SOME KINETIC AND REGULATORY PROPERTIES OF MAMMALIAN LACTATE, GLUTAMATE, AND RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES

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LIST OF SYMBOLS AND ABBREVIATIONS

α	- Greek alpha
β	- Greek beta
Δ	- Greek delta, to indicate difference
κ	- Greek kappa
λ	- Greek lambda, wavelength
Σ	- Greek sigma, to indicate a summation
θ	- Greek theta, to indicate an angle
τ	- Greek tau, to represent turbidity
%	- Percent sign
$\Delta G^{o'}$	- Gibbs standard free-energy change at 37^{o} C
$>$	- Is greater than
$<$	- Is less than
$\geq$	- Greater than or equal to
$\sim$	- Approximately equal to
LDH	- Lactate dehydrogenase
GDH	- Glutamate dehydrogenase
NADH	- Reduced nicotinamide adenine dinucleotide
NAD	- Oxidized nicotinamide adenine dinucleotide
G3PD	- Glyceraldehyde-3-phosphate dehydrogenase
G3P	- Glyceraldehyde-3-phosphate
1,3-DPG	- 1,3-diphosphoglycerate
α-KG	- α-Ketoglutaric acid
ATP	- Adenosine triphosphate

ADP - Adenosine diphosphate

GDP - Guanosine diphosphate

EDTA - Ethylenediaminetetraacetic acid

DTT - Dithiothreitol

CHAPTER I

INTRODUCTION

This thesis reports studies of three different mammalian enzymes, lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), and glyceraldehyde-3-phosphate dehydrogenase (G3PD). These projects are part of investigations of the catalytic and regulatory properties of these enzymes at physiological concentrations (0.2 - 2 mg enzyme/ml) which were in progress in our laboratory. The relation of this thesis to the overall investigations is described in detail in the separate sections devoted to each enzyme.

An aspect of enzyme regulation which has not been explored until recently is the occurrence in vivo of many enzymes at concentrations about 10^3 times those conventionally used to determine enzyme kinetic properties (1). Several such enzymes exist at substrate concentrations and possess sufficient binding capacity to bind a significant amount of their substrates. Enzymes at high concentration are subject to increased protein-protein interactions and as a consequence, it is reasonable to expect the distribution of different molecular weight species will be shifted in the direction of higher states of association. It is also possible that higher molecular weight forms exhibit kinetic and ligand binding constants different from lower molecular weight species. Thus, regulatory properties of physiological concentrations of enzymes may differ markedly from regulatory properties determined at dilute enzyme

concentrations. Extrapolation from results *in vitro* to metabolic consequences *in vivo* cannot be made with confidence. A better understanding of metabolic regulation should be based on data at cellular enzyme concentrations, however.

CHAPTER II

A BRIEF INTRODUCTION TO THE ENZYMES

LDH

In the last step of anaerobic glycolysis, lactic acid dehydrogenase (E.C.1.1.1.27) catalyzes the reduction of pyruvate to lactate

$$\text{Pyruvic Acid} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{Lactic Acid} + \text{NAD}^+$$

The overall equilibrium of this reaction lies far to the right and has a large negative value of $\Delta G^{o\prime}$.

When muscle cells of higher organisms function anaerobically during short bursts of strenuous activity, this reaction is utilized by the cell to reoxidize NADH to NAD which is needed for the oxidative phosphorylation of glyceraldehyde-3-phosphate. The coupled relationship between these two redox reactions provides a continued regeneration of NAD, present in limited quantities compared with the carbohydrate supply, and thus permits skeletal muscle to continue functioning during periods of limited oxygen supply.

Unlike the phosphorylated intermediates of glycolysis, lactate and pyruvate may diffuse across the cellular plasma membrane. Thus lactate does not accumulate during anaerobic glycolysis, but is carried by the circulatory system to the heart or to the liver. In these tissues, which function under aerobic conditions, pyruvate is oxidized to acetyl CoA, which enters the Krebs cycle, or in liver may be utilized in part for

gluconeogenesis. Under aerobic conditions, NADH from the oxidative phosphorylation of glyceraldehyde-3-phosphate is reoxidized to NAD by the mitochondrial electron transport system via the glycerol phosphate or malate shuttle instead of the LDH reaction. Thus, while LDH permits anaerobic glycolysis and generation of ATP (2 moles ATP/mole glucose), aerobic oxidation of pyruvate is much more efficient (36 moles ATP/mole glucose) since it not only allows complete oxidation of pyruvate, but also involves the mitochondrial electron transport system.

In mammals and birds there are 5 LDH isozymes with essentially the same molecular weight which catalyze the same reaction, but with different kinetic and ligand binding constants. The enzymes are tetramers of α or β subunits; the isozymes correspond to the 5 different permutations of the two subunits within the tetramer. The isozyme predominating in heart tissue is an α tetramer designated as LDH-1 (formerly labeled H_4 or H-LDH) and is characterized by inhibition of its activity by high concentrations of pyruvate.

The isozyme found predominantly in skeletal muscle and liver is designated as LDH-5, (a β_4 tetramer) and is much less sensitive to pyruvate inhibition. In both cases, pyruvate inhibition is thought to be the result of a pyruvate-NAD-enzyme dead-end complex; the equilibrium for complex formation with heart isozyme was presumed higher than with muscle isozyme. As a result of the higher sensitivity of the heart isozyme to substrate inhibition by pyruvate, and related properties Kaplan (2) has proposed that LDH-5 functions essentially as a pyruvate reductase; whereas, LDH-1 is primarily involved in dehydrogenation of lactate. This situation would allow pyruvate to be oxidized by the Krebs cycle in aerobic tissue such as heart and thus would be more

energetically favorable for aerobic tissue than reduction of pyruvate to lactate. Griffin and Criddle (3) have recently described marked enzyme concentration dependence of kinetics and equilibria for formation of the pyruvate-NAD-enzyme dead-end complex with the muscle isozyme. Data are lacking, however, on the heart isozyme which according to Kaplan's theory is the only LDH isozyme appreciably affected by this complex in vivo. Another deficiency in available data is that Kaplan's (4) latest measurements, from which they greatly extend their original theories, are confined to chicken enzymes, although chemical differences have been claimed between chicken and mammalian heart enzymes (5).

Wuntch et al. (6) have objected to Kaplan's view of LDH isozyme function and present data which indicate that substrate inhibition of LDH-1 and LDH-5 by maximum physiological concentrations of pyruvate ($\leq$ 0.8 mM in skeletal muscle) does not occur at physiological enzyme concentrations. However, at dilute, unphysiological enzyme concentrations, the activity was severely inhibited. Wuntch et al. concluded that pyruvate inhibition of LDH is probably not physiologically significant. The original conclusions of Wuntch et al. (7) have been questioned since their measurements probably corresponded to an unphysiological transient conditon prior to formation of the pyruvate-NAD-enzyme dead-end complex. Wuntch et al. (8) replied with additional data, but their reasoning is questionable and experimental design inadequate; their conclusions regarding the extent of inhibition when LDH is incubated with pyruvate and NAD do not appear to us to be supported by their own data, for example.

Stambaugh and Post (9) have presented data on substrate and product inhibitions of LDH by lactate and pyruvate from which they have concluded

that lactate exerts the more significant influence on LDH in vivo. However, their data are too limited to confidently extrapolate to physiological levels of pyruvate and lactate and were confined to enzyme concentrations less than 1 μg/ml. In addition, these data must be reinvestigated in light of the slowness of dead-end complex formation (3). Most studies have not initiated the catalytic reaction from equilibrium with respect to dead-end complex formation even though it may be assumed that the simultaneous presence in vivo of both pyruvate and NAD would produce considerable dead-end complex.

Due to conflicting viewpoints and lack of data, we have attempted to characterize the properties of LDH with physiological ranges of substrates, products and enzyme concentrations at both 25 and 37°. As part of this project, this thesis reports measurements characterizing pyruvate product inhibition of LDH-catalyzed lactate oxidation. Other measurements from our laboratory on LDH are reported in a manuscript in preparation.

GDH

Bovine liver glutamate dehydrogenase (E.C.1.4.1.3) catalyzes the following reaction:

$$H_2O + \text{Glutamate} + NAD(P)^+ \rightleftharpoons \alpha\text{-KG} + NAD(P)H + H^+ + NH_3$$

The reaction is particularly important for all species since it is essentially the only pathway for the formation of α-amino acids directly from NH_3. Thus, GDH provides a link between amino acid and carbohydrate metabolism in animal tissues. This reaction also fulfills an important function in the regulation of ammonium ion concentration in liver. Toxic concentrations of ammonium ion may be disposed of by three methods:

(a) by direct excretion of ammonium ion, or (b) by utilizing the reverse direction of the above reaction as part of a salvage pathway for amino acid biosynthesis, or (c) it may be mobilized by various transaminations into glutamate, which provides NH_3 for urea synthesis via its GDH-catalyzed oxidative deamination. Rapid and extensive reversal of pathway (c) is toxic in that the equilibrium position of the GDH-catalyzed reaction strongly favors reductive amination of α-ketoglutarate. This can quite effectively remove α-KG from the Krebs cycle in the absence of compensating anaplerotic reactions, resulting in inhibition of respiration, as well as excess ketone body formation from acetyl CoA in liver. Thus, the concentration of ammonium ion in liver is carefully regulated by GDH and the urea cycle.

The enzymatically active monomer of GDH which predominates at concentrations below 10 μg/ml has a molecular weight of 313,000 daltons (10) and is composed of six apparently identical subunits of molecular weight 52,000. Further dissociation requires denaturing agents and is largely irreversible. The enzyme monomer (a hexamer of subunits) undergoes self-association with increasing enzyme concentration *in vitro* above 0.1 mg/ml forming long, linear oligomers of increasing size (10). The concentration of the enzyme in liver mitochondria is considered to be in excess of 2 mg/ml.

Spivey (11) has reported previously that when GDH concentration *in vitro* is increased to greater than 0.75 mg/ml substrate inhibitions by NADH and α-ketoglutarate are entirely abolished; inhibitions by the hormones progesterone, thyroxine, estradiol and its analog diethylstilbestrol, which are effective at μM levels of dilute enzyme are greatly reduced, as is inhibition by the allosteric modifier, GDP (12). In

contrast to the altered inhibitions, activation by the allosteric modifiers ADP and ATP as well as kinetic parameters determined with saturating levels of substrates and no inhibitors are not affected by increasing enzyme concentration above 0.75 mg/ml (13).

If the concentrations of reactants and modifiers of an enzyme are sufficiently greater than the enzyme concentration even when the reaction is studied at high enzyme concentration (1 mg/ml = 20 μM in GDH binding sites) then there exist only two mechanisms by which the enzymic reaction rates could significantly differ between dilute and concentrated enzyme solutions. The first mechanism is through a hysteretic transition (e.g., a slow response to a rapid change in ligand concentration) in an enzyme form such that at the lower enzyme concentrations and slower catalytic turnover, only the catalysis with transformed enzyme is observed. With higher enzyme concentrations, however, only untransformed enzyme exists during the observed initial velocity period because of the slowness of transformation relative to catalytic turnover. The second mechanism for obtaining enzyme-dependent specific activities requires an enzyme association-dissociation equilibrium. It is necessary to distinguish between these two mechanisms to evaluate the physiological significance, if any, of observed changes in enzyme activities with enzyme concentration.

If the hysteretic transition occurs from an enzyme form other than the central enzyme complex, incubation of enzyme with the appropriate combination of substrates and products prior to initiating the catalytic reaction should eliminate or grossly alter transitions during the catalytic reaction. Incubation of LDH with NAD and pyruvate permits the hysteretic transition to the ternary dead-end complex to occur, for example. Catalytic reaction rates observed after subsequent addition of

NADH to the concentrated LDH reaction mixtures are therefore extensively inhibited. Substrate inhibition of concentrated LDH by pyruvate will not be apparent without the pre-incubation; that is, pre-incubation of LDH with NAD and pyruvate restores the substrate inhibition observed with dilute LDH.

It was not possible to restore NADH and GDP inhibition of concentrated GDH by any possible permutation of incubations, including products with substrates which might form dead-end complexes, except possibly with an equilibrium mixture of substrates and products, which has not yet been tried (14). A hysteretic transition from a central enzyme complex or enzyme association therefore remained as possible reasons for the loss of substrate inhibition at high enzyme concentrations. A transition from uninhibited to inhibited enzyme had always been observed within the expected initial velocity extent of reaction with 0.2 - 0.3 mg enzyme/ml in support of a hysteretic transition. Evidence on the effects of enzyme association on the substrate inhibition, however, were lacking. The recent report that saturation of buffers with toluene or benzene greatly enhances GDH association, without affecting its specific activity, provided us with a direct and potentially conclusive method for correlating changes in enzyme inhibition with extents of enzyme association (15). These measurements were therefore made as part of this thesis as described in the following chapter.

G3PD

D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating) (E.C.1.2-1.12) catalyzes the oxidation and phosphorylation of the C-1 aldehyde of D-glyceraldehyde-3-phosphate to give 1,3-diphosphoglycerate,

a mixed anhydride of a carboxylic acid and orthophosphoric acid. The reducing equivalents are transferred from D-G3P to the B side of the nicotinamide ring of NAD.

The enzyme is a tetramer of molecular weight 145,000 and is considered to have four subunits, each of molecular weight 36,000. The enzyme contains sulfhydryl groups necessary for enzymatic activity and 14 of these are titratable. Four of the sulfhydryls are particularly reactive and are located at the active site of each subunit.

Conflicting claims have been made for the steady-state mechanism of G3PD. Furfine and Velick (16) had concluded from equilibria of partial reactions and from extensive steady-state kinetic studies of the rabbit muscle enzyme that random addition and release of substrates and products best describes the kinetic mechanism. These authors also report that the rate-limiting step occurs in the reaction of a kinetically important quaternary enzyme-substrates complex, namely, acyl group transfer from the enzyme to an external acceptor. Orsi and Cleland (17) have criticized these conclusions and presented data which they claim are compatible only with an ordered ter-bi reaction, with NAD, aldehyde and phosphate or arsenate adding in this order. Release of acid precedes the rate-limiting step, NADH release, in their proposed mechanism.

Stopped-flow data from Trentham's lab (18) on the lobster enzyme and from our lab on the rabbit muscle enzyme suggest a reason for the conflicting views. The rates of NADH release from enzyme-NADH complex are essentially independent of pH. At low pH, rates of catalytic turnover are less than rates of NADH release, but increase with pH and exceed the rates of NADH release at pH values greater than 7.8. Thus, the NADH release step becomes rate-limiting only at pH values above 7.8

and an alternative pathway may be used at lower pH values. A similar shift in mechanism has been reported for aldose reductase (19). Orsi and Cleland's data were confined to pH 8.6 and the data of Furfine and Velick which appear in conflict with the ordered mechanism were confined to pH 7.4. Clarification of the mechanism is of importance for two reasons. First, to assist in the interpretation of some of our stopped-flow data, we need to know whether NADH can be released from acylated enzyme which is produced by NAD oxidation of D-G3P in the absence of acyl acceptor (20). The ordered mechanism claimed by Orsi and Cleland would not permit this. In a wider context, however, it would be of interest to know to what extent an apparent ordered mechanism is but a special case of random pathways, depending on pH and other factors (alternate substrate, modifiers, etc.). Steady-state kinetic measurements designed to help answer this question are reported in Chapter III.

CHAPTER III

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials

Rabbit muscle and beef heart lactic acid dehydrogenases were obtained as the ammonium sulfate suspensions from Sigma Chemical Company, (types II and III, respectively). Disc-gel electrophoresis of the heart isozyme, and further purification by preparative polyacrylamide electrophoresis had not altered its kinetic properties (21). The enzymes were therefore used without further purification. Also obtained from Sigma were the lithium salt of L-(+)-lactic acid, imidazole (Grade III, low fluorescence blank), bicine and dithiothreitol. DL-G3P was obtained from Sigma, either as the free acid, or prepared from its diethylacetal according to the procedure of Furfine and Velick (16). Ammonium sulfate suspensions of bovine liver glutamate dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase were purchased from Boehringer-Mannheim Corporation. G3PD was also obtained from the white skeletal muscle of the legs and spine of New Zealand White rabbits according to a procedure by Ferdinand (22). This latter source of G3PD was from a preparation of Dr. B. C. Peczon in our laboratory. The product, 1,3-diphosphoglyceric acid used for product inhibition studies of G3PD was prepared by oxidative phosphorylation of G3P using the procedure of Negelein (23) as modified by Furfine and Velick (16). The sodium salt of pyruvic acid and α-ketoglutaric acid were obtained from Calbiochem;

NAD (free acid) and disodium NADH, both Chromatopure grade, were purchased from P-L Biochemicals. Disodium EDTA was obtained from Schwarz-Mann, and glutaric acid was purchased from Eastman Organic Chemicals. K_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, benzene, triethanolamine, $Na_4P_2O_7$, KCl and Na_2PO_4, all reagent grade, were obtained from Fisher. Na_2HAsO_4 was supplied by Mathieson, Coleman and Bell. Benzene was distilled prior to use and only the fraction collection at 59° C was used.

Solution Preparations

Potassium phosphate buffer, 0.05 M, pH 7.5, containing 0.2 M NaCl, and $1x10^{-4}$ M EDTA, was prepared from stock solutions of 0.2 M potassium dihydrogen phosphate and 0.2 M dipotassium hydrogen phosphate. Enzyme and reagent stock solutions used in all LDH measurements and some GDH measurements were prepared in this buffer. Saturation of phosphate buffer with benzene for use in other GDH measurements was accomplished by adding an excess of the amount of benzene needed to saturate the buffer and stirring vigorously for 24 hours at room temperature prior to use (15). Enzyme and reagent stock solutions used in G3PD kinetic measurements performed at pH 6.0 and pH 8.6 were prepared in imidazole buffer, 0.05 M, pH 6.0, which contained 0.1 M KCl, $1x10^{-3}$ M EDTA, and $1x10^{-3}$ M dithiothreitol, or in bicine-acetate buffer, 0.02 M, pH 8.6, which contained $5x10^{-4}$ M EDTA. Enzyme, substrate and coenzyme stock solutions used in a standard assay (Table I) of G3PD activity were prepared in triethanolamine buffer 0.04 M, pH 8.7, which contained 0.05 M Na_2HPO_4 and $2x10^{-4}$ M EDTA (22). Nucleotide coenzyme stock solutions were prepared fresh daily. The purity of NAD was determined prior to its first use and was not assayed again (24). Concentrations of NADH

TABLE I

ENZYME STANDARD ASSAY CONDITIONS

	LDH	GDH	G3PD
Buffer	$KPO_4^{=}$, 0.05 M, pH 7.5, NaCl, 0.2 M, EDTA, 0.1 mM	$KPO_4^{=}$, 0.05 M, pH 7.5, NaCl, 0.2 M, EDTA, 0.1 mM	$N(CH_2CH_2OH)_3$, 0.04 M, pH 8.7
Substrates and their concentration	Lactate, 1.5 mM	NH_4Cl, 0.1 M α-KG, 5 mM	G3P, 20 mM Na_2HPO_4, 0.5 M
Coenzymes and their concentration	NAD, 1 mM	NADH, 0.15 mM	NAD, 0.83 mM
Enzyme concentration	0.2-0.4µg/ml	0.5 µg/ml	0.17 µg/ml
Specific Activity Obtained	100 units/mg	60 units/mg	130 units/mg

stock solutions were verified by measuring UV absorbance at 340 nm. The pH of all reagent stock solutions was adjusted to the desired value with KOH or HCl, if necessary. All pH measurements were made with a Fisher Model 320 pH meter.

Enzyme stock solutions were prepared by centrifugation of the enzymes from their ammonium sulfate suspensions and dialyzed four times with 10 volumes of buffer and a minimum of two hours between buffer changes. Protein concentrations of all enzyme stock solutions were determined spectrophotometrically using absorptivities of 1.45 ml mg^{-1} cm^{-1} at 280 nm for LDH, 0.973 ml mg^{-1} cm^{-1} at 279 nm for GDH, and using absorptivities determined at 260 nm and 280 nm by Dandliker and Fox (25) for G3PD. GDH stock solutions were prepared in benzene-free phosphate buffer and purposely maintained at > 10 mg enzyme/ml during preparation to minimize the effects of diluting the benzene in the phosphate buffers to which it was subsequently transferred (15). Enzyme and reagent stock solutions used in light-scattering measurements were filtered through 0.45μ pore-size Millipore MF filters, obtained from the Millipore Corporation, Bedford, Massachusetts. GDH was treated with charcoal to remove bound nucleotides and centrifuged, then dialyzed and filtered prior to determining protein concentrations. Solutions of substrates and coenzymes at greater than final concentration were prepared from reagent stock solutions and filtered. The desired amount of this solution was then transferred into a light-scattering cell and diluted to the final concentration by addition of filtered enzyme. Mixing of the light-scattering solution was achieved by gently inverting the light-scattering cell. All glassware used in light-scattering measurements, including pipets and light-scattering cells, were rinsed with dust-free glass-distilled water prior to each use.

Kinetic Measurements

Enzymic activity in these studies was determined spectrophotometrically by measuring the rate of production or disappearance of NADH at 340 nm. Enzyme specific activity is defined as the amount (μmoles) of product produced per unit time (minute) divided by the quantity of enzyme (mg) used to catalyze the reaction.

All measurements of LDH and G3PD activity, as well as measurements of GDH activity at protein concentrations $\leq$ 0.5 μg/ml were performed with a Coleman 124 ultraviolet-visible spectrophotometer equipped with a Coleman 165 recorder and Coleman No. 801 scale expander. Reaction rates were determined by measuring the slope of a tangent drawn to the most linear portion of the initial velocity trace. All enzyme stock solutions were routinely assayed prior to and at the end of each experiment. Standard assay conditions for the enzymes studied are given in Table I.

Effects of the pyruvate-NAD-enzyme dead-end complex on initial velocity were measured by incubating LDH with pyruvate, NAD, and buffer in the cuvet prior to initiating the reaction with lactate. Other kinetic measurements, however, were started by addition of a small volume (0.01 - 0.05 mls) of enzyme stock solution as the last reagent. Stock G3PD solutions were maintained at concentrations $\geq$ 10 mg enzyme/ml because of the tendency of the enzyme to dissociate into monomers and denature upon dilution (26). Fresh serial dilutions of enzyme stock solution were used to initiate each reaction. Final enzyme concentrations ranged from 0.05 μg/ml to 0.20 μg/ml in the reaction. Measurement of GDH specific activities at concentrations greater than 0.5 μg/ml were made with a Durrum-Gibson Stopped-Flow Spectrophotometer, made by

the Durrum Instrument Company, Palo Alto, California, using a 0.234 cm path length cell. Instrument calibrations have been previously described (20). Reaction velocity traces were monitored on a Tektronix type 564 storage oscilloscope. Data were permanently recorded by photographing stored oscilloscope traces using the projected graticule Tektronix No. 100. Solutions to be placed in a specified drive syringe of the stopped-flow were pre-mixed in a beaker, then loaded into the drive syringe. The solutions were prepared so that one drive syringe contained buffer, enzyme, nucleotide coenzyme and one substrate, while the other drive syringe contained buffer, enzyme, nucleotide coenzyme and the second substrate.

Product concentrations were calculated from the stopped-flow records by application of Beer's law with voltages assumed proportional to transmittances. Specific activities were calculated from the initial reaction velocities.

Molecular Weight Determinations

Apparent weight-average molecular weights of GDH were determined from light-scattering measurements with a Brice-Phoenix Model 2000 instrument and digital galvanometer (Phoenix Model DMR-200). Solutions were at room temperature (about 25° in an air-conditioned room). Cylindrical (C-105), micro (T-108) and 24x24 mm rectangular (T-104) cells were used. The primary data are galvanometer deflections, G_θ, obtained with the detector at an angle θ from the incident beam. Readings were taken 5 seconds after opening the shutter in the sequence θ = 0, 45, 58, 69, 80, 90, 100, 111, 122, and 135° for five cycles of this sequence. Average G_θ values were corrected for a small dark

current deflection and used to calculate $\kappa c/R_\Theta$ at each Θ from

$$\frac{\kappa c}{R_\Theta} = \frac{4.80\times10^{-9} \times 0.918}{a(\frac{r}{r'})} f(\Theta)c \times \frac{1}{\Delta(F\frac{G_\Theta}{Go}) - \Delta(F\frac{G_{\Theta-180}}{Go})} \quad (3\text{-}1)$$

In this equation, $f(\Theta) = (1 + \cos^2 \Theta)/\sin \Theta$, c is protein concentration in mg/ml, F is the product of transmittances of filters used at $\Theta = 0$, Δ denotes difference between solution and buffer values and $\underline{a}$ and $(\frac{r}{r'})$ are constants determined by the user. Values obtained in this study are $\underline{a} = 0.052$, and $(\frac{r}{r'}) = 1.60$ or 2.06 for the cylindrical and micro-cells, respectively. The numerical factors in equation 3-1 are a combination of constants, including the refractive index increment $\frac{\partial n}{\partial c}$, of the macromolecule in the solvent used. A value of $\frac{\partial n}{\partial c} = 0.195$ ml/mg for GDH in phosphate buffers was used (27). Some of the other constants in Equation 3-1 vary from one individual instrument to another and with different cell types. The specific data, therefore, supplied with each instrument and opal glass reference standard need to be used in the more general equations described in the Operations Manual, in order to obtain correct numerical factors in Equation 3-1. Measurements made at 90° only in the micro-cell to conserve enzyme utilized a simpler form of the above equation,

$$\frac{\kappa c}{R_{90}} = \frac{4.80\times10^{-9}c}{a(\frac{r}{r'})} \times \frac{1}{\Delta(F\frac{G_{90}}{Go})} \quad (3\text{-}2)$$

Molecular weights were determined from Equations 3-1 and 3-2 by the fundamental relation,

$$\frac{\kappa c}{R} = \frac{1}{M_{a_{pp}}} \left[1 + k R_G^2 \sin^2 (\frac{\Theta}{2}) \right]$$

described in the Appendix. When the cylindrical cell was used permitting measurements at all angles, Kc/R_Θ was plotted against $\sin^2(\frac{\Theta}{2})$ to give the weight average molecular weight as the reciprocal of the intercept at $\Theta = 0$. When measurements were made in the micro-cell at 90° only, the change in Kc/R_Θ from 90 to 0° determined from previous measurements was substracted from Kc/R_{90} to give the intercept value.

CHAPTER IV

RESULTS AND DISCUSSION

LDH

Figure 1 summarizes initial velocity of lactate oxidation as a function of pyruvate concentration. Bovine heart and rabbit muscle enzymes are compared. The two lactate concentrations used for these measurements correspond to levels measured from exhaustively exercised and resting muscle, respectively (28). Table II summarizes literature values of LDH isozyme and substrate concentration in the specified tissues. These conditions are most pertinent to this work, as the majority of lactate oxidation occurs in liver and heart.

It is reasonable to invoke formation of the ternary dead-end complex of pyruvate-NAD-enzyme to understand the data presented in Figure 1 because half lives, $t_{\frac{1}{2}}$, for complex formation under these conditions are on the order of a few seconds (29). In addition, LDH is continuously incubated with NAD and pyruvate in vivo, therefore, considerable ternary complex formation could be expected to occur.

Figure 1 indicates that with normal cellular levels of lactate in heart (<1.5 mM) but no pyruvate, the heart isozyme is considerably more efficient in lactate oxidation than the muscle isozyme as a consequence of its lower K_m for lactate (9 mM vs. 25 mM) (30). However, extensive inhibition by pyruvate develops with the heart isozyme at physiological levels of pyruvate and little advantage of one isozyme over another can

Figure 1. Pyruvate Inhibition of Lactate Oxidation. Pyruvate concentrations are near physiological levels (6). The rate of NADH production was followed at 340 nm with 1 mM NAD and 0.2-0.4 µg LDH/ml. LDH-1 and LDH-5 are represented as (○) and (△), for 0.15 mM (shaded symbols) and 20 mM (unshaded symbols) lactate, respectively.

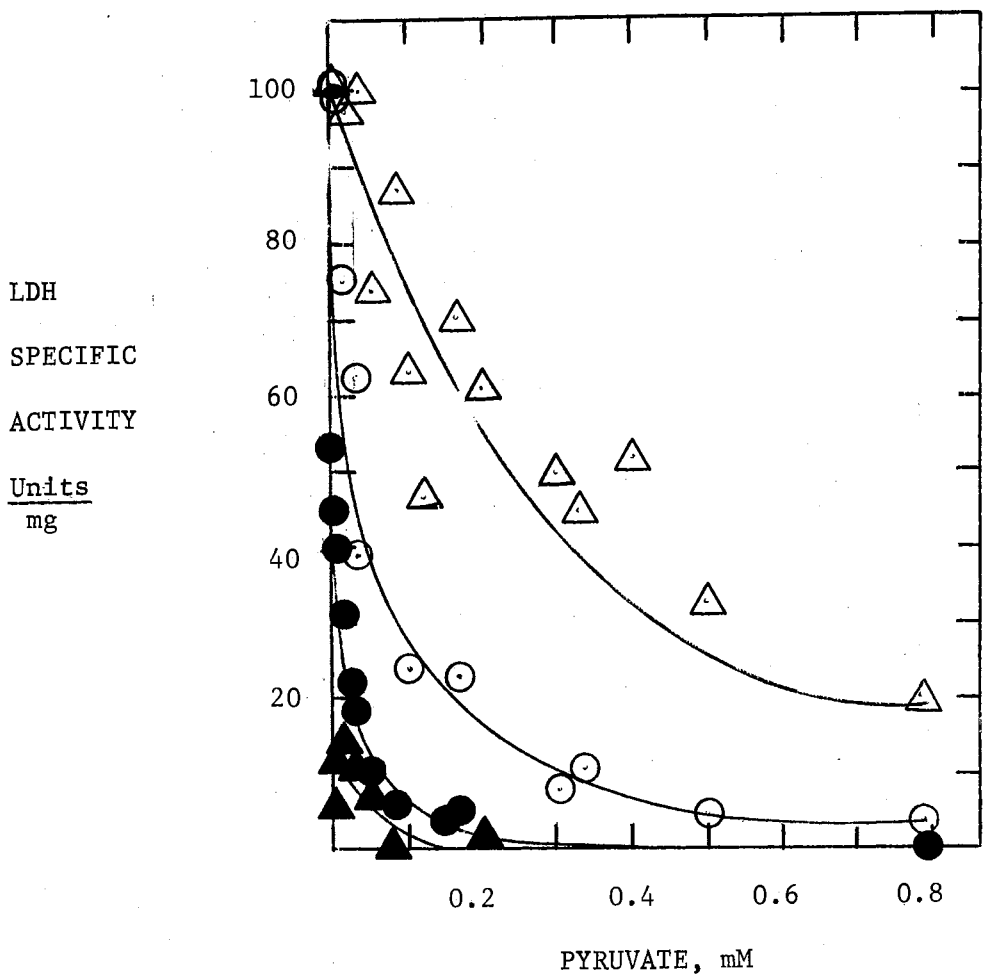

LDH
SPECIFIC
ACTIVITY
Units
mg
100
80
60
40
20
0.2
0.4
0.6
0.8
PYRUVATE, mM

TABLE II

LDH ENZYME AND SUBSTRATE
LEVELS IN TISSUES

	Concentrations in mM and (nmoles/g of dry tissue)[a]		mg/ml[a]	
	Pyruvate	Lactate	LDH-1	LDH-5
Rat Heart[b]	0.05 (130)	0.27[c] (700) 0.87 (2,300)[d]	0.33	0.0055
Rat Skeletal Muscle[e]			0.0073	0.24
Canine Skeletal Muscle[e]				
Resting	0.33	1.5		
Ischemic and exhaustively exercised	0.80	20		
Rat Liver[f]	0.50 (1,250)	(1.5)[g]	essentially none	predominantly LDH-5
Rat Renal Medula			0.105	0.088

[a]Wuntch, Vesell, and Chen (1970) Science 167, 63-65.

[b]Williamson, J. R. (1965) J. Biol. Chem. 240, 2308-2321.

[c]normalized to control

[d]10 mM acetate infusion

[e]Vesell, and Pool (1966) Proc. Nat. Acad. Sci., U.S. 55, 756-762.

[f]Williamson, J. R. (1966) Biochem. J. 101, 11c; Exton, and Park (1969) J. Biol. Chem. 244, 1424-1433.

[g]Estimation

be discerned. No evidence was obtained in these or other measurements that lactate dissociates the dead-end complex and hence abolishes pyruvate inhibition, as Kaplan (4) has described for chicken heart LDH (29). On the other hand, a small reduction in heart pyruvate concentrations would cause a large increase in lactate utilization, in contrast to the results with muscle isozyme. Thus, our observations are only partially in agreement with Kaplan's (4) latest views; they agree in demonstrating a potential advantage of the heart isozyme in heart at low pyruvate concentrations, but disagree in that we observe considerable inhibition of the enzyme at pyruvate concentrations at, or near those estimated to exist in heart tissue (Table II). Uncertainties from these measurements result from our ignorance of pyruvate concentrations and distributions within cells. Everse and Kaplan (4) attempted to measure the extent of LDH dead-end complex formation in chicken heart and breast muscle. They measured LDH activity in these tissues after dilution of the cellular contents by homogenization with three volumes of buffer per gram of tissue. Since a steady increase in activity was observed in the heart homogenates (but not breast muscle) with a time dependence qualitatively similar to rates of dissociation of enzyme dead-end complex ($t_{\frac{1}{2}} \sim 30$ minutes), they conclude that an extensive fraction of LDH exists in the dead-end complex in heart. We have not been able to reproduce these observations, however. Furthermore, if appreciable dead-end complex does exist, our measurements, in contrast to Kaplan's, suggest that lactate oxidation and pyruvate reduction would be equally inhibited.

The conclusion of Wuntch et al. (6) that pyruvate inhibition of LDH is not physiologically significant may be valid; however, we do not

agree with their results because the measurements of specific activity which they report were made without pre-incubation of enzyme, pyruvate, and NAD. Under the conditions of enzyme concentration used by Wuntch, (10^{-6} M), the catalytic reaction would be over before ternary complex formation could occur, since at this enzyme concentration, $t_{\frac{1}{2}}$ for complex formation would be approximately 20 minutes. Using the same concentration of NAD, the time required for complex formation would be even longer when large concentrations of enzyme are used (29).

The conclusions of Stambaugh and Post seem even more doubtful to us because lactate concentrations are unlikely to exist above 20 mM even in exhaustively exercised skeletal muscle, and muscle LDH is much less inhibited by lactate than heart isozyme (28). For rat heart perfused with 10 mM glucose, lactate concentrations of 0.3 mM were recorded which increased to only 0.9 mM after addition of 10 mM acetate to the perfusing solution (30). Thus, it is doubtful that lactate concentrations would ever reach inhibiting concentrations <u>in vivo</u>.

GDH

Table III compares molecular weights of GDH in benzene-free phosphate buffer, in benzene-containing phosphate buffer, and in benzene-containing phosphate buffer with inhibitory concentrations of coenzyme (0.8 mM NADH), 0.1 M NH_4Cl, and 25 mM glutaric acid. Glutaric acid, a substrate analog was used instead of α-ketoglutarate because we did not wish to have NADH depleted by enzyme-catalyzed reaction during the measurements. Examination of molecular weights obtained in presence of benzene and inhibitory levels of NADH indicate a 20% decrease in molecular weight relative to values obtained in absence of NADH. This is in keeping with

TABLE III

APPARENT MOLECULAR WEIGHTS (M_{app}) OF GDH

Solution Composition[a]	Enzyme Concentration (mg/ml)	M_{app}[b] ($\times 10^{-6}$)
1a	0.20	0.88
1a	0.40	1.01
1a	1.0	1.21
1b	0.10	1.45
1b	0.20	1.31
1b	0.40	1.72
1b	0.4	1.81
1b	1.0	1.65
2a	0.4	1.16
2b	0.4	2.32

[a]Numbers denote solution composition; letters indicate the absence or presence of benzene, as follows:

SYMBOL	DESIGNATION
1	0.05 M potassium phosphate, pH 7.5 containing 0.2 M NaCl, 0.1 mM EDTA, 0.6 mM NADH, 0.1 M NH_4Cl, 25 mM glutaric acid.
2	0.05 M potassium phosphate, pH 7.5 containing 0.2 M NaCl, 0.1 mM EDTA.
a	no benzene
b	Solution is at least 90% saturated with benzene.

[b]The estimated precision in M_{app} is 0.1 x M_{app}.

data obtained in absence of benzene from our lab and Frieden's (32). These values are also in good agreement with those of Reisler and Eisenberg (15) although M_{app} in benzene solutions are a little lower.

Figure 2 summarizes inhibition of NADH oxidation by NADH and α-ketoglutarate in benzene-free and benzene-containing systems at dilute enzyme concentration ($\leq$ 0.5 μg/ml). Benzene does not alter the activities of GDH which are maximal at 0.15 mM NADH and 5 mM α-ketoglutarate and approximately 75% inhibition of activity occurs at 0.8 mM NADH and 25 mM α-KG.

Figure 3 summarizes release of inhibition by NADH and increase in molecular weight of GDH as a function of enzyme concentration. In the presence of benzene, association of enzyme subunits occurs to a much greater extent; however, the presence of benzene has little or no effect on the specific activity of GDH and the loss of enzyme inhibition occurs over the same concentration range (0.1 - 0.4 mg/ml) in the presence or absence of benzene. Although molecular weights obtained in the presence of benzene are somewhat erratic (Figure 3), additional data (Table III) substantiates our observations that benzene significantly enhances the extent of subunit association in the concentration range where the kinetic transition occurs (0.2 - 0.4 mg/ml). Thus, release of NADH inhibition with increasing enzyme concentration occurs independently of the protein association-dissociation equilibrium. The inability to demonstrate inhibition by incubation of high concentrations of GDH with substrates and products prior to initiation of enzymic reaction indicates that high NADH concentrations promote a transition in a central enzyme complex which is only present transiently during the enzymic reaction.

Figure 2. NADH Inhibition of Dilute GDH in Presence and Absence of Benzene. The rate of NADH disappearance was monitored at 340 nm, with 0.1 M NH_4Cl, 5 mM α-KG (unshaded symbols), 25 mM α-KG (shaded symbols), and 0.5 μg GDH/ml. Measurements were performed in benzene-free (○) and benzene-containing (△) phosphate buffers.

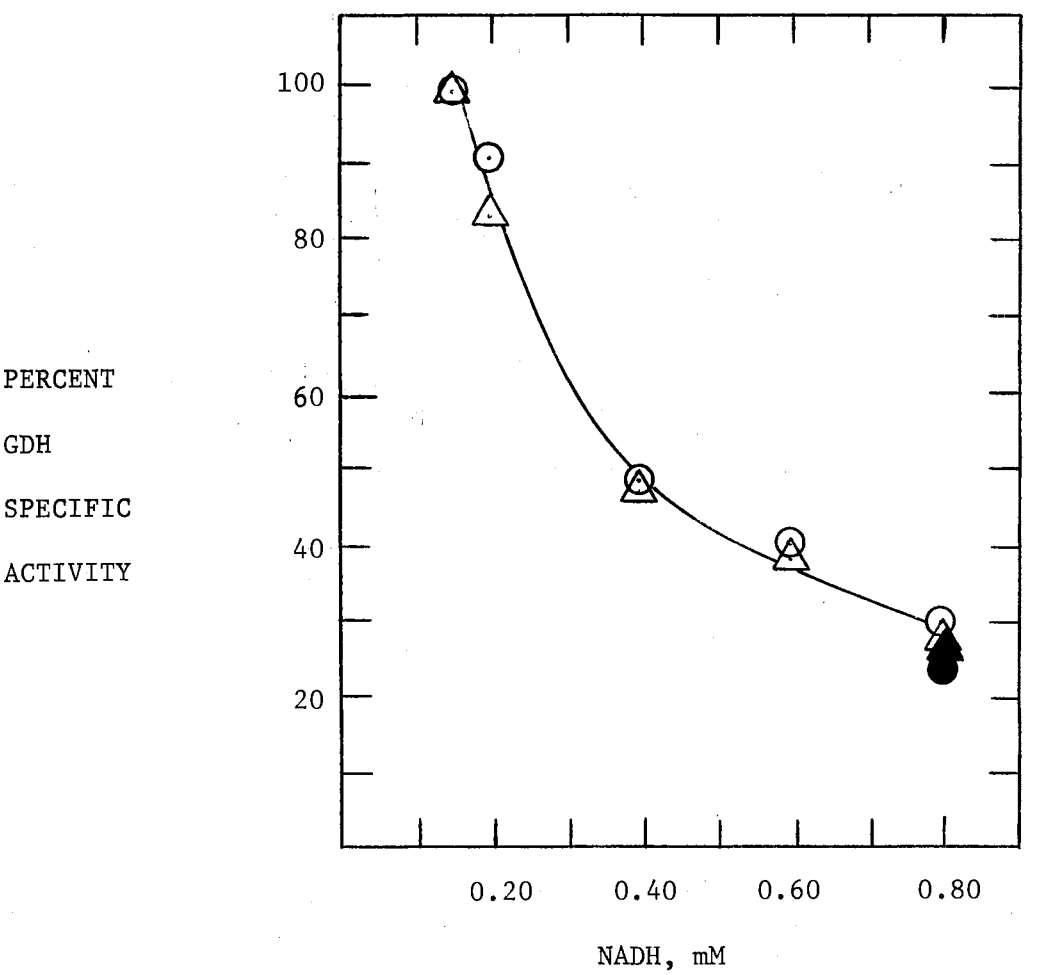
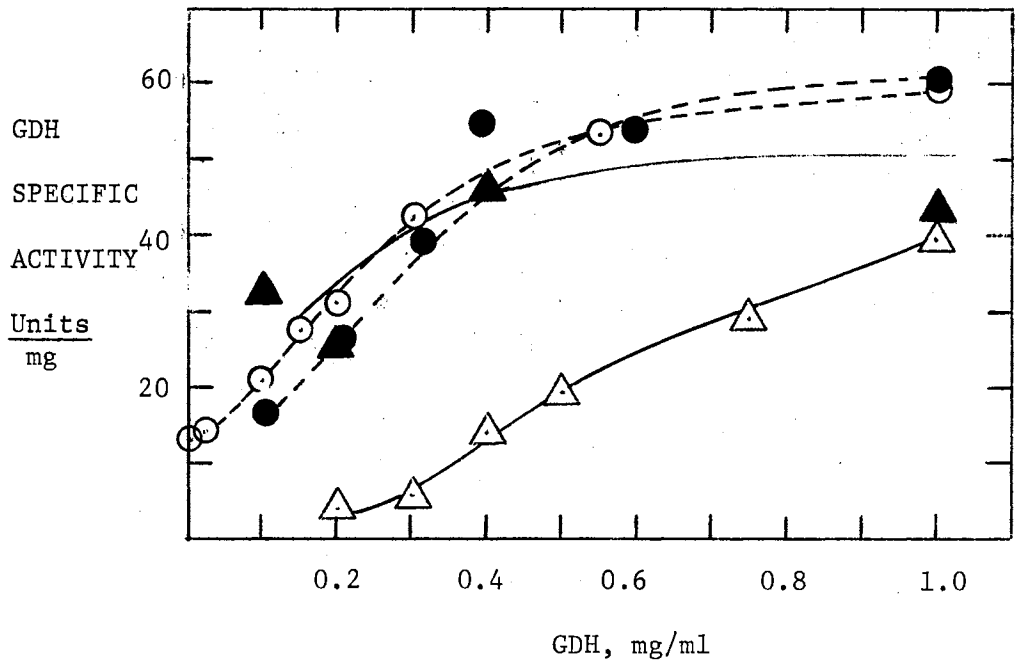

PERCENT
GDH
SPECIFIC
ACTIVITY
100
80
60
40
20
0.20
0.40
0.60
0.80
NADH, mM

Figure 3. Specific Activity and Molecular Weight of GDH as a Function of Concentration in Benzene-Free and Benzene-Containing Buffer. Disappearance of NADH was monitored at 340 nm with 0.6 mM NADH, 0.1 M NH_4Cl, and 25 mM α-KG. Specific activity (◯) and molecular weight (△) data are shown for benzene-free (open symbols) and benzene-containing (shaded symbols) phosphate buffers. Reasonable theoretical smooth curves are drawn for specific activity (---) and molecular weight data (——).


GDH
SPECIFIC
ACTIVITY
Units
mg
60
40
20
0.2
0.4
0.6
0.8
1.0
GDH, mg/ml

G3PD

Orsi and Cleland (17) have concluded that substrate oxidation at pH 8.6, catalyzed by rabbit muscle G3PD proceeds by an ordered ter-bi mechanism in which NADH cannot disociate from enzyme-product complexes until after release of the acyl product. This view conflicts with results from our lab at pH 7.0 under conditions where little deacylation occurs (20), Trentham (18) has shown that the rate of deacylation increases with pH until it exceeds the NADH dissociation rate constant at pH 8.0 and above. If this occurs with the rabbit muscle enzyme, release of products from the enzyme could be random at low pH with the sequence, acyl phosphate, NADH becoming predominant near and above pH 8.0.

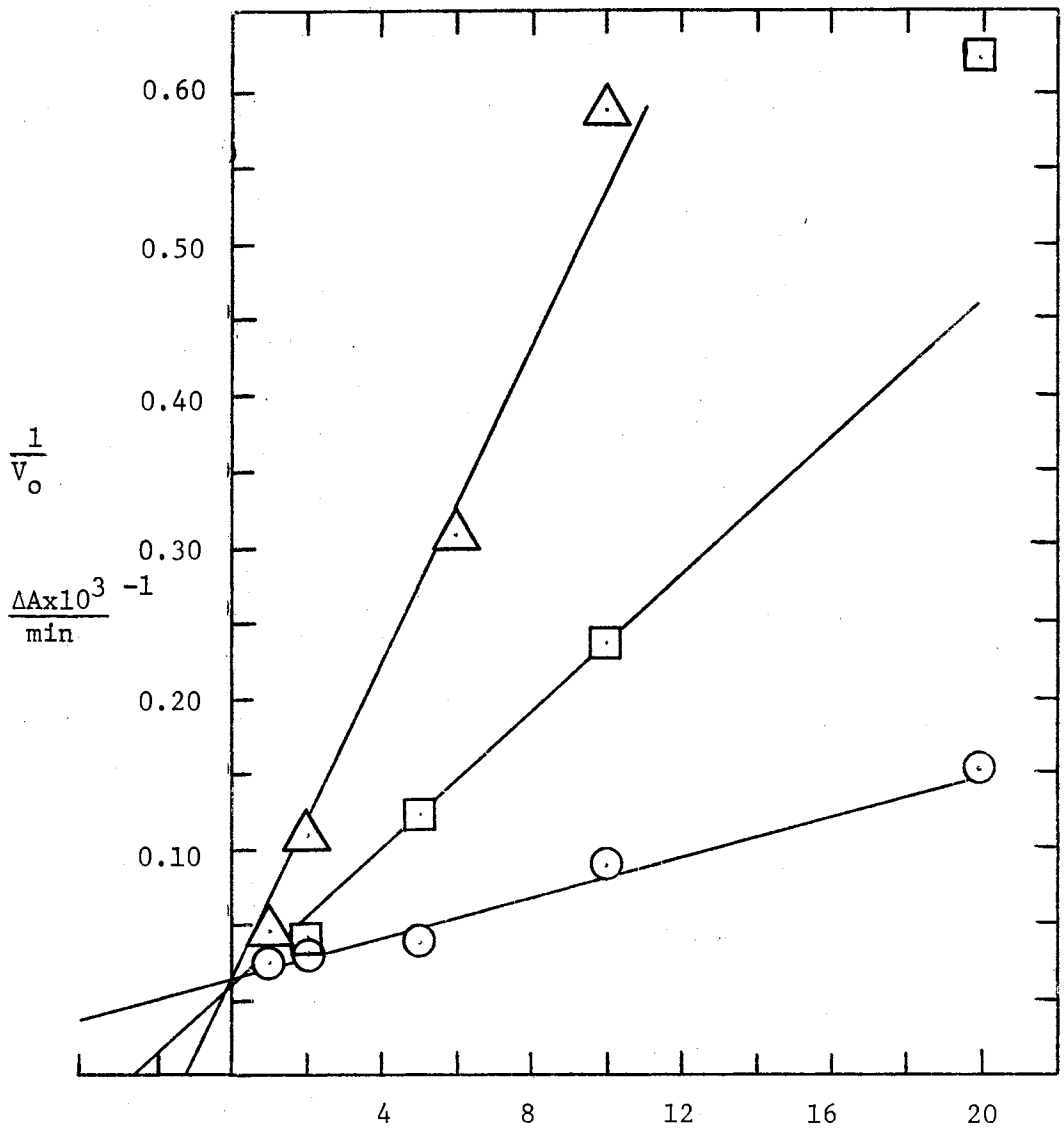
Under steady-state conditions, a random mechanism may be distinguished from an ordered mechanism by studies of product inhibition of the reaction initial velocity. Prior to these studies, initial velocity measurements were made to determine optimal concentrations of all substrates. Results of initial velocity measurements at pH 6.0 are summarized for both enzyme preparations in Table IV. Discrepancies in optimal concentrations of substrates for the two enzyme preparations may be due in part to the difficulty of obtaining reproducible initial velocities at pH 6.0. At pH 6.0, enzyme turnover is very slow and this is reflected in the initial velocities obtained. With both enzyme preparations, double reciprocal plots reveal product inhibition by NADH to be competitive with NAD, (Figure 4a). This inhibition pattern alone is consistent with either a random or an ordered mechanism of addition and release of substrates and products from the enzyme. The fact, however, that 1,3-diphosphoglycerate is also a competitive product inhibitor of NAD

TABLE IV

OPTIMAL G3PD SUBSTRATE CONCENTRATIONS AT 6.0

Enzyme Preparation	NAD	D-G3P	Na_2HAsO_4
Ferdinand Preparation	1.0 mM	4.0 mM	50 mM
Boehringer Preparation	1.0 mM	1.0 mM	150 mM

Figure 4a. Product Inhibition of G3PD Initial Velocity: NADH vs. NAD. NADH product inhibition of G3PD with NAD as variable substrate was monitored at 340 nm with 5 mM D-G3P, 125 mM Na_2HAsO_4 and 0.05–0.2 µg G3PD/ml. NADH concentrations are designated as: 0, (○); 0.01 mM, (□); and 0.02 mM, (△). Measurements were performed in 0.05 M imidazole, pH 6.0, containing 0.1 M KCl, 1 mM EDTA, 1 mM DTT.


0.60
0.50
0.40
0.30
0.20
0.10
$\frac{1}{V_o}$
$\frac{\Delta A \times 10^3}{min}^{-1}$
4
8
12
16
20

(Figure 4b) is compatible with only the random mechanism.

Similar product inhibition studies were planned at pH 8.6, but initial velocity measurements revealed activation and inhibition ranges for arsenate which we would prefer to avoid in order not to introduce possible artifacts in our subsequent measurements. To avoid these complications, product inhibition studies were planned on the reverse reaction in the absence of arsenate. Time was not available, however, for me to complete these measurements.

Spivey and Blanton (33) have recently completed the necessary steady-state measurements of the reduction of 1,3-diphosphoglycerate at pH 8.6 and 7.0. It is possible to report, therefore, that the results indicate that the steady-state mechanism of G3PD enzyme changes from random to ordered as pH is increased to 8.6. The patterns observed and expected for these mechanisms are summarized in Table V. Both mechanisms include the dead-end complex of enzyme-NADH-glyceraldehyde-3-phosphate as demonstrated at pH 8.6 (17) and generally expected for rapid equilibrium random patterns (34).

We would caution, however, against the assumption that the G3PD mechanism is a purely sequential one. Good evidence exists for the additional sequence in which NAD recombines with acyl-enzyme immediately after release of NADH and prior to release of the acyl group from the enzyme complex (18). This alternative sequence should be preferred at high NAD concentrations. With very low substrate concentrations, the enzymatic mechanism is apparently still more complex. Under these conditions, differing extents of substrate binding and catalytic activities may occur at each enzyme subunit with very complex interactions among them, depending on the degrees of saturation of each subunit with

Figure 4b. Product Inhibition of G3PD Initial Velocity: 1,3-DPG vs. NAD. 1,3-DPG product inhibition of G3PD with NAD as variable substrate was followed at 340 nm, with 1 mM D-G3P, 50 mM Na_2HAsO_4 and 0.4 µg G3PD/ml. 1,3-DPG concentrations are designated as: 0, (○); 0.123 mM, (△); 0.307 mM, (□). Measurements were performed in 0.05 M imidazole, pH 6.0, containing 0.1 M KCl, 1 mM EDTA, and 1 mM DTT.

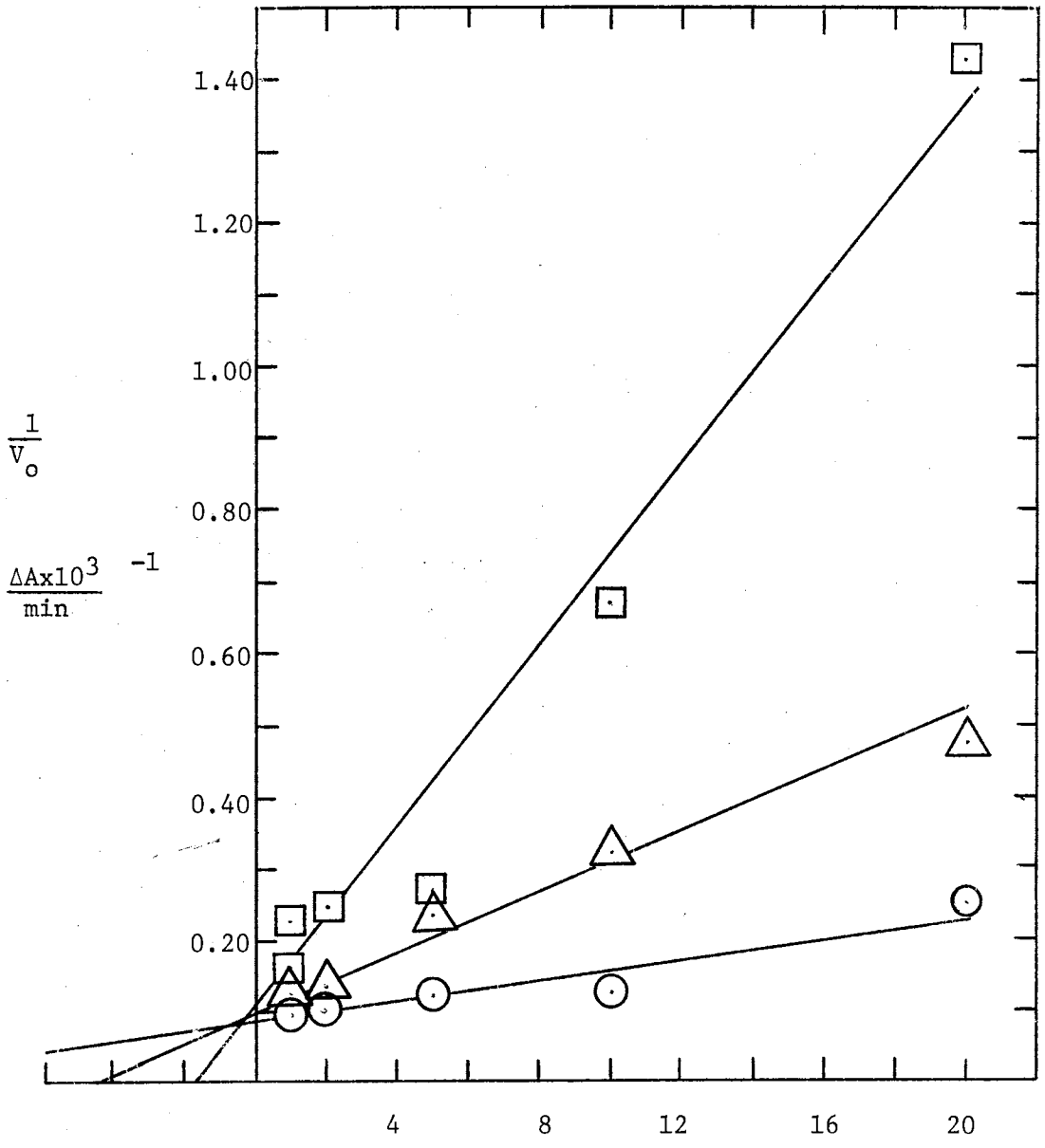

1.40
1.20
1.00
0.80
0.60
0.40
0.20
$\frac{1}{V_o}$
$\frac{\Delta A \times 10^3}{min}^{-1}$
4
8
12
16
20

TABLE V

EXPECTED AND OBSERVED STEADY-STATE PATTERNS FOR RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE[a]

EXPECTED					OBSERVED		
Var Sub	Prod Inh	Sat Sub	Rand	Ord	8.6[b]	7.0[b]	6.0[c]
NADH	NAD	None or DPG	C	C	C	C	
NADH	G3P	None	NC	UC	UC	C	
		or DPG	C	UC			
NAD	DPG	AsO_4+ G3P	C	UC			C
NAD	NADH	AsO_4+ G3P	C	C			C

[a]Abbreviations: Var Sub, Variable substrate; Prod Inh, Product Inhibitor; Sat Sub, Saturating substrate; Rand, Random; Ord, ordered.

[b]Data from Spivey and Blanton (33).

[c]Data from this thesis.

substrates and products (35). A general steady-state mechanism explaining data under all of these conditions has not been proposed as yet. We do feel confident, however, in having demonstrated that a shift with pH in preferred catalytic pathways does occur with G3PD near pH 8.0. Although this shift could permit dissociation of NADH from the enzyme prior to the acyl group (as in a random mechanism), possible complexities of the real mechanism prevent us from considering this NADH dissociation as proven by the steady-state data in this thesis.

CHAPTER V

SUMMARY

Only recently has the significance of the occurrence *in vivo* of many enzymes at substrate concentration been recognized. Enzymes at such concentrations would be subject to increased protein-protein interactions and may exhibit ligand binding and kinetic constants different from those of dilute enzymes assayed under conventional conditions *in vitro*. Thus, enzyme regulatory properties determined at dilute enzyme concentrations *in vitro* may not reflect metabolic situations *in vivo*. The measurements reported in this thesis are parts of studies in our lab to characterize enzyme kinetic and regulatory properties at physiological enzyme concentrations.

LDH heart isozyme is more efficient in lactate oxidation than muscle isozyme, with physiological lactate levels in heart (< 1.5 mM), because of its lower K_m for lactate (9 mM vs. 25 mM). However, use of physiological pyruvate concentrations results in extensive pyruvate inhibition of heart enzyme, and little advantage of one isozyme over the other can be discerned. No evidence was obtained that lactate abolishes pyruvate inhibition by dissociating the ternary pyruvate-NAD-enzyme complex. On the other hand, a small reduction in heart pyruvate concentration would result in a large increase in lactate oxidation.

Saturation of buffer with benzene or toluene leading to enhanced enzyme association provides a method for characterization of GDH kinetic

properties at different states of association. Substrate inhibitions of GDH by NADH and α-KG are unaltered by addition of benzene and resulting increased association of enzyme. Maximal activity occurs at 0.15 mM NADH and 5 mM α-KG, and approximately 75% inhibition occurs at 0.8 mM NADH, and 25 mM α-KG. It has not been possible to restore NADH and GDP inhibitions of concentrated GDH by any possible combination of incubations of enzyme, substrates, and products which might form dead-end complexes. Results with enzymes whose association was enhanced in benzene-containing buffers, therefore demonstrated that the release of substrate inhibition by NADH and α-KG when GDH is concentrated to 0.75 mg/ml and above, is due to a hysteretic (slow) transition of an enzyme central complex, and not due to increased enzyme association. Thus, with 0.8 mM NADH and 25 mM α-KG, one observes only the inhibited reaction with transformed enzyme when GDH concentration < 0.5 μg/ml. However, when GDH is concentrated to approximately 1 mg/ml, only the uninhibited rate with untransformed enzyme is observed, because NADH is depleted by the reaction before the inhibitory complex can form in significant concentrations. Molecular weights obtained in the presence of benzene agree with those reported in the literature.

A study of concentrated G3PD at pH 6.0 in our lab has shown that NADH can be released from acylated or unacylated enzyme, and in the absence of acyl acceptor during G3P oxidation. Competitive inhibitions of G3P oxidation by NADH and 1,3-DPG at dilute (< 0.2 μg enzyme/ml) enzyme concentrations support a random mechanism at pH 6.0. Spivey and Blanton completed measurements of 1,3-DPG reduction and found product inhibitions by NAD and G3P to be non-competitive. We have concluded that the ordered mechanism at pH 8.6 is a special case of more general

random mechanism, and that the predominant pathway of addition and release of substrates and products from the enzyme shifts as pH is increased.

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APPENDIX

An excellent treatment of light scattering theory is presented by Tanford (36). A brief summary and interpretation of the final working equations is presented here to facilitate an evaluation of our results.

The intensity of light i_Θ scattered from a macromolecular solution at an angle Θ relative to the incident beam is given by

$$i_\Theta = \frac{\kappa I_o (1 + \cos^2 \Theta)\, c}{r^2} \times M_{app} \qquad \text{(A-1)}$$

for macromolecules whose size in any direction is less than 1/20 of the wavelength of the incident light ($\sim$ 200 Å for the 436 nm Hg line radiation used). In Equation (A-1), I_o is the intensity of the incident beam, κ is known constant of the system, c is the macromolecular concentration in g/ml, r is the constant distance between sample and detector, and M_{app} is the apparent molecular weight. Thus, i_Θ is symmetrical about $\Theta = 90^o$. It is convenient to express Equation (A-1) as

$$\frac{\kappa c}{R_\Theta} = \frac{1}{M_{app}} \qquad \text{(A-2)}$$

utilizing the definition of Rayleigh's ratio,

$$R_\Theta = \frac{i_\Theta}{I_o} \frac{r^2}{1 + \cos^2\Theta} \qquad \text{(A-3)}$$

a constant independent of Θ. Experimental measurement of R_Θ and calculation of $\kappa c/R_\Theta$ are described under MATERIALS AND EXPERIMENTAL PROCEDURES, p. 12.

Equation (A-2), when applied to an associating protomer system or heterogeneous polymer will necessarily give the weight average molecular weight,

$$M_w = \Sigma c_i M_i / c \tag{A-4}$$

where $c = \Sigma c_i$ is the total weight concentration of the macromolecules in g/ml and the subscripts refer to values of the individual polymers with i = 1, 2, 3 ... protomer or monomer units (36).

For macromolecules larger than 0.05λ, however, destructive interference occurs for all scattering angles except at $\Theta = 0$, such that scattered intensities, i'_Θ are reduced below those expected in the absence of destructive interference, i_Θ. For particles between 0.05 and 0.5λ, the equation

$$\frac{i_\Theta}{i_\Theta'} = 1 + kR_G^2 \sin^2 \left(\frac{\Theta}{2}\right) \tag{A-5}$$

is sufficiently accurate where k is a known constant and R_G is the radius of gyration of the particles. Consequently, Equation (A-2) becomes

$$\frac{Kc}{R_\Theta'} = \frac{1}{M_{app}} \left[1 + kR_G^2 \sin^2 \left(\frac{\Theta}{2}\right) \right] \tag{A-6}$$

and a plot of $\frac{Kc}{R_\Theta'}$ vs. $\sin^2 \left(\frac{\Theta}{2}\right)$ will give $\frac{1}{M_{app}}$ at its intercept. The condition of significant destructive interference in i_Θ is easily detected by the dissymetry in i_Θ as required by the $\sin^2$ term in Equation (A-5). This disymmetry is very small for glutamate dehydrogenase solutions (10).

Although R_Θ corresponds more directly to the experimentally measured value, i_Θ, the turbidity, τ, is used in the older literature. For

$i_\Theta \ll I_o$, τ is related to R_Θ by $\tau = 16\ \pi\ R_\Theta/3$. Thus, $\frac{Kc}{R}$ or $\frac{Hc}{\tau}$ are equivalent with $K = 3H/(16\ \pi)$ (36, p. 288). They are often interconverted in this way although the conversion is not accurate in principle for high turbidity values. It would be preferable to drop use of τ altogether. For non-associating macromolecules, the apparent molecular weight is related to the true molecular weight, M, through

$$\frac{i}{M_{app}} = \frac{1}{M} + Bc + Cc^2 + \ldots \qquad \text{(A-7)}$$

where B and C are the virial coefficients in the power series expansion of solvent chemical potential. Thus, extrapolation of $\frac{1}{M_{app}}$ to $c = 0$ gives $\frac{1}{M}$. In the case of glutamate dehydrogenase, changing extents of enzyme association preclude extrapolation to zero concentration. Independent measurements or estimates reveal that the virial coefficients and terms are small in our experiments (27), however, and M_{app} is a sufficient approximation of the weight average molecular weight of glutamate dehydrogenase.

VITA

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Candidate for the Degree of

Master of Science

Thesis: SOME KINETIC AND REGULATORY PROPERTIES OF MAMMALIAN LACTATE, GLUTAMATE, AND RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES

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