THE EFFECT OF PROTEIN AND HYDROGEN ION CONCENTRATION ON THE ACTIVITY OF RABBIT MUSCLE 5'-AMP AMINOHYDROLASE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

ROSA MARIA HEMPHILL

1971

ABSTRACT

THE EFFECT OF PROTEIN AND HYDROGEN ION CONCENTRATION ON THE ACTIVITY OF RABBIT MUSCLE 5'-AMP AMINOHYDROLASE

By

Rosa Maria Hemphill

The effect of hydrogen ion and protein concentration on the kinetics of rabbit muscle 5'-AMP aminohydrolase were examined. Spectrophotometric assays were used to determine specific activity and the kinetic parameters, Km, Vmax, and Hill slope. Results of this study indicate that specific activity decreased with increasing enzyme dilution in a pH-dependent manner. Hydrogen ion (pH 6.3) protected against the dilution-induced loss of activity observed at pH 7.0, as did saturating substrate concentration. At suboptimal substrate concentration, bovine serum albumin stabilized the enzyme against the effects of dilution at pH 7.0, while solvents glycerol and $(CH_3)_2SO$ enhanced these effects. ADP activation of the enzyme also exhibited pH dependence. Further, a lag in time-transmittance curves in ADP activation was observed at pH 7.0, but not at pH 6.3. The results point to an allosteric role for hydrogen ion in ADP activation and perhaps in the dissociation process of the enzyme. A second implication of this study is that caution must be used in the interpretation of sigmoidal kinetics observed at enzyme concentrations in the range of the enzyme subunit dissociation constant.

THE EFFECT OF PROTEIN AND HYDROGEN ION CONCENTRATION ON THE ACTIVITY OF RABBIT MUSCLE 5'-AMP AMINOHYDROLASE

By

Rosa Maria Hemphill

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1971

In peace and in joy,
to Del

ACKNOWLEDGMENTS

The author wishes to thank Mr. Norbert Feliss for assistance with the computer program used in some calculations and Dr. Carol Zielke for her help with stopped-flow data calculations and for many helpful suggestions. I am grateful to my parents and especially to my husband for their support and encouragement during this work. I particularly wish to thank Dr. Clarence H. Suelter, my thesis director, for his guidance in this study and for sharing his thoughts on the philosophy of science.

The author acknowledges and thanks the Danforth Foundation for their financial support during this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
GMP	guanosine 5'-monophosphate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
IMP	inosine 5'-monophosphate
ITP	inosine 5'-triphosphate
XMP	xanthine 5'-monophosphate
Tris	tris(hydroxylmethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
Cacodylic	dimethylarsenic acid
MES	2-(N-morpholino)ethanesulfonic acid
n_H	Hill slope
K_A	concentration of activator required for 50% activation
K_D	apparent subunit dissociation constant
BSA	bovine serum albumin
MSH	2-mercaptoethanol
F	a factor obtained as the difference in molar absorptivity of AMP and IMP at a given wavelength

INTRODUCTION

This study is an examination of the effect of hydrogen ion concentration on rabbit muscle 5'-AMP aminohydrolase activity and ADP activation. The effect of protein concentration encountered in this work was also studied. These effects were examined kinetically using spectrophotometric methods, including stopped-flow spectrophotometry, by observing changes in the kinetic parameters, specific activity, Hill slope, Km, and Vmax. This thesis presents data which indicate a role of hydrogen ion and substrate in the protection of 5'-AMP aminohydrolase against protein concentration-dependent, dilution-induced inactivation.

LITERATURE REVIEW

A. Brief Review of 5'-AMP Aminohydrolase.

5'-AMP aminohydrolase (EC 3.5.4.6, also AMP deaminase), first isolated by Schmidt in 1928 (1), is of widespread occurrence in nature. The enzyme has been purified from a variety of organisms including carp (2), frog (3,4), snail (5), chicken (6), pigeon (4), elasmobranch fish (7), and Erlich ascites tumor cells (8). The enzyme also occurs in mammals such as the rat (4,9-13), mouse (9), calf (14-18), rabbit (9,10,19-22), guinea pig (9,10), human (23-26), cat (24), dog (24,27), and ox (28). Tissues possessing 5'-AMP aminohydrolase activity include brain (13-18), lung (9), liver (9,13), spleen (9), intestine (9), heart (9), skeletal muscle (4,9,12,19-22), and erythrocyte tissue (23-25). Skeletal muscle contains the highest activity (2,4,13). The enzyme has been identified in nuclear, mitochondrial, microsomal, and other fractions of rat brain and liver preparations (13). It is usually isolated as a soluble enzyme (1,2,10,16,21,23). However, the enzyme is membrane-bound in human erythrocytes (23,25), and certain experiments have shown the muscle enzyme to be tightly bound to the muscle surface (29).

The rabbit muscle enzyme has been assigned a molecular weight ranging from 270,000 to 320,000 (30-32); the brain enzyme has provisional molecular weights of 310,000 and 560,000 for monomeric and dimeric forms, respectively (18). The rabbit muscle enzyme is a tetramer of four subunits of about 60,000 molecular weight each (30). Both rabbit and rat muscle enzymes are zinc metalloenzymes with 2.6 and 2.0 moles zinc per mole enzyme, respectively (33,34).

5'-AMP aminohydrolase from various sources exhibits allosteric properties, requiring or being activated by

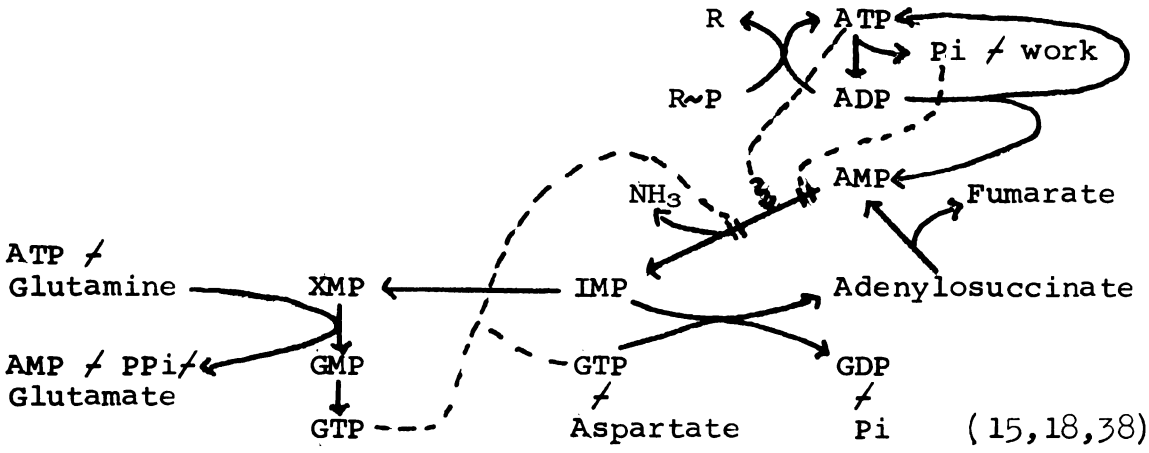
monovalent cations (2,6,7,16,21,24-26,35,36). It is activated or inhibited by both ATP and GTP depending on parameters such as concentration and source (8,11,12,15-17,22,24). The enzyme from various sources is activated by ADP and GDP (12, 35,36) and inhibited by phosphate (6,12,13,20,36), fluoride (2,6,7,10,13,36), other anions (12,20), ethacrynic acid (25), 2,3-diphosphglycerate (6,26), and detergents (13).

Several workers have attempted to coordinate the varying effects obtained with nucleotides. Costello and Brady (9) demonstrated that ATP activated 5'-AMP aminohydrolase in lung, liver, spleen, intestines, heart, and skeletal muscle of mouse, rat, and guinea pig; GTP activated the enzyme only in skeletal muscle. They also noted that the response of rabbit muscle enzyme to ATP in crude and purified preparations varied with substrate and ATP concentration (9). Ronca-Testoni et al. (4) prepared the enzyme from frog, pigeon, guinea pig, rabbit, and rat muscle by the same method and found that the enzyme from all sources was activated by KCl. At pH 6.5, ATP activated the guinea pig and rabbit muscle enzymes below 100mM KCl, but inhibited all systems above 100 mM KCl; at pH 7.1 ATP inhibited even at low KCl concentrations (4). GTP, ITP, and phosphate inhibited at all concentrations of KCl at both pH's (4). ADP strongly activated at low KCl concentrations but only weakly or not at all at high KCl concentrations; it also counteracted nucleotide triphosphate and phosphate inhibitions(4).

The reaction catalyzed by 5'-AMP aminohydrolase,

$$AMP + H_2O \longrightarrow IMP + NH_3 \quad (36,37),$$

functions in purine biosynthesis and interconversions (37,38) and is under allosteric control by monovalent cations and, in a complex way, by nucleotides. Physiologically, the role of 5'-AMP aminohydrolase may be seen as follows:



(15,18,38).

The activation-inhibition control of 5'-AMP aminohydrolase suggests a system self-regulated by relative purine nucleotide levels (15). The enzyme, according to Setlow et al. (16), exists in two forms, one active, one inactive, in equilibrium. The active form, with an accessible active site, would be stabilized by substrate, activator, or monovalent cation (16). This model simply assumes two symmetrical subunits which rearrange during the change from inactive to active form induced by substrate or activator binding (16). In this model, the control of 5'-AMP aminohydrolase, and indirectly of the AMP/ATP ratio (8), is apparently regulated by an ATP:GTP ratio (17) or an AMP:ATP:GTP ratio (11) rather than by absolute quantities of activators.

Recently, hydrogen ions have been suggested to activate 5'-AMP aminohydrolase (39), perhaps in a manner similar to the hemoglobin Bohr effect (40).

B. Role of Hydrogen Ion in the Classic Bohr Effect.

Hydrogen ions may act as general acid catalysts in many biochemical reactions (41). They may have a marked effect on affinity for substrate, inhibitor, or other ligand due to ionization of ligand, enzyme, or enzyme-ligand complex (42). One specific effect of the hydrogen ion on biochemical systems gives rise to what is called a Bohr effect, first defined for hemoglobin (40).

The Bohr effect in mammalian hemoglobin (40,43-45) may be briefly summarized as follows. Mammalian hemoglobin,

which possesses four heme groups, binds ligands in four steps; each association has a different equilibrium constant (43-45). This situation yields a sigmoidal binding curve (44), which may be described by Hill's empirical equation

$$KX^n = Y/(1-Y) \qquad (43,44,46)$$

where K is an equilibrium constant, X the activity of the ligand, and Y the fractional saturation of the ligand. n, also noted as n_H, is referred to as the Hill slope and has been defined mathematically as

$$n = d\ \ln(Y/(1-Y))/d\ \ln X = (1/Y(1-Y)(d\ Y/d\ \ln X) \quad (43).$$

For mammalian hemoglobin, in the middle pH range, there is a significant dependence of the hemoglobin-ligand equilibrium and thus of its equilibrium constant on hydrogen ion concentration: pH as well as the equilibrium constant decreases as oxygen binds hemoglobin in an unbuffered system(45). The variation in affinity of hemoglobin for oxygen with pH change is known as the classic oxygen Bohr effect (43). Wyman (43) defined the Bohr effect mathematically in terms of a linkage function, linking the binding of hydrogen ion as one ligand to the binding of oxygen as the second ligand, in the following equations:

$$\left(\frac{\partial \bar{H}\not}{\partial \bar{Y}}\right)_{\alpha_H} = -\left(\frac{\partial \ln p}{\partial \ln \alpha_H}\right)_{\bar{Y}} \quad \text{or} \quad \left(\frac{\partial \ln \alpha_H}{\partial \bar{Y}}\right)_{\bar{H}\not} = \left(\frac{\partial \ln p}{\partial \bar{H}\not}\right)_{\bar{Y}} \qquad (43)$$

where $\bar{H}\not$ and $\bar{Y}$ are fractional saturation of a macromolecule by hydrogen ion and ligand (oxygen), respectively, and α_H and p refer to the activities of hydrogen ion and ligand, respectively.

It is noteworthy that Wyman also related the oxygen linkage function to the hydrogen ion involved in the hemoglobin dissociation process since dissociation of hemoglobin in acid solution appears to be dependent on oxygen-binding (43). In this case there is no Bohr effect for either native or dissociated hemoglobin; any observed Bohr effect is due entirely to dissociation (43). Wyman notes that dissociation may be understood as an extreme conformational change (43).

C. Role of Hydrogen Ion as an Enzyme Allosteric Effector.

The Bohr effect may be considered a particular type of allosteric linkage effect in which binding of hydrogen ion affects ligand binding via a structural alteration. This phenomenon has been defined for multisubunit enzymes by Monod et al. (47), by Koshland et al. (48), and by Whitehead (49). The hemoglobin-ligand equilibrium serves as a model for allosteric phenomenon, the Bohr effect pointing to the role of hydrogen ion as an activator in allosteric enzyme systems (43).

The concept of the hydrogen ion as a heterotropic effector, capable of binding to a given site on a protein and inducing conformational change, has been further developed by Garel and Labousse (50). They suggest that when catalytic steps do not directly involve hydrogen ion, pH-dependent reversible conformational changes qualify as allosteric effects of hydrogen ion binding if linkage exists between substrate and hydrogen ion, i.e., binding of one influences binding of the other through mediation of protein structural change (50). The hydrogen ion-linked phenomenon could apply to ligands other than substrate (50). This allosteric action of hydrogen ion has been demonstrated in several enzymes.

Chymotrypsin, in particular, has been extensively studied with respect to hydrogen ion effects on both activity and conformational change (50-60). Chymotrypsin exhibits a bell-shaped pH-rate curve for acylation (k_2) and a sigmoidal profile for the de-acylation step (k_3) in the following scheme:

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} \begin{matrix} ES' \\ + Pi \end{matrix} \xrightarrow{k_3} E + P_2 \quad (51,54,57,60).$$

The ionizable group in both acylation and de-acylation steps appears to be a histidine (pK~6.8) which must be de-protonated for activity (58). The N-terminal isoleucine (pK~8.9) is involved in the acylation step but not in the bond-breaking process (50,52,54,57,60,61). Protonation of

the α-amino group of this isoleucine induces an active conformational form, allowing substrate binding and catalysis--perhaps via salt bonding with aspartate (60,61). Thus, the pH dependency of chymotrypsin-catalyzed reactions in the alkaline pH range may be explained in terms of a pH-dependent equilibrium between two chymotrypsin conformations (50,52,61). One conformation is active and capable of binding substrate and the other inactive; the equilibrium between them is controlled by the pK 8.9 isoleucine (52,61). The pH dependency of this equilibrium in the overall reaction (not including the dependency of acylation and de-acylation on the pK 6.8 catalytic group) could be expressed:

$$K^*_H \quad \begin{matrix} E^*H \\ \rightleftarrows \\ E^* \end{matrix} \quad \begin{matrix} \xrightarrow{K_{TH}} \\ \xleftarrow[K_T]{} \end{matrix} \quad \begin{matrix} EH\not \\ \rightleftarrows \\ E \end{matrix} \quad K_H \quad \underset{K_S}{\xrightarrow{S}} \quad E(H\not)S \quad \xrightarrow{k_{cat}} \quad E \not + P \qquad (51)$$

where E* and E are non-binding and binding conformations of the enzyme, respectively; $K_T = E^*/E$ and $K_{TH} = E^*H\not/EH\not$ (51). Chymotrypsin thus is an example of a linkage function with hydrogen-ion binding linked to binding of substrate (or other ligand) through a conformational change in the enzyme (50) analogous to the hemoglobin Bohr effect. The acidic counterpart, the deprotonation of a carboxyl group resulting in conformational change that prevents substrate binding has been noted for chymotrypsin (50,62) and for trypsin (62,63). Two related enzymes, ficin and papain, exhibit kinetics similar to chymotrypsin and may also involve a conformational change in the acylation step (64).

The activation of pyruvate kinase by fructose-1,6-diphosphate also appears to be a pH-dependent process (65-67), in the rat (65,66), in human erythrocytes (67), and in yeast (68). Wieker et al. (68) have indicated that hydrogen ions are allosteric effectors of yeast pyruvate kinase; the Hill slope is independent of pH in the presence of fructose-1,6-diphosphate while in its absence the Hill

slope increases to a pH-independent maximum. Wieker et al. (68) propose an equilibrium between active (protonated) and inactive (deprotonated) enzyme conformations mediated by protonation of a pK 6.2 group. Allosteric liver pyruvate kinase (type L) exhibits a pH dependence of substrate binding (65) and in its activation by potassium ion (66). The liver enzyme exhibits hyperbolic kinetics with phosphoenolpyruvate at acidic pH but exhibits sigmoidal kinetics at higher pH (65). This sigmoidal response at pH's above 7.2 is lacking in the presence of fructose-1,6-diphosphate (65). Allosteric activation by potassium ion is markedly decreased as pH is removed from neutrality in either direction; the Hill slope varies from 1.2 to 2.2 to 1.4 as pH is varied from 6.0 to 7.5 to 8.35, respectively (66). Staal et al.(67) reported a similar response to hydrogen ion concentration, i.e., hyperbolic kinetics at pH 5.9 but sigmoidal kinetics at pH 7.6, with purified erythrocyte pyruvate kinase. ATP inhibition of the erythrocyte enzyme also exhibits pH dependence (67). In the presence of fructose-1,6-diphosphate, the erythrocyte enzyme, like rat liver pyruvate kinase, exhibits hyperbolic kinetics at both acidic pH and pH 7.6 (67). In the absence of fructose-1,6-diphosphate, the erythrocyte enzyme exhibits sigmoidal kinetics at pH 7.6 (67).

Heart phosphofructokinase also exhibits pH-dependent allosteric phenomenon (69-73). At pH 6.9 the enzyme exhibits sigmoidal kinetics with respect to fructose-6-phosphate while at pH 8.9 hyperbolic kinetics are observed (70,73). Transition between the two states of the enzyme occurs through a conformational change, perhaps mediated by protonation of a histidine residue (70,72,73). Further, heart phosphofructokinase has been shown to dissociate into subactive protomers at pH 6.5, an effect reversed by alkaline pH (70).

Rabbit liver fructose 1,6-diphosphatase appears to exhibit linkage between binding of substrate or allosteric effector, AMP, and binding of hydrogen ion to tyrosyl residues, through a conformational change (74,75). Recently,

Taketa et al. (76) reported that fructose 1,6-diphosphatase activity decreased reversibly with decreasing pH due to a conformational change to a low activity conformation with no change in molecular weight. The Hill slope for AMP changed from 1.8 for the active enzyme at neutral pH to 1.0 for the enzyme inactivated at pH 6.5 (76). Fructose-1,6-diphosphatase from rabbit muscle (77) also exhibits pH-dependent allosteric behavior with sigmoidal kinetics at pH 7.5 and hyperbolic kinetics at pH 9.3. Taketa et al. (76) noted the similarity in the response of substrate binding to pH in the fructose 1,6-diphosphatase system to the response of oxygen binding to pH in hemoglobin.

Similarly, for aspartate transcarbamylase, interactions among subunits at pH 7 were reversibly lost at pH 10.2 through a change in conformation perhaps due to ionization of side groups, yielding a negative charge (78). This loss of subunit interaction occurs without dissociation (78).

Another enzyme which may exhibit allosteric effects of hydrogen ion is maltodextrin phosphorylase which exhibits pH dependence of both Km and Vmax between pH 5.6 and 8.5 (79). The ionization of two groups affects substrate binding, one possibly through a conformational change.

Several enzymes undergo a pH-dependent association-dissociation process affecting ligand binding in which dissociation is considered an extreme form of conformational change (43). Proteins which may be in this class include transcarboxylase (80), cytochrome c oxidase (81), phosphofructokinase (70,82), phycoerythrin (83), catalase (84), rabbit muscle aldolase (85), myosin (86), lysine-2,3-aminomutase (87), human carbonic anhydrase (88), and glutamate dehydrogenase (89-91). Glutamate dehydrogenase may undergo a conformational change prior to dissociation (91).

MATERIALS AND METHODS

A. Reagents and Materials.

1. Enzyme preparation. The enzyme source, frozen young rabbit muscle, type 1, deboned, was obtained from Pel Freeze Biological, Inc., Roger, Arkansas. KCl, KOH, KH_2PO_4, and HCl were analytical grade reagents from Mallincrodt Chemical Works, St. Louis, Missouri. Disodium EDTA and Trizma base were from Sigma Chemical Co., St. Louis, Missouri. Mannex-P cellulose phosphate, lot T-4648L, (1.15 meq/g capacity, 80 sec. flow rate, 9.5 ml/g wet bulk) was obtained from Mann Research Laboratories, New York, New York. 2-Mercaptoethanol was purchased from Matheson, Coleman, and Bell, East Rutherford, New Jersey.

2. Enzyme assays. Reagents required for the assays were the highest purity available. 5'-AMP (crystalline preparation, type V, of the free acid from equine muscle) as well as Trizma base, Cacodylic acid, and MES were purchased from Sigma Chemical Co., St. Louis, Missouri. KCl and HCl were from Mallincrodt Chemical Works, St. Louis, Missouri. Chelex-100, sodium form, 50-100 mesh, was obtained from BioRad Laboratories, Richmond, California. Chelex-100 was washed with three cycles of 1 N HCl and glass-distilled water, then titrated to neutral pH with Tris base before use. All reagents used in assays, except Tris base, were passed over Tris-Chelex-100. The desired pH's were obtained by titration with Tris base or HCl.

3. Other reagents and materials. $NaHCO_3$, reagent grade, was obtained from Baker and Adamson, Morristown, New Jersey. Dialysis tubing, from Union Carbide Corp., Chicago, Illinois, was boiled three times each with $NaHCO_3$, Tris-EDTA, and glass-distilled water prior to use and stored at 4° C. in glass-distilled water. BSA, crystalline,

obtained from Sigma Chemical Co., St. Louis, Missouri, was dialyzed against three changes (100 volumes) of 10^{-2} M Tris-EDTA and six changes (100 volumes) of glass-distilled water and then stored at 4° C. Glycerol, analytical grade (95%), and NaOH, analytical grade, were obtained from Mallincrodt Chemical Works, St. Louis, Missouri. $(CH_3)_2SO$, redistilled under vacuum, was from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. ADP, obtained from Sigma Chemical Co., St. Louis Missouri, as the sodium salt, was converted to the Tris salt by passage over Dowex 50W-X8, reagent grade from J. T. Baker Co., Phillipsburg, New Jersey. Deionized, glass-distilled water was used for enzyme preparations and assays.

For stopped flow scans, Polaroid Polaline Land Projection Film Type 146-L and Polaroid Dippit No. 646, stored at 4° C., were used.

A Sargent Model LS pH meter was used for pH measurements. Standardizations were made with Mallincrodt analytical reagent standard buffers.

B. Enzyme Preparation.

The enzyme isolation procedure was based on that of Smiley et al. (35). Usually, two pounds of muscle were used. The cellulose phosphate used was washed with 80 volumes each of 0.5 N NaOH, glass-distilled water, 0.5 N HCl, and glass-distilled water and then soaked three days in 10^{-2} M Tris-EDTA, before washing exhaustively with glass-distilled water. The enzyme preparation was taken through the cellulose phosphate column step. Elution from the column was accomplished by block elution using 1 M KCl and 10^{-3} M MSH at pH 7, rather than gradient elution. The enzyme was stored at 4° C.

C. Protein Concentration.

Concentrations of 5'-AMP aminohydrolase and BSA were routinely determined by the method of Warburg and Christian (92) or according to Zielke (30) for the

enzyme. For mole-gram conversions, a molecular weight of 278,000 as determined by Zielke and Suelter (32) was used.

D. Kinetic Assays.

1. Reaction.

a. Components. The assay contained 5'-AMP, KCl, Tris-MES or Tris-cacodylate, MSH, and enzyme at the appropriate pH.

b. Conversion factors. AMP deamination was determined by following the change in optical density at 265 mμ, 285 mμ, or 290 mμ and dividing by the conversion factors 8.86, 0.30, or 0.12 optical density units per μmole per ml, respectively, to obtain molar quantities of AMP deaminated.

c. Activity. One unit of activity is defined as the amount of enzyme which catalyzes deamination of one μmole of AMP per minute. Specific activity is the amount of AMP deaminated in μmoles per minute per mg protein.

2. Assays.

a. Standard Assay. The standard assay was used for determining activity during enzyme purification and for monitoring specific activity in various preparations and experiments. It consisted of 0.15 M KCl, 0.05 M Tris-cacodylate, pH 6.3, and 5.0×10^{-5} M Tris-AMP, pH 6.3, in a one-ml reaction volume, final pH 6.3. Reaction was initiated by addition of 5-20 μl enzyme being prepared or by addition of 5 μl of an enzyme solution which consisted of 0.5 M KCl, 0.05 M Tris-MES, pH 7.0, 10^{-3} M MSH, and 0.1 mg/ml of enzyme, usually in a 2-ml volume. The assay mixture was incubated for 5 minutes and the reaction run at 30° C. Optical Density measurements were made with a Beckman DU Model 2400 spectrophotometer and a Sargent Recorder Model SRL. A one centimeter light path was used. Specific activities usually ranged from 110 to 130 μmoles $min^{-1} mg^{-1}$.

b. Basic assay. The basic assay was employed for specific activity determinations, obtaining substrate-velocity curves, and enzyme protection studies. It consisted

of Tris-AMP at various concentrations, 0.1 M KCl, 0.05 M Tris-MES, and enzyme in a one-ml volume, at the experimental pH. The assay varied for different experiments by the inclusion, in the same volume, of BSA, glycerol, or $(CH_3)_2SO$. Enzyme solutions consisted of 0.1 M KCl, 0.05 M Tris-MES, 1 mM MSH, and enzyme at the appropriate pH.

c. Stopped-flow assay. This assay was used for high protein concentrations that produced rates not measurable with a normal spectrophotometer. The reaction mixture in a final volume of about 0.4 ml contained varying concentrations of Tris-AMP, 0.1 M KCl, 0.05 M Tris-MES, 1 mM MSH, and enzyme at the appropriate pH. Solutions were made using degassed glass-distilled water. Both enzyme and substrate reservoir solutions contained salt, buffer, and MSH at final concentration. Enzyme and substrate were at concentrations two-fold the final concentrations. Appropriate dilutions were made with buffer containing all components at final concentrations except protein or substrate. Reaction was initiated by mixing equal volumes of substrate and enzyme solutions in the mixing chamber. The rate was followed with a Durram-Gibson stopped-flow spectrophotometer (2 msec. mixing dead time, 2-cm light path, 30 ml/sec syringe drive rate), employing a Beckman DU Model 2400 monochromater (slit width, 0.4). All solutions and measurements were at 30° C. Transmittance changes were recorded on a storage oscilloscope and photographic transparencies were made of the reaction scans. Conversion to optical density was accomplished as described in appendix 1.

3. Calculation of Kinetic Parameters.

a. Hyperbolic kinetics. For reactions exhibiting normal Michaelis-Menten kinetics, Km and Vmax were usually determined from Lineweaver-Burk plots (93).

b. Sigmoidal kinetics. For reactions exhibiting sigmoidal substrate-velocity curves, the kinetic parameters

Km, Vmax, and n_H were determined either graphically from plots resulting from treatment of the data according to the Hill equation (94) or by application of a linear equation for these parameters developed to fit the Dye and Nicely program (95). The application of this program for determination of kinetic parameters is given in appendix 2. Hill slopes and kinetic parameters were also calculated by use of the Hill n program developed by William I. Wood (personal communication).

RESULTS

A. Hydrogen Ion Concentration Effect on 5'-AMP Aminohydrolase Reaction.

The influence of hydrogen ion concentration on the 5'-AMP aminohydrolase reaction, suggested by Suelter et al. (39), was studied at pH 6.3 and pH 7.0. The effect of hydrogen ion concentration was observed by determining its influence on substrate-velocity curves and on the kinetic parameters, Km, Vmax, and Hill slope, n_H, at these two pH's. Enzyme for these experiments was stored as a stock solution of 0.1 mg ml^{-1} at the proper pH and diluted 200-fold in the assay mix to a final concentration of 0.5 µg ml^{-1}. The basic assay (see Materials and Methods) was employed. Substrate was varied at each pH from 5×10^{-5} M to 5×10^{-3} M. The Hill slope changes from unity at pH 6.3 to 1.81 at pH 7.0 (Table 1), a figure indicating sigmoidal kinetics and usually implying some cooperative interactions among substrate binding sites for AMP or the possibility of non-catalytic binding sites.

However, similar studies with the stopped flow apparatus at enzyme concentrations of about 0.5 mg ml^{-1} did not yield similar differences in Hill slope at the two pH's; n_H remained near unity, Vmax did not change, and Km almost doubled from pH 6.3 to pH 7.0 (C. Zielke, personal communication). Thus it appeared that although at pH 6.3 the Hill slope remained at unity at both high and dilute enzyme concentrations, at pH 7.0 the Hill slope varied from unity to 1.81 at high and dilute enzyme concentrations, respectively. Such differing results led to studies of the protein concentration dependency of the 5'-AMP aminohydrolase reaction at these two pH values.

TABLE 1

Kinetic Parameters for 5'-AMP Aminohydrolase at pH 6.3 and pH 7.0 in the Presence and Absence of MSH or BSA.

pH	Km (mM)	Vmax (μmoles $min^{-1} mg^{-1}$)	n_H
6.3	0.28-0.30	1400	1.03
7.0	0.34-0.36	1200	1.81
6.3 (1 mM MSH)	0.25	1000	1.08
7.0 (1 mM MSH)	0.32	925	1.75
6.3 (0.1 mg BSA ml^{-1})	0.12	1500	1.01
7.0 (0.1 mg BSA ml^{-1})	0.24	1000	1.30

Assay mixtures contained Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, and enzyme. Tris-AMP was varied from 5×10^{-5} M to 5×10^{-3} M. Enzyme stock concentration was 0.1 mg ml^{-1} (0.05 M Tris-MES, 0.1 M KCl, and 1 mM MSH). Reaction was initiated by addition of 5 μl aliquot of enzyme stock solution. BSA had been dialyzed against 3 changes 0.01 M Tris-EDTA and 6 changes glass-distilled H_2O. Reaction temperature was 30^0 C.

B. Protein Concentration Effect on the 5'-AMP Aminohydrolase Reaction at Two Hydrogen Ion Concentrations.

1. Variation of specific activity at high protein concentration. The variation of 5'-AMP aminohydrolase specific activity at pH 6.3 in the protein concentration range suitable for study with a stopped-flow spectrophotometer was investigated using the stopped flow assay (see Materials and Methods). Reservoir protein concentrations ranged from 0.45 mg ml^{-1} to 8.7 x 10^{-4} mg ml^{-1}. Reservoir substrate concentration was 8.85 x 10^{-5} M Tris-AMP. Assays monitored transmittance changes at 265 mμ. There was no loss in activity of the enzyme during the experiment. Two determinations were made for each protein concentration point. The data were reproducible at the same protein concentration for different sweep times. The results are plotted in Figure 1A. At pH 6.3, the specific activity remains fairly constant over the entire protein concentration range measured, falling off at higher protein concentrations. The possibility of a decrease in specific activity due to protein aggregation at this pH is not eliminated.

The variation of specific activity with protein concentration was also examined at pH 7.0 (Figure 1B). The reservoir protein solution ranged from 0.51 mg ml^{-1} to 9.1 x 10^{-3} mg ml^{-1}; the reservoir substrate concentration was at 1.17 x 10^{-4} M Tris-AMP. Assays were followed at 265 mμ. There was no loss of enzyme activity during the experiment. Each point is the result of duplicate scans at each protein concentration. Again, specific activity remained fairly constant over most of the protein concentration range studied but did begin to fall off at lower protein concentrations. Specific activity values at pH 7.0 were lower than at pH 6.3. For both pH's specific activity is not altered over a fairly wide protein concentration range, despite a two-fold dilution in the assay. This indicates the protein solutions at each concentration were not losing activity as a result of dilution.

Figure 1. Effect of Enzyme Concentration on Specific Activity of 5'-AMP Aminohydrolase in Stopped Flow Studies.

A, pH 6.3. Assay mixture contained 4.43×10^{-5} M Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1×10^{-3} M MSH, and enzyme, at pH 6.3. Final volume was approximately 0.4 ml. Reaction was followed at 265 mμ. Reaction temperature was 30^{0} C.

B, pH 7.0. Assay components and conditions were as in A, but at pH 7.0. Tris-AMP was 5.86×10^{-5} M.

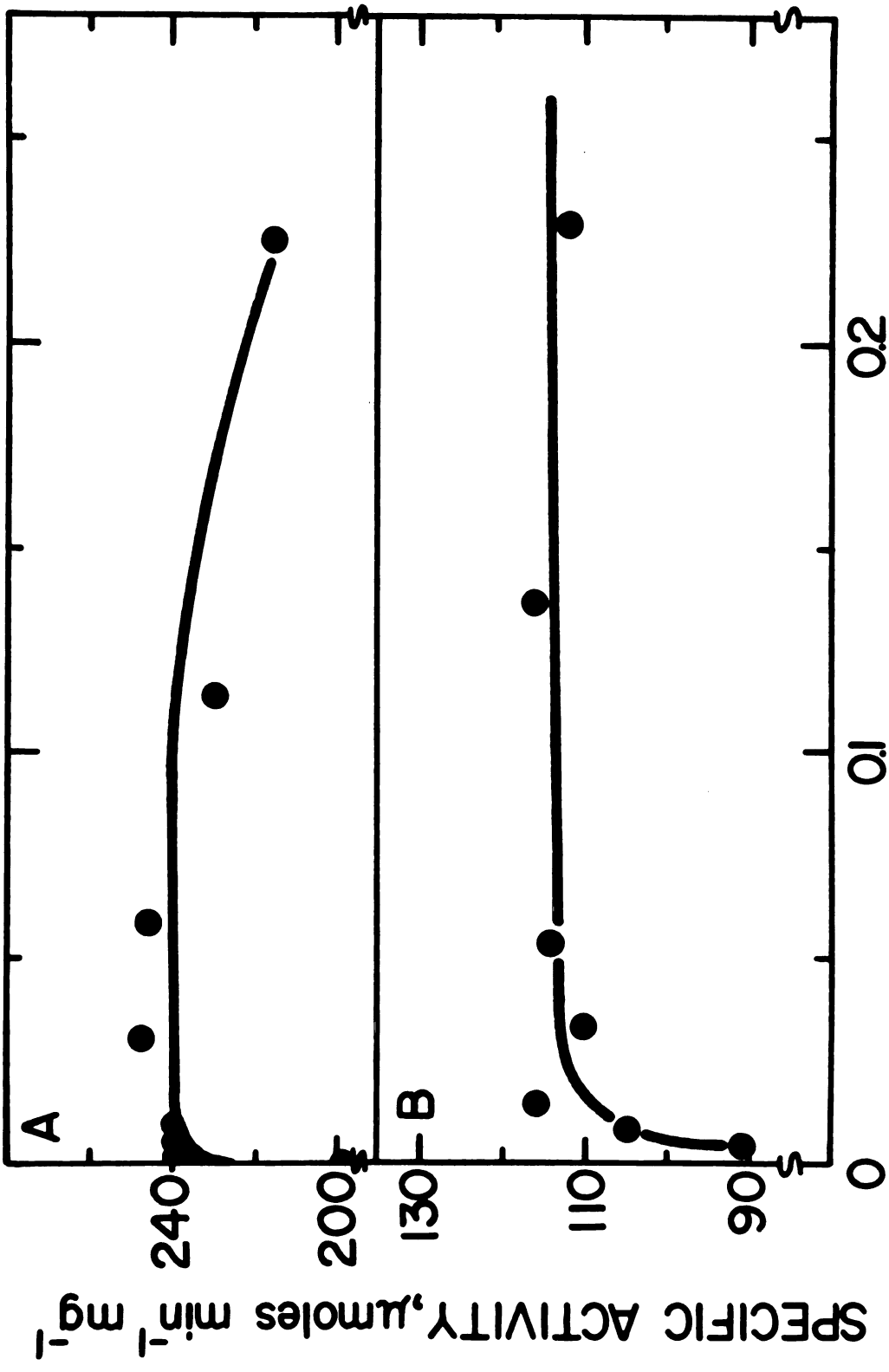

A
B
240
200
130
110
90
0
0.1
0.2
SPECIFIC ACTIVITY, μmoles min⁻¹ mg⁻¹
ENZYME CONCENTRATION IN ASSAY, mg ml⁻¹

Figure 1.

2. Variation of specific activity at dilute protein concentrations. The specific activity of 5'-AMP aminohydrolase at pH 6.3 at enzyme concentrations ranging from 2.25 μg ml^{-1} to 8.8 x 10^{-3} μg ml^{-1} was examined in the Beckman DU spectrophotometer. The basic assay (see Materials and Methods) was employed. Substrate concentration at pH 6.3 was 4.45 x 10^{-5}M Tris-AMP. MSH was present at a final concentration of 1 mM to allow for comparison with studies made under stopped flow conditions. Protein stock solutions, from which 5 μl aliquots were taken for the assays, were varied by dilution over the concentration range from 0.45 mg ml^{-1} to 8.8 x 10^{-4} mg ml^{-1}. Data were collected in duplicate and averaged. The results (Figure 2A (●)) show that specific activity remained fairly constant at protein concentrations greater than 0.5 to 0.7 μg ml^{-1} but decreased as the enzyme was further diluted.

The relationship of specific activity to assay protein concentration was studied at pH 7.0 under similar conditions. Assay enzyme concentration varied from 2.54 μg ml^{-1} to 1.2 x 10^{-1} μg ml^{-1}. Stock protein concentrations varied from 0.51 mg ml^{-1} to 9.1 x 10^{-3} mg ml^{-1}. Tris-AMP was 5.85 x 10^{-5} M with MSH at 1 mM. Data were taken in duplicate and averaged. A plot of specific activity versus protein concentration is presented in Figure 2B (●).

A similar set of studies at both pH 6.3 and pH 7.0 over a comparable protein concentration range was made in the absence of MSH in the assay mixture to control for the presence of MSH in the above experiments. At pH 6.3, protein concentration in the assay ranged from 3.2 μg ml^{-1} to 1.25 x 10^{-2} μg ml^{-1} and in stock solutions from 6.4 x 10^{-1} mg ml^{-1} to 2.5 x 10^{-3} mg ml^{-1}. Substrate at pH 6.3 was 5.06 x 10^{-5}M. At pH 7.0, the protein concentration in the assay ranged from 2.85 μg ml^{-1} to 4.45 x 10^{-2} μg ml^{-1} and in the protein stock solutions from 0.57 mg ml^{-1} to 8.9 x 10^{-3} mg ml^{-1}. The substrate concentration at pH 7.0 was 5.03 x 10^{-5} M. Results of these experiments are

Figure 2. Effect of Dilution on Specific Activity of 5'-AMP Aminohydrolase.

A, pH 6.3. Assay mixture contained 4.43×10^{-5} M Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, and enzyme, at pH 6.3. 5 µl aliquots of enzyme stock solutions ranging from 0.64 to 8.7×10^{-4} mg ml^{-1} were used to initiate the reaction. Assays were made in the presence (●) and absence (□) of 1×10^{-3} M MSH. Reaction volume was 1.0 ml. Reaction was followed at 265 mµ. Reaction temperature was 30^{0} C.

B, pH 7.0. Assay components and conditions were as in A, but at pH 7.0. Tris-AMP was 5.86×10^{-5} M. Enzyme stock solutions varied from 0.57 to 8.9×10^{-3} mg ml^{-1}.

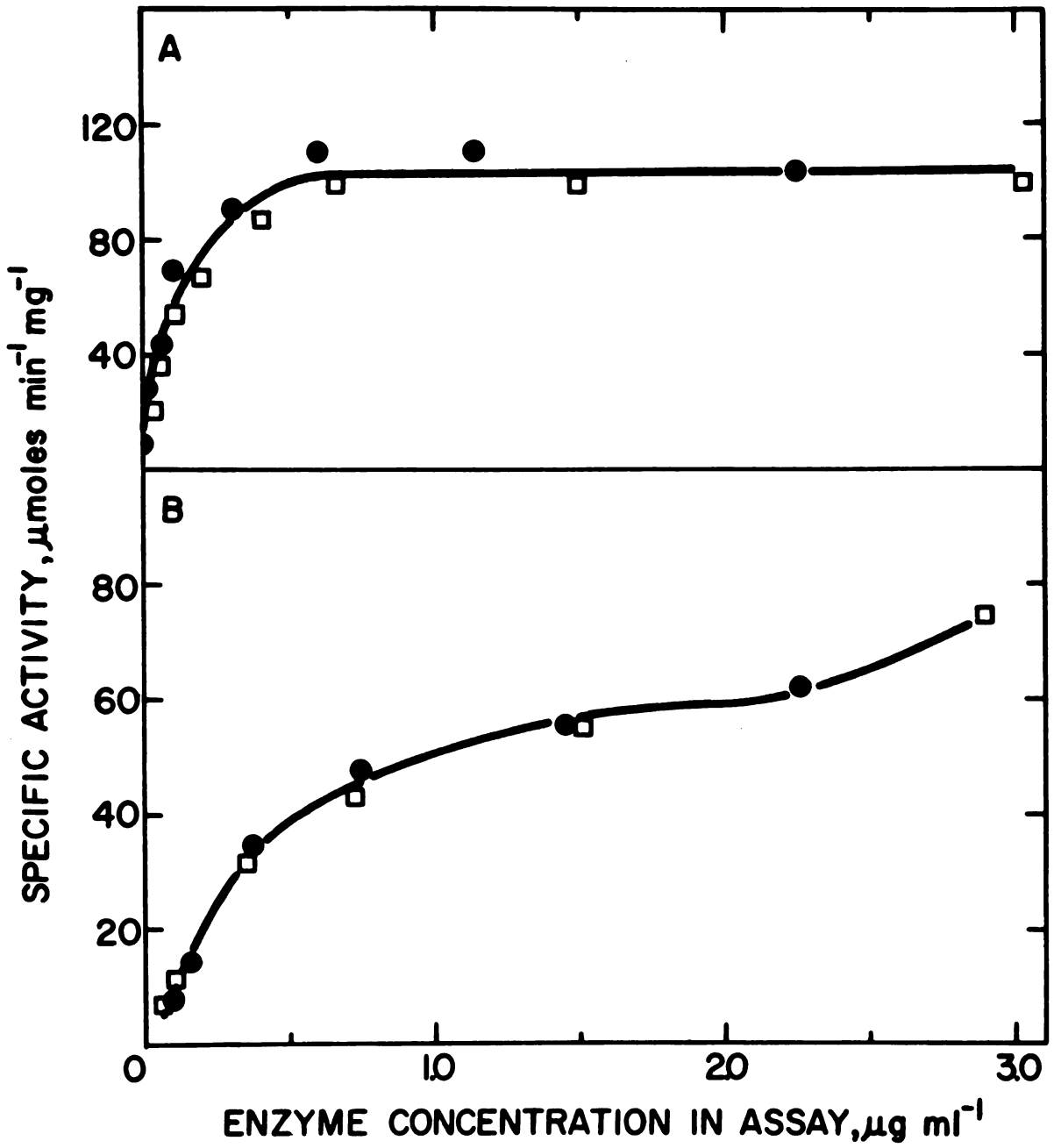

A
B
120
80
40
80
60
40
20
0
1.0
2.0
3.0
SPECIFIC ACTIVITY, μmoles min⁻¹ mg⁻¹
ENZYME CONCENTRATION IN ASSAY, μg ml⁻¹

Figure 2.

presented for pH 6.3 and pH 7.0 in Figures 2A (□) and 2B (□), respectively. These results indicate that the rates at which specific activity decreases with decreasing enzyme concentration are similar in the presence and absence of MSH and are consistent with the results in Table 1 indicating similar kinetic parameters in the presence or absence of 1 mM MSH.

Comparison of curves in Figure 2B (pH 7.0) against those in Figure 2A (pH 6.3) shows that the specific activity of 5'-AMP aminohydrolase at a given enzyme concentration in the range studied is lower at pH 7.0. The results of these experiments indicate that in the range 0.5 to 1.0 μg ml^{-1} specific activity remains fairly constant at pH 6.3 but is decreasing at pH 7.0.

The results in Figures 2A (●) and 2B (●), compared with those in Figures 1A and 1B, emphasize that, while little loss in specific activity occurs in two-fold dilution of the stock solutions, there is a significant loss of specific activity after a 200-fold dilution of aliquots from the same or similar stock solutions. These results demonstrate a protein concentration dependency of the reaction consistent with a dilution-induced dissociation of the enzyme at the dilute concentrations used in the spectrophotometric assay. If this is the case, as suggested for other diluted enzymes (96), a rough calculation of dissociation constants based on the data presented in Figures 2A and 2B and assuming a dimeric dissociating species can be made, giving subunit dissociation constants of approximately 2.88×10^{-10} M at pH 6.3 and greater than 1.94×10^{-9} M at pH 7.0.

Specific activity at dilute enzyme concentrations was also tested using a single stock solution and varying the amount of enzyme used to initiate the reaction. At pH 6.3, stock protein was kept at 5.9×10^{-2} mg ml^{-1}. Enzyme in the assay was varied from 5.9 μg ml^{-1} to 1.88×10^{-2} μg ml^{-1}. The basic assay (see Materials and

Methods) was used. MSH at 1 mM was included in the assay mix. Substrate concentration was 4.92×10^{-5} M. Specific activity versus enzyme concentration is presented in Figure 3A.

At pH 7.0, under similar conditions, stock solution of enzyme was at a concentration of 5.9×10^{-2} mg ml^{-1}. Protein in the assay varied from 2.95 µg ml^{-1} to 2.95×10^{-2} µg ml^{-1}. Substrate was 5.09×10^{-5} M and the assay included 1 mM MSH. Results at this pH are presented in Figure 3B.

These experiments serve as controls for those represented in Figure 2. At both pH's, although specific activities are somewhat higher, the response of specific activity to enzyme concentration is similar.

3. Substrate protection at dilute enzyme concentrations. The possibility that substrate protected the enzyme against the effects of high dilution was considered. An apparent substrate activation at dilute enzyme concentration at pH 7.0 (Table 1), absent at high enzyme concentration (Dr. Carol Zielke, personal communication) and the marked dilution effects on specific activity at this pH at protein concentrations where a Hill slope greater than unity is obtained, made a study of the role of 5'-AMP at this pH as well as at pH 6.3 interesting.

In the dilute enzyme concentration range, the effect of substrate at suboptimal and at saturating concentrations was examined, at both pH 6.3 and pH 7.0. The basic assay was used (see Materials and Methods) and the assay included 1 mM MSH. At pH 6.3, enzyme concentrations were varied from 5.9 µg ml^{-1} to 1.88×10^{-2} µg ml^{-1}, by varying aliquots from a stock solution at a concentration of 5.9×10^{-2} mg ml^{-1}. Tris-AMP was 4.92×10^{-5} M for studies at suboptimal substrate concentration (Figure 3A) and 6 mM for studies at saturating concentrations (Figure 4A). At pH 7.0, the stock enzyme solution was at a concentration of 5.9×10^{-2} mg ml^{-1} from which aliquots were drawn to provide a range in the assay from 2.95 µg ml^{-1} to 2.95×10^{-2} µg ml^{-1}.

Figure 3. Effect of Suboptimal Substrate Concentration on Specific Activity of Dilute 5'-AMP Aminohydrolase.

A, pH 6.3. Assay mixture contained 4.92×10^{-5} M Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1×10^{-3} M MSH, and enzyme, at pH 6.3. Enzyme stock concentration was 0.059 mg ml^{-1}. Reaction volume was 1.0 ml. Reaction was followed at 265 mμ. Reaction temperature was 30° C.

B, pH 7.0. Assay components and conditions were as in A, but at pH 7.0. Tris-AMP was 5.09×10^{-5} M. Enzyme stock concentration was 0.059 mg ml^{-1}.

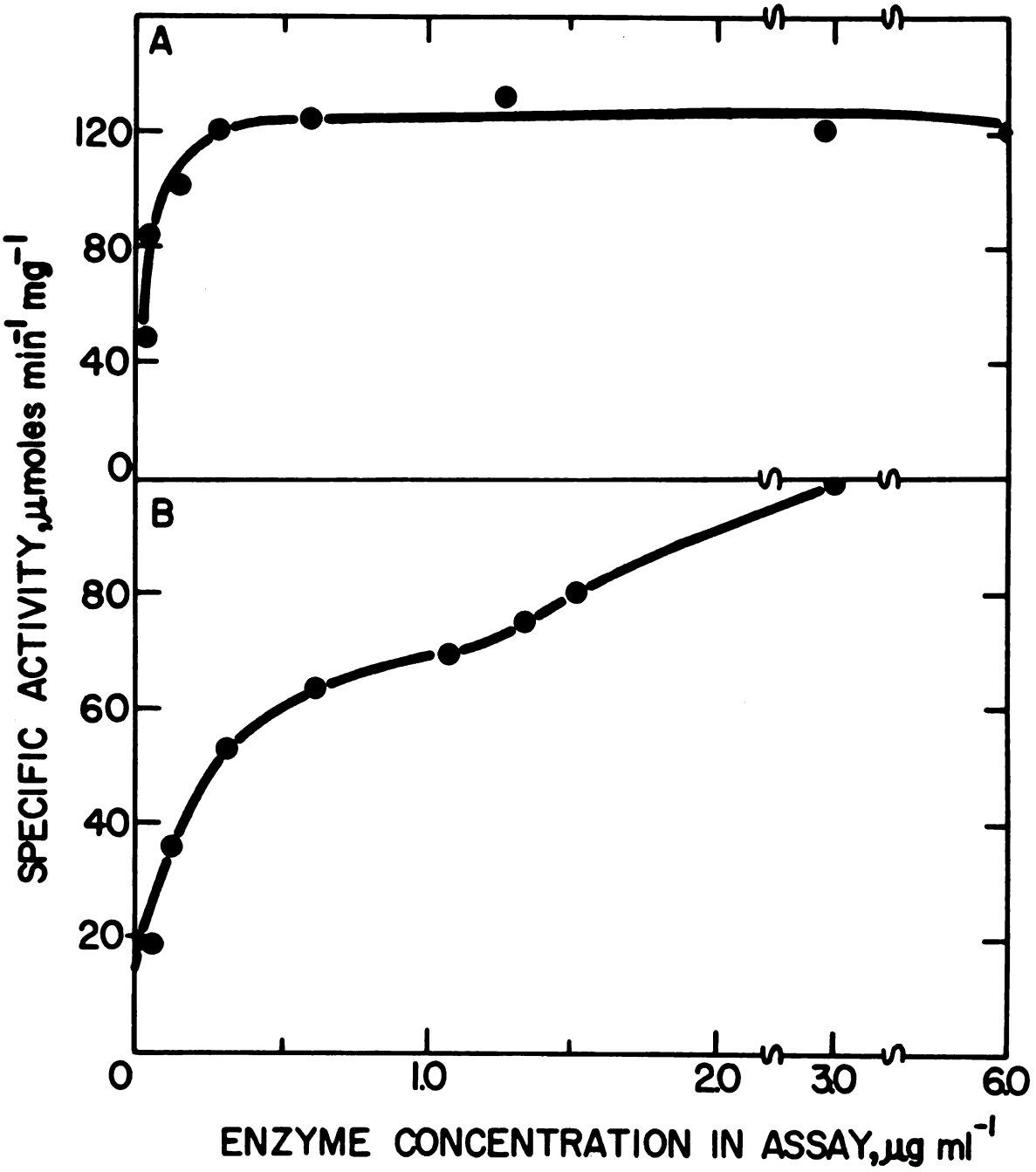

A
B
120
80
40
0
80
60
40
20
0
1.0
2.0
3.0
6.0
SPECIFIC ACTIVITY, μmoles min⁻¹ mg⁻¹
ENZYME CONCENTRATION IN ASSAY, μg ml⁻¹

Figure 3.

Figure 4. Effect of Saturating Substrate Concentration on Specific Activity of Dilute 5'-AMP Aminohydrolase.

A, pH 6.3. Assay mixture contained 6.0×10^{-3} M Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1×10^{-3} M MSH, and enzyme, at pH 6.3. The enzyme stock concentration was 0.059 mg ml^{-1}. Reaction volume was 1.0 ml. Reaction was followed at 290 mμ. Reaction temperature was 30° C.

B, pH 7.0. Assay components and conditions were as in A, but at pH 7.0. Concentration of Tris-AMP was 6.0×10^{-3} M and of enzyme stock solution was 0.059 mg ml^{-1}.

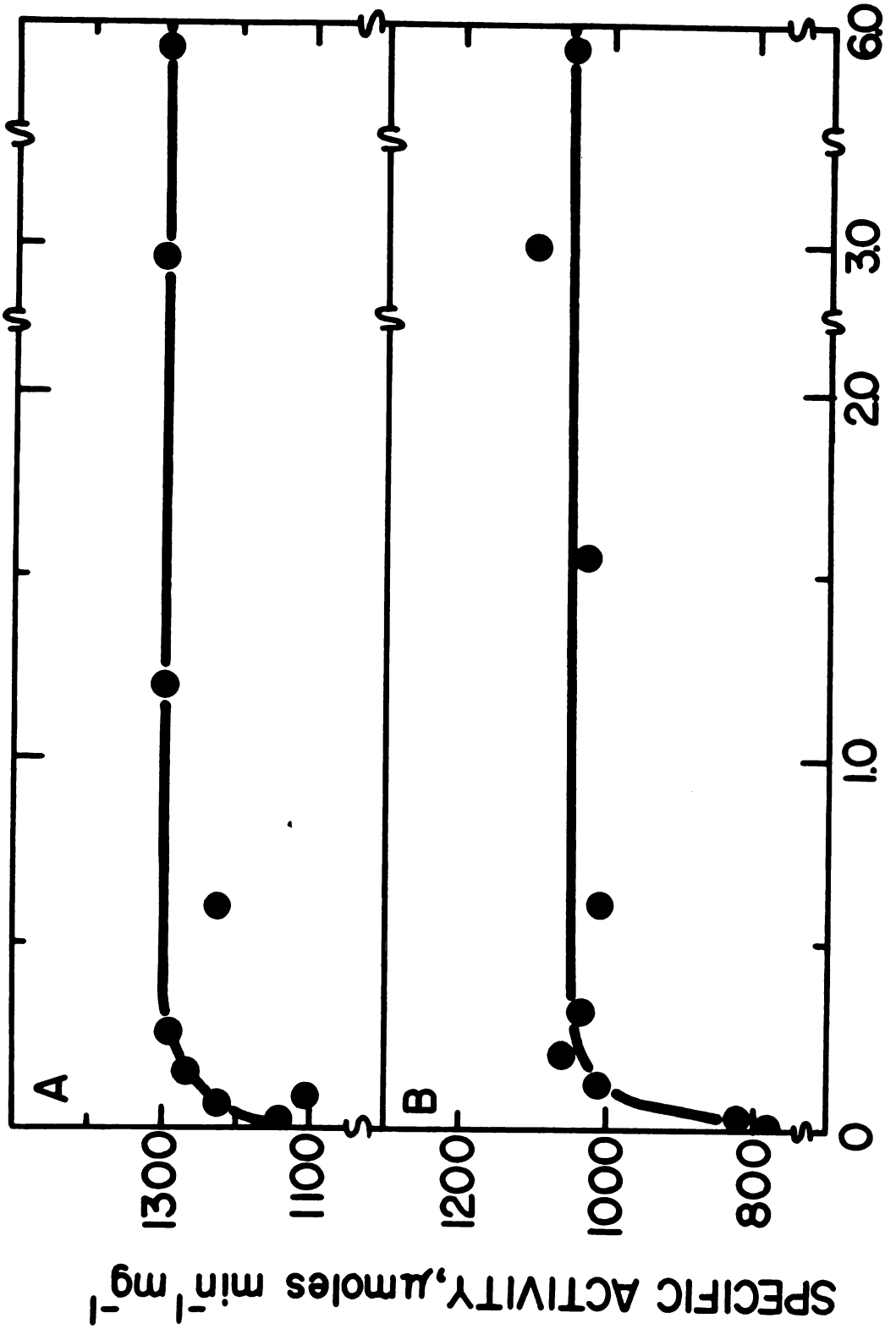

A
B
1300
1100
1200
1000
800
0
1.0
2.0
3.0
60
SPECIFIC ACTIVITY, μmoles min⁻¹ mg⁻¹
ENZYME CONCENTRATION IN ASSAY, μg ml⁻¹

Figure 4.

The Tris-AMP concentration for the suboptimal substrate work was 5.09×10^{-5} M (Figure 3B) and 6 mM for the saturating level of substrate (Figure 4B).

Results, at both pH 6.3 and pH 7.0, of specific activity variation with dilute enzyme concentration at suboptimal substrate concentration (Figures 3A and 3B) are similar, as already noted, to those obtained in Figures 2A and 2B, respectively. However, the effect of saturating substrate concentration at both pH's on specific activity was marked, especially at pH 7.0. At pH 6.3 (Figure 4A) specific activity remained constant over most of the protein concentration range studied and decreased slightly at the lowest protein concentrations. Specific activity appeared to fall off at about the same enzyme concentration as at suboptimal substrate concentration, although not as rapidly (Figure 3A). At pH 7.0 (Figure 4B) the effect of saturating substrate concentration was more pronounced; specific activity remained constant over the large majority of the protein concentration range in which it was decreasing at low substrate (Figure 3B). If substrate did protect the enzyme from the effect of dilution, a similar decrease in specific activity at both saturating and suboptimal substrate concentrations would be expected. It should be noted that the protection by hydrogen ion and by substrate appear essentially similar. These results are consistent with protection by substrate against dissociation caused by dilution at the dilute enzyme concentrations studied.

4. Effects on the 5'-AMP aminohydrolase reaction of other compounds.

a. Effect of BSA. As indicated by other workers (96), BSA is known to protect enzymes against dilution or to activate them. The effect of BSA on the Hill slope at pH 6.3 and pH 7.0 was therefore examined. The basic assay, with BSA at a final concentration of 0.1 mg ml^{-1}, was used. Enzyme concentration at pH 6.3

and at pH 7.0 was 0.5 μg ml^{-1}. Substrate concentration at each pH varied from 5 x 10^{-5} M to 5 x 10^{-3} M. Kinetic parameters obtained from these experiments are given in Table 1. It is to be noted that, whereas n_H at pH 6.3 remained at unity, at pH 7.0 n_H decreased from 1.81 to 1.30. Km at both pH's decreased. Since the results were consistent with BSA functioning to protect the enzyme against dilution, its effect was studied further.

The effect of BSA on 5'-AMP aminohydrolase at the two pH's was looked at by studying the effect of varying amounts of BSA on the enzyme at enzyme concentrations used in the Hill slope determinations. The basic assay was used (see Materials and Methods), with BSA as a variable component ranging in concentration from 0 to 6.0 mg ml^{-1} at pH 6.3 and from 0 to 3.0 mg ml^{-1} at pH 7.0. Substrate concentration at pH 6.3 was 4.67 x 10^{-5} M and at pH 7.0, 4.68 x 10^{-5}M. The results at pH 6.3 are presented in Figure 5A, those at pH 7.0 in Figure 5B. Dashed lines indicate control values in the absence of BSA. At pH 6.3, BSA does not affect specific activity significantly up to about 0.2 mg ml^{-1} above which it appears to inhibit the reaction. In contrast, at pH 7.0, BSA stimulates activity significantly up to 0.5 mg ml^{-1}, almost two-fold; above 0.5 mg ml^{-1} activity decreases to control values. These responses of specific activity are consistent with results obtained at the two pH's for the Hill slope. At the BSA concentration used to obtain the Hill slopes (0.1 mg ml^{-1}), at pH 6.3 there is no stimulation of activity, while at pH 7.0 there is marked stimulation. The almost two-fold increase in specific activity at pH 7.0 approaches the specific activity level at pH 6.3. These results at this BSA concentration at pH 7.0, as well as the reduction in Hill slope, are consistent with a protection by BSA of the enzyme against the effects of high dilution.

Figure 5. Effect of BSA on Specific Activity of Dilute 5'-AMP Aminohydrolase.

A, pH 6.3. Assay mixture contained 4.67×10^{-5} M Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1×10^{-3} M MSH, 0.5 μg ml^{-1} enzyme, and BSA, at pH 6.3. Enzyme stock solution was 0.1 mg ml^{-1}. BSA was dialyzed against 3 changes of 0.01 M Tris-EDTA and then 6 changes of glass-distilled deionized H_2O. Reaction volume was 1.0 ml. Reaction was followed at 265 mμ. Reaction temperature was 30° C.

B, pH 7.0. Assay components and conditions were as in A, but at pH 7.0. Tris-AMP was 4.68×10^{-5} M.

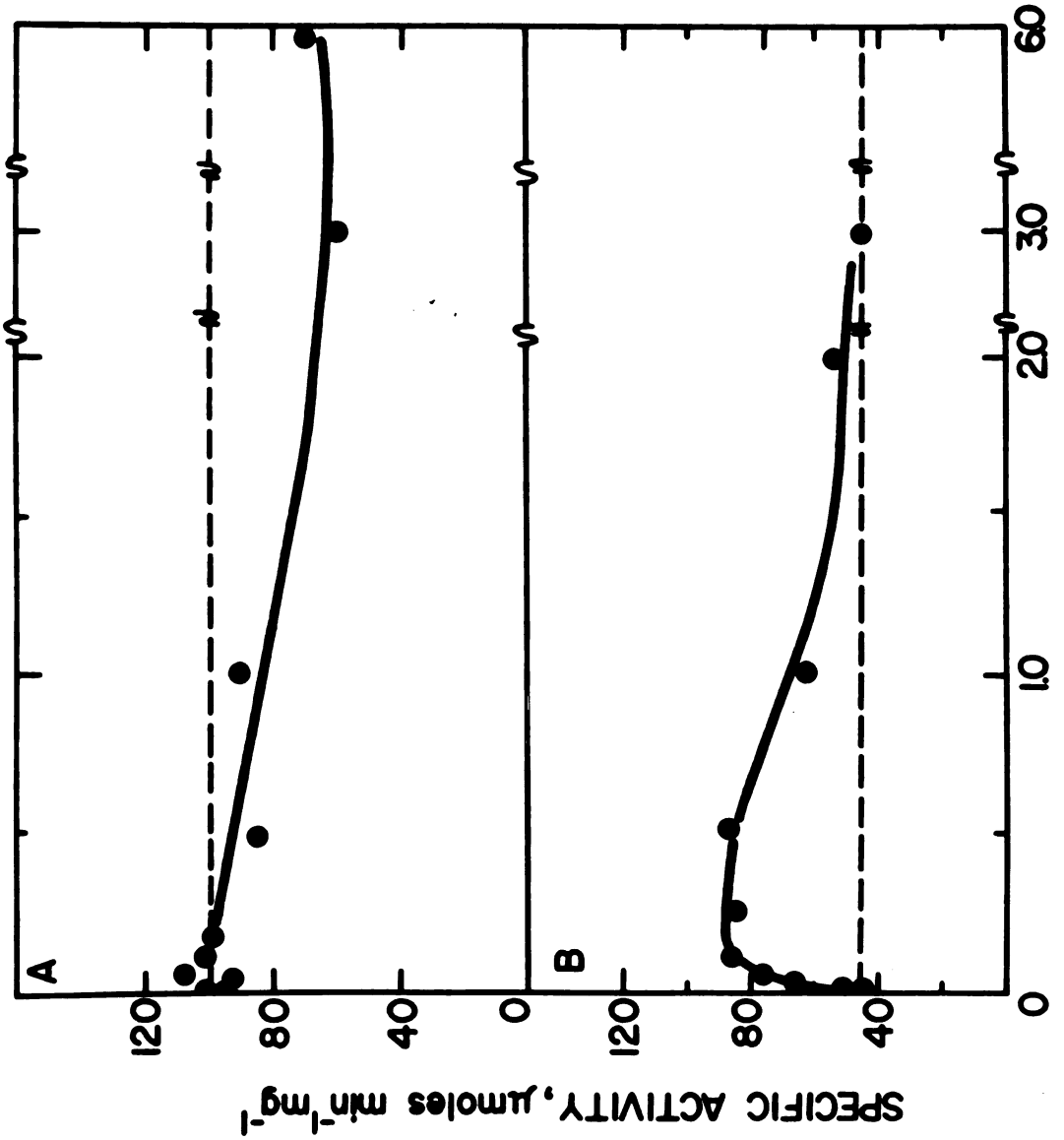

A
B
120
80
40
0
120
80
40
0
1.0
2.0
3.0
6.0
BSA IN ASSAY, mg ml^{-1}
SPECIFIC ACTIVITY, μmoles min^{-1}mg^{-1}

Figure 5.

b. Effect of glycerol and $(CH_3)_2SO$. Two organic compounds, glycerol and $(CH_3)_2SO$, are responsible for changes in the kinetic parameters in several enzyme systems (80, 97, C.H. Suelter and M. Ruwart, personal communication). Their effects on the 5'-AMP aminohydrolase system were studied to determine if changing solvent polarity resulted in a protective or an inhibitory effect on specific activity and to determine if the pH effect were dependent on solvent polarity. The percentage of glycerol and $(CH_3)_2SO$ used was in the range which resulted in activation of yeast pyruvate kinase (C.H. Suelter and M. Ruwart, personal communication). Controls for experiments at both pH 6.3 and pH 7.0 in both solvents are presented in Table 2. Stock protein concentration at pH 6.3 was 4.9×10^{-2} mg ml^{-1}, at pH 7.0, 5.9×10^{-2} mg ml^{-1}. High and low enzyme concentrations at pH 6.3 were 4.9×10^{-1} µg ml^{-1} and 4.9×10^{-2} µg ml^{-1}, respectivly; at pH 7.0 high and low enzyme concentrations were 1.18 µg ml^{-1} and 5.9×10^{-2} µg ml^{-1}, respectively. Substrate concentrations were 4.98×10^{-5} M and 6 mM for suboptimal and saturating levels at pH 6.3; at pH 7.0, suboptimal and saturating levels of substrate were 4.91×10^{-5} M and 6 mM, respectively. There was no loss in specific activity during these experiments as monitored by the standard assay.

The effect of 20% (v/v) glycerol on specific activity at pH 6.3 was noted at both suboptimal and saturating substrate concentrations. The basic assay (see Methods and Materials) was used; 1 mM MSH was included. The effect of glycerol was observed at protein concentrations that yielded low and high specific activities, 4.9×10^{-2} µg ml^{-1} and 4.9×10^{-1} µg ml^{-1}, respectively, at pH 6.3. At pH 6.3 in the presence of saturating substrate, glycerol had little effect on specific activity at either protein concentration (Table 2). However, at suboptimal substrate concentrations, there is a marked reduction in specific activity at both high and low enzyme concentrations, less

TABLE 2

Specific Activity of 5'-AMP Aminohydrolase as a Function of Enzyme and Substrate Concentration at pH 6.3 and pH 7.0 in the Presence and Absence of Glycerol or $(CH_3)_2SO$.

pH	Specific Activity (μmoles $min^{-1} mg^{-1}$)			
	Saturating Substrate		Suboptimal Substrate	
	Enzyme Conc. ($\mu g\ ml^{-1}$)		Enzyme Conc. ($\mu g\ ml^{-1}$)	
	0.049	0.49	0.049	0.49
6.3	1204	944	135	80
6.3 (glycerol, 20% v/v)	1296	1056	48	51
6.3 ($(CH_3)_2SO$, 20% v/v)	1759	1407	84	67
	Enzyme Conc. ($\mu g\ ml^{-1}$)		Enzyme Conc. ($\mu g\ ml^{-1}$)	
	0.059	1.18	0.059	1.18
7.0	918	847	103	41
7.0 (glycerol, 20% v/v)	989	833	66	37
7.0 ($(CH_3)_2SO$, 20% v/v)	1589	1342	70	31

Reaction mixture contained Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1 mM MSH, and enzyme. At pH 6.3, saturating Tris-AMP was 6.0×10^{-3} M and suboptimal was 4.98×10^{-5} M; enzyme stock solution was 0.49 mg ml^{-1} (0.05 M Tris-MES, 0.1 M KCl, 1 mM MSH, pH 6.3). At pH 7.0, saturating Tris-AMP was 6.0×10^{-3} M and suboptimal was 4.91×10^{-5} M; enzyme stock solution was 0.59 mg ml^{-1} (0.05 M Tris-MES, 0.1 M KCl, 1 mM MSH, pH 7.0). Reaction was followed at 30°C. at 265 mμ or 290 mμ.

marked at the more dilute enzyme concentration. The results at pH 7.0 (Table 2) are similar to those at pH 6.3. However the glycerol effect at low enzyme concentration and suboptimal substrate concentration is not as marked as at pH 6.3.

The effect of 20% (v/v) $(CH_3)_2SO$ was observed under conditions similar to those in the glycerol experiment. The basic assay (see Materials and Methods) was used with $(CH_3)_2SO$ at a final concentration of 20% (v/v) and MSH present at 1 mM. At pH 6.3, dilute and high enzyme concentrations were 4.9×10^{-2} μg ml^{-1} and 4.9×10^{-1} μg ml^{-1}, respectively. Suboptimal and saturating levels of substrate were 4.6×10^{-5} M and 6 mM, respectively. At pH 7.0, dilute and high enzyme concentrations were 5.9×10^{-2} μg ml^{-1} and 1.18 μg ml^{-1}, respectively; suboptimal and saturating levels of substrate were 4.54×10^{-5} M and 6 mM, respectively. At pH 6.3 (Table 2), $(CH_3)_2SO$ stimulated the system in the presence of saturating substrate, to about the same degree at both dilute and high enzyme concentrations. As in the glycerol experiments, there was inhibition of specific activity at the suboptimal substrate concentration for both enzyme concentrations studied, more marked at the higher enzyme concentration. At pH 7.0, results were similar with slightly greater activation at saturating substrate concentrations than at pH 6.3; inhibition at suboptimal substrate concentration was similar at both pH's.

C. Hydrogen Ion Concentration Effect on ADP Activation of the 5'-AMP Aminohydrolase Reaction.

Since results of the above experiments indicated that substrate activation observed at pH 7.0 at dilute protein concentration was due to the effects of high dilution and perhaps dissociation rather than true activation, the activation of this system by ADP was studied at high protein concentration of the stopped flow apparatus. The stopped flow assay (See Materials and Methods)

was used. At pH 6.3 and assay concentrations of 1.79×10^{-4} M Tris-AMP and 5×10^{-2} mg ml^{-1} enzyme, Tris-ADP was varied from 0 to 1.79×10^{-4} M in the assay. Maximally activated velocity at this substrate concentration was 340 µmoles min^{-1} mg^{-1}. At pH 7.0 and assay concentrations of 1.65×10^{-4} M Tris-AMP and 5×10^{-2} mg ml^{-1} enzyme, Tris-ADP in the assay varied from 0 to 2.0×10^{-4} M; maximally activated velocity under these conditions was 390 µmoles min^{-1} mg^{-1}. Optical densities were calculated as described in appendix 1. Kinetic parameters were determined both from Hill plots (94) and from use of the Hill n program (William I. Wood, personal communication). Calculation of initial velocities were made excluding any lag present. Calculation of activation parameters was made after subtraction of velocity in the absence of Tris-ADP. There was a notable increase in both K_A and the Hill slope for ADP with pH (Table 3). In these experiments, a lag was observed in the transmittance-time curves at pH 7.0 that was not present at pH 6.3 at the same time sweep. This lag at pH 7.0 was apparent at low ADP concentrations and was removed at highest ADP concentrations. Photographic records of two time-transmittance curves for the ADP activation experiments at pH 6.3 and pH 7.0 are shown in Figures 6A and 6B, respectively. The scans were obtained for similar substrate, enzyme, and ADP concentrations at similar instrument settings, with 100 msec sweep time at both pH's. One may note the apparent lag at pH 7.0 (see Figure 6B).

TABLE 3

Kinetic Activation Parameters for ADP Activation of 5'-AMP Aminohydrolase at pH 6.3 and pH 7.0 Determined by Stopped Flow Spectrophotometry.

pH	K_A (μM)	n_{H_A}
6.3	13.9	0.8
7.0	46.7	2.9

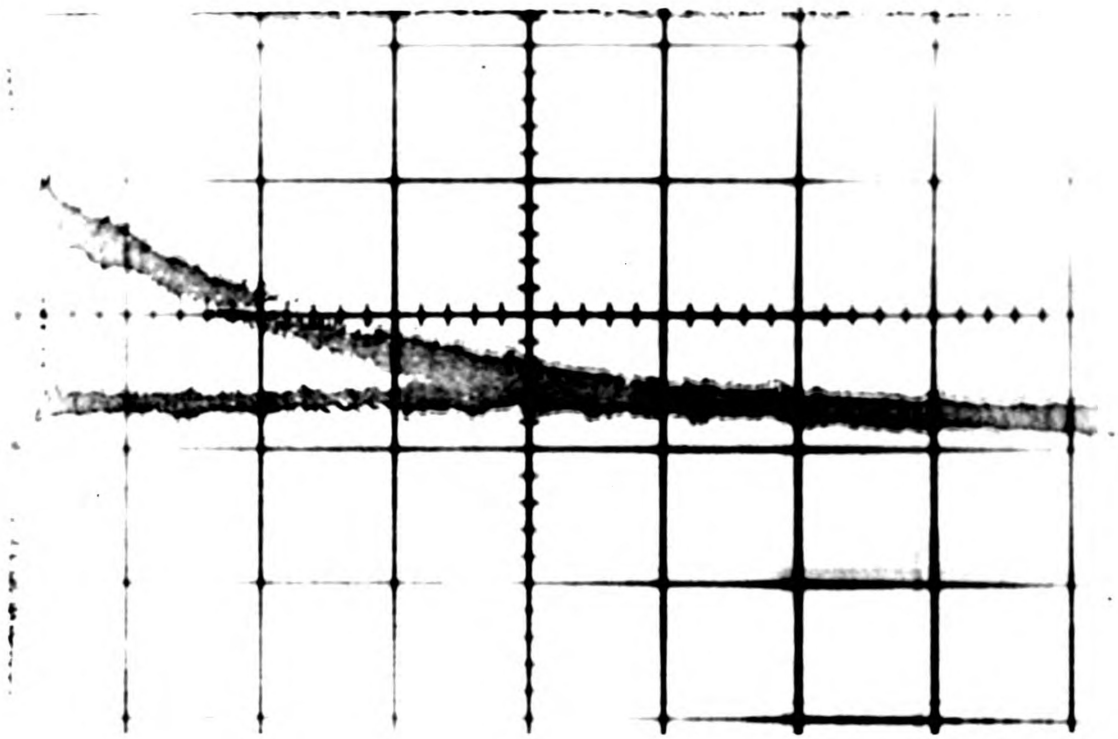
Reaction mixtures contained Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1 mM MSH, 0.05 mg ml^{-1} enzyme, and Tris-ADP, at the appropriate pH. At pH 6.3, Tris-AMP was 1.79×10^{-4} M and Tris-ADP varied from 0 to 1.79×10^{-4} M. At pH 7.0, Tris-AMP was 1.65×10^{-4} M and Tris-ADP varied from 0 to 2.0×10^{-4} M. Reaction was followed at 285 mμ at 30° C.

Figure 6. Transmittance-Time Curves for ADP Activation of 5'-AMP Aminohydrolase in Stopped Flow Studies.

A, pH 6.3. Assay mixture contained 1.79×10^{-4} M Tris-AMP, 0.05 M Tris-MES, 0.1 M KCl, 1×10^{-3} M MSH, 0.05 mg ml^{-1} enzyme, and 4.48×10^{-5} M Tris-ADP. Reaction volume was approximately 0.4 ml. Reaction was followed at 285 mμ. Reaction temperature was 30° C. Sweep time was 100 msec $division^{-1}$.

B, pH 7.0. Assay components and conditions were as in A, but at pH 7.0. Tris-AMP was 1.66×10^{-4} M and Tris-ADP was 4.0×10^{-5} M.

A.



B.

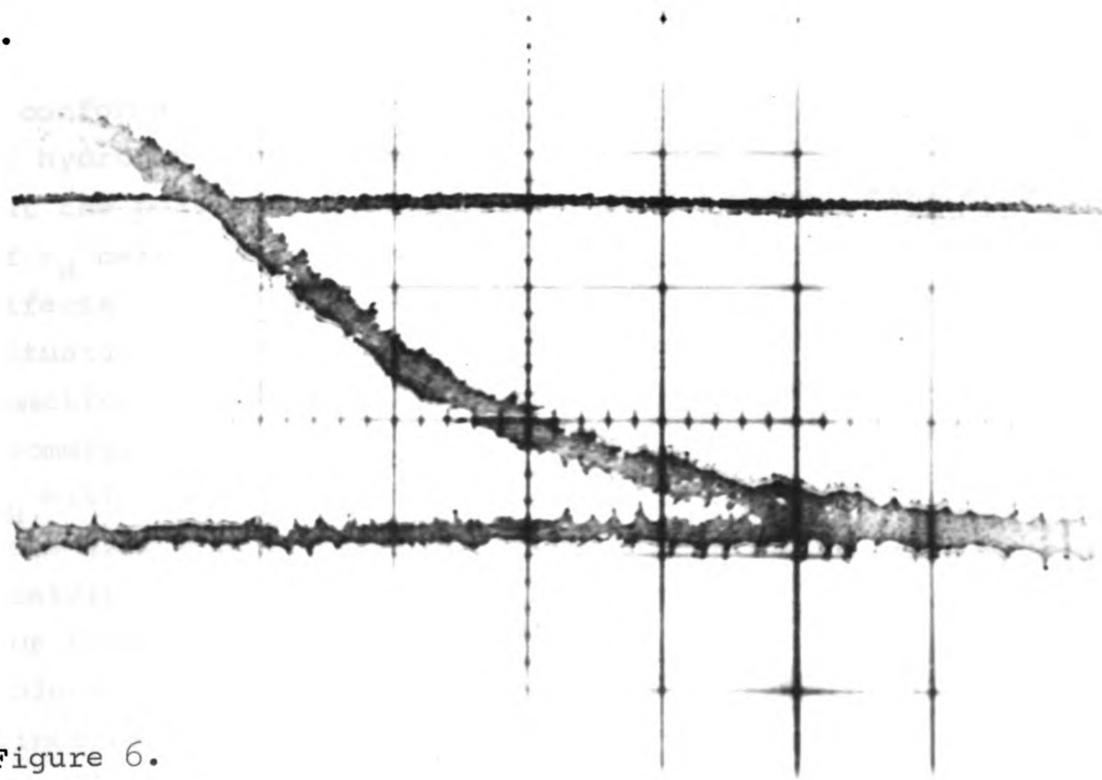


Figure 6.

DISCUSSION

A. Protein Concentration Effect on the 5'-AMP Aminohydrolase Reaction at Two Hydrogen Ion Concentrations.

Several points may be drawn from analysis of the results presented here. Results in Table 1 indicate a change in Hill slope from near unity at pH 6.3 to 1.81 at pH 7.0 at dilute concentrations of enzyme. At higher enzyme concentrations, n_H is near unity at both pH's (C. Zielke, personal communication). The Hill slope, an empirical term, has been assigned various types of physiological significance (44,45) including a role as a measure of the interactions between binding sites of a ligand (45), where such interactions are understood in terms of a structurally mediated response to the ligand. Thus, the Hill slope could monitor pH-dependent changes in substrate binding linked to hydrogen ion binding through a conformational alteration, in an allosteric effect of hydrogen ion analogous to the hemoglobin Bohr effect (44). But the possibility of protein concentration dependency of n_H makes the role of n_H as a measure of allosteric effects of hydrogen ion less acceptable. In this situation, n_H loses its significance in terms of allosteric homotropic or heterotropic interactions resulting from isomeric forms of the protein. However, the change in n_H with decreasing protein concentration coupled with loss in specific activity may acquire significance as a measure of inter-subunit interactions responsible for holding an oligomeric protein intact. Binding of small molecules to a polymerizing protein system to give sigmoidal kinetics has been studied by Nichol *et al.* (98) as a model for allosterism, although for a system in which the dissociated species is the active form; Frieden (99) and

Wyman (100) have also considered allosteric properties not uniquely the result of binding equilibria among different conformations, but also the result of differing responses to ligands of proteins in different states of association. Thus, the change in Hill slope over a protein concentration range remains interesting relative to changes in specific activity with protein concentration.

Results in Figures 1 and 2 offer strong support for a protein concentration dependent activity of 5'-AMP aminohydrolase. It is again interesting to observe that the protein concentration used in the Hill slope study at pH 6.3 is in the range in which specific activity is not decreasing, while at pH 7.0, this same protein concentration is in the range where specific activity is decreasing. If a decrease in specific activity can be used as an indication of the extent of dissociation of an oligomeric enzyme into inactive or subactive species (96,101-105), specific activity loss with decreasing enzyme concentration is consistent with dilution-induced dissociation on 200-fold dilution in the spectrophotometric assay used in Figure 2. The results in Figure 1 argue against a loss in activity in the stock solutions.

From data in Figure 2, estimates of dissociation constant based on half-maximal specific activity were calculated to be 2.88×10^{-10} M at pH 6.3 and 1.94×10^{-9} M at pH 7.0. These values may be compared with molar dissociation constants calculated from the data of Klotz et al (106) presented in Table 4. Interestingly, dissociation of zinc from α-amylase in subunit dissociation has a dissociation constant in the 10^{-10} M range.

Whether Michaelis-Menten or allosteric kinetics are observed, a point to be examined is the relationship of kinetic properties and protein concentration. This is particularly important in systems which can dissociate, especially if dissociation is to inactive subunits as in transcarboxylase (80) or to a less active subunit as

TABLE 4

Molar Dissociation Constants for Various Proteins*.

Protein*	Molar Dissociation Constant** K_D
Bacillus subtilis	
∝-Amylase, pH 7.0, 20°	
$A_2(Zn\not\not) \rightleftharpoons 2A_1 \not + Zn\not\not$	5×10^{-10}
Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase, pH 7, 20°	
$A_4 \rightleftharpoons 2A_2$	5×10^{-7}
Insulin, pH 2, 25°	
$A_2 \rightleftharpoons 2A_1$	9.9×10^{-5}
Hemoglobin, pH 6.9, 25°	
$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$	1×10^{-6}
pH 7, 3°	
$\alpha\beta \rightleftharpoons \alpha \not + \beta$	1.61×10^{-8}
$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$	3.15×10^{-6}
β-Lactoglobulin, pH 4.4, 4.5°	
$A_8 \rightleftharpoons 4A_2$	3.57×10^{-12}
β-Lactoglobulin A, pH 6.9, 20°	
$A_2 \rightleftharpoons 2A_1$	2.05×10^{-5}
β-Lactoglobulin B, pH 6.9, 20°	
$A_2 \rightleftharpoons 2A_1$	7.04×10^{-6}
Lactate Dehydrogenase, pH 2, 20°	
$A_2 \rightleftharpoons 2A_1$	3.7×10^{-5}
$A_4 \rightleftharpoons 4A_1$	3.63×10^{-15}
$A_8 \rightleftharpoons 8A_1$	3.44×10^{-33}
Tryptophanase, pH 8, 5°	
$A_4 \rightleftharpoons 2A_2$	8.34×10^{-5}

*These proteins and the subunit equilibria are from Klotz *et al.* (106); the subunit equilibria have been depicted for dissociation.

**Molar dissociation constants were calculated from molar association constants listed by Klotz *et al.* (106).

suggested for heart phosphofructokinase (70). Dissociation due to high dilution (of the type that may be involved in certain assays) may result in variation, such as a decrease, in specific activity. Dennis (107) has pointed out that the sigmoidicity of the substrate saturation curve in a system containing inactivated enzyme may be an artefact of enzyme inactivation.

Bernfield et al. (96) have cited crystalline rabbit muscle aldolase and lactate dehydrogenase, porcine pancreatic α-amylase and sweet potato β-amylase as enzymes that dissociate reversibly into inactive species at high dilution. In this study each enzyme had a characteristic concentration at which specific activity was half the maximal value-- 2, 1.5, 0.005, and 1.0 μg ml^{-1}, respectively for the enzymes cited (96). Similar findings were observed with bovine testicular hyaluronidase (103,104) and with bovine spleen and liver β-glucuronidase (104,105).

Similarly, for rabbit muscle phosphofructokinase at pH 6.7, dilution below 100 μg ml^{-1} caused a protein concentration dependent loss of specific activity, while at pH 8.0 dilution to 1 μg ml^{-1} caused no decrease of specific activity (82). Studies with rabbit liver phosphofructokinase at pH 8.0 also indicate that high dilution of enzyme results in markedly reduced specific activity; the loss is reduced in the presence of activator, Na_2SO_4 (108). For this enzyme, in a stock solution in the presence or absence of the activator, fructose-6-phosphate, the Hill slope is one, while for the diluted enzyme, the Hill slope is 1.58 in the absence of fructose-6-phosphate and unity in its presence (108).

Wurster and Hess (109) in their stopped flow studies (pH 6.4) suggested that rabbit muscle lactate dehydrogenase existed as an active tetramer to a concentration of 0.28 μg ml^{-1} (2×10^{-9} M, assuming a molecular weight of 140,000 g $mole^{-1}$ (113)), and that below this concentration partial dissociation to an inactive subunit might be occurring,

resulting in decreased specific activity. Lineweaver-Burk treatment of data at various protein concentrations suggests on inspection that loss of specific activity is also occurring for Aerobacter aerogenes D-lactate dehydrogenase (110) at protein concentrations in the range around 0.17 μg ml^{-1} (0.00125 μM) at pH 5.7 and around 0.35 μg ml^{-1} (0.0025 μM) at pH 7.0. For this enzyme, at acid pH, Lineweaver-Burk plots yield straight lines down to 0.17 μg ml^{-1} enzyme, while at pH 7.0 upward curvature occurs, indicating sigmoidal kinetics; at pH 7.0, raising the protein concentration to 0.1 μM erased the sigmoidal response seen at dilute protein concentrations (110). Both Sawula and Suzuki (110) and Griffin and Criddle (111) suggest the importance of considering protein concentration in evaluating a system yielding sigmoidal kinetics. Sawula and Suzuki (110) argue that D-lactate dehydrogenase is present in the cell at concentrations which would show hyperbolic kinetics. Griffin and Criddle (111) on the other hand, suggest that rabbit muscle lactate dehydrogenase may exist in a particulate form in the cell and that compartmentalization may complicate in vivo considerations; they refer to an enzyme concentration in rabbit and rat tissues of 0.2 mg ml^{-1}.

Cross and Fisher (112) reported that glutamate dehydrogenase is fully dissociated at 0.5 μg ml^{-1}, an assay concentration.

Phenylalanine hydroxylase also appears to exhibit a dependence of activity on protein concentration (113).

Echerichia coli alkaline phosphatase, a dimeric zinc metalloenzyme, is reported to dissociate under assay conditions at protein concentrations less than 0.3 μg ml^{-1}, although the resultant monomer is postulated to be more active (101). For this enzyme, dissociation of zinc is thought to play a role in protein dissociation (101) with zinc dissociation constants of 20 nM and 60 pM for the first and second zinc, respectively (114).

5'-AMP aminohydrolase from calf brain also appears to lose specific activity with decreasing protein concentration at pH 7.5; specific activity decreases from a maximal value (8 μmoles min^{-1} mg^{-1}) at an assay protein concentration of 0.1 mg ml^{-1} to a half-maximal value (4 μmoles min^{-1} mg^{-1}) at a concentration around 0.01 mg ml^{-1} (18).

Another point to be drawn from results presented here refers to the role of substrate in the rabbit muscle 5'-AMP aminohydrolase assay system. Since protein dissociation at high dilution may occur in this system, the sigmoidal kinetics at pH 7.0 (Table 1) may be interpreted as protection of the enzyme by high substrate concentration against dissociation by high dilution. This interpretation is supported by the results in Figures 3 and 4 which show response of specific activity to enzyme concentration at suboptimal and saturating substrate concentrations. At suboptimal substrate concentrations (Figure 3), little protection is offered as the enzyme is diluted, especially at pH 7.0. However, at saturating substrate concentrations (Figure 4), specific activity remains fairly constant over most of the protein concentration range studied; at pH 7.0 in particular, saturating substrate appears to protect over the protein concentration range in which decreasing specific activity is observed at suboptimal substrate concentrations. This observation points to the role of substrate in protecting against dilution-induced dissociation . Substrate may function by shifting the equilibrium of the dissociated system toward the associated, fully active form. Two conformations of the enzyme may exist in which substrate binds the enzyme in the more active conformation which is less susceptible to the effects of high dilution. The decrease in specific activity of some enzymes resulting from high dilution is a phenomenon protected against or reversed by the presence of substrate (108,115) or other activators (108,115-117).

These possibilities are supported by results presented in Figure 5 and in Table 1 for experiments carried out with BSA. At pH 7.0, BSA (0.1 mg ml^{-1}) causes a reduction in the sigmoidicity of the velocity-substrate curve with a change in Hill slope from 1.81 to 1.30 (Table 1) and stimulates specific activity almost two-fold (Figure 5). These results were obtained at a 5'-AMP aminohydrolase concentration of 0.5 μg ml^{-1} at which specific activity at pH 7.0 is markedly reduced. Thus, BSA may function to protect the enzyme against the effects of high dilution.

BSA appears to stabilize many enzymes including swine kidney microsomal aminopeptidase (118), phenylalanine hydroxylase from Comanona (119), phosphofructokinase (108), and lactate dehydrogenase (120). For dilution-induced dissociation of rabbit muscle aldolase and lactate dehydrogenase, porcine α-amylase, and sweet potato β-amylase as described by Bernfield et al. (96), BSA, as well as other polycations and polycationic proteins, functions as an "activator" of the diluted enzymes, reversing or preventing dissociation. These observations, and the finding that the Hill slope at high protein concentration remains at unity (C. Zielke, personal communication) suggest that a dilution-induced dissociation of 5'-AMP amino-hydrolase into in- or sub-active species may be occurring and is protected against by high substrate concentration as well as by BSA.

In this study, the effects of the organic solvents, glycerol and $(CH_3)_2SO$, on the reaction kinetics at dilute enzyme and high enzyme concentration were also examined in the presence of suboptimal and saturating substrate concentrations. Glycerol, $(CH_3)_2SO$, as well as other organic compounds, i.e., dioxane and sucrose, as employed in assays or extraction procedures may have an activating or inhibitory effect on the specific activity of the enzyme, possibly by affecting its association-dissociation equilibrium (80, 92-95, 97-99) and may thus

alter the effects of high dilution. Glycerol and $(CH_3)_2SO$ at 25% (v/v) concentration are known to protect yeast pyruvate kinase against inactivation by dilution (C.H. Suelter and M. Ruwart, personal communication). Mayer and Avi-Dor (97) suggest that glycerol and $(CH_3)_2SO$ stimulate the soluble K^+-stimulated enzymes, p-nitrophenyl phosphatase and muscle pyruvate kinase, and inhibit the membranal Na^+, K^+-activated adenosine triphosphatase in a dilution-reversible phenomenon. They attribute the solvent effects to both alterations in enzyme structure and changes in degree of solvation of the activating cation(97). In the 5'-AMP aminohydrolase system, glycerol and $(CH_3)_2SO$ (both 20%, v/v) presented differing effects depending on the concentration of substrate. The effect (Table 2) at suboptimal substrate concentration at both dilute and high enzyme concentrations was inhibition, indicating that these solvents enhance the effect of dilution at low substrate concentrations, perhaps by shifting a dissociation equilibrium in the direction of the dissociated inactive species. However, at saturating substrate concentrations where protection is already occurring, at both dilute and high enzyme concentrations, there was a slight stimulatory effect by glycerol and a marked stimulation of specific activity with $(CH_3)_2SO$. Response to the solvent is similar at both pH 6.3 and pH 7.0. These results are difficult to explain. They might be interpreted as a stabilization of the groups responsible for dissociation. At high substrate concentrations these groups may be buried in the conformation stabilized by high substrate and inaccessible to the solvents, where at low substrate concentrations, these groups may be accessible to the solvents, resulting in dissociation and decrease in specific activity.

B. Hydrogen-Ion Concentration Effect on the 5'-AMP Aminohydrolase Reaction.

The effect of pH on the Hill slope change at dilute enzyme concentrations (Table 1) may be explained as the result of a protein concentration dependence of the reaction. However, there is a hydrogen-ion concentration effect evident in the protein concentration dependency of the reaction. A hydrogen-ion concentration dependent difference in the response of specific activity to protein concentration is seen in Figures 1 and 2. In stopped-flow experiments (Figure 1) over the same protein concentration range, specific activity is more constant in the range below 0.04 mg ml^{-1} at pH 6.3 than at pH 7.0. This trend is stronger in Figure 2; specific activity at pH 6.3 remains fairly constant to a concentration of 0.5 to 0.7 $\mu g\ ml^{-1}$ below which it begins to decrease, while at pH 7.0 specific activity decreases over the entirety of the same protein concentration range. Thus, the dissociation constant appears to be pH-dependent, the estimate at pH 7.0 being about ten-fold greater than at pH 6.3. The same trend is visible in Figures 3 and 4 in the effects of substrate protection. At suboptimal substrate levels (Figure 3), with enzyme varied from a constant stock solution, pH dependence results are basically those of Figure 2. At saturating substrate concentration (Figure 4) the results at pH 6.3 and pH7.0 are essentially similar with a slightly slower decrease in specific activity at pH 6.3. The effect of BSA on specific activity (Figure 5) also points to this pH difference. At pH 6.3, low concentrations of BSA have no effect while higher concentrations are inhibitory. At pH 7.0 specific activity is enhanced almost two-fold by low concentrations of BSA; this stimulation decreases at increasing BSA concentrations.

The differing results at the two pH's may be explained in terms of different states of dissociation

which in turn point to a pH-dependent dissociation process and dissociation constants at suboptimal substrate concentrations. For dilution-induced protein dissociation, indicated by decrease in specific activity, a pH dependence implies the existence of groups involved in the dissociative process, which are at different ionization states at the two pH's. This difference in response to pH implies an allosteric hydrogen ion effect in which dissociation may be considered an extreme form of conformational change, analogous to the dissociation Bohr effect (43). The binding of substrate is influenced by binding of hydrogen ion through dissociation; substrate and hydrogen ion are linked functions of the ionizable groups involved in the dissociation process. The pH studies reported here indicate these ionizable groups might have a pK between 6.3 and 7.0, with the imidazole of histidine being the most likely candidate (125). Such groups might be buried in the form stabilized by saturating substrate concentrations.

The results of stopped-flow studies with ADP (Table 3), together with the lag observed at pH 7.0 but not at pH 6.3 (Figure 6) point to a pH dependency in ADP activation of 5'-AMP aminohydrolase. ADP binding may be considered linked to hydrogen-ion binding through a conformational change mediated by groups with a pK between 6.3 and 7.0. The pH effect changes over the same range as that observed for the apparent dissociation phenomenon, although at a protein concentration where dissociation is not indicated. Thus the same group(s) may be responsible for both effects.

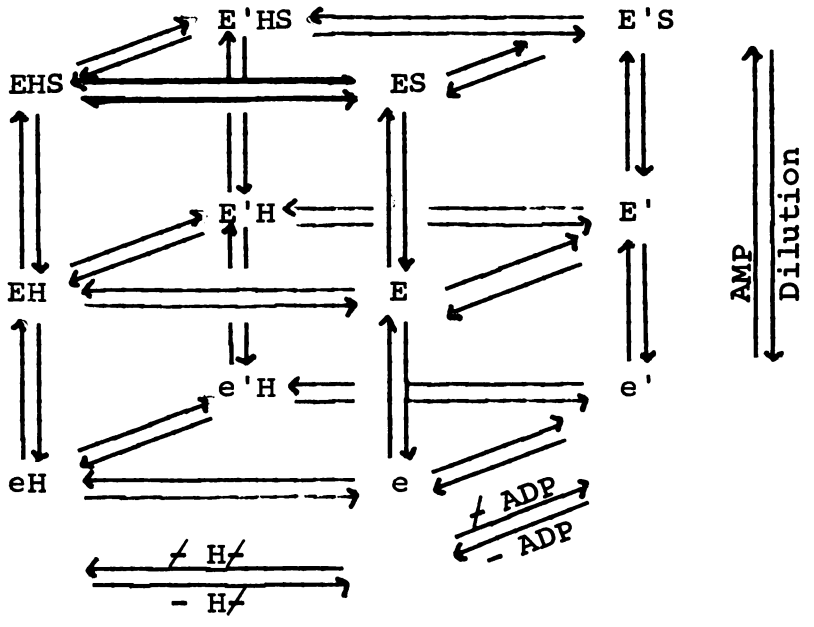
C. Physiological Significance of Protein and Hydrogen-Ion Concentration Effects of the 5'-AMP Aminohydrolase Reaction.

The effect of hydrogen-ion concentration on the association-dissociation state of 5'-AMP aminohydrolase may be significant *in vivo*. The *in vivo* enzyme concentration in rabbit muscle is at least 0.15 mg ml^{-1}, as calculated from yields of preparations used in this study (R.M. Hemphill, unpublished observation). If all isolated enzyme were

soluble *in vivo*, hyperbolic kinetics could be expected. However, there are several indications that the enzyme is also particulate. Weil-Malherbe and Green (27) reported its activity associated with insoluble particles from brain. The human erythrocyte enzyme exists in both soluble and particulate forms, exhibiting sigmoidal and hyperbolic kinetics, respectively (23,25). The muscle enzyme also may be membrane associated (29). A particulate form of the enzyme and the resulting compartmentalization would indicate a more complicated kinetic scheme *in vivo*. There may be an equilibrium between particulate and soluble enzyme with the concentration of the soluble form in a range subject to association or dissociation with pH changes. There may also be a pH-dependent ADP activation and control of the enzyme *in vivo*. The role of hydrogen-ion concentration in muscle as a controlling factor is supported by reports of large pH changes during muscle contraction (126) and calcium release (127).

D. Interrelation of Protein and Hydrogen-Ion Concentration Effects on the 5'-AMP Aminohydrolase Reaction.

The effects of protein and hydrogen-ion concentration as well as the effects of substrate, ADP, BSA, and dilution suggest the following theoretical model:



This model assumes an oligomeric enzyme existing in two conformational states, E and E', both capable of binding and reacting with substrate, E' being the more active form. Each form may be protonated; the protonated forms are more stable against dissociation. Each form of the enzyme is subject to the effects of dilution, dissociating to forms, e and e', which bind but do not react with substrate and which can be protonated. Hydrogen ion binding shifts the dissociative equilibrium toward the associated, active form. Thus, for a given enzyme concentration in the dissociative range, the greater the hydrogen ion concentration, the larger the proportion of the associated, active species. Substrate binding also protects the enzyme by shifting the equilibrium toward the associated, active form, locking the enzyme in this form. Hydrogen ion and substrate both act by making groups responsible for dissociation less accessible to solvent and to dilution effects, although in different ways. ADP activates the enzyme by shifting the equilibrium toward the more active form, E'(H). Hydrogen ion binding is linked to ADP binding through a conformational change; hydrogen ion binding is linked to substrate binding through a dissociative change. BSA functions to stabilize the associated, active form of the enzyme. Glycerol and $(CH_3)_2SO$ act to stabilize the dissociated form.

Such a hypothetical model remains to be confirmed by ultracentrifugal analysis of the dissociative properties of the system at different pH's and by direct conformational studies in the presence and absence of ADP. The possibility of zinc involvement in dissociation remains to be examined.

CONCLUSIONS

Several conclusions may be drawn from the work presented here. 5'-AMP aminohydrolase is inactivated by high dilution as monitored by loss in specific activity, probably by dissociation into in- or sub-active subunits. The effect of enzyme dilution is pH-dependent, perhaps due to a pH-dependent dissociation constant of group(s) involved in dissociation. Hydrogen ion (pH6.3) protects against the effects of dilution, as does saturating substrate concentration. BSA also protects the enzyme against the effects of high dilution in a pH-dependent manner. ADP activation at high enzyme concentration (0.05 mg ml^{-1}) is pH-dependent; there is a lag at pH 7.0 not observed at pH 6.3 under similar conditions.

The primary implication of these conclusions is that caution must be applied to the interpretation of sigmoidal kinetics for enzymes assayed in protein concentration ranges similar to those used in this work. Estimates for molar dissociation constant for 5'-AMP aminohydrolase dissociation at pH 6.3 and pH 7.0 are 2.88×10^{-10} M and 1.94×10^{-9} M, respectively, assuming dimeric dissociation. In general, for kinetic assays carried out in the range of the dissociation constant of the enzyme, non-hyperbolic kinetics should be analyzed in terms of depolymerization as well as an equilibrium between isomeric forms of the enzyme.

A second point drawn from these conclusions is the allosteric role of hydrogen ion in this reaction. An allosteric heterotropic effect of hydrogen ion is noted in ADP activation of 5'-AMP aminohydrolase. Considering dissociation an extreme conformational change, an allosteric

response to hydrogen ion is also observed for substrate binding. The role of hydrogen ion in the allosteric control of 5'-AMP aminohydrolase as indicated in this study deserves further examination.

APPENDICES

APPENDIX 1

TREATMENT OF DATA COLLECTED IN STOPPED FLOW EXPERIMENTS

Data collected by Durram-Gibson stopped-flow spectrophotometry was initially recorded on a storage oscilloscope and photographed with a Polaroid camera using Polaroid Polaline Projection Film, Type 146-L. The transmittance-time transparency scans were projected, enlarged, and traced on graph paper, zero and end-of-reaction lines being noted. Readings were made at various times of the reaction run. Transmittance values were calculated basically by the equation

$$\text{Transmittance} = I/I_o$$

where I is transmitted light and I_o is incident light (128, 129). Calculations were made according to Gibson (130) as modified for use in this laboratory (personal communication, C.H. Suelter and C. Zielke). For decreasing transmittance (increasing optical density), taken at 285 mμ or 290 mμ (see Figure 7A), transmittance values were calculated by the relation

$$\text{Transmittance} = (R \not\prec H)/T$$

where R is the actual reading in mm between the end-of-reaction line and the reaction curve at a given time, H is the base deflection, the difference between the zero line and the end-of-reaction line, in mm, multiplied by an amplification factor, if any, and T is the total deflection, represented by the sum $H \not\prec R_o$ at zero time, in mm. For increasing transmittance (decreasing optical density), at 265 mμ (see Figure 7B), transmittance values were calculated by the relation

$$\text{Transmittance} = (T-R)/T$$

where R is the reading between the end-of-reaction line and the reaction curve, in mm, and T is the total deflection from the zero line to the end-of-reaction line, in mm.

Optical density values were calculated from transmittance values using the POLFIT*** program from Applied Computer Time Share, Inc., Detroit, Michigan, as modified by Howard Brockman (131) for use on the Burroughs 5500 Computer of the Philco-Ford Corporation Time Sharing System, Detroit, Michigan. The program was designed to fit data to a polynomial of form

$$y = a + bx + cx^2 + \ldots lx^{11}$$

which could be reduced to linear form

$$y = a + bx.$$

Transmittance values were converted to optical density values through application of this program to the linear conversion of transmittance to optical density:

optical density = -log transmittance

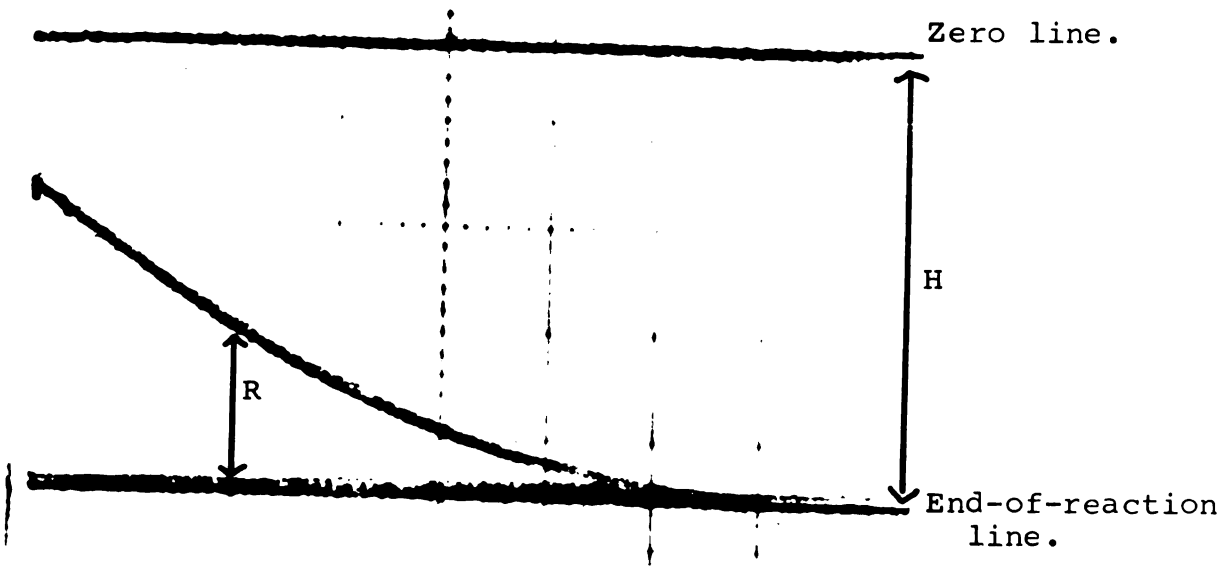
where transmittance was defined for increasing and decreasing systems as given above.

Optical density-time values were plotted and initial slopes noted as the change in optical density per msec. Activities were calculated by the following relation:

$$\mu\text{mole min}^{-1} = \frac{(\text{OD msec}^{-1} \times 1000 \text{ msec sec}^{-1} \times 60 \text{sec min}^{-1})2}{F}$$

where OD is optical density and F is a conversion factor with the values 8.86, 0.30, and 0.12 optical density units $\mu\text{mole}^{-1}\ \text{ml}^{-1}$ at wavelengths 265 mμ, 285 mμ, and 290 mμ, respectively (35,39). Multiplication by a factor of two was made to correct for a two-cm light path used in the stopped flow experiments. Specific activity is defined as activity per mg protein as $\mu\text{moles min}^{-1}\ \text{mg}^{-1}$.

A. Decreasing Transmittance



B. Increasing Transmittance

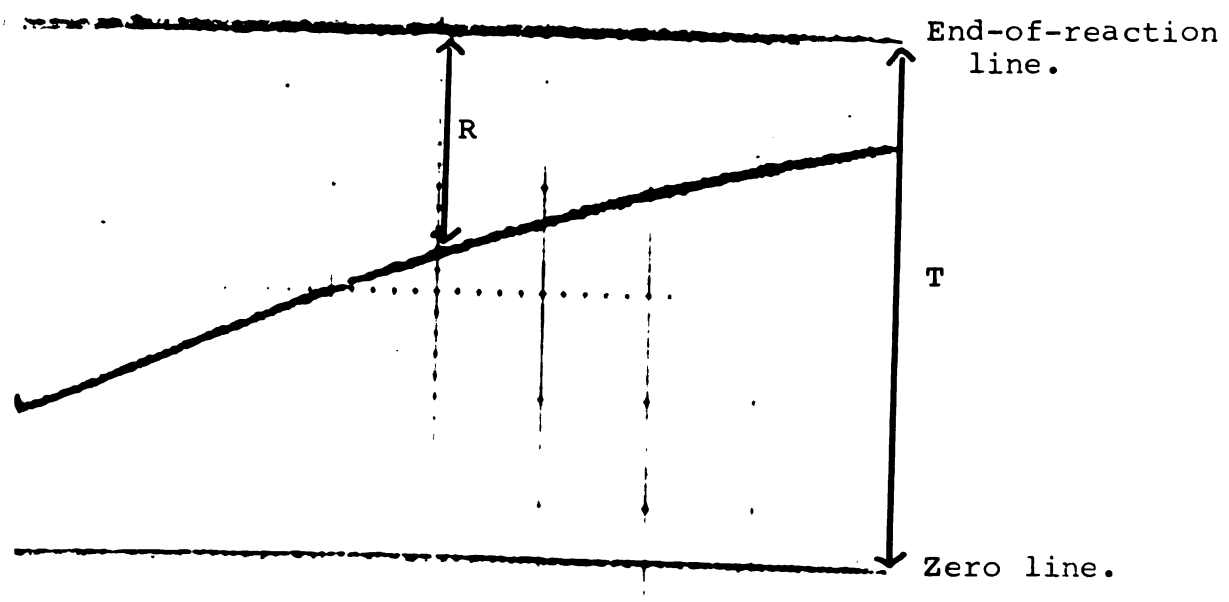


Figure 7. Stopped Flow Transmittance-Time Curves for Decreasing and Increasing Transmittance.

APPENDIX 2

CALCULATION OF KINETIC PARAMETERS

Dye and Nicely (95) have written a program for class and research use designed as a general curve-fitting program. A general linear equation for this program which would allow fitting of kinetic data obtained from both hyperbolic and sigmoidal velocity-substrate (or velocity-modifier) curves was derived. The kinetic parameters Km, Vmax, and n_H may be evaluated using this equation in the Dye-Nicely program.

For a system exhibiting Michaelis-Menten kinetics, the system may be described as follows (132):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P.$$

After forward and reverse velocities are equated and rearranged

$$Km = \frac{k_{-1} + k_2}{k_1} \qquad (1)$$

and

$$[ES] = \frac{[E][S]}{Km + [S]} \qquad (2).$$

Since initial velocity, v, is $k_2[ES]$ and maximal velocity, Vmax, is $k_2[E]$, then by substitution

$$v = \frac{Vmax\ [S]}{Km + [S]} \qquad (3).$$

A system with n interacting substrate binding sites may be described as follows

$$E + nS \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} ES_n \xrightarrow{k'_2} E + nP$$

where

$$[ES_n] = \frac{[E][S]^n}{Km + [S]^n} \qquad (4)$$

where the rate constants and Km are complex terms. For this system

$$v = \frac{Vmax\ [S]^n}{Km + [S]^n} \qquad (5).$$

One form of the Hill equation (94) may be derived from this relation which yields

$$(Vmax-v)/v = Km/[S]^n \qquad (6)$$

which after taking logarithms becomes

$$\log((Vmax-v)/v) = -n \log [S] + \log Km \qquad (7)$$

the form of the Hill equation used in manual calculation of Hill slopes.

One may also derive from the linear equation $y = ax + b$ a linear equation of form

$$1/y = 1/(ax + b) \qquad (8).$$

From equation 5 it follows that

$$v = \frac{1}{(Km/Vmax)(1/[S]^n) + 1/Vmax} \qquad (9)$$

which is of the form of equation 8 with $v = 1/y$, $1/[S]^n = x$, $Km/Vmax = a$, and $1/Vmax = b$; n is the Hill slope, n_H.

The Dye-Nicely program (95) functions by minimizing the sum of the squares of the residuals of the data points. The residual is determined for a given set of data as the difference between actual velocity and the velocity calculated from the substrate concentration using estimates of the parameters (Km/Vmax), n, and Vmax provided initially by the operator and, subsequently, by the program through reiteration. A weighting system is available for using weights at the beginning, not using weights, or converging without the use of weights, then using weights.

Equation 9 was defined for the program for use with two variables and three parameters as follows

$$v = \frac{1}{A(1/[S]^n) + 1/B} \qquad (10)$$

where $A = Km/Vmax$ and $B = Vmax$. The two variables were represented as $1/v = VEL$, and $[S] = XX(1)$. The three parameters were represented: $A = U(1)$, $n = U(2)$, and $B = U(3)$. The equation was written

$$VEL = 1/(U(1)*((1/XX(1))**U(2) + 1/U(3)) \qquad (11)$$

with the residual = VEL - XX(2). The subroutine used is presented in Figure 8.

The Dye-Nicely program (95) was tested for fitting data to this equation with theoretical data calculated

manually for expected Hill slopes of 1.0, 1.3, 1.7, and 2.0. The parameters resulting from this test as well as the initiallizing parameters are presented in Table 5. Results obtained with various weighting procedures did not change resulting parameters greatly. The system was also tested for velocity-substrate stopped flow data gathered at pH 6.8; manual calculation yielded a Hill slope of 1.1 while subjection of data to equation 9 in the Dye-Nicely program (95) yielded a value of 1.2 (R.M. Hemphill, unpublished observation). These results indicate that equation 9 is suitable for use with the Dye-Nicely curve-fitting program (95) to obtain adequate values for the kinetic parameters, Km, Vmax, and n_H.

```
SUBROUTINE EQN      FORTRAN EXTENDED VERSION 2.0   (210)   12/14/70          .11.01.08.
      SUBROUTINE EQN
      COMMON KOUNT,ITAPE,JTAPE,IWT,LAP,XINCR,NOPT,NOVAR,NCUNK,X,U,ITMAX,EQN 1282
     1WTX,TEST,I,AV,RESID,IAR,EPS,ITYP,XX,RXTYP,DX11,FOP,FU,FU,P,ZL,TO,EEQN 1283
     2IGVAL,XST,T,DT,L,M,JJJ,Y,DY,VECT,NCST,CONST                        EQN 1284
05    DIMENSION X(4,100), U(20), WTX(4,100), XX(4), FOP(100), FO(100), FEQN 1285
     1U(100), P(20,21), VECT(20,21), ZL(100), TO(20), IGVAL(20), XST(10EQN 1286
     20), Y(10), DY(10), CONST(16)                                       EQN 1287
      GO TO (2,3,4,5,1), ITYP                                            EQN 1295
    1 CONTINUE                                                           EQN 1296
10    ITAPE=60                                                           EQN 1300
      JTAPE=61                                                           EQN 1302
      WRITE (JTAPE,6)                                                    EQN 1303
    6 FORMAT ( * ACTIVATION RATES * )
      NOUNK=3
15    NOVAR=2
      RETURN
    2 CONTINUE
C 3 PARAMETERS U(1)=A, U(2)=N,U(3)=B
C EQN 1/Y=A(1/X)**N + 1/B
20  C EQN IN PROGRAM Y = 1/(A(1/X)**A + 1/B)
      VEL=1/(U(1)*((1/XX(1))**U(2)) + 1/U(3))
      RESID=VEL-XX(2)
      RETURN
    3 CONTINUE
25    4 CONTINUE
    5 CONTINUE
      RETURN
      END
```

Figure 8. Subroutine Used in Dye-Nicely Program.

TABLE 5

Kinetic Parameters Used for Data Generation, Estimated for Input, and Obtained as Output in a Test of a Curve-Fitting Program[1].

No. Data Pts	Parameters for Data Generation			Input					Output				
				Estimates					Calculated Values				
	Km^2	n_H	$Vmax^2$	$\frac{Km^2}{Vmax}$	n_H	$Vmax^2$	Wt[3]	Var[4]	$\frac{Km^2}{Vmax}$	n_H	$Vmax^2$	IR[5]	NE[6]
11	100	1.0	1500	0.074	1.1	1700	1	1:1	0.067	1.00	1500	4	60
11	100	1.0	1500	0.074	1.1	1700	1	1:100	0.067	1.00	1500	4	60
11	100	1.0	1500	0.066	1.0	1500	0	1:100	0.067	1.00	1500	4	56
11	100	1.3	1500	0.072	1.4	1650	1	1:1	0.060	1.26	1504	4	54
11	100	1.3	1500	0.066	1.3	1500	0	100:1	0.067	1.30	1500	4	56
13	100	1.7	1500	0.072	1.6	1700	1	1:1	0.061	1.70	1490	5	69
14	100	2.0	1500	0.063	2.15	1700	1	1:1	0.067	2.00	1500	4	60

[1] The general curve-fitting program used was that of Dye and Nicely (95).

[2] Km is in μM; Vmax is in μmoles min^{-1} mg^{-1}.

[3] Weighting system employed (95): 0 = converge without weights, then use weights.
1 = do not use weights

[4] Relative variance of variables.

[5] Iterations required.

[6] Number of times the sum of the squares of the residuals was evaluated.

LITERATURE CITED

1. Schmidt, G., Z. Physiol. Chem. 179, 243 (1928).
2. Purzycka-Preis, J. and Zydowo, M., Acta Biochim. Polon. 16, 235 (1969).
3. Dunkley, C. R., Manery, J. F., and Dryden, E. E., J. Cellular Physiology 68, 241 (1966).
4. Ronca-Testoni, S., Raggi, A., and Ronca, G., Biochim. Biophys. Acta 198, 101 (1970).
5. Fujiwara, T. and Spencer, B., Biochem. J. 85, 19P (1962).
6. Sammons, D. W., Henry, H., and Chilson, O. P., J. Biol. Chem. 245, 2109 (1970).
7. Makarewicz, W., Comp. Biochem. Physiol. 29, 1 (1969).
8. Atkinson, M. R. and Murray, A. W., Biochem. J. 104, 10C (1967).
9. Costello, J. and Brady, T.G., Biochem. J. 119, 6P (1970).
10. Currie, R. D. and Webster, H. L., Biochim. Biophys. Acta 64, 30 (1962).
11. Smith, L. D. and Kizer, D. E., Biochim. Biophys. Acta 191, 415 (1969).
12. Ronca, G., Raggi, A., and Ronca-Testoni, S., Biochim. Biophys. Acta 167, 626 (1968).
13. Lee, Y.-P. and Wang, M.-H., J. Biol. Chem. 243, 2260 (1968).
14. Cunningham, B. and Lowenstein, J. M., Biochim. Biophys. Acta 96, 535 (1965).
15. Setlow, B., Burger, R., and Lowenstein, J. M., J. Biol. Chem. 241, 1244 (1966).
16. Setlow, B. and Lowenstein, J. M., J. Biol. Chem. 242, 607 (1967).
17. Setlow, B. and Lowenstein, J. M., J. Biol. Chem. 243, 3409 (1968).
18. Rhoades, D. G., Ph. D. Thesis. Brandeis University, 1970.
19. Kalckar, H.M., J. Biol. Chem. 167, 461 (1947).
20. Nikiforuk, G. and Colowick, S. P. in Methods in Enzymology 2, S. P. Colowick and N. O. Kaplan, Eds., New York: Academic Press (1955), p. 469.

21. Smiley, K. L., Jr., Berry, A. J., and Suelter, C. H., J. Biol. Chem. 242, 2502 (1967).

22. Tomozawa, Y. and Wolfenden, R., Biochemistry 9, 3400 (1970).

23. Askari, A., Science 141, 44 (1963).

24. Askari, A. and Franklin, J. E., Jr., Biochim. Biophys. Acta 110, 162 (1965).

25. Rao, S. N., Hara, L., and Askari, A., Biochim. Biophys. Acta 151, 651 (1968).

26. Askari, A. and Rao, S. N., Biochim. Biophys. Acta 151, 198 (1968).

27. Weil-Malherbe, H. and Green, R. H., Biochem. J. 61, 218 (1955).

28. Mendicino, J. and Muntz, J. A., J. Biol. Chem. 233, 178 (1958).

29. Manery, J. F., Riordan, J. R., and Boegman, R. J., Federation Proc. 26, 591 (1967).

30. Wolfenden, R., Tomozawa, Y., and Bamman, B., Biochemistry 7, 3965 (1968).

31. Zielke, C. L., Ph.D. Thesis. Michigan State University, 1970.

32. Lee, Y.-P., J. Biol. Chem. 227, 993 (1957).

33. Zielke, C. and Suelter, C. H., J. Biol. Chem., in press (1971).

34. Raggi, A., Ranieri, M., Taponico, G., Ronca-Testoni, S., Ronca, G., and Rossi, C. A., FEBS Letters 10, 101 (1970).

35. Smiley, K. L., Jr., and Suelter, C. H., J. Biol. Chem. 242, 1980 (1967).

36. Lee, Y.-P. in The Enzymes 4, P. D. Boyer, H. Lardy, and K. Myrbach, Eds., New York: Academic Press (1960), p. 279.

37. Lowenstein, J. and Tornheim, K., Science 171, 397 (1971).

38. Seegmiller, J. E., Rosenbloom, F. M., and Kelly, W. N., Science 155, 1682 (1967).

39. Suelter, C. H., Kovacs, A. C., and Antonini, E., FEBS Letters 2, 65 (1968).

40. Antonini, E. and Brunori, M., Ann. Rev. Biochem. 139, 977 (1970).

41. Cantarow, A. and Schepartz, B., Biochemistry, Philadelphia: W. B. Saunders Company (1967), p.64.

42. Dixon, M., Biochem. J. 55, 161 (1953).

43. Wyman, J., Jr., Advan. Protein Chem. 19, 223 (1964).

44. Rossi-Fanelli, A., Antonini, E., and Caputo, A., Advan. Protein Chem. 19, 73 (1964).

45. Wyman, J., Advan. Protein Chem. 4, 407 (1948).

46. Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., and Kilmartin, J. V., Nature 222, 1240 (1969).

47. Monod, J., Wyman, J., and Changeux, J. P., J. Mol. Biol. 12, 88 (1965).

48. Koshland, D. E., Jr., Nemethy, G., and Filmer, D., Biochemistry 5, 365 (1966).

49. Whitehead, E., Biochemistry 9, 1440 (1970).

50. Garel, J. R. and Labousse, B., J. Mol. Biol. 47, 41 (1970).

51. Bender, M. L., Gibson, M. J., and Whelan, D. J., Proc. Nat. Acad. Sci. U. S. 56, 833 (1966).

52. Himoe, A, Parks, P. C., and Hess, G. P., J. Biol. Chem. 242, 919 (1967).

53. Cuppett, C. C. and Canady, W. J., J. Biol. Chem. 245, 1069 (1970).

54. Bender, M. L., Clement, G. E., Kezdy, F. J., and Zerner, B., J. Am. Chem. Soc. 85, 358 (1963).

55. Zerner, B. and Bender, M. L., J. Am. Chem. Soc. 85, 356 (1963).

56. Zerner, B. and Bender, M. L., J. Am. Chem. Soc. 83, 2267 (1961).

57. Bender, M. L. and Kezdy, F. J., J. Am. Chem. Soc. 86, 3704 (1964).

58. Valenzuela, P. and Bender, M. L., Proc. Nat. Acad. Sci. U. S. 63, 1214 (1969).

59. Valenzuela, P. and Bender, M. L., Biochemistry 9, 2440 (1970).

60. Hess, G. P., Brookhaven Symp. Biol. 21, 155 (1968).

61. Hofstee, B. H. J., Biochem. Biophys. Res. Comm. 41, 1141 (1970).

62. Bellon, B. R. and Delaage, M. A., European J. Biochem. 17, 43 (1070).

63. Bechet, J.-J. and d'Albis, A., Biochim. Biophys. Acta 178, 561 (1969).

64. Lake, A. W. and Lowe, G., Biochem. J. 101, 402 (1966).

65. Rozengurt, E., Jimenez de Asua, L., and Carminatti, H., J. Biol. Chem. 244, 3142 (1969).

66. Jimenez de Asua, L., Rozengurt, E., and Carminatti, H., J. Biol. Chem. 245, 3901 (1970).

67. Staal, G. E. J., Koster, J. F., Kamp, H., Van Milligen-Boersma, L., and Veeger, C., Biochim. Biophys. Acta 227, 86 (1971).

68. Wieker, H.-J., Johannes, K.-J., and Hess, B., Hoppe-Seyler's Z. Phys. Chem. 351, 273 (1970).

69. Mansour, T. E., J. Biol. Chem. 240, 2165 (1965).

70. Mansour, T. E. and Ahlfors, C. E., J. Biol. Chem. 243, 2523 (1968).

71. Ahlfors, C. E. and Mansour, T. E., J. Biol. Chem. 244, 1247 (1969).

72. Setlow, B. and Mansour, T. E., J. Biol. Chem. 245, 5524 (1970).

73. Lorenson, M. Y. and Mansour, T. E., J. Biol. Chem. 244, 6420 (1969).

74. Tamburro, A. M., Scatturin, A., Grazi, E., and Pontremoli, S., J. Biol. Chem. 245, 6624 (1970).

75. Pontremoli, S., Grazi, E., and Accorsi, A., J. Biol. Chem. 244, 6177 (1969).

76. Taketa, K., Watanabe, A., Sarngadharan, M. G., and Pogell, B. M., Biochemistry 10, 565 (1971).

77. Fernando, T., Horecker, B. L., and Pontremoli, S., Arch. Biochem. Biophys. 136, 515 (1970).

78. Gregory, D. S. and Wilson, I. B., Biochemistry 10, 154 (1971).

79. Chao, J. and Graves, D. J., Biochem. Biophys. Res. Comm. 38, 1398 (1970).

80. Jacobson, B., Gerwin, B. I., Ahmad, F., Waegell, P., and Wood, H. G., J. Biol. Chem. 245, 6471 (1970).

81. Love, B., Chan, S. H. P., and Stotz, E., J. Biol. Chem. 245, 6664 (1970).

82. Paetkau, V. and Lardy, H. A., J. Biol. Chem. 242, 2035 (1967).

83. Fujimori, E. and Pecci, J., Biochim. Biophys. Acta 207, 256 (1970).

84. Jones, P., Pain, R. H., and Suggett, A., Biochem. J. 118, 319 (1970).

85. Deal, W. C., Rutter, W. J., Massey, V., and Van Holde, K. E., Biochem. Biophys. Res. Comm. 10, 49 (1963).

86. Godfrey, J. E. and Harrington. W. F., Biochemistry 9, 886 (1970).

87. Zappia, V. and Barker, H. A., Biochim. Biophys. Acta 207, 505 (1970).

88. Taylor, P. W., King, R. W., and Burgen, A. S. V., Biochemistry 9, 3894 (1970).

89. Fisher, H. F., McGregor, L. L., and Cross, D. G., Biochim. Biophys. Acta 65, 175 (1962).

90. Fisher, H. F., McGregor, L. L., and Power, U., Biochem. Biophys. Res. Comm. 8, 402 (1962).

91. Fisher, H. F. and Bard, J. R., Biochem. Biophys. Res. Comm. 37, 581 (1969).

92. Warburg, O. and Christian, W., Biochem. Z. 310, 384 (1941).

93. Dixon, M. and Webb, E. C., Enzymes, New York: Academic Press (1964), pp. 67-70.

94. Atkinson, D. E., Hathaway, J. A., and Smith, E. C., J. Biol. Chem. 240, 2683 (1965).

95. Dye, J. L. and Nicely, V. A., J. Chem. Educ., in press (1971).

96. Bernfield, P., Berkeley, B. J., and Bieber, R. E., Arch. Biochem. Biophys. 111, 31 (1965).

97. Mayer, M. and Avi-Dor, Y., Biochem. J. 116, 49 (1970).

98. Nichol, L. W., Jackson, W. J. H., and Winzor, D. J., Biochemistry 6, 2449 (1967).

99. Frieden, C., J. Biol. Chem. 242, 4945 (1967).

100. Wyman, J., J. Am. Chem. Soc. 89, 2202 (1967).

101. Winzor, D. J., Biochim. Biophys. Acta 200, 423 (1970).

102. Bernfield, P. and Tuttle, L. P., Federation Proc. 18, 191 (1959).

103. Bernfield, P., Tuttle, L. P. and Hubbard, R. W., Arch. Biochem. Biophys. 92, 232 (1961).

104. Bernfield, P., Bernfield, H. C., Nisselbaum, J. S., and Fishman, W. H., J. Am Chem. Soc. 76, 4872 (1954).

105. Bernfield, P., Jacobson, S., and Bernfield, H. C., Arch. Biochem. Biophys. 69, 198 (1957).

106. Klotz, I. M., Langerman, N. R., and Darnall, D. W., Ann. Rev. Biochem. 39, 25 (1970).

107. Dennis, D. T., Biochim. Biophys. Acta 191, 719 (1969).

108. Ramaiah, A. and Tejwani, G. A., Biochem. Biophys. Res. Comm. 39, 1149 (1970).

109. Wurster, B. and Hess, B., Hoppe-Seyler's Z. Phys. Chem. 351, 869 (1970).

110. Sawula, R. V. and Suzuki, I., Biochem. Biophys. Res. Comm. 40, 1096 (1970).

111. Griffin J. H. and Criddle, R. S., Biochemistry 9, 1195 (1970).

112. Cross, D. G. and Fisher, H. F., J. Biol. Chem. 245, 2612 (1970).

113. Fisher, D. B. and Kaufman, S., Biochem. Biophys. Res. Comm. 38, 663 (1970).

114. Cohen, S. R. and Wilson, I. B., Biochemistry 5, 904 (1966).

115. Eisenkraft, B., Ph. D. Thesis. University of Amsterdam, 1969.

116. Tomkins, G. M. and Yielding, D. L., Cold Spring Harbor Symp. Quant. Biol. XXVI, 331 (1961).

117. DeVincenzi, D. L. and Hedrick, J. L., Biochemistry 9, 2048 (1970).

118. Dehm, P. and Nordwig, A., FEBS Letters 9, 225 (1970).

119. Guroff, G., Karadbil, M, and Dayman, T., Arch. Biochem. Biophys. 141, 342 (1970).

120. Wuntch, T., Chen, R. F., and Vesell, E. S., Science 169, 480 (1970).

121. Henderson, T. R., Henderson, R. F., and Johnson, G. E., Arch. Biochem. Biophys. 132, 242 (1969).

122. Yielding, K. L., Biochem. Biophys. Res. Comm. 38, 546 (1970).

123. Churchich, J. E. and Wood, F., Biochemistry 2, 781 (1963).

124. Perlman, R. L. and Wolff, J., Science 160, 317 (1968).

125. Mahler, H. R. and Cordes, E. H., Biological Chemistry, New York: Harper and Row, Inc. (1966), p. 53.

126. Wadell, W. J. and Bates, R. G., Physiol. Rev. 49, 285 1969).

127. Nakamaru, Y. and Schwartz, A., Biochem. Biophys. Res. Comm. 41, 830 (1970).

128. Klingenberg, J. J. and Reed, K. P., Introduction to Quantitative Chemistry, New York: Reinhold Publishing Corporation (1965), p. 216.

129. Maron, S. H. and Prutton, C. F., Principles of Physical Chemistry, New York: The Macmillan Company (1967), pp. 715-716.

130. Gibson, G. H. in Methods in Enzymology 16, K. Kustin, Ed., New York: Academic Press (1969), pp. 187-228.

131. Brockman, H., Ph. D. Thesis. Michigan State University, 1971.

132. White, A., Handler, P., and Smith, E., Principles of Biochemistry, New York: McGraw-Hill Book Company (1968), pp. 226-227.