aminobutyrate (\(\alpha\)AB) in the presence and absence of methionine sulfoximine. Glutamate and MgATP\(^2\) were held constant at 16 mM and 4 mM respectively. \(\alpha\)-Aminobutyrate (\(\alpha\)AB) was varied from 1.25 mM to 5.0 mM. Methionine sulfoximine concentrations were 0 (\(\circ\)); 0.5 (\(\times\)); 1 mM (\(\circ\)). Other experimental details are described under "Experimental Procedure".
Fig. 23. Plot of reciprocal of initial reaction velocity ($v$) with respect to the reciprocal of the concentration of MgATP$^{2-}$ in the presence and absence of methionine sulfoximine. 

$\alpha$-Aminobutyrate and glutamate were held constant at 6 mM and 4 mM respectively. MgATP$^{2-}$ was varied from 0.37 to 1.7 mM. Methionine sulfoximine concentrations were 0 (o); 0.5 mM (x); 1 mM (o). Other experimental details are described under "Experimental Procedure".
Fig. 24. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of glutamate in the presence and absence of α-methyl glutamate. α-Aminobutyrate and MgATP$^2-$ were held constant at 8 mM and 2 mM respectively. Glutamate was varied from 0.29 mM to 2 mM. α-Methyl glutamate concentrations were 0 (o); 1.5 mM (x); 3 mM (c). Other experimental details are described under "Experimental Procedure".
Fig. 25. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of \(\alpha\)-aminobutyrate in the presence and absence of \(\alpha\)-methyl glutamate. Glutamate and MgATP\(^{2-}\) were held constant at 8 mM and 3 mM respectively. \(\alpha\)-Aminobutyrate was varied from 0.125 mM to 5 mM. \(\alpha\)-Methyl glutamate concentrations were 0 (o); 1.5 mM (x); 3 mM (o). Other experimental details are described under "Experimental Procedure".
Fig. 26. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the concentration of MgATP\(^{2-}\) in the presence and absence of α-methyl glutamate. α-Aminobutyrate and glutamate were held constant at 6 mM and 4 mM respectively. MgATP\(^{2-}\) was varied from 0.167 mM to 0.5 mM. α-Methyl glutamate concentrations were 0 (○); 1 mM (△); 2 mM (○). Other experimental details are described under "Experimental Procedure".
proline (100), N-propylurea (10), \( \beta \)-aminobutyrate (10),
\( \gamma \)-aminobutyrate (10), \( \alpha \)-aminoisobutyrate (10), taurine (10)
and butanol (10). A comparison of the structures of the compounds
tested to the structures of cysteine and \( \alpha \)-aminobutyrate is
listed in Table III. These compounds were all found to be
inactive as substrates as shown by Orlowski and Meister (1971a),
Rathbun (1967) and confirmed by the present studies. Phosphoserine
at 10 mM concentration did cause approximately 50% inhibition of the
reaction. This inhibition by phosphoserine is of interest
relative to the mechanism of the reaction but lability of the
compound in the assay system has precluded further investigations
on the details of the inhibition. In general, the compounds
that were tested either required very high concentrations of the
inhibitor for significant inhibition or did not appear to be
competitive with \( \alpha \)-aminobutyrate. The high concentration required
for some of the inhibitor might cause inhibition simply from ionic
strength changes, making interpretation of the results impossible.

A summary of the inhibition studies is presented in Table IV.
TABLE III
Structures of Cysteine and Analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>HOOC-CH-CH₂-SH</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
<tr>
<td>α-Aminobutyrate</td>
<td>HOOC-CH-CH₂-CH₃</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
<tr>
<td>γ-Aminobutyrate</td>
<td>HOOC-CH₂-CH₂-CH₂-NH₂</td>
</tr>
<tr>
<td>β-Aminobutyrate</td>
<td>HOOC-CH₂-CH-CH₃</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
<tr>
<td>1,2-Butanediol</td>
<td>HO-CH₂-CH-CH₂-CH₃</td>
</tr>
<tr>
<td></td>
<td>[\text{OH}]</td>
</tr>
<tr>
<td>2-Bromobutyric acid</td>
<td>HOOC-CH-CH₂-CH₃</td>
</tr>
<tr>
<td></td>
<td>[\text{Br}]</td>
</tr>
<tr>
<td>2-Amino-1-butanol</td>
<td>HO-CH₂-CH-CH₂-CH₃</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
<tr>
<td>Serine</td>
<td>HOOC-CH-CH₂-OH</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>HOOC-CH-CH₂-SO₃H</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
<tr>
<td>N-propylurea</td>
<td>[\text{NH}_2-C-NH-CH₂-CH₂-CH₃]</td>
</tr>
<tr>
<td></td>
<td>[\text{O}]</td>
</tr>
<tr>
<td>2-Amino-1-propanol</td>
<td>HO-CH₂-CH-CH₃</td>
</tr>
<tr>
<td></td>
<td>[\text{NH}_2]</td>
</tr>
</tbody>
</table>
### TABLE IV

**INHIBITION OF γ-Glutamylcysteine Synthetase**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Apparent Inhibition constant* (mM)</th>
<th>MgATP</th>
<th>α-aminobutyrate</th>
<th>Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgADP</td>
<td>1.4</td>
<td>competitive</td>
<td>non-competitive</td>
<td>non-competitive</td>
</tr>
<tr>
<td>NADH</td>
<td>0.8</td>
<td>competitive</td>
<td>non-competitive</td>
<td>non-competitive</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.2</td>
<td>un-competitive</td>
<td>non-competitive</td>
<td>competitive</td>
</tr>
<tr>
<td>Sulfoximine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Methyl Glutamate</td>
<td>0.35</td>
<td>un-competitive</td>
<td>non-competitive</td>
<td>competitive</td>
</tr>
</tbody>
</table>

* Calculated from the equation $K_I = (I)(S_0)/(S_1 - S_0)$, where $K_I$ is the apparent inhibition constant, $I$ is the concentration of inhibitor, $S_0$ is the slope of the uninhibited line from the competitive inhibition plot, and $S_1$ is the slope of the inhibited line on the same graph for a particular (I).
DISCUSSION

When initial rate studies with a three substrate enzyme are performed as described by Fromm (1967), a Ping Pong mechanism will yield one or more Lineweaver-Burke plots that exhibit parallel lines. If however, a quaternary enzyme-substrate complex is an obligatory intermediate, all three double reciprocal plots will intersect on or to the left of the $1/v$ axis. Since all three initial rate plots have lines that intersect to the left of the $1/v$ axis, the possibilities of a discrete covalent enzyme substrate intermediate, i.e., all Ping Pong mechanisms, and of the rapid equilibrium ordered mechanism, are eliminated. That the slope replot from the $1/MgATP^2-$ reciprocal plot has a zero intercept indicates in the kinetic binding mechanism of this enzyme, that one ternary complex is missing (Clayton and Rudolph, 1976). Thus, only four types of partially random mechanisms are consistent with this conclusion from the initial rate and replot data. These four mechanisms are shown in fig. 27.

From the inhibition studies, there is one uncompetitive inhibition pattern observed. An uncompetitive inhibition pattern means that the kinetic binding order occurs in such a way that the varied substrate in the uncompetitive inhibition pattern binds to the enzyme first, before the inhibitor can bind. That is, in this case, MgATP$^2-$ binds before glutamate. Thus, only the steady state ordered, the rapid equilibrium ordered, and the random BC mechanisms are consistent with the observed inhibition data. These mechanisms are illustrated in fig. 28.
Fig. 27. Possible mechanisms consistent with initial rate data. Dashed lines indicate pathways which do not affect the rate equation.
Fig. 28. Possible mechanisms consistent with the competitive inhibition studies.
A comparison of the possible mechanisms deduced from initial rate and replot data, shown in fig. 27, and deduced from inhibition studies, shown in fig. 28, shows that the only three substrate sequential binding mechanism consistent with the data obtained is the random BC mechanism. This mechanism is illustrated in fig. 29 with the rate equation.

As was discussed in the introduction, based on the partial reaction studies, Meister (1974) has proposed a tentative mechanism which can be visualized either as a Ping Pong, if \( P_1 \) is released before cysteine binds to the enzyme, or as an ordered sequential, if \( P_1 \) is not released. However, the random BC mechanism presented in this report can explain all previous experimental findings. The different effects of glutamate and \( \alpha \)-aminobutyrate on the partial reactions, ATP → ADP + \( P_1 \) and ATP + \( P_1 \) → ADP + PF, are consistent with the random BC mechanism. The binding of glutamate to the enzyme shifts its form to EAC, presumably a conformation which is less active in carrying out the ATP hydrolysis and pyrophosphate formation. The water or \( P_1 \) acceptor site may be blocked by glutamate. \( \alpha \)-Aminobutyrate binding shifts the enzyme form to EAB, presumably a form which is more active in carrying out the partial reactions. The activation of ATP hydrolysis by \( \alpha \)-aminobutyrate is prevented by the presence of L-glutamate or its analogues. Thus, in the presence of glutamate, enzyme forms EAC and EABC will be present and the activation effect of \( \alpha \)-aminobutyrate will be diminished. This is also supported by the experimental finding that the rate of exchange of \( \alpha \)-aminobutyrate
Kinetic Mechanism of γ-Glutamylcysteine Synthetase

A = MgATP; B = α-aminobutyrate; C = glutamate

\[
\frac{E_0}{V} = \frac{1}{k_1} + \frac{K_5}{k_1(B)} + \frac{K_4}{k_1(C)} + \frac{K_2K_4}{k_1(B)(C)} + \frac{K_1K_2K_4}{k_1(A)(B)(C)}
\]

Fig. 29. The kinetic mechanism of γ-glutamylcysteine synthetase and the rate equation.
into the dipeptide $\gamma$-glutamyl-$\alpha$-aminobutyrate is twice as fast as the exchange of glutamate, which would indicate that glutamate binds at least twice as tight as $\alpha$-aminobutyrate. The effect of $\alpha$-aminobutyrate on the partial reaction, glutamate + ATP $\rightarrow$ 5-oxoproline + ADP + $P_i$, can also be explained by similar interactions.

$\gamma$-Glutamylcysteine synthetase belongs to a general class of enzymes that catalyze the formation of an amide bond with cleavage of a nucleotide triphosphate to a diphosphate and inorganic phosphate. $\gamma$-Glutamylcysteine synthetase is closely related to glutamine synthetase and, to some extent, glutathione synthetase, where the general reaction is:

\[
R-\text{NH}_2 + \text{HOOC-R'} \xrightleftharpoons[\text{Mg}^{2+}]{\text{ATP}} \xrightarrow{\text{ADP} + P_i} R-\text{NH-C-R'}
\]

where for glutamine synthetase, $R = \text{H}$, $R' = \text{glutamate}$;

for $\gamma$-glutamylcysteine synthetase, $R = \text{HOOC-C-H}$, $R' = \text{glutamate}$;

for glutathione synthetase $R = \text{H}_2\text{C-COCH}$, $R' = \text{dipeptide}$.

A comparison of some of the catalytic properties of $\gamma$-glutamylcysteine synthetase and glutamine synthetase is summarized in Table V. It can be seen that their properties, and also those of glutathione synthetase, are very similar and it has generally been assumed that their mechanism of action is the same. Although glutamine synthetase has been studied extensively, there is still controversy over the mechanism of catalysis. Wedler and Boyer (1972),
TABLE V
COMPARISON OF THE CATALYTIC PROPERTIES OF OVINE BRAIN
GLUTAMINE SYNTHETASE AND \( \gamma \)-GLUTAMYLCYSTEINE SYNTHETASE

<table>
<thead>
<tr>
<th>Glutamine synthetase</th>
<th>( \gamma )-Glutamylcysteine synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No exchange between ATP and ( P_i ) or ATP and ADP in absence of other substrate</td>
<td></td>
</tr>
<tr>
<td>2. L-methionine-S-sulfoximine + ATP + Enz ( \rightarrow ) Enz-(L-methionine-S-sulfoximine phosphate)(ADP)</td>
<td></td>
</tr>
<tr>
<td>3. Glutamate + ATP ( \rightarrow ) 5-Oxoproline + ADP + ( P_i )</td>
<td></td>
</tr>
<tr>
<td>4. Hydroxylamine + Glutamate + ATP ( \rightarrow ) ( \gamma )-Glutamylhydroxamate + ADP + ( P_i )</td>
<td></td>
</tr>
<tr>
<td>5. Not active with cysteine</td>
<td>Not active with ( \text{NH}_3 )</td>
</tr>
<tr>
<td>6. (- * -)</td>
<td>ATP ( \rightarrow ) ADP + ( P_i )</td>
</tr>
<tr>
<td>7. (- * -)</td>
<td>ATP + ( P_i ) ( \rightarrow ) ( P Pi ) + ADP</td>
</tr>
<tr>
<td>8. Carbamyl phosphate + ADP ( \rightarrow ) ATP + ( \text{CO}_2 ) + ( \text{NH}_3 ) (- * -)</td>
<td></td>
</tr>
<tr>
<td>9. ( \beta )-Glutamyl phosphate + ADP ( \rightarrow ) ( \beta )-Glutamate + ATP (- * -)</td>
<td></td>
</tr>
<tr>
<td>10. Enz + Cycloglutamate + ATP ( \rightarrow ) Enz(Cycloglutamyl phosphate)(ADP) (- * -)</td>
<td></td>
</tr>
</tbody>
</table>

\[ a \] Listed in Meister, 1974

\(- * -\) Not reported
using isotope exchange studies, found the mechanism of adenylation
E. Coli glutamine synthetase to be totally random and argued that
acyl phosphate is not a significant intermediate. Wedler (1974)
has extended these isotope exchange studies to glutamine synthetases
from different sources. With ovine brain enzyme, he concluded
that the kinetic patterns of equilibrium exchange are consistent
with a partially ordered sequence of substrate binding where ATP
binds before NH$_3$ but glutamate binds randomly. With pea seed
enzyme, Mn$^{2+}$-activated adenylation E. Coli enzyme and Mg$^{2+}$-
activated unadenylated E. Coli enzyme, Wedler (1974) found that
the order of substrate binding was totally random. On the other
hand, Krishnaswany et al. (1962) have provided much evidence with
ovine brain glutamine synthetase to support the formation of
$\gamma$-glutamyl phosphate as an enzyme bound intermediate and argued
that the mechanism of substrate binding cannot be totally random.
The reaction mechanism proposed by Meister (1974) for glutamine
synthetase is similar to that for $\gamma$-glutamylcysteine synthetase.

With glutathione synthetase, the problem is similar. Wendel
and Heinle (1975) found that the kinetic mechanism is totally
random, while Meister and co-workers (Meister, 1969; Nishimura
et al., 1984) showed that acyl phosphate is an intermediate in
the reaction.

As shown in the present report, the kinetic mechanism of
$\gamma$-glutamylcysteine synthetase is different from those proposed for
glutamine synthetase and glutathione synthetase, being partially
random BC instead of totally random as was generally assumed.
Here, the kinetic mechanism fits the data from studies on the
partial reactions, but there is still the question of \( \gamma \)-glutamyl phosphate as an intermediate. However, as discussed elsewhere (Meister, 1974), the postulation that acyl phosphate is formed as an intermediate is not entirely inconsistent with the kinetic mechanism if the acyl phosphate formed does not dissociate from the enzyme until all the substrates are bound or if the acyl phosphate is not formed unless all the substrates are bound on the enzyme and is then further reacted with the third substrate to give the final products.
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