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To the Graduate Council:

I am submitting herewith a thesis written by Cecilia Lee Hsu entitled "Inhibition of Glucose-6-Phosphate Dehydrogenase from Yeast by Aurintricarboxylic Acid." I recommend that it be accepted for eighteen quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

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13

INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM YEAST BY AURINTRICARBOXYLIC ACID

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Cecilia Lee Hsu

March 1974

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ABSTRACT

Inhibition of glucose 6-phosphate (G6P) dehydrogenase from yeast by aurintricarboxylic acid (ATA) was competitive with respect to glucose-6-phosphate, Ki being 2.5×10^{-7} M and 1.32×10^{-6} M in the absence and presence of Mg^{2+}. With respect to $NADP^{+}$, inhibition was a mixed type, Ki being 3.5×10^{-5} M and 2.0×10^{-6} M in the presence and absence of Mg^{2+}.

The Dixon plots and the Johnson Eyring plots indicated that at low concentrations of ATA, one molecule of ATA was bound per molecule of enzyme. At higher concentrations of ATA, more than one molecule of ATA per enzyme molecule participated in the formation of enzyme inhibitor complex.

Several enzymes were tested for inhibition by ATA. Alcohol dehydrogenase from horse liver and malic dehydrogenase from pig heart, both requiring NAD^{+} as coenzyme, were not inhibited by ATA. Xanthine oxidase, a dehydrogenase requiring no coenzyme for its activity, was inhibited by ATA with a Ki of 2.6×10^{-5} M. Phosphoglucomutase from yeast and rabbit muscle and alkaline phosphatase from *E. coli* were not inhibited by ATA.

Kinetic constants at pH values ranging from 5 to 9 were studied. The plots of pKm and pKi against pH suggested that an ionization group with pK of 8.3 or 8.4 might be involved in the glucose 6 phosphate binding site of the enzyme. It was proposed that the sulfhydryl group of cysteine is involved in the binding of glucose 6 phosphate

and ATA to G6P dehydrogenase.

The effect of temperature on inhibition of G6P dehydrogenase by ATA was studied. Association constants (k), change of free energy (ΔF), enthalpy (ΔH) and entropy (ΔS) for the reaction of ATA inhibition were determined at 19, 25, 34, 38 and 42°C. The nature of kinetic behavior at various temperatures was also investigated and expressed in Johnson Eyring plots and percentage inhibition curves. When two molecules of ATA were bound per one molecule of enzyme, the formation of enzyme-ATA complex was characterized by a highly exothermic and exergonic reaction with a large negative entropy change. At low temperature (19°C) ATA inhibition was by a simple Michaelis reaction but at high temperatures (38 and 42°C) allosteric mechanism was observed. At 34°C a combination of the two occurred.

Difference spectra between ATA and G6P dehydrogenase were obtained. The optical density of difference spectra of E-ATA complex was greatly increased with the addition of $NADP^+$.

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CHAPTER I

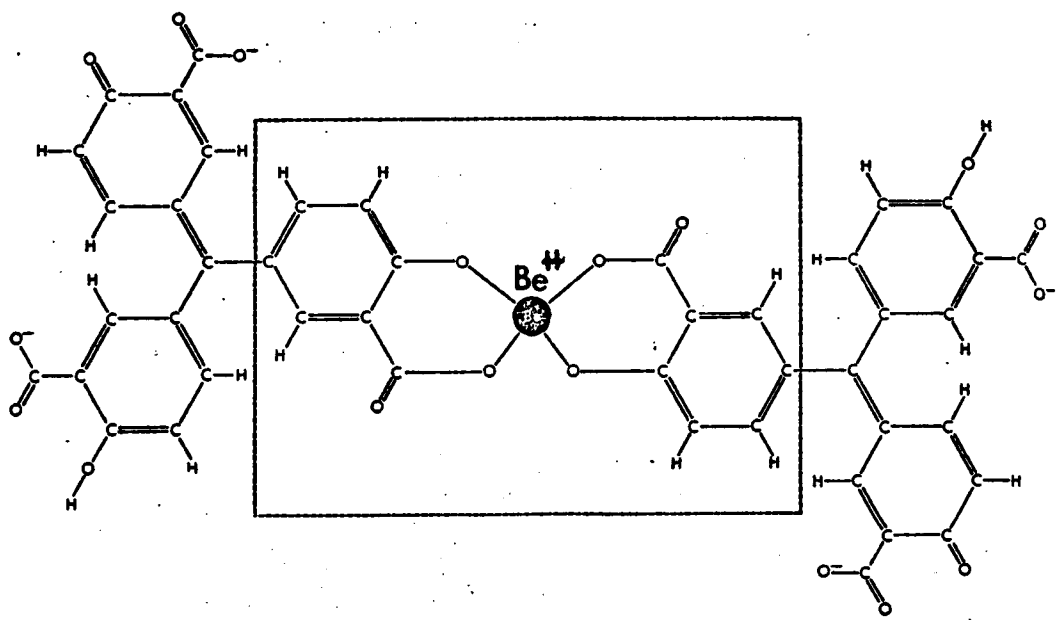
INTRODUCTION

A. Aurintricarboxylic Acid

Aurintricarboxylic acid (ATA, "aluminon") is a triphenylmethane dye, commonly used by analytical chemists, for the analysis of aluminum. Biologically, it has been used to alleviate acute beryllium poisoning in mice and rats (1). Available data indicate that the toxicity of beryllium is due to the inhibition of two magnesium-activated enzymes, alkaline phosphatase and phosphoglucomutase (1, 2, 3, 4). By using steady-state kinetic analysis, it was demonstrated that beryllium competes with magnesium (4). It was further shown, in vivo as well as in vitro, that the ability of ATA to reverse this inhibition and toxicity is due to chelate formation between Be^{++} and orthocarboxyl, hydroxyl groups in the ATA molecule and concomitant formation of a non-diffusable, colloidal lake (5).

Over seventy different compounds have been studied to determine the functional groups of the ATA and to ascertain the relationship between molecular structure, functional groups and the effectiveness of a compound in chelating Be^{++} (6). These studies showed that compounds that protected at least 50% of the population of Be^{++} poisoned mice possessed two ortho-hydroxyl groups or ortho-carboxyl, hydroxyl groups attached directly to the benzene nucleus. Examples are salicilic, gentisic, sulfosalicilic, aurintricarboxylic and

2, 4-dihydroxybenzoic acids. All compounds that produced over 40% reversal contained functional groups attached directly to a structure stabilized by resonance--the benzene nucleus. An aliphatic and saturated ring structures produced a lesser degree of reversal. Compounds such as benzoic acid and phenol that contain only one functional group were ineffective. Compounds in which the functional groups were in such a position that they were capable of forming only seven or eight membered chelate rings, such as m- and p- hydroxybenzoic acid, were also ineffective. Based on these observations the following diagram of ATA chelation was suggested.



The effectiveness of the ATA as an antidote for acute experimental beryllium poisoning is characterized not only by chelation between Be^{++} and the ATA but also by its ability to bind plasma alkaline phosphatase (7). The binding constants were determined to

be 850, 60 and 20 for ATA, sulfosalicilic acid and salicilic acid. The binding constants correlated well with ability to reverse Be^{++} poisoning.

Additional work on the relative contribution of prosthetic groups and molecular size to protein binding involved quantitative measurement of the binding of a well characterized protein, serum albumin, with ATA and related compounds (8). In general, the degree of protein binding followed the same trend as the reversal of the beryllium--induced inhibition of alkaline phosphatase. Several factors which contributed to the binding of ATA and protein were elucidated in this study.

(1) Interaction with the water solvent. The increased binding by albumin of salicilic acid (SA) over m- or p- hydroxybenzoic acid was ascribed to the formation of an intramolecular hydrogen bond by SA, thus reducing the reactivity of the OH groups with the aqueous solvent.

(2) Ionization. The greater the degree of ionization of the anion the greater the electrostatic attraction for the cationic center in albumin. Hence, the binding between ATA and albumin at low pH was strengthened.

(3) Van der Waals Force. This becomes a significant factor as molecular size of the anion increases. Thus, ATA which consists of three molecules of salicilic acid bound by a methyl group is bound to albumin 25 times more strongly than simple SA. The increased binding was due to the additional rings in the ATA molecule

and to the probability that ATA exists in solution as a polymerized species (9).

Recently ATA was used to study initiation of protein synthesis in bacteria and eukaryotes. It was reported that ATA inhibits the attachment of mRNA to the ribosome in cell free extracts prepared from E. coli (10, 11, 12).

The effect of ATA on hemoglobin synthesis in reticulocyte lysate resembles that observed in extracts of E. coli. Experiments with purified transfer factors and washed reticulocyte ribosomes have shown that ATA has no direct effect on peptide bond formation (13). Initiation of protein synthesis on certain plant ribosomes, with tobacco mosaic virus as the source of mRNA, was also inhibited by ATA (14). It appears that ATA affects eukaryotes as well as prokaryotes.

B. Glucose 6-Phosphate Dehydrogenase from Yeast

Glucose 6-phosphate dehydrogenase, originally called Zwischenferment, catalyzes the following reaction:

$$\text{Glucose 6-phosphate} + NADP^+ \rightleftharpoons \text{6-phosphogluconate} + NADPH + H^+$$

Great effort was expended to purify glucose 6-phosphate dehydrogenase from a variety of sources, including yeast, mold, bacteria, mammalian erythrocytes, liver, adrenal tissue, and mammary gland. The first crystalline preparation of this protein from brewer's yeast was reported by Noltman and Kuby (15). The enzyme isolated from yeast was

conveniently crystallized as the $NADP^+$ compound. The sedimentation behavior of the ultracentrifugally homogeneous protein was dramatically influenced by the presence of $NADP^+$. Evidence (15, 16, 17) indicated an association phenomenon induced by the $NADP^+$. Thus, the addition of $NADP^+$ to the enzyme crystallized without the coenzyme resulted in a considerable increase in the sedimentation velocity. By choosing the appropriate NADP concentrations, the change from one form into another could be demonstrated, and it was possible to separate the $NADP^+$-enzyme compound and free apoenzyme by ultracentrifugation. With an excess of $NADP^+$ added to convert the enzyme entirely into the NADP compound, only a single boundary again was observed. Values for S_{20w}, extrapolated to zero protein concentration were found to be 9.6 Svedberg units for the $NADP^+$-enzyme and 6.3 Svedberg units for the $NADP^+$-free enzyme.

The apoprotein molecular weight has been estimated first, by sedimentation velocity and translational diffusion and second, by equilibrium sedimentation as 101,600 and 101,700 respectively (16, 17). Molecular weight of the $NADP^+$-enzyme compound estimated by low speed equilibrium sedimentation, in the presence of excess $NADP^+$ yielded a calculated MW of 2.12×10^5 (17). Sedimentation equilibrium studies of the apoprotein conducted in the presence of 6M guanidinium chloride pH 3.5, yielded MW of 5.1×10^4. These observations led to the conclusion that glucose 6-phosphate dehydrogenase was composed of two polypeptide chains and that the $NADP^+$-enzyme complex was the "dimer" of the apoprotein, i.e. it consisted of four subunits.

Data on the role of metal in this enzyme appear contradictory.

For example, analyses for elemental metal was conducted by Vallee, et al. (18), who reported considerable amounts of Pb, Fe, Zn, Cu, Ca and Mg. On the basis of inhibition studies with metal chelator, Vallee, et al. (18) suggested Zn^{++} as a possible metal constituent of the enzyme from yeast. Noltman, et al., however, reported that atomic absorption analyses failed to reveal any significant concentration of a metal constituent. EDTA inhibited the enzyme activity, but its effect was not due to its chelation properties, but rather to its ability to prevent the $NADP^+$-induced association of enzyme subunits (17). Thus, the danger of utilizing inhibition by a specific chelator as the means to prove the presence of metal co-factor in an enzyme preparation appears noteworthy.

The distinguishing feature between the enzymes from different sources appears to be the relative affinity and requirement for the pyridine nucleotide. Thus, the enzyme from Leuconostoc mesenteroids may utilize either NAD^+ or $NADP^+$ equally well (19) and other bacterial and mold enzymes which oxidize glucose 6-phosphate with NAD^+ have been described (20). On the other hand, the yeast enzyme specifically requires NADP (21). Product NADPH was a competitive inhibitor of the variable substrate $NADP^+$ for the forward reaction, NADH did not act as an inhibitor for yeast enzyme (22). Orthophosphate inhibited the enzyme but the inhibition was reversed by NADP (22).

One report (21) showed that Vmax, as well as the kinetic parameters of this enzyme, are influenced by a variety of univalent and divalent anions. Although earlier assay procedures included 0.15 M Mg^{++}

in the assay mixture (15), subsequent data showed no requirement for any specific metal for catalytic activity (17).

C. Statement of Problem

Until now, ATA has been used in biochemical studies to reverse poisoning by Be^{++} by restoring alkaline phosphatase activity and as an inhibitor of protein synthesis. In addition, during our studies on the effect of Be^{++} on phosphoglucomutase, it was discovered that ATA inhibited yeast G6P dehydrogenase, an enzyme used in a coupled assay system for measuring G6P, the product of the phosphoglucomutase reaction.

The experiments described below were designed to study in some detail

(1) The specificity of ATA inhibition,

(2) Kinetic and thermodynamic properties of ATA inhibition, and

(3) The nature and localization of binding sites.

CHAPTER II

EXPERIMENTAL

A. Materials

Glucose 6-phosphate dehydrogenase from yeast and malic dehydrogenase from pig heart were purchased from Sigma; xanthine oxidase from milk and alcohol dehydrogenase from horse liver were products of Worthington Biochemical Corporation; alkaline phosphatase from E. coli and phosphoglucomutase from rabbit muscle and yeast were isolated as described before (22, 23). Aurintricarboxylic acid (ATA) was obtained from Fisher Scientific Company; and nicotinamide-adenine dinucleotide phosphate ($NADP^+$), nicotinamide-adenine dinucleotide (NAD^+), Sigma Chemical Company. Other chemicals used were of analytical grade.

B. Methods

Activities of various enzymes were measured as follows:

1. G6P Dehydrogenase

Dehydrogenase activity was routinely measured at room temperature by recording the change in absorbancy at 340 nm in a Gilford multiple absorbance recorder (Model 2000).

In a typical assay, the standard reaction mixture contained 2 mM glucose 6-phosphate, 0.15 mM $NADP^+$, and 50 mM Tris-HCl buffer (pH 7.5) with or without 10 mM $MgSO_4$ added in a final volume of 1 ml,

The reaction was started by adding 5 µl to 10 µl of enzyme, and initial reaction rate determined by measuring increase in the absorbance at 340 mµ as NADPH was formed.

2. Xanthine Oxidase

Oxidase activity was measured as described by Fridovich (24). The rate of conversion of hypoxanthine to uric acid was followed by absorption at 295 mµ increase in a 1 ml. reaction volume containing 0.05 mM hypoxanthine, 0.01 mM EDTA and 50 mM potassium phosphate buffer at pH 7.8.

3. Alkaline Phosphatase

Phosphatase was assayed in a mixture containing 3.6 mM p-nitrophenyl phosphate, 0.25 mM $MgCl_2$ and 0.5 mM Tris-HCl buffer at pH 9.0. The release of p-nitrophenol was measured by following the increase in absorbance at 410 mµ(25).

4. Phosphoglucomutase

The measurement of phosphoglucomutase activity was based on the fact that glucose 1-phosphate has acid-labile phosphate while glucose 6-phosphate has acid stable phosphate. The rate of reaction can be followed by measuring the reduction in the hydrolyzable phosphate. In a 4 ml. reaction volume containing $MgSO_4$ (6 mM), glucose 1-phosphate (0.2 mM) and 0.1 M Tris buffer pH 7,5 was placed in room temperature. The reaction was started by adding 1 ml. of the enzyme. At various time intervals, 1 ml. aliquots were withdrawn and placed in test tubes containing 1 ml. of 5N H_2SO_4. Phosphoglucomutase

activity was determined by measuring changes in acid stable and acid labile phosphates as described by Fiske and SubbaRow (26).

5. Alcohol Dehydrogenase

Activity was determined as described by Theorell and Bonnichsen (27). The assay is based on spectrophotometric measurement of the amount of NAD^+ reduced in 3 min. at pH 9.5 in the presence of excess alcohol. The reaction mixture contained 3 ml. of 0.1 M glycine-sodium hydroxide buffer (pH 9.5), 0.1 ml. of NAD^+ (5 mM) and 0.1 ml. of 95% ethanol. The reaction was started by addition of enzyme and the increase in absorbance at 340 mμ was measured in a Gilford multiple absorbance recorder (Model 2000).

6. Malic Dehydrogenase

The activity assay was based on the measurement of the rate of oxidation of NADH (decrease in optical density at 340 mμ) in the presence of the enzyme and excess oxalacetate. The reaction mixture contained 0.3 ml. of 0.25 M glycylglycine buffer (pH 7.4), 0.1 ml. of NADH (0.15 μM), 0.1 ml. of oxalacetate (0.76 μM) enzyme and water to a final volume to 3.0 ml. The reaction was started by the addition of oxalacetate. The decrease in optical density at 340 mμ was measured in a Gilford multiple absorbance recorder (Model 2000).

7. Determination of Kinetic Constants

(a) The Michaelis constant (Km), the equilibrium constant of the reversible combination of an enzyme with its substrate, was determined by the graphic method of Lineweaver and Burk (28). The

Michaelis equation is:

$$v = \frac{VS}{Km + S} \qquad (1)$$

Here v is the reaction velocity with the substrate concentration S, and V is the maximum velocity obtained at saturating substrate concentrations. A plot of 1/v against 1/S yields a straight line. This straight line intercepts the absissa at a point giving -1/Km, as shown in Figure 1. This is shown by rearranging equation (1) to obtain:

$$\frac{1}{v} = \frac{Km}{V} \cdot \frac{1}{S} + \frac{1}{V} \qquad (2)$$

which then gives 1/S = -1/Km at 1/v = 0.

(b) The inhibitor constant, Ki, is the equilibrium constant of the reversible binding of the enzyme with a competitive inhibitor. Ki was determined by the graphic Lineweaver and Burk plot (28) method in which the reciprocal of the initial velocity is plotted against the reciprocal of the substrate concentration (Figure 1). Ki was calculated by the formula

$$Ki = \frac{[I] \times Km}{(Kp - Km)} \qquad (3)$$

where [I] is the concentration of inhibitor, Km is the Michaelis constant and Kp is the apparent Michaelis constant in the presence of the particular concentration of inhibitor used.

(c) The apparent number of inhibitor molecules reacting per

enzyme molecule to form an inactive enzyme complex is n. If one assumes the following overall reaction,

$$E + nI \rightleftharpoons E - In$$

then

$$K = \frac{[E - In]}{[E]\,[I]^n}$$

and

$$\log \frac{[E - In]}{[E]} = \log K + n \log [I]$$

where E = active enzyme,

I = inhibitor,

E-I= inactive enzyme-inhibitor complex,

n = apparent number of inhibitor molecules reacting per enzyme molecule to form an inactive enzyme complex,

K = apparent overall association constant.

In practice $\log (V_0 - V_I)/V_I$ or $\log (V_0/V_I - 1)$ is used as $\log [E - In]/[E]$ where V_0 is the control activity without inhibitor and V_I is the enzyme activity in the presence of inhibitor. Johnson and Eyring (29) found that in many biological systems, if $\log (V_0/V_I - 1)$ was plotted against log I (i.e., log concentration of inhibitor) at constant temperature and pressure, a straight line relation resulted. When n is obtained graphically, the apparent association of constant K can be obtained by calculating $\log (V_0/V_I - I)$ at log I.

8. Determination of Thermodynamic Parameters

For the reaction,

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES,$$

$$\Delta F^{\circ}_{1} = - RT \log_e \frac{K_{+1}}{K_{-1}} = -2.303 \times RT \log \frac{K_{+1}}{K_{-1}}$$

where ΔF°_{1} is the standard free energy change. This is the free energy change (in calories per mole) of the reaction $E + S \rightleftharpoons ES$ when E, S and ES are all at unit activity and is positive when the formation of ES is endothermic. R is 1.98 cal. per deg. per mole. From the effect of temperature on K_{+1}/K_{-1} the heat change may be obtained by means of the equation:

$$\Delta H^{\circ}_{1} = 2.303 \times RT^2 \cdot \left\{ \frac{d \log \left[\frac{K_{+1}}{K_{-1}} \right]}{dT} \right\}$$

The most convenient way is to plot $\log_{10} (K_{+1}/K_{-1})$ against 1/T. The slope of the straight line obtained is then $- \Delta H^{\circ}_{1}/2.303\ R$. Since $\log K \left(= \log \frac{K+1}{K-1} \right)$ can be obtained from the Johnson-Eyring plot as explained earlier, ΔH (enthapy) can be calculated from this relationship. The thermodynamic parameters, ΔF and ΔS (entropy) are calculated from this information by using the equations $\Delta F = -RT \ln K$ and $\Delta S = (\Delta H - \Delta F)/T$.

9. Spectrophotometric Measurements

Studies on difference spectra between ATA and G6P dehydrogenase

or Mg^{++} and ATA were recorded on a Cary 15 spectrophotometer using split cells with a total path length of 1.0 cm. The detailed procedure is described elsewhere.

10. Reaction Rates

A Gilford Model 2000 recording spectrophotometer was used.

CHAPTER III

RESULTS

A. Kinetic Nature of ATA Inhibition

Detailed kinetic measurements were taken after it was established that the inhibition of G6P dehydrogenase by ATA was instantaneous, reversible by dilution and independent of the order of the addition of reagents. Thus no inactivation was observed when the enzyme was preincubated in 0.05 M Tris buffer pH 7.5, with or without 10 mM Mg^{++} and ATA at concentrations up to 4×10^{-6} M for as long as 40 minutes but assayed after 100 fold dilution.

1. The Lineweaver-Burk Plots

All the kinetic studies reported here were conducted with and without Mg^{2+} ion because (1) Mg^{++}, although not required for catalysis, stimulates G6P dehydrogenase and (2) ATA, as mentioned before, is a chelating agent.

With glucose 6-phosphate as the varied substrate and $NADP^{+}$ in excess, linear competitive inhibition was obtained (Figure 1A, B). When $NADP^{+}$ was the varied substrate, the inhibition was a mixed type (Figure 1C, D). The kinetic constants of Km and Ki are given in Table 1.

Neither the types of inhibition nor Km were affected by the presence of Mg^{2+}. However, Mg^{2+} did increase Ki about one order of magnitude for both types of inhibition.

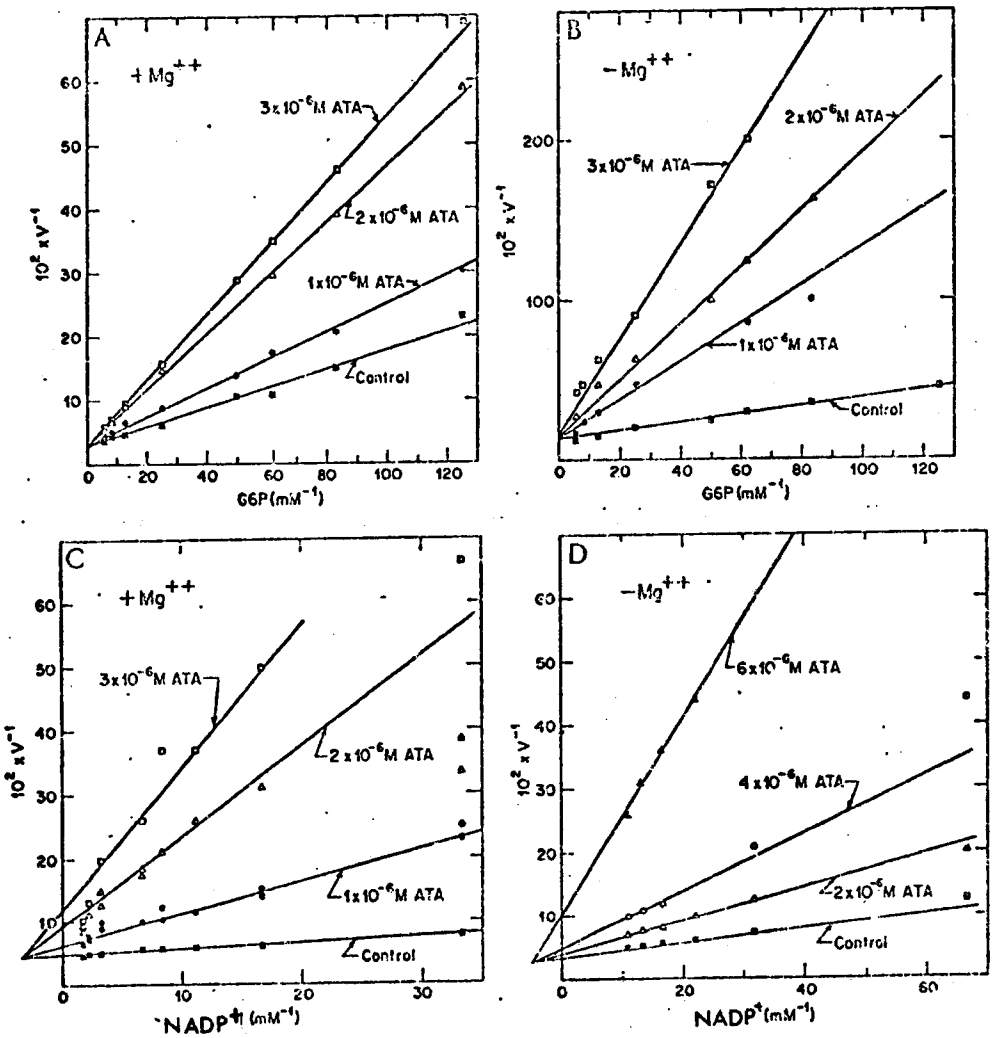


Figure 1. Lineweaver-Burk Plots of the Inhibition of G6P Dehydrogenase by ATA.

TABLE I

KINETIC CONSTANTS OF G6P DEHYDROGENASE

	Km (M)	Ki (M)
$NADP^+$	2.0×10^{-5}	1.6×10^{-7}
$NADP^+ + Mg^{+2}$	3.5×10^{-5}	2.00×10^{-6}
G6P	1.9×10^{-5}	2.5×10^{-7}
$G6P + Mg^{+2}$	5.55×10^{-5}	1.32×10^{-6}

2. The Dixon Plots

The initial velocity was measured at varied concentrations of ATA and a saturating concentration of substrates. The biphasic inhibition (Figure 2) was highly reproducible, therefore, the result was not attributable to experimental errors. Indeed, it was even more prominent when the substrate concentration of $NADP^+$ was increased from 0.15 mM (Figure 2) to 0.75 mM (Figure 3A, 4A). Dixon plots of these data (insert of Figure 2) indicated that more than one ATA molecule was bound per enzyme molecule.

3. The Johnson Eyring Plot

Figures 3B, 4B and 5 were plotted from the data of Figures 3A, 4A and 2 as described by Johnson, Eyring and Williams (29). Accordingly, the slope of the plot of log $\frac{V_0}{V_I} - 1$ versus log ATA gives the number of the ligand molecules combining with the enzyme. V_0 and V_I are the velocities with and without ATA (Figures 3B, 4B and 5). Again, the plot was biphasic. These data suggested that at lower concentrations of ATA, one molecule of ATA was bound per molecule of enzyme, thus yielding a straight line with a slope of about one. Within this concentration of ATA, initial velocity versus varied ATA concentrations gave lines of rectangular hyperbolas as expected from first order kinetics. However, at higher concentrations of ATA, the inhibition curves were sigmoidal, suggesting that more than one molecule of ATA per enzyme molecule participated in the formation of the enzyme inhibitor complex. Thus, at higher concentrations of ATA, Johnson Eyring plots gave a straight line with a slope of about 2.

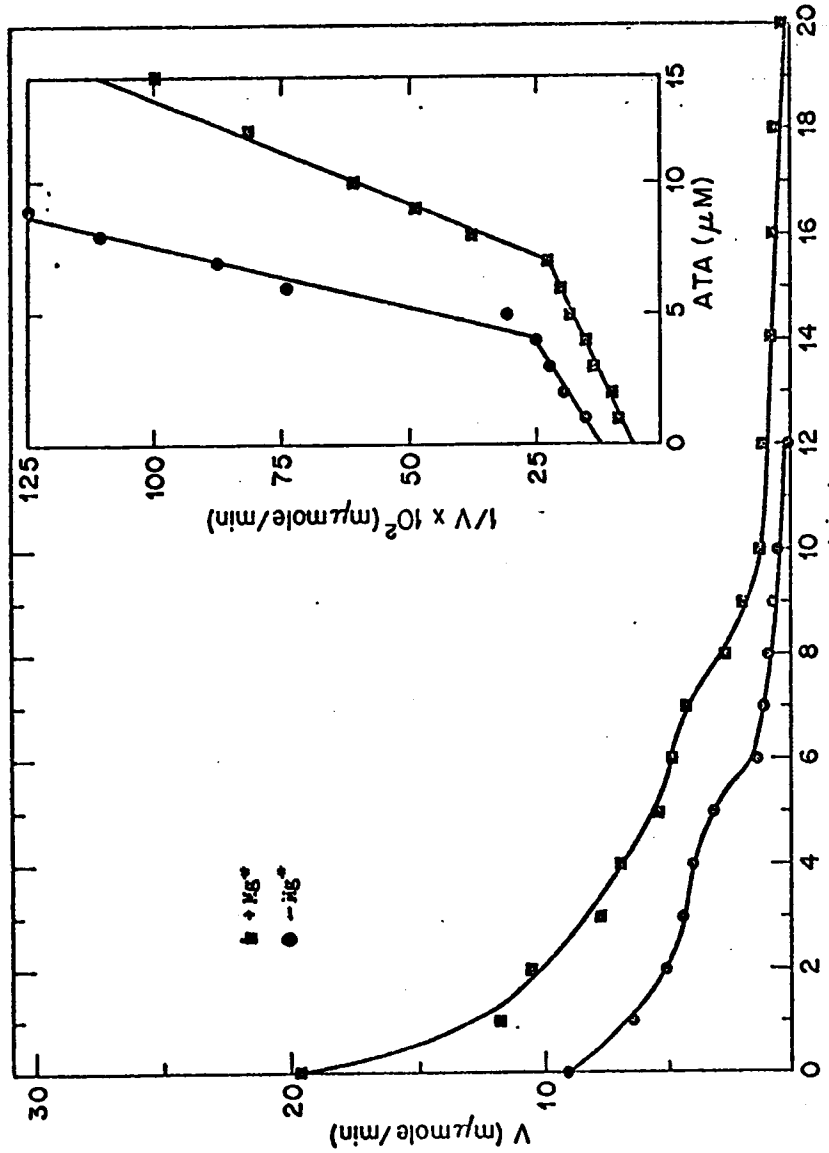


Figure 2. The Dixon Plot of the Inhibition of G6P Dehydrogenase by ATA.

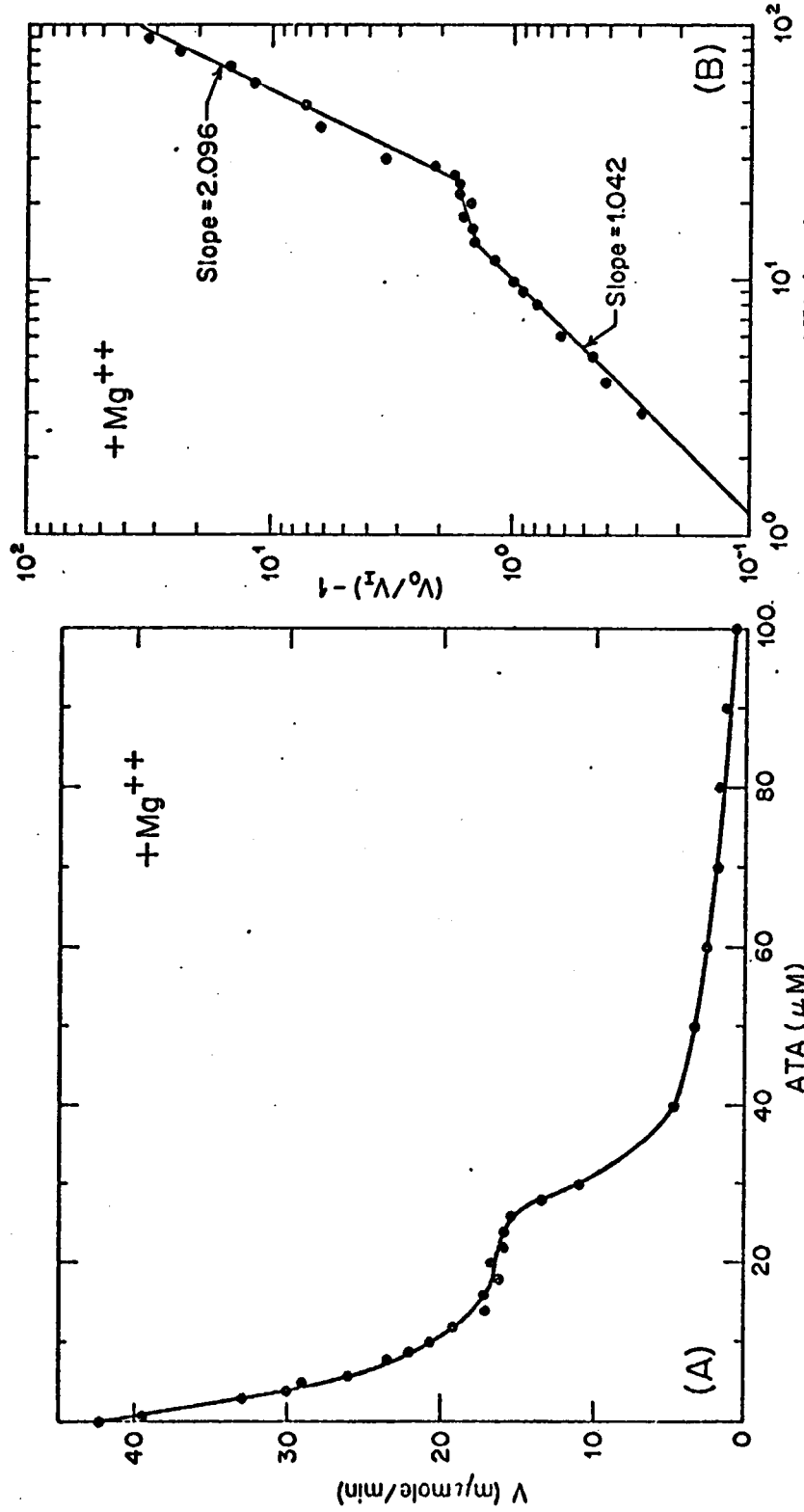


Figure 3. The Effect of ATA on (A) the Velocity of G6P Dehydrogenase and (B) Johnson-Eyring Plots in the Presence of Mg^{++}.

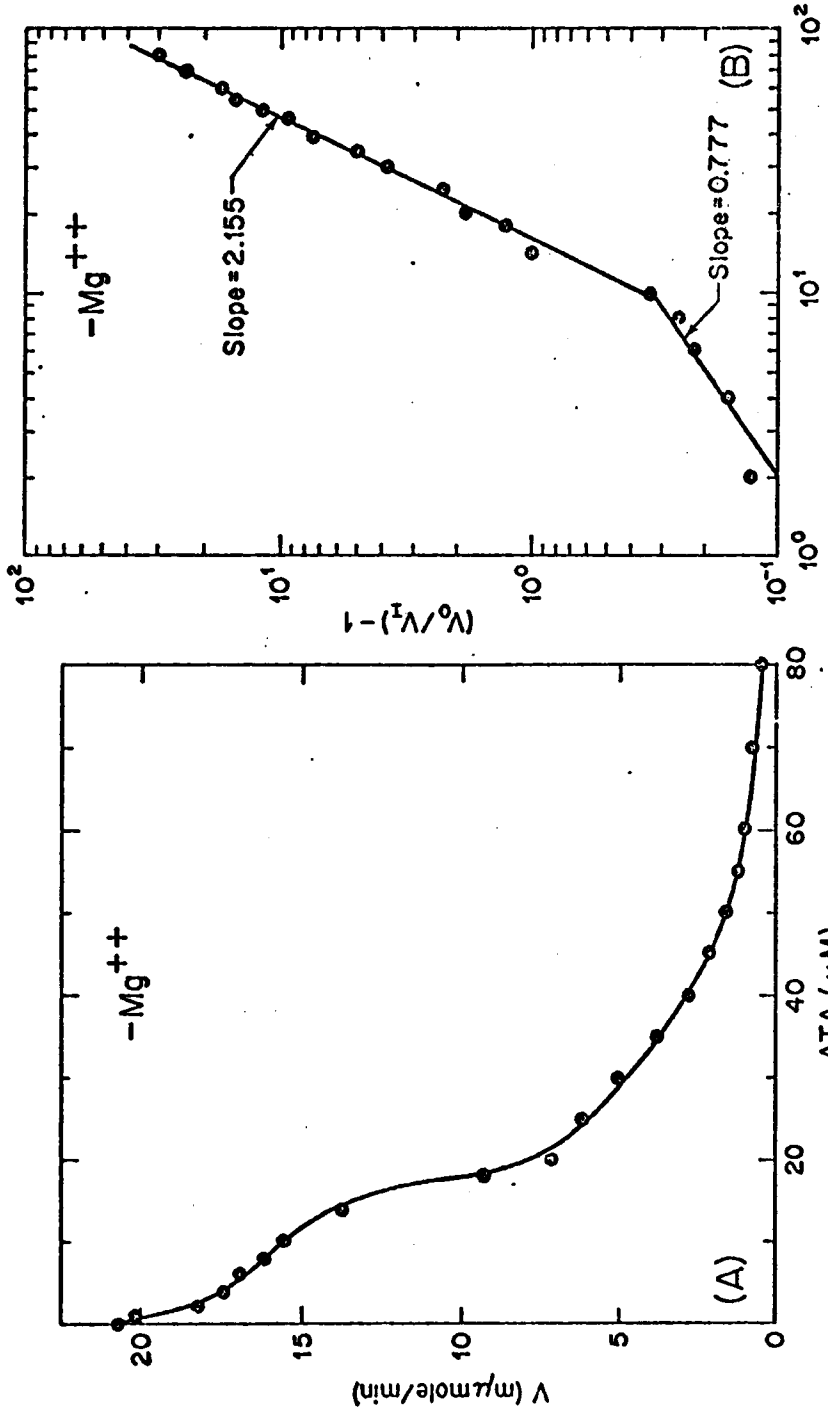


Figure 4. The Effect of ATA on (A) the Velocity of 3GP Dehydrogenase and (B) Johnson-Eyring Plots in the Absence of Mg^{++}.

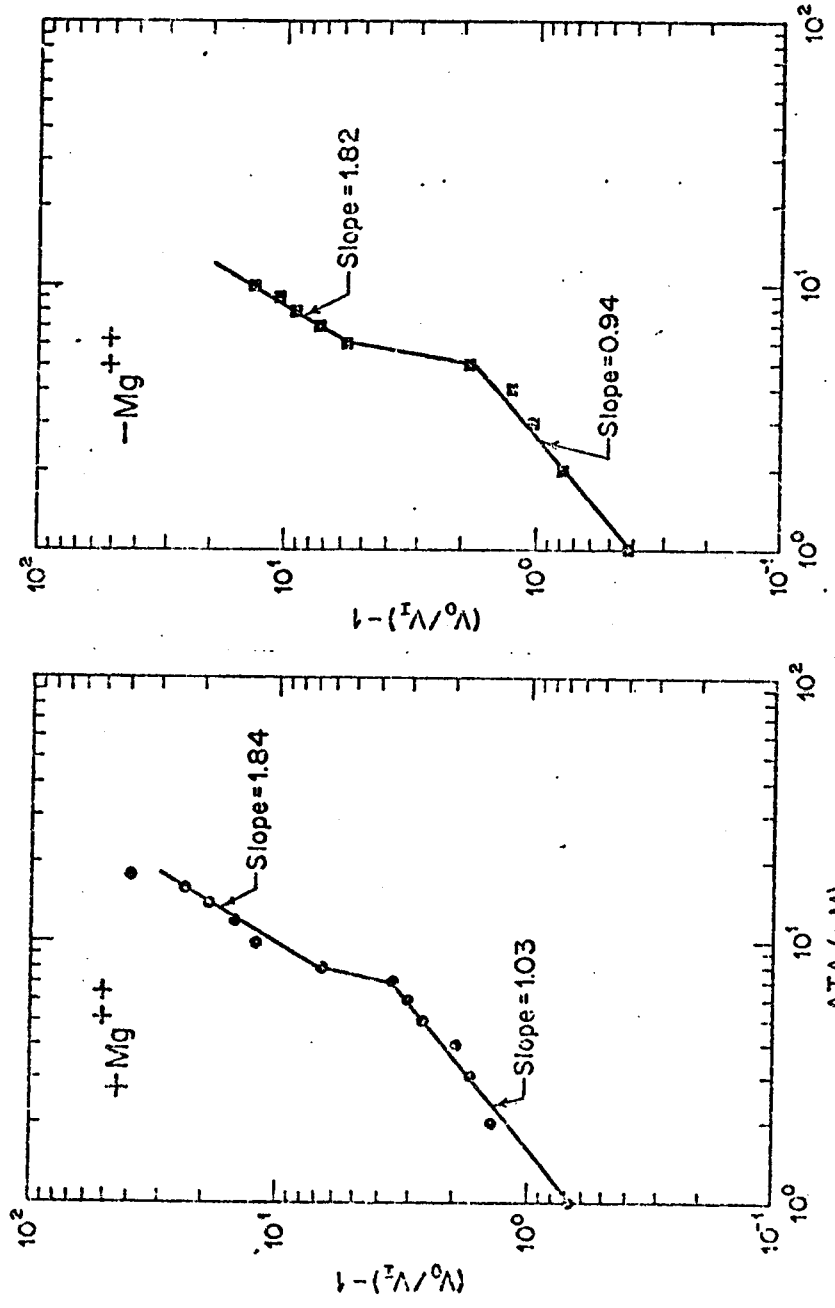


Figure 5. The Johnson-Eyring Plots of the Inhibition of G6P Dehydrogenase by ATA.

It is noteworthy that higher concentrations of ATA were required to initiate the sigmoidal curve in the presence of Mg^{2+} than in its absence. This observation appears consistent with the Mg^{2+} induced increase in Ki for ATA with either substrate (Figure 1).

B. Specificity of Inhibition of G6P Dehydrogenase by ATA

In order to investigate the degree of specificity by which G6P dehydrogenase was inhibited by ATA, several enzymes in coenzyme linked dehydrogenase systems were tested for their inhibition by ATA. Concentrations of ATA ranging from 10^{-6} M to 10^{-3} M were employed for inhibition studies. The results are shown in Table II. Alcohol dehydrogenase from horse liver and malic dehydrogenase from pig heart which required NAD^{+} as coenzyme were not affected by ATA. Xanthine oxidase, a dehydrogenase requiring no coenzyme, was inhibited by ATA, with a Ki of 2.6×10^{-5} M. Although ATA inhibited the activity of G6P dehydrogenase by competing with the glucose 6-phosphate substrate, ATA did not affect the activity of phosphoglucomutase from either yeast or rabbit muscle, which catalized the conversion of glucose 1-phosphate to glucose 6-phosphate.

It was noteworthy that plasma alkaline phsophatase from a rat was reported to be 50% inhibited by ATA at a concentration of 1×10^{-3}M (5), but as shown here, alkaline phosphatase from E. coli was unaffected.

TABLE II

EFFECT OF ATA ON VARIOUS ENZYMES

Enzymes	Source	Cofactor	ATA Inhibition	Ki (μM)	Subunit
Glucose 6 Phosphate Dehydrogenase	Yeast	TPN	+	1.32 2.50	2
Xanthine Oxidase	Milk	---	+	26.2	4
Phosphoglucomutase	Yeast	---	-	----	1
	Rabbit Muscle	---	-	----	1
Alcohol Dehydrogenase	Horse Liver	DPN	-	----	2
Malic Dehydrogenase	Pig Heart	DPN	-	----	2
Alkaline Phosphatase	E. coli	---	-	----	2

C. Effect of pH on Inhibition of G6P Dehydrogenase by ATA

Initial velocity experiments were performed in 0.05 M Tris-acetate buffers with pH ranging from 5.0 to 9.5. Altogether twelve experiments were performed, each composed of duplicate assays at six concentrations of G6P. The kinetic constants obtained from this series of experiments are summarized in Table III and ploted in Figures 6 and 7 according to the procedure outlined by Dixon and Webb (30). Although anions affected the catalytic activities of G6P dehydrogenase (31), CH_3COO^- up to 0.05 M concentrations did not affect Km and Vmax.

The double reciprocal plots of the pH studies were linear and gave lines which intersected on the ordinate above the baseline.

Dixon (30) gave a general treatment with a number of rules for interpreting the data. His treatment relates to Km, but applies equally to Ki for inhibitors. Dixon pointed out that if a graph of pKm (- log Km) is plotted against pH the graph of pKm will consist of a straight line section joined by short curved parts. Each bend indicates the pK of an ionizing group in one of the components (free enzyme, ES complex or substrate). Each pK of a group situated in the ES complex produces an upward bend, a bend with the concave side upwards. Each pK of a group situated in either the free enzyme or the free substrate produces a downward bend.

In our studies, a plot of pKm on pH (Figure 6), revealed bends at pH 6.0, 7.9 and 8.4, indicating three ionizing groups in this system. The upward bends of pK 6.0 and 7.9 were due to the ionizing group of ES complex while the downward bend of pK 8.4 was

TABLE III

KINETIC CONSTANTS AT VARIOUS pH VALUES

pH	Vmax m M/min.	G6P Km (μM)	G6P Ki (μM)
5.0	5.25	66.0	0.083
5.5	6.66	31.2	0.108
6.0	8.33	20.2	0.086
6.5	11.1	19.0	0.100
7.0	14.3	22.0	0.136
7.5	19.88	19.2	0.235
8.0	50	34.0	0.360
8.5	66.6	500	0.700
9.0	66.6	770	0.700
9.5	66.6	770	0.700

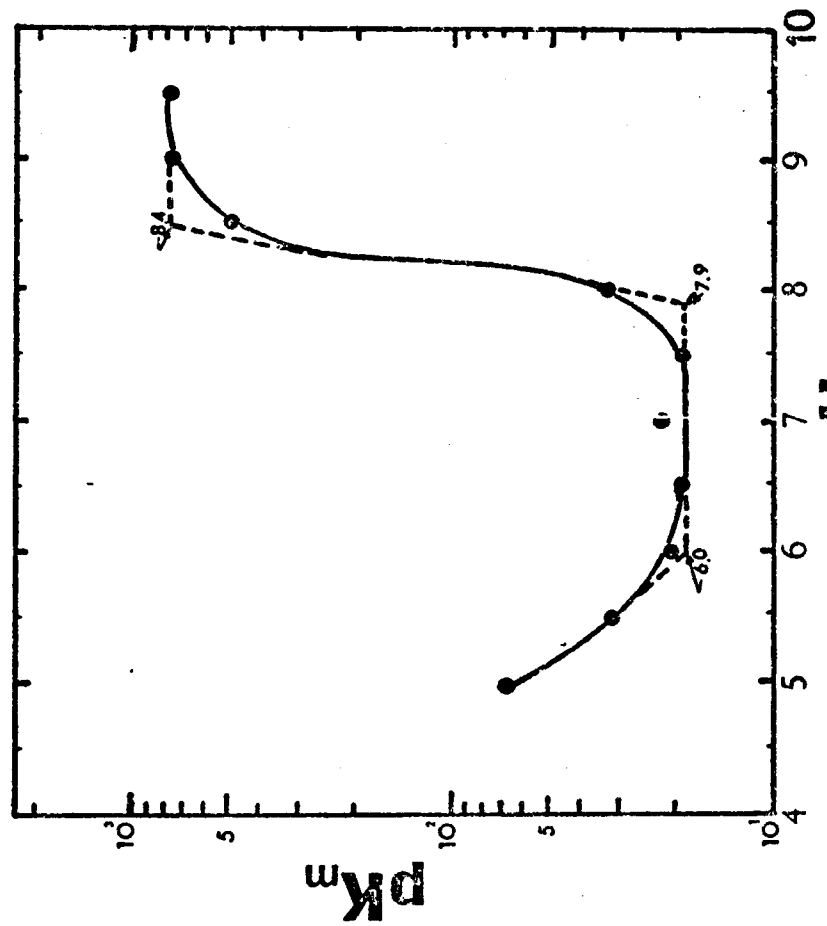


Figure 6. The Effect of pH on pK_m of G6P Dehydrogenase.

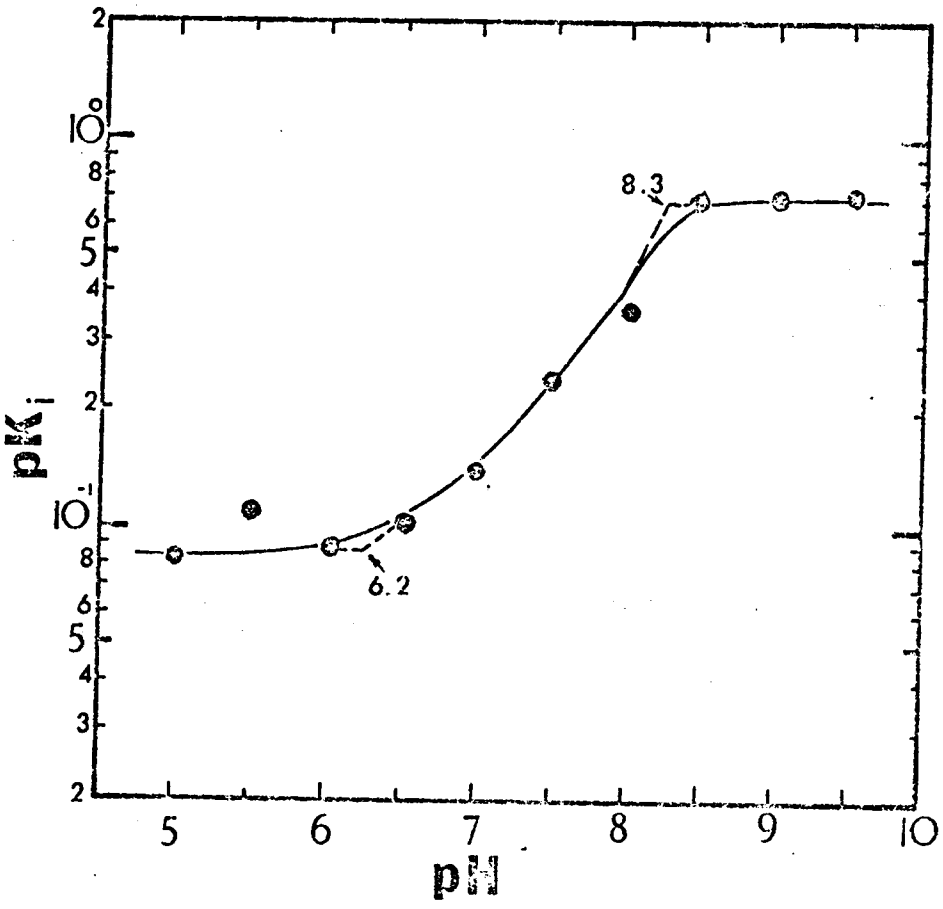


Figure 7. The Effect of pH on pK_i of G6P Dehydrogenase.

assumed to be situated in either the free enzyme or the free substrate. The pK values of the substrate glucose 6-phosphate occur at 0.9 and 6.13 (32), therefore, the pK of 8.4 belonged to the ionizing group of free G6P dehydrogenase.

Two bends occurred in the plot of pKi on pH (Figure 7). One was an upward bend with pK of 6.2, presumably belonging to the ionizing group of ES enzyme, while the other was a downward bend with pK of 8.3 apparently situated in free G6P dehydrogenase. The glucose 6-phosphate and ATA were probably competing for binding to G6P dehydrogenase at an active center which had an ionizing group with a pK of approximately 8.3 or 8.4. These observations suggested that the group involved in binding of substrate was a sulfhydryl of cysteine which has a pK of 8.3.

D. Effect of p-OH-Mercuribenzoate on Inhibition of G6P Dehydrogenase by ATA

The pH studies of ATA inhibition on G6P dehydrogenase suggested that a group on the enzyme with pK = 8.4 (Figure 6) or pK = 8.3 (Figure 7) was involved in binding glucose 6-phosphate. Since the sulfhydryl of cysteine falls in this range, we tested the effect of p-hydroxymercuribenzoate (PHMB) on the activity of G6P dehydrogenase. The results, presented in Figure 8, showed that PHMB inhibited G6P dehydrogenase instantly. Ki was 7.1×10^{-5} M. PHMB, like ATA, also competitively inhibited G6P dehydrogenase when glucose 6-phosphate was varied substrate and $NADP^+$ kept at a constant level. However, in the presence of both ATA (10^{-6} M) and PHMB (10^{-4} M), the

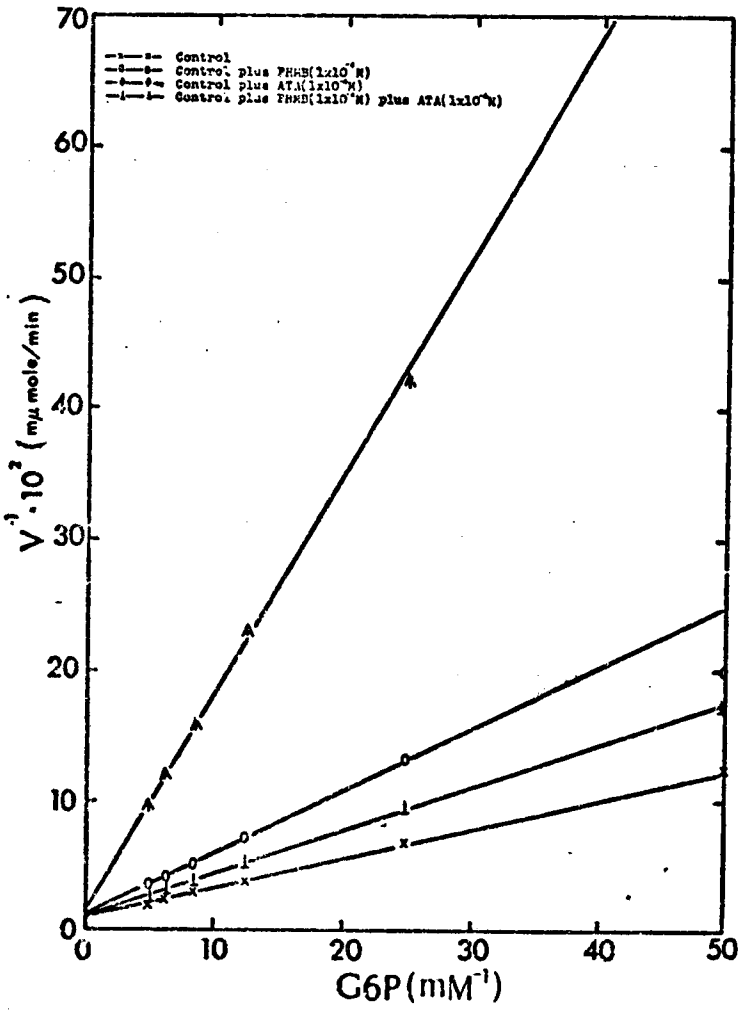


Figure 8. The Double Reciprocal Plots of the Inhibition by PHMB and ATA.

degree of inhibition was less than inhibition by either one individually.

Reversal of ATA (10^{-6} M) and PHMB (10^{-4} M) inhibition by cysteine was investigated in the absence of Mg^{2+}. The results are presented in Figure 9. Inhibition of G6P dehydrogenase by PHMB was completely reversed in two hours by incubating cysteine (10^{-3} M) with PHMB enzyme complex. Inhibition of G6P dehydrogenase by ATA was slowly reversed by cysteine. In three hours incubation with cysteine, inhibition by ATA was reduced from 90% to 65%.

E. Effect of Temperature on Inhibition of G6P Dehydrogenase by ATA

Determination of thermodynamic constants were made by studying the effect of temperature on kinetic constants in the presence of varying concentration of ATA.

The data obtained is presented in Table IV and Figures 10 through 14. The following features appear noteworthy.

(1) At low temperature, the curves were sigmoid. At intermediate temperatures, the curves appeared to possess both rectangular hyperbolas and sigmoid curves.

(2) At ATA concentrations up to 4 μM inhibition decreased with increase in temperature. However, at high ATA concentration no such simple relationship was apparent.

(3) As seen earlier, ATA was more inhibitory in the absence of Mg^{++}.

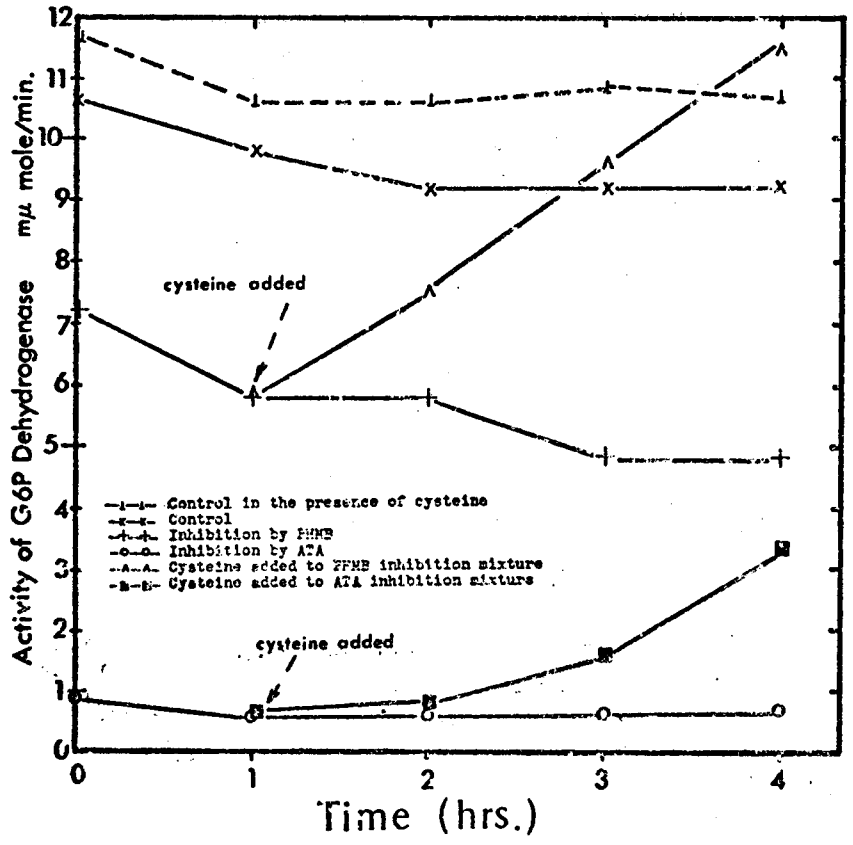


Figure 9. The Reversible Inhibition of ATA and PHMB by Cysteine.

TABLE IV

KINETIC AND THERMODYNAMIC PROPERTIES FOR OVERALL ASSOCIATION REACTIONS

$$E + nATA \rightleftharpoons E.ATA_n$$

ΔH = -10.75 Kcal when n = 1; ΔH = -18.30 Kcal and -32.03 Kcal when n = 2 in the presence of Mg^{++}
ΔH = -7.69 Kcal when n = 1; ΔH = -10.51 Kcal and -23.79 Kcal when n = 2 in the absence of Mg^{++}

Temperature	Association Constant K		ΔF Kcal/mole		ΔS Cal/mole/degree	
	$+Mg^{++}$	$-Mg^{++}$	$+Mg^{++}$	$-Mg^{++}$	$+Mg^{++}$	$-Mg^{++}$
19°C	n = 0.90 $K = 3.16 \times 10^5$	n = 0.93 $K = 3.98 \times 10^5$	-319	-3.24	-25.9	-15.2
25°C	n = 0.90 $K = 2.14 \times 10^5$	n = 0.93 $K = 3.19 \times 10^5$	-3.15	-3.26	-25.6	-17.2
	n = 1.55 $K = 7.99 \times 10^8$		-5.03		---	
34°C	n = 0.90 $K = 1.25 \times 10^5$	n = 0.93 $K = 2.3 \times 10^6$	-3.11	-3.26	-24.4	-14.4
	n = 2.2 $K = 1 \times 10^{12}$	n = 2.2 $K = 2.89 \times 10^{12}$	-7.32	-7.60	-80.5	-49.4
38°C	n = 2.2 $K = 2.24 \times 10^{12}$	n = 2.2 $K = 1.67 \times 10^{13}$	-7.59	-8.17	-79.3	-50.2
42°C	n = 2.2 $K = 1.36 \times 10^{12}$	n = 2.2 $K = 1.1 \times 10^{13}$	-7.59	-8.17	-79.3	-50.2

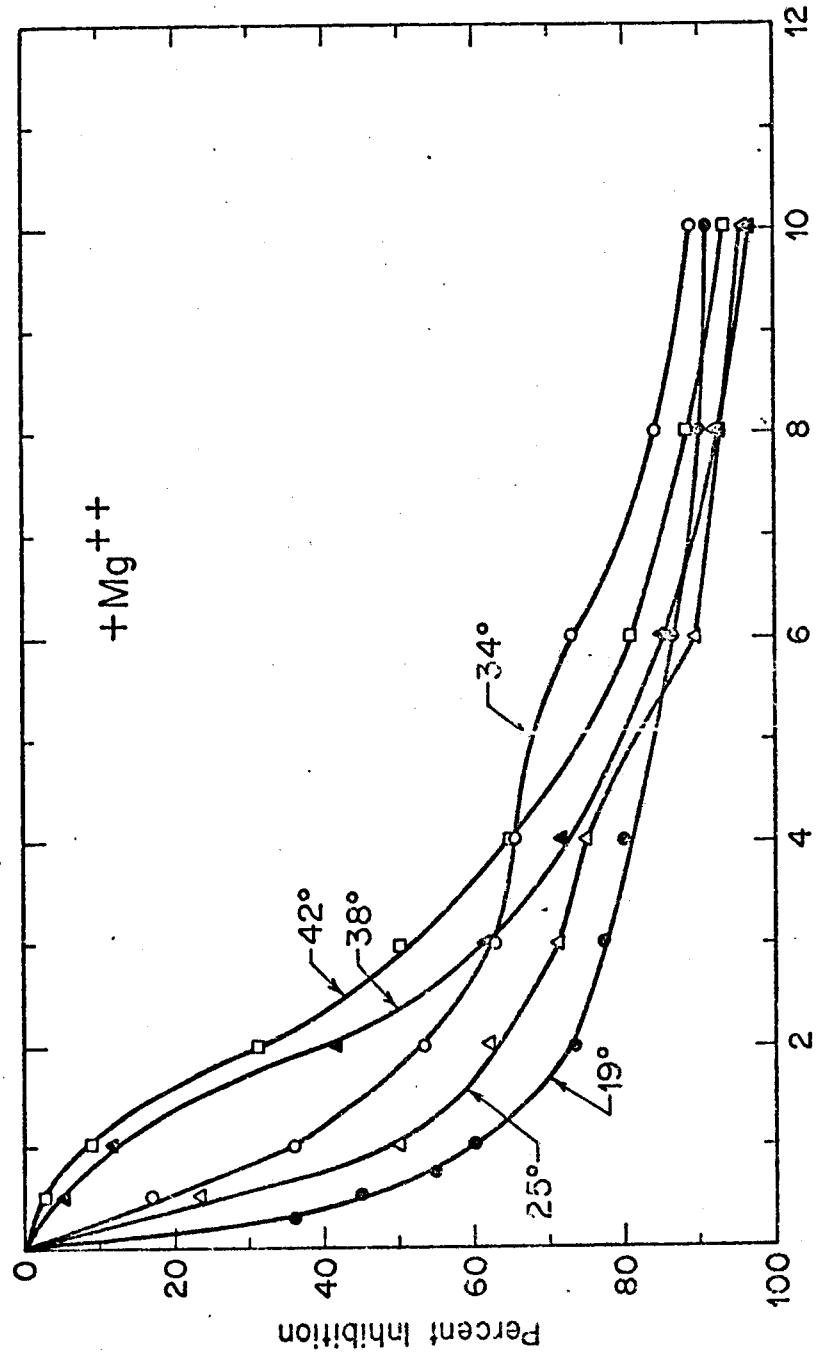


Figure 10. The Effect of Temperature on Percent Inhibition of G6P Dehydrogenase by ATA in the Presence of Mg^{++}.

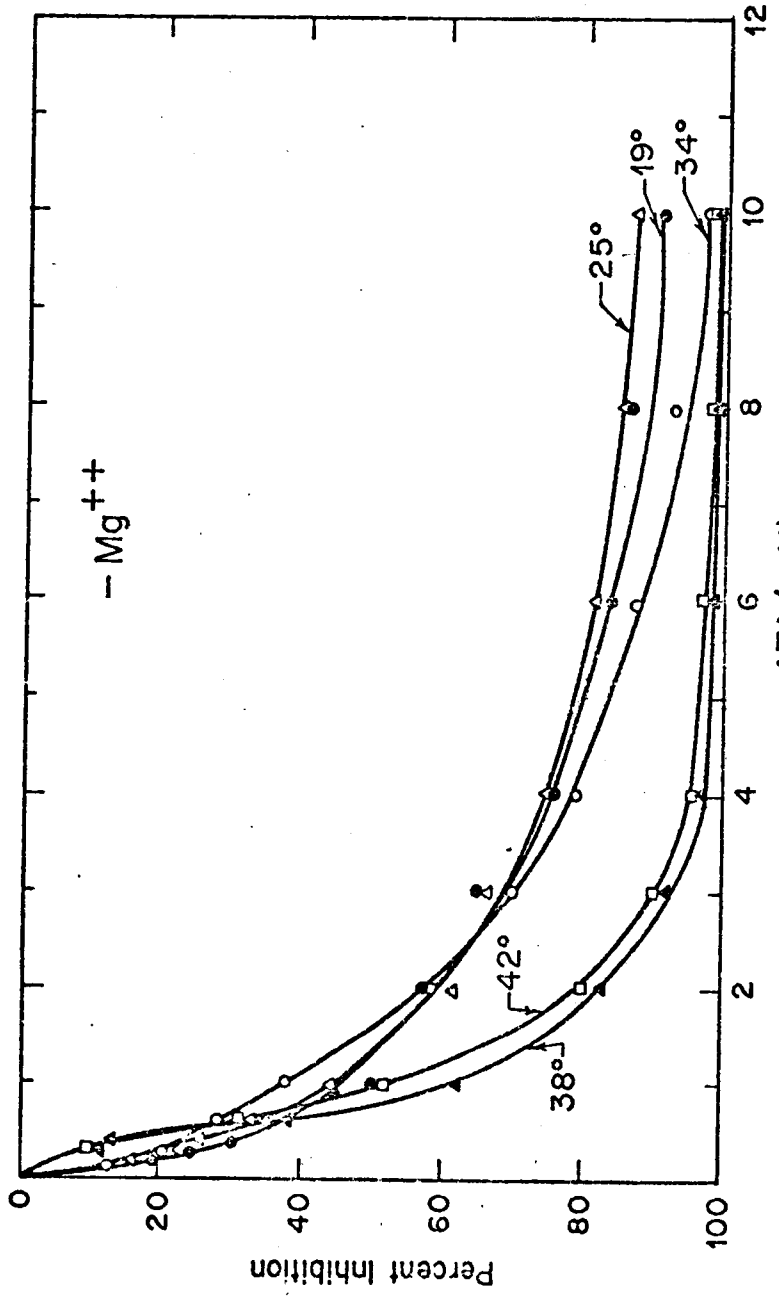


Figure 11. The Effect of Temperature on Percent Inhibition of G6P Dehydrogenase by ATA in the Absence of Mg^{++}.

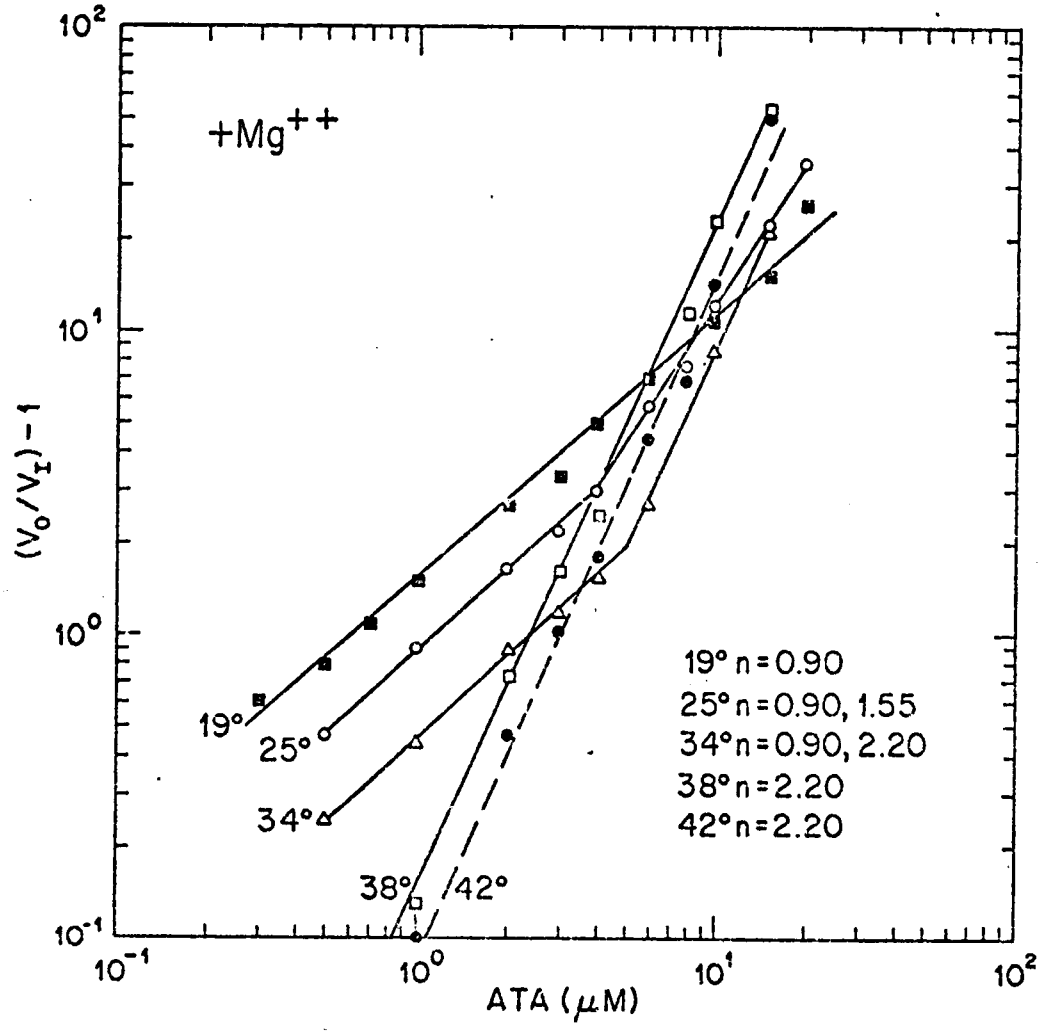


Figure 12. The Effect of Temperature on the Inhibition of G6P Dehydrogenase by ATA (Johnson-Eyring Plots) in the Presence of Mg^{++}.

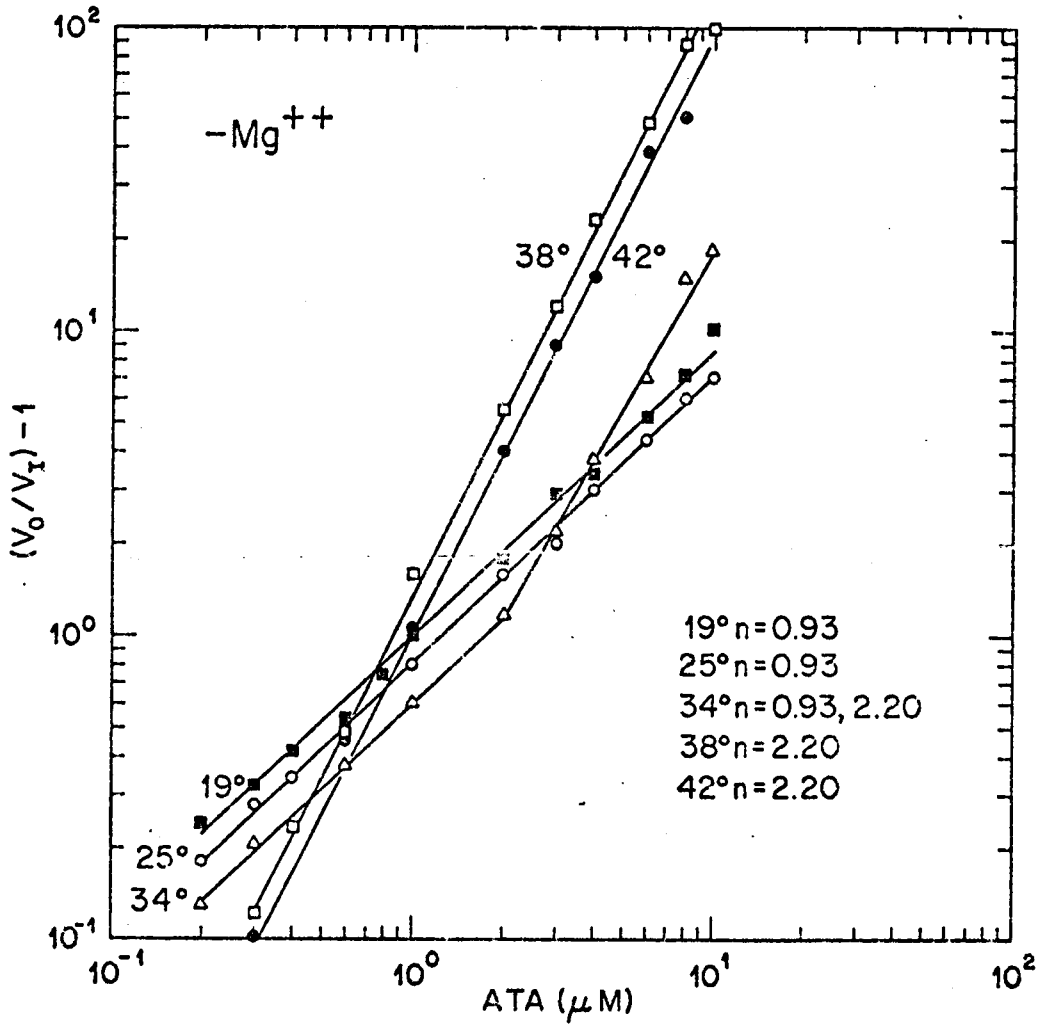


Figure 13. The Effect of Temperature on the Inhibition of G6P Dehydrogenase by ATA (Johnson-Eyring Plots) in the Absence of Mg^{++}.

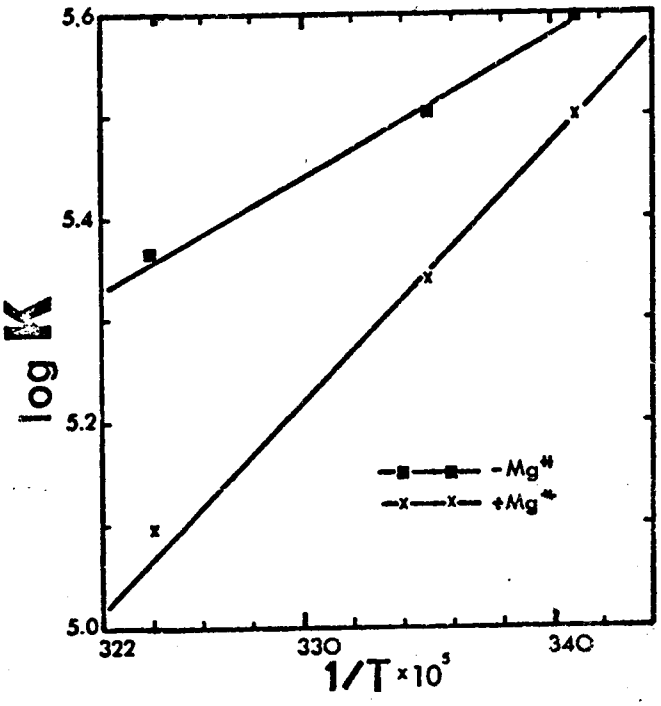


Figure 14. A Plot of log K against 1/T.

From these data, the association constants (K) and changes of free energy (ΔF), enthalpy (ΔH) and entropy (ΔS) for the reaction of ATA inhibition on G6P dehydrogenase were determined. The results are summarized in Table IV. The association constant for the binding of enzyme and ATA was determined from Figures 11 and 12, which showed plots of $\log\left[\frac{V_0}{V_I} - 1\right]$ versus $\log [ATA]$. From the overall reaction

$$E + I \rightleftharpoons EI, \quad \log K = \log [EI]/[E] - \log [I]$$

At equilibrium, when $\log I = 0$, $\log K = \log ([EI]/[E])$, which is measured by $\log\left[\frac{V_0}{V_I} - 1\right]$. Thus, in the plot of $\log [V_0/V_I - 1]$ versus $\log[ATA]$, the intercept where $\log [ATA] = 0$ is the value of $\log K$.

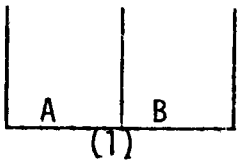
The plots of the log K values against 1/T were straight lines (Figure 14). According to Taketa and Pogell (33), the molecular enthalpy change ΔH = - slope x 2.303R where R is the gas constant. The changes in free energy and entropy were calculated from the equations $\Delta F = -2.303R\ T \log K$ and $\Delta S = (\Delta H - \Delta F)/T$.

F. Spectral Studies of Enzyme-ATA Complex

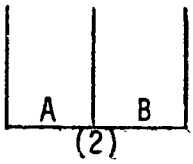
From the studies reported so far it became apparent that the binding of ATA to the enzyme was dependent not only on the concentration of the inhibitor, but also on the concentration and the species of anions present in the environment. Since such binding

was reversible it seemed reasonable that the conformational change, if any, caused by the inhibitor could also be reversible. In order to get some insight into the binding site and conformational changes, the technique of difference spectroscopy was used. Although it was realized that such studies would be limited by the quantities of enzyme available, some preliminary experiments conducted are reported below.

In a typical procedure, two double sector cells were used as shown in the following figure. In each compartment,



Reference Cell



Sample Cell

A contained enzyme and buffer while B contained reagents to be mixed with the contents of A to elicit conformation change. After the reference spectrum of these identical cells were recorded, the contents of sample cell (2) were mixed and the difference spectrum of mixed against unmixed control was recorded. Accordingly, ATA (10^{-5} M) and G6P (20 mM) or $NADP^{+}$ (1.5 mM) had no detectable reaction, whereas ATA (10^{-5} M) and Mg^{++} (10^{-2} M) showed time dependent difference spectrum, notably in the visible region (Figure 15).

To investigate the possible difference spectrum between ATA and enzyme, the molar ratios of ATA/enzyme at 10, 20, and 100 were studied. 1.2 mg of G6P dehydrogenase (5.92×10^{-9} M) was used. Sample and reference cuvetts both contained 0.05 M Tris-buffer at pH 7.5.

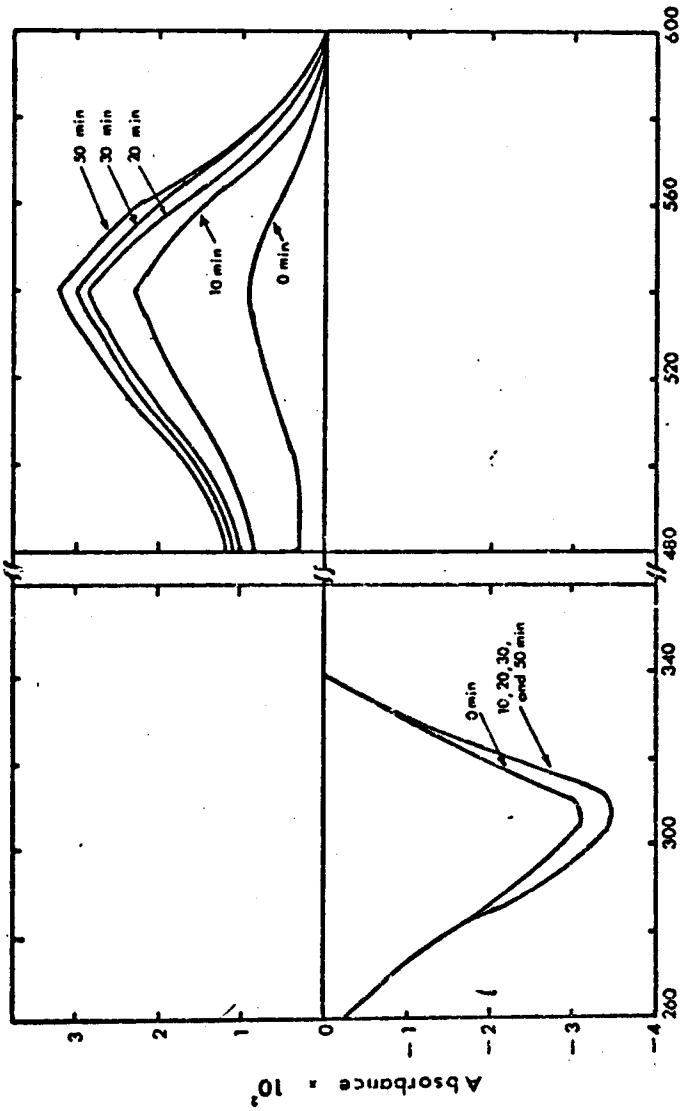


Figure 15. Difference Spectra between the Mg^{++}-ATA Complex and Free ATA plus Free Mg^{++}.

ATA and enzyme were injected separately into two cells of each sample and reference cuvetts. Sample cuvetts were mixed and read in a Cary 15 spectrophotometer against reference cuvetts which were not mixed. No difference in spectra were observed at molar ratios of 10 or 20 between ATA and enzyme. Very slight negative difference spectra at 280 mμ were observed at a molar ratio of 100 between ATA and enzyme (Figure 15). When $NADP^+$ (4.5×10^{-5} M) was added to the cell of the reference cuvett containing only enzyme and to the sample cuvett containing the ATA-Enzyme mixture, a stronger negative absorbance and spectrum at 275 mμ was obtained (Figure 16). Additional $NADP^+$(7.5×10^{-5}) resulted in a further shift of the difference spectra to 265 mμ. When $NADP^+$ was kept constant at this concentration and ATA was increased from the 1:100 molar ratio of E/ATA to 1:400, 1:800 and 1:1600, the optical density of negative absorbance increased accordingly though not proportionally. The concentration of enzyme was kept constant throughout this experiment.

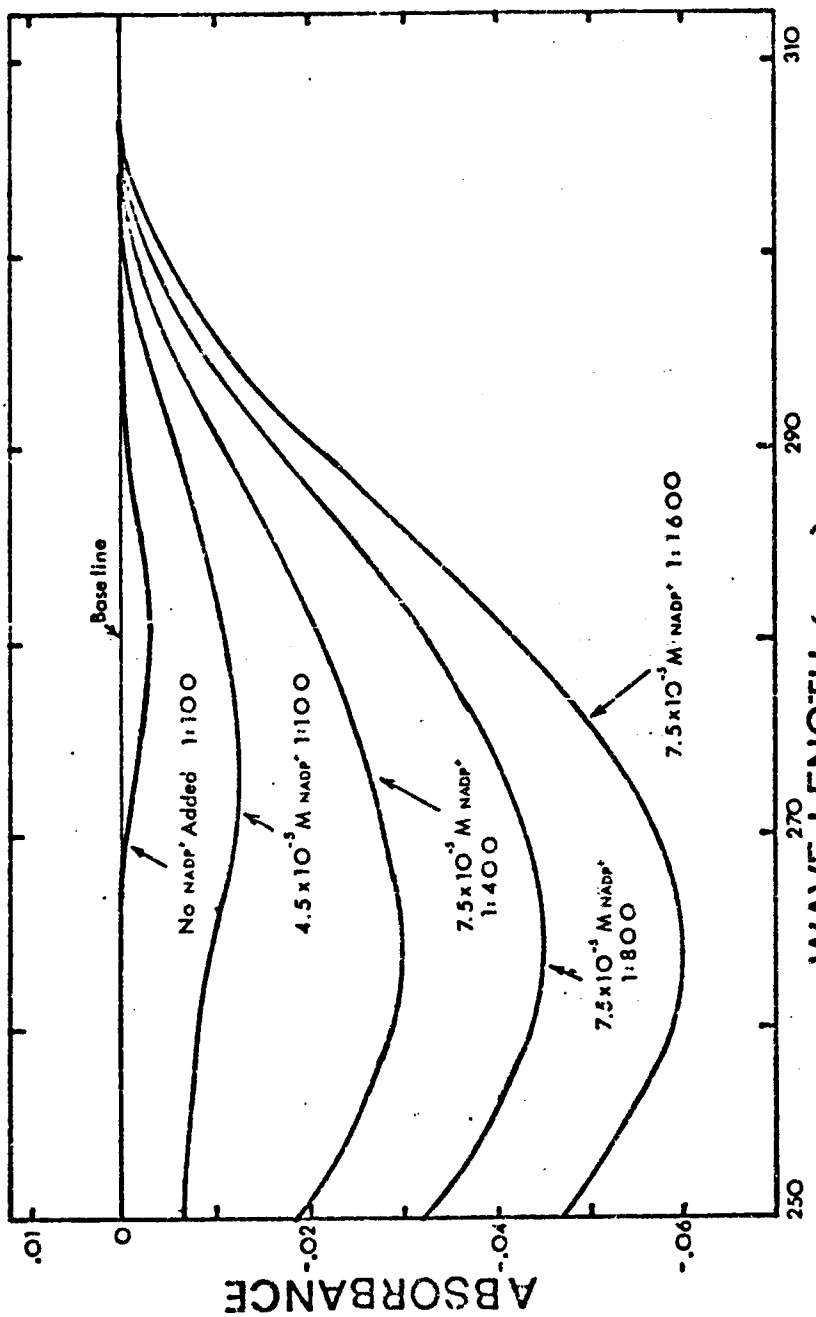


Figure 16. Difference Spectra between the G6P Dehydrogenase-ATA Complex and Free ATA plus Free Enzyme.

CHAPTER IV

DISCUSSION

Results presented here show that the inhibition of yeast G6P dehydrogenase by ATA is "competitive" with respect to G6P and "mixed" with respect to $NADP^+$ (Figure 1). The number of ATA molecules bound per enzyme molecule vary with the concentration of the inhibitor. Indeed, biphasic Johnson Eyring plots (Figure 2) and sigmoidal Dixon plots (Figure 2) indicate that ATA is an allosteric inhibitor where the first molecule bound creates the binding site for the second.

As stated before (page 6), G6P dehydrogenase from a variety of sources was shown to be a polymeric enzyme (17) where NADP was required for catalytic activity as well as for the stability of polymeric structure. Indeed, Luzzatto (34) using kinetic data showed that human erythrocyte G6P dehydrogenase had a total of four $NADP^+$ binding sites. Only two of these were required for catalytic activity, whereas all four were required to stabilize the protein against dissociation during sedimentation by ultracentrifugation (17). If a similar phenomenon is assumed for the yeast enzyme, differential binding of ATA molecules becomes plausible. It appears that ATA serves as an enzyme modulator at two different functional levels, each responsive to a different concentration of the inhibitor. Thus, inhibitor binding to the G6P substrate site at the lower concentration induces the first conformational change which in turn results in exposure of the second modulator binding site. When ATA binds to the

second modulator site, there is a second conformational change resulting in a sharp decrease of binding affinity of the enzyme for $NADP^+$.

One might argue that the second ATA binding site was there all the time, not due to the conformational change but due to its lower affinity for ATA. In such a case the second molecule of ATA would not bind until the concentration of ATA was high enough to saturate the first site. The association constants between ATA and enzyme calculated from kinetic data of Figures 12 and 13 argue against this effectively. The K values were much higher when two molecules of ATA were bound per one molecule of enzyme than when one molecule of ATA was bound per one molecule of enzyme. These were in the order of 10^{12} and 10^5 respectively. This indicates that the second ATA binding site was nonexistent before ATA was introduced into the reaction mixture. If the two ATA binding sites were independent of each other, Johnson Eyring plots would be linear rather than biphasic.

Further support in favor of the ATA induced conformational change in the protein molecule comes from the difference spectroscopy studies between ATA-enzyme complex and free ATA. Difference spectra of ATA and enzyme at concentration ratios of 100 to 1 show a negative absorbance at 280 mμ. This indicates that aromatic amino acids are being shielded by the conformational change of the protein. In the presence of $NADP^+$ there is a shift of negative absorbance from 280 mμ toward 265 mμ. This could be interpreted as the folding in of the adenine group in $NADP^+$ caused by the conformational change of the enzyme.

Grollman et al. (13) showed that ATA can induce conformational

changes in ribosomes. In the presence of ATA the sedimentation coefficient of single ribosomes from E. coli increased from 70S to 78S. Admittedly, in this work more definitive evidence for the conformational changes could be obtained by sedimentation equilibrium studies under a variety of conditions. However, these could not be done due to an insufficient amount of enzyme available.

At 19°C, the number of ATA molecules bound per enzyme molecule was one, regardless of the ATA concentration, indicating that unfolding of the second site for ATA binding was not only ATA dependent but also temperature dependent. At temperatures of 38 and 42°C the affinity of the protein for the second ATA molecule is ATA independent. The number of ATA moles bound per one mole of enzyme is two as soon as the low concentration of ATA is added (Figures 12 and 13).

Entropy change of the enzyme at 19°C was -25.9 and -15.2 calorie/mole/degree in the presence and absence of Mg^{2+} respectively, compared to 79.3 and -50.2 calorie/mole/degree at 42°C. The large increase in entropy was indicative of the conformational change due to increase in temperature. The data of Roughton, Otis and Lyster (35) for the overall reaction, hemoglobin + $4O_2$ → hemoglobin $(O_2)_4$, indicated a total entropy change of -159 calorie/mole/degree. Considering the fact that only two molecules of ATA were bound to a molecule of enzyme, our entropy change in the presence of Mg^{+2} (79.3 calorie/mole/degree) is comparable to their data which was for binding four molecules of O_2 per one molecule of hemoglobin.

Requirement for a free SH group for the catalytic activity of this enzyme from diverse origins was reported earlier. Thus DeMoss

et al. (38) showed involvement of an SH group in the active site of G6P dehydrogenase from erythrocyte. During the purification procedure, a portion of this enzyme was spontaneously transformed from the native "fraction I" (Km = 81 μM) to a "fraction II" having different kinetics (Km = 272 μM). The conversion of fraction I and II was reproduced easily by treatment with iodosobenzoate, and fraction II was converted back to fraction I by treatment with dithioglycol. They suggested that the oxidation-reduction of sulfhydryl group was involved in the interconversions.

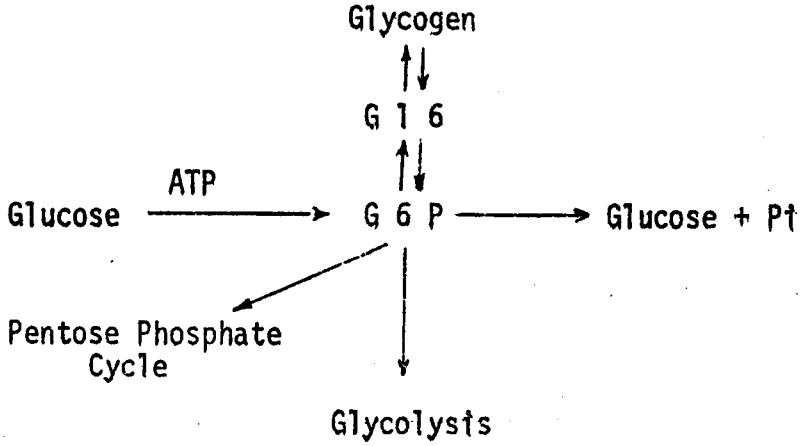
Involvement of an SH group in G6P dehydrogenase from yeast was indicated by the inhibition of the enzyme in the presence of P-OH mercuribenzoate (PHMB). Also, double reciprocal plots (Figure 8) showed that PHMB was competitive with respect to glucose 6-phosphate and ATA. Inhibition by either ATA or PHMB was reversed (Figure 9) at least partially by addition of cysteine.

Other evidence supporting the involvement of an SH group in the binding of substrate to the enzyme was the sharp increase in Km value at pH 8.5 or above (Table III). Since the pK of the thiol group of crysteine is 8.3 it is possible that an increase in the S^- species as pH increased above the ionization constant resulted in favorable conditions for s^- to oxidize to the disulfide linkage S - S. This might impose constraints on the conformation of the molecule, reducing in turn the affinity of the active site for glucose 6-phosphate. Such an explanation appears in accord with Babalola, et al. (38). While they could easily convert high affinity species to low affinity species of enzyme by means of an oxidizing agent (iodosobenzoate) and

a reducing agent (dithioglycol), we were able to demonstrate this conversion by means of protonation of the SH group at low pH and deprotonation of the SH group at high pH.

With the exception of phosphoglucomutase all the enzumes tested (Table II) were sulfhydryl enzymes (40-44). However, only two, namely milk xanthine oxidase and yeast G6P dehydrogenase, were inhibited by ATA which indicates that ATA may not be useful as a general "SH blocking agent." However, the fact that the latter was inhibited by very low concentrations of ATA ($K_i = 10^{-6} \sim 10^{-7}$) suggests that an enzyme with a specific steric structure is necessary to accommodate ATA.

As mentioned in Chapter I, ATA was able to reverse the toxicological properties of Be^{++} in the mouse (4). Considering the importance of G6P dehydrogenase as a key enzyme involved in the Embden-Meyerhof-Parnas pathway of glycolysis and through the pentose phosphate cycle, our findings that ATA is a potent inhibitor of G6P dehydrogenase makes it questionable that ATA could be used in biological systems for antitoxic purposes.



However, there was a dramatic demonstration conducted by a group of scientists in Argonne National Laboratory (39). They injected mice with enough beryllium salt to kill them with a few days, then injected half the animals with a small dose of ATA and left the others untreated. The results were significant: Virtually every animal treated with ATA survived and lived normally, while all of the untreated animals died. This experiment was repeated with hundreds of animals of different species with the same degree of protection. These observations raised the following questions concerning our observations:

(1) While ATA inhibits G6P dehydrogenase *in vitro*, does it inhibit the enzyme *in vivo*?

(2) While ATA inhibits G6P dehydrogenase from yeast, does it inhibit the enzyme from other species?

(3) Is the inhibition of xanthine oxidase significant? This enzyme contains FAD, MO^{+5}, Fe^{+2} and acid labile sulfide as integral part of the enzyme. All of these are involved in the electron transfer from xanthine to O_2. Since it can oxidize a variety of purines (45, 46) and aldehydes (47), it is considered of importance in interconversion of nucleic acids. What is the mechanism of inhibition of xanthine oxidase by ATA?

These questions constitute a good research area for future investigations.

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