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Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M. Sc.

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METABOLIC REGULATION OF
GLUTAMATE DEHYDROGENASE

by

© KEITH BRADFORD MALE, B.Sc. (Hons. Biochemistry)

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

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ABSTRACT

Glutamate dehydrogenase (GDH), a key control enzyme interrelating carbohydrate and amino acid metabolism, was investigated with respect to coenzyme utilization and regulatory control via allosteric effectors. GDH from gall fly larva, Eurosta solidaginis, was purified and kinetic studies suggested that it was preferentially NADPH-linked. Temperature effects on the regulatory properties of this GDH, including ATP/GTP interactions, may be responsible for the increase in proline seen at low temperature in the overwintering larva. GDH from the intertidal sea anemone, Anthopleura xanthogrammica, was purified and found to be NADP(H)-specific. This GDH was not regulated by nucleotide effectors but was strongly affected by ions. The dual coenzyme specific GDH from bovine liver was investigated with respect to its coenzyme preference. The data suggested that under increasing glutamate concentrations the preferred coenzyme was NADP, a preference which was promoted by the action of metabolic modulators.

Acknowledgments

I wish to express my gratitude to Dr. Ken Storey for his enthusiasm and guidance throughout this research project, and Janet Storey for her patience and technical assistance. The advice and criticism of my advisors Dr. C.S. Tsai and Dr. J.M. Neelin was much appreciated. Technical assistance supplied by Cathy Shay with regard to amino acid analysis was gratefully accepted. I am also grateful to Dr. T.W. Moon and Lise Belanger for the use of facilities and assistance in measuring inorganic ions. I wish to acknowledge Pat Walsh for useful discussions on sea anemones. Helpful discussion and friendship was offered by my colleagues in the lab. A special thanks goes out to my parents for all their encouragement over the years. I would also like to thank all my friends at the R.A. Badminton Club for providing the needed social life to complement this endeavour. Finally I thank Susan Buske for the word processing and printing of this thesis.

The research was supported by an NSERC grant to Dr. Storey.

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List of Abbreviations and Terms

- A₂₈₀ - Absorbance at 280 nanometers
- ADP - Adenosine diphosphate
- AMP - Adenosine monophosphate
- ATP - Adenosine triphosphate
- cal - calories
- cm - centimeters
- Direct linear plot - velocity versus substrate concentration
- GDH - Glutamate dehydrogenase
- GDP - Guanidine diphosphate
- gln - Glutamine
- glu - Glutamate
- GTP - Guanidine triphosphate
- h - hours
- Hanes plot - substrate conc./velocity versus substrate conc.
- Hill plot - log velocity/(V_{max} - velocity) versus log substrate conc.
- I₅₀ - inhibitor concentration producing 50% decrease in the enzyme velocity
- IMP - Inosine monophosphate
- K_{av} - represents the fraction of the stationary gel volume which is available for diffusion of a given solute species
- K_{eq} - equilibrium constant - for GDH
$$\frac{[\text{NAD(P)H}][\alpha\text{-KG}][\text{NH}_4^+]}{[\text{Glu}][\text{NAD(P)}]}$$
- α KG - α -ketoglutarate
- lys - Lysine
- M - molar
- mA - milliamperes
- mg - milligrams
- min - minutes

ml - millilitres
 mM - millimolar
 mOsm - millosmolar
 M_r - molecular weight
 n - Hill coefficient slope of plot of Hill plot
 NAD - Nicotinamide adenine dinucleotide
 NADH - Nicotinamide adenine dinucleotide reduced
 NADP - Nicotinamide adenine dinucleotide phosphate
 NADPH - Nicotinamide adenine dinucleotide phosphate reduced
 NAD(P)(H) - NAD, NADP, NADH or NADPH
 nm - nanometers
 Pi - inorganic phosphate
 Q_{10} - ratio of enzyme's velocity for a set temperature decrease of 10°C . ie. $(V+10^\circ\text{C})/V$
 S.A. - specific activity
 $S_{0.5}$ or K_m - substrate concentration producing 50% of enzyme's maximal velocity
 SDS - Sodium dodecyl sulphate
 sec - seconds
 S-NAD - Thionicotinamide adenine dinucleotide
 S-NADP - Thionicotinamide adenine dinucleotide phosphate
 $t_{1/2}$ - half life
 Tris - tris(hydroxymethyl)aminomethane
 μM - micromolar
 $V_{\text{max}(\text{app})}$ or $K_{\text{m}(\text{app})}$ - apparent K_m or V_{max} values obtained at subsaturating substrate concentrations
 V_{max} - maximum enzyme velocity

GENERAL INTRODUCTION

Glutamate dehydrogenase (GDH) (E.C.1.4.1.3) catalyzes the reaction:

The enzyme is a key locus interrelating carbohydrate and amino acid metabolism in the cell (Fig. 1) and is a major site for the incorporation of ammonium ions into amino acids in animals. Glutamate, produced by the reverse reaction, subsequently serves as the amino group donor for a variety of transamination reactions which are responsible for the biosynthesis of some other amino acids.

In animals, glutamate dehydrogenase is found solely in the matrix of the mitochondria. However the enzyme in yeast is located in the cytoplasm and some plants contain an NADP specific enzyme activity associated with the chloroplasts in addition to the mitochondrial NAD specific activity (Smith *et al.*, 1975). GDH from most sources is a hexamer with a molecular weight of about 350,000 daltons. The K_{eq} for the bovine enzyme is approximately 7×10^{-15} M such that equilibrium favours glutamate synthesis (Frieden, 1963). However, the activity ratio of the forward to reverse reactions in vitro varies from organism to organism: mammals 1:15; insect flight muscle 1:6 (Bursell, 1975); and squid mantle muscle 1:1 (Storey *et al.*, 1978). To date, tissue specific isozymes of GDH in animals have not been detected.

Bovine GDH has pH optima of 7.5 and 8.5 for the reverse and forward directions, respectively. In the forward direction the enzyme can catalyze the oxidative deamination of leucine, alanine, norvaline, and α -aminobutyrate as alternative substrates to

Fig. 1 Integration of amino acid metabolism and carbohydrate metabolism by glutamate dehydrogenase.

glutamate (Smith et al., 1975). The in vivo significance of deamination of these alternative substrates is unlikely to be important, however, since the pH optima for the reactions are extremely high. In the reverse direction, glutamine as well as asparagine can serve as alternative substrates for ammonium ion by donating their terminal amino groups. These reactions usually have a higher pH optima of around 8.4. In Neurospora the glutamine linked activity is only associated with the NADP linked reaction and not the NAD linked reaction (Smith et al., 1975).

The coenzyme specificity of GDH varies with its source. Most animal GDHs show a dual specificity utilizing either NAD(H) or NADP(H) in vitro (Smith et al., 1975). However, some invertebrate tissues such as insect flight muscle or squid mantle muscle show a strong preference for NAD in the catabolic reaction (Bursell, 1975; Storey et al., 1978). The only known NADP specific GDHs from animal sources are found in marine coelenterates such as sea anemones (Bishop et al., 1978; Walsh, 1981). Most bacteria and fungi studied have either a NAD(H) or NADP(H) specific GDH or both activities as separate enzymes. With a few exceptions these bacterial or fungal GDHs are not affected by purine nucleotides (Kimura et al., 1977). Plant GDHs are generally either NAD specific or show dual specificity.

Early studies suggested that the mechanism of the GDH reaction was ordered, with the binding of substrates and coenzymes being NADH, NH_4^+ , and α -KG (Frieden, 1963), but it is now thought that the reaction is random (Engels and Dalziel, 1970). The formation of α -iminoglutarate is enzyme mediated as indicated by the finding that competitive inhibitors prevent its formation (Smith et al., 1975). Residue Lys-126 is involved in the substrate binding, a

condensation arising between the uncharged γ -amino group and α -KG to form a Schiff base with the release of water. The attack of ammonium ion on the Schiff base intermediate yields α -imino-glutarate which is then reduced by NAD(P)H to form glutamate. With regard to the binding of the coenzyme, the only difference in the behaviour of the two coenzymes NAD versus NADP appears to be at the level of binding of the reduced coenzymes NAD(P)H, where there appears to be an additional non active binding site for NADH (Merzi-D'Eril and Dalziel, 1973). It is postulated that NADPH cannot fit into this regulatory site due to steric hindrance caused by the extra phosphate group. In the case of the GDH-NADPH complex only the nicotinamide ring interacts, while in the GDH-NADH complex the adenine group also contacts the enzyme and thus there is an additional mode of binding. The regulatory site in question is believed to be the ADP regulatory site.

GDH from most sources is under tight regulatory control. Modes of control for glutamate dehydrogenase isolated from various tissues include: substrate availability; differential use of the coenzymes NAD(H) and NADP(H); allosteric effects by GDP, GTP, AMP, ADP, ATP and leucine; covalent modification by phosphorylation-dephosphorylation; and enzyme aggregation-disaggregation.

Substrate availability is an important factor in the regulation of cellular pathways, since competition arises between enzymes for common substrates. Control of GDH by substrate availability is quite likely linked to microcompartmentation in the mitochondria. There is evidence for two separate pools of glutamate in the mitochondria of rat kidney. One pool is related to glutamate oxaloacetate transaminase and is located near the inner

mitochondrial membrane. This pool is not, apparently, associated with GDH. A second glutamate pool is located in the matrix in proximity to GDH and glutaminase. Under conditions of metabolic acidosis, glutamine entering the mitochondria is catabolized via these two enzymes through the intermediary of the matrix glutamate pool resulting in the production of 2 moles of ammonia per mole of glutamine converted to α -ketoglutarate (Schoolwerth and Lanoue, 1980).

Little is known about the relation between the coenzyme used by GDH and the metabolic function in vivo. Evidence from in vivo studies suggests that NADPH is linked to the biosynthetic function. In vivo studies carried out on the dual specific GDH from rat liver indicated that the reductive amination of α -KG utilizes NADPH as the primary source of reducing equivalents (Tischler et al., 1977; Sies et al., 1974). Ratios of NAD/NADH and NADP/NADPH in the cytosol and mitochondria were measured. Upon ammonium perfusion of the cells leading to glutamate formation, there was a large increase in the ratio of total NADP/NADPH in the mitochondria. On the other hand the NAD/NADH ratios were unaffected indicating that NADH is not serving as a reducing equivalent.

Allosteric control of GDH is vested in adenine and guanine nucleotide effects as well as effects by leucine. It is now believed that there are at least three separate sites - one active and two regulatory- on each subunit of glutamate dehydrogenase in animals. ADP has its own regulatory binding site and causes activation. Initial studies indicated that GTP interacted at this site also but it is now clear that there are two distinct and mutually exclusive regulatory sites such that GTP has its own binding site (Dieter et al., 1981) and inhibits enzyme activity.

Activation and inhibition by these allosteric modifiers results in conformational changes in the enzyme and the interaction between subunits. Kinetic parameters such as the K_m (indicating substrate affinity), the Hill coefficient (n) and the V_{max} are all altered by these allosteric effectors. The ADP and GTP effects produce inhibition of enzyme activity when energy supply is high and activation when energy supply is low. Occupation of the ADP site in the presence of NADH totally reverses the conformational changes brought about by GTP. (Chen and Engel, 1975). Leucine is often a potent activator of the reverse direction of GDH and is thought to also have its own regulatory site. ATP is unique in that it activates with respect to NADP(H) and inhibits with respect to NAD(H) (Goldin and Frieden, 1971).

ADP and GTP, in addition to their allosteric effects on the enzyme's kinetics, cause aggregation-disaggregation of the enzyme at constant enzyme concentration. ADP activation enhances enzyme aggregation while GTP inhibition enhances disaggregation so that activation-inactivation is attributed to aggregation-disaggregation. In the aggregated state GDH oligomers form long linear rods. Disaggregation also occurs in dilute solution with polymerization at concentrations above 0.1mg/ml (Smith et al., 1975).

Covalent modification by phosphorylation-dephosphorylation has been shown for the NAD-linked GDH of the yeast Candida utilis (Hemmings, 1981). The active form of the enzyme is dephosphorylated whereas the inactive form is phosphorylated such that inactivation can be achieved by reversible phosphorylation. This form of control is not found in the NADP-linked GDH of Candida utilis which seems to have an activation-inactivation control based instead on

degradation and resynthesis of the enzyme (Hemmings, 1978).

The enzyme is a unique control enzyme in that it catalyzes a reversible reaction in vitro whereas most control enzymes are thought to catalyze essentially irreversible reactions both in vitro and in vivo. However it is thought that in vivo metabolic conditions make GDH essentially irreversible. The role of glutamate dehydrogenase in vivo is still controversial. Early studies on GDH function in mammals suggested that the reaction was the major pathway for ammonium production in vivo and thus was involved in nitrogen excretion (Braunstein, 1957). However this proposed role for GDH in furnishing the ammonia required for carbamoyl phosphate synthesis and urea production has been criticized by McGiven and Chappell (1975). They suggested that the ammonia required for carbamoyl phosphate came instead from the deamination of AMP to IMP and NH_3 and that the primary role of the enzyme is in glutamate synthesis. This function in nitrogen storage is also supported by Palailogos and Felig (1976). Supporting this theory was the effect of leucine. While leucine activates GDH, it inhibits the synthesis of urea (McGiven and Chappell, 1975). It would be unusual for an activator of an enzyme to also be an inhibitor of the pathway in which the enzyme is involved.

It seems probable that GDH has multiple roles in tissues with metabolic function closely regulated by substrate availability, choice of coenzyme utilized and the action of allosteric effectors (Goldin and Frieden, 1971). Although much work has been done on allosteric effectors little has been done on coenzyme choice. The reversibility of the enzyme in vitro would suggest that in vivo there could be two possible roles for the enzyme one in amino acid synthesis and another in amino acid oxidation. The roles of GDH

appear to vary from organism to organism as well as from tissue to tissue. For example, insect GDH is probably involved in both catabolic and anabolic functions and in particular may play a key role in the regulation of proline metabolism as shown in Fig. 2. GDH function in adult flight muscle during the early phases of flight is involved in the oxidation of proline, a major flight muscle fuel in many species. The α -ketoglutarate derived from proline is introduced into the Krebs cycle. This GDH from insect flight muscle was found to be NAD-linked (Bursell, 1975). On the other hand, GDH function in insect fat body or larvae is probably involved largely in the de novo synthesis of amino acids a function which is demonstrated in this thesis and appears to be NADPH linked. Other evidence for this idea of oxidative versus biosynthetic function of GDH comes from plants where there are often two separately functioning GDHs in the same plant, one NAD and one NADP specific (Smith et al. 1975).

The purpose of this study was to investigate metabolic regulation of glutamate dehydrogenase paying particular attention to two modes of GDH control, allosteric regulation and coenzyme specificity. It was also important to try to relate GDH regulation to the role of the enzyme in vivo. GDH from the gall fly larva, Eurosta solidaginis, was investigated with regard to its apparent biosynthetic role in the production of proline as an adaptation to low temperature survival during overwintering. The NADP specific GDH from the intertidal sea anemone, Anthopleura xanthogrammica, was examined with particular attention to potential regulation of the enzyme for a role in amino acid metabolism during osmoregulation. The dual coenzyme specific GDH from bovine liver was investigated to try to establish the choice of coenzyme, NAD or NADP, used in

Fig. 2 Role of glutamate dehydrogenase in proline metabolism.

vivo by the technique of thioderivatives of the natural coenzymes of NAD and NADP.

CHAPTER ONE

PURIFICATION AND PROPERTIES OF GLUTAMATE DEHYDROGENASE
FROM THE COLD-HARDY GALL FLY LARVA, EUROSTA SOLIDAGINIS

INTRODUCTION

The third instar larvae of the goldenrod gall fly, Eurosta solidaginis, overwinter inside stem galls on goldenrod plants, Solidago canadensis, and display a cold tolerance which allows survival of temperatures as low as -40°C (Morrisey and Baust, 1976). The eggs are laid by the female adult in the unfolded leaves of the terminal bud of the growing goldenrod plant. After hatching the young larvae bore through the leaves to the base of the bud and into the meristematic tissue. The galls of the plant are first visible three to four weeks after the eggs have been deposited and there is still controversy as to whether gall formation arises from a secretion left by the female or from salivary materials secreted by the larva (Uhler, 1951). The larvae are enclosed in the galls throughout their period of growth and enlarge a chamber for themselves within the galls in addition to boring an exit tunnel for emergence of the adult in the spring.

The gall enclosed larvae must face and survive the rigors of full Canadian winters as they are often exposed above the snow line. The gall does not have any insulating value to the larva but does protect it against body contact with rain, ice, snow, and sunlight (Uhler, 1951). The insect larvae display a range of properties which enable them to survive subzero temperatures. The amount of water bound by both high and low molecular weight subcellular components during low temperature acclimation, is increased limiting intracellular dehydration and protecting proteins from denaturation (Morrisey and Baust, 1976; Storey et al., 1981a). Supercooling of the hemolymph will result in freezing only below the supercooling point of -10°C (Morrisey and Baust, 1976). High levels of the polyhydric alcohols, sorbitol and

glycerol, are synthesized at the expense of stored glycogen as the temperature drops. These compounds serve as cryoprotectants for the cells (Storey et al., 1981b).

Glycerol accumulates first in the larva at high temperatures in early autumn outdoors. During laboratory low temperature acclimation glycerol reaches a plateau of 235 mM when temperature had decreased to 5°C. Below this temperature glycerol synthesis halts and sorbitol synthesis is initiated, reaching a plateau of 145 mM by -10°C (Storey et al., 1981b). Among amino acids the only substantial changes occurring during low temperature acclimation were increases in the levels of proline and alanine, along with a transient increase in glutamate (Storey et al., 1981b). Accumulations of proline and alanine accompany low temperature acclimation in the cold-hardy insect Anthergea pernyi may have a cryoprotectant function as well (Mansingh, 1967).

During low temperature acclimation of the larvae, proline synthesis is activated. Proline concentration increases from 32 to 56 mM with a temperature decrease from 15°C to -5°C which represents a 50% increase in the total free amino acid pool. (Storey et al., 1981b). Synthesis of proline, which requires mitochondrial function, argues strongly for the maintenance of mitochondrial activity at temperatures at least as low as -5°C in these larvae. The transient increase in glutamate levels during the period suggests a precursor role for glutamate in proline synthesis and the possible function of GDH as the site of incorporation of ammonium ion into the de novo production of this amino acid. Metabolite changes during laboratory low temperature acclimation are shown in Figure 3.

In this chapter the kinetic and regulatory properties of

Fig. 3 Levels of glycogen, polyols, and the amino acids glutamate and proline in Eurosta solidaginis larvae acclimated to low temperatures. Data was obtained from Storey and Storey (1981). (A) Levels of glycogen ($\square$), glycerol (O), and sorbitol ($\diamond$). (B) Levels of proline ($\square$) and glutamate ($\circ$). Acclimation temperatures are expressed in degrees centigrade while concentrations are in $\mu\text{mol/g}$ wet weight.

purified GDH from Eurosta solidaginis larvae have been studied. The study represents the first purification of the insect larval enzyme. Like many animal GDHs, but unlike adult insect flight muscle GDH (Bursell, 1975), the larval form can make significant use of either coenzyme, NAD(H) or NADP(H). Substrate affinities and modulator effects by ADP, ATP, and GTP suggest that glutamate synthesis in vivo may be NADPH linked. The activity ratios, forward/reverse, for the larval enzyme favour the reverse or glutamate synthesizing reaction much more strongly than does the enzyme from the adult flight muscle. Temperature effects on ADP/GTP regulatory interactions may be important in activating the synthesis of glutamate by GDH, leading to accumulation of proline, during low temperature acclimation of the larvae.

MATERIALS AND METHODS

Animals and Chemicals: Round galls containing third instar larvae of the gall fly, Eurosta solidaginis, were collected and acclimated to low temperature (Storey et al., 1981b). Larvae were then removed from their galls, killed by immersion in liquid nitrogen and stored at -80°C until use.

All biochemicals were purchased from Boehringer Mannheim Corp., phosphocellulose and molecular weight marker proteins were from Sigma Chemical Co. and Sephacryl S-300 was from Pharmacia Fine Chemicals.

Purification of GDH: Whole larvae (3 g per preparation) were homogenized in 5 vol ice-cold 20 mM Tris-HCl buffer, pH 7.0 containing 114 mg/ml (20% saturation) ammonium sulfate using a Polytron PT 10-35 homogenizer at full speed for 3 x 5 sec. The homogenate was then centrifuged at 27,000 g for 30 min at 4°C . The supernatant was saved and the pellet was resuspended in a second

volume of 20 mM Tris-HCl buffer, pH 7.0, rehomogenized and recentrifuged as above. The second supernatant was added to the first producing a crude enzyme preparation at 10% saturation with ammonium sulfate. Additional ammonium sulfate (183 mg/ml) was then added to bring the solution to 40% saturation. The solution was stirred at room temperature for 30 min., then centrifuged as above. The protein pellet was discarded and the supernatant was brought to 50% saturation (by addition of 63 mg/ml) with ammonium sulfate, stirred and centrifuged. The pellet, containing GDH, was resuspended in 15 ml 10 mM imidazole-HCl buffer, pH 6.5 containing 15 mM 2-mercaptoethanol and layered onto a phosphocellulose column (5 ml) equilibrated in the same buffer. The column was then washed with buffer until the A_{280} dropped to a minimum value. GDH was eluted from the column using a 0 to 4 M gradient of KCl run at 30 ml/h. Peak fractions were pooled and concentrated to approximately 1 ml (to increase enzyme stability) using an Amicon Centriflo membrane cone with a molecular weight cut-off at 25,000 daltons. The concentrated enzyme was stable at 4°C for at least 1 week.

Molecular weight determination: Molecular weight

determinations were performed using a column (50 x 0.7 cm.) of Sephacryl S-300 with 20 mM imidazole-HCl buffer, pH 7.0 as the equilibration/elution buffer. One ml fractions were collected and assayed for GDH activity. Molecular weight was determined from a plot of K_{av} versus $\log M_r$ for standard proteins: ferritin ($M_r = 440,000$), catalase ($M_r = 240,000$) and aldolase ($M_r = 158,000$).

SDS polyacrylamide gel electrophoresis: Electrophoresis using

10% slab gels was carried out at 40 mA constant current for 4 h at 4°C using Tris-glycine (46 mM/35 mM), pH 8.0 containing 1% SDS as the electrode buffer. Prior to running, protein samples were

preincubated with 1% SDS at 80°C for 20 min. Protein was detected using the silver stain of Switzer et al. (1979). Subunit molecular weight was determined from a plot of R_f versus molecular weight for the protein standards: trypsinogen (24,000), ovalbumin (45,000) and bovine glutamate dehydrogenase (60,000).

Protein assay: Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad Laboratories prepared reagent and bovine gamma globulin as the standard.

Enzyme assay and kinetics: GDH activity was monitored by following NAD(P)(H) utilization at 340 nm using a Pye Unicam SP 8-100 recording spectrophotometer with water jacketed cell holder for temperature control. Optimal assay conditions (final volume = 1 ml) for GDH in the forward direction were: 50 mM Tris-HCl buffer, pH 8.0, 25 mM L-glutamate, 2 mM NAD(P) with 1 mM ADP for the NAD-linked activity and 0.5 mM ADP for the NADP linked activity. In the reverse direction, optimal conditions were: 50 mM imidazole-HCl buffer, pH 7.5, 300 mM NH_4Cl , 25 mM α -ketoglutarate, 0.15 mM NADH and 0.5 mM ADP for the NADH-linked activity and 50 mM imidazole-HCl buffer, pH 6.75, 100 mM NH_4Cl , 5 mM α -ketoglutarate, 0.20 mM NADPH and 0.1 mM ADP for the NADPH linked activity. One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol NADH per min at 25°C.

Kinetic constants, $S_{0.5}$ and n , were determined from Hill plots using experimentally measured V_{max} values in place of calculated V_{max} values as a result of extrapolation difficulties for V_{max} calculated due to substrate inhibition. I_{50} 's, the inhibitor concentration producing a 50% decrease in enzyme rate at constant substrate concentrations, were determined from plots of $v_{(\text{uninhibited})} / v_{(\text{inhibited})}$ versus inhibitor concentrations by the method of Job et al. (1978).

For low temperature studies, buffers were pH adjusted at 25 °C and buffer pH was allowed to change with changing temperature (for imidazole this results in an approximately 0.02 pH unit increase in pH per 1 °C decrease in temperature (Somero, 1978)).

RESULTS

Purification and physical properties: The maximal activity of GDH in E. solidaginis was 6.7 units/g wet weight of larvae. Previously GDH was reported as about 3 units/g in the larvae (Storey and Storey, 1981); yield in the present study was greatly improved by the inclusion of ammonium sulfate in the homogenizing medium. The purification of E. solidaginis GDH is summarized in Table 1. Under the conditions used, phosphocellulose, a cation exchanger functioned essentially like an affinity resin, enzyme binding probably resulting through interactions of the phosphate residue with the nucleotide binding sites of GDH. The reason it was so effective is that at the pH of the run, the majority of the protein in the sample except for GDH was negatively charged and ran straight through the column. As a result the purification at this one step was over 250 fold. As can be seen by the elution profile, figure 4, the salt concentration which was needed to elute the enzyme from the column is extremely high at about 1100 mM KCl. The final preparation was purified about 500 fold to a final specific activity of 37.5 units/mg protein compared to specific activities of 40 units/mg for ox liver and brain GDH (McCarthy et al., 1980) and 43-50 units/mg for commercial bovine liver GDH from Sigma Chemical Co. The enzyme was judged to be >95% pure by SDS electrophoresis.

Figure 5 indicates that the molecular weight of Eurosta solidaginis GDH was $350,000 \pm 30,000$ ($n = 7$) similar to an average

Table 1 Purification of Glutamate Dehydrogenase from the Gall Fly Larva, Eurosta solidaginis

Step	Total Activity (units)	Total Protein (mg)	Yield (%)	Purification	Specific Activity (units/mg protein)
Crude homogenate	17.9	244	-	-	0.073
Ammonium sulfate	15.0	115	84	1.8	0.130
Cellulose phosphate	9.0	0.24	50	514	37.5

Activities were measured under the following conditions: 25mM α -ketoglutarate, 0.15mM NADH, 300mM NH_4Cl and 0.5mM ADP. Proteins were measured using the Bio-Rad protein assay. GDH was eluted from the cellulose phosphate column using a 0-4 M KCl gradient.

Fig. 4 Purification of glutamate dehydrogenase from Eurosta solidaginis via a phospho-cellulose column eluted with a 0-4M KCl gradient. A 40-50% ammonium sulphate pellet solution was layered onto the phospho-cellulose column (5ml) equilibrated in 10mM imidazole-HCl buffer pH 6.5 and eluted by KCl in the same buffer. GDH activity ($\square$) is expressed as a percentage of the peak tube activity, KCl gradient ($\bullet$) is expressed in mM and the fraction size was 2.1 ml.

Fig. 5   Molecular weight determination of glutamate dehydrogenase from Eurosta solidaginis by gel filtration with Sephacryl S-300. Molecular weight determinations were performed using a column (50 x 0.7 cm.) of Sephacryl S-300 with 20mM imidazole-HCl buffer pH 7.0 as equilibration/ elution buffer. One ml. fractions were collected and assayed for GDH activity while standard proteins were measured by A_{280} . Molecular weight was determined from a plot of K_{av} versus $\log M_r$ for standard proteins: ferritin ($M_r = 440,000$), catalase ($M_r = 240,000$) and aldolase ($M_r = 158,000$). $K_{av} = (V_e - V_o) / (V_t - V_o)$ where V_e is the elution volume of the solute, V_o is the void volume of the column and V_t is the total volume of the column. Solid line drawn by the method of least square.

molecular weight of about 330,000 reported for most species (Smith et al., 1975). The subunit size of $57,000 \pm 5,000$ ($n = 4$) derived from figure 6 suggests that the enzyme like all other animal GDH's, is a hexamer (See plate 2).

pH optima and coenzyme utilization: Figure 7 shows pH profiles for E. solidaginis GDH. In the reverse direction (NAD(P)H utilizing), the enzyme has pH optima of 7.5 and 6.75 for the NADH and NADPH reactions, respectively while in the forward direction, the optima are 8.0 and 7.5 for NAD and NADP, respectively. The acidic shift in the pH optimum in the presence of NADP or NADPH was also reported for the Drosophila larval enzyme (Bond and Sang, 1968). Enzyme activity with NAD(H) as the coenzyme was significantly higher than that with NADP(H). In the reverse direction, the activity ratio, NADH/NADPH was 11 at pH 7.5 but dropped to 3.5 at pH 6.75. In the forward direction, the ratio NAD/NADP was 15 at pH 8 and 7 at pH 7.5.

The ratio of enzyme activity in the forward versus reverse directions (at the respective pH optima) was 1:10 for NAD/NADH and 1:20 for NADP/NADPH.

The Reverse reaction:

A. Substrate affinities: Tables 2 and 3 show the substrate affinities ($S_{0.5}$) and Hill coefficients (n) for E. solidaginis GDH for the NADH and NADPH linked reactions, respectively. Enzyme affinity for both NH_4^+ and α -ketoglutarate was significantly higher ($S_{0.5}$'s 4 and 5 fold lower, respectively) for the NADPH linked reaction versus the NADH linked reaction. The lower $S_{0.5}$'s for the NADPH reaction probably more closely resemble in vivo levels of these substrates, perhaps suggesting that NADPH is the preferred coenzyme for the reductive amination reaction of GDH. The $S_{0.5}$

Fig. 6 Subunit molecular weight determination of glutamate dehydrogenase from Eurosta solidaginis by SDS gel electrophoresis. Electrophoresis using 7.5% acrylamide and 1% SDS slab gels was carried out at 40mA constant current for 4 hours at 4⁰C using Tris-glycine (46mM/35mM) pH 8.0 containing 1% SDS as the electrode buffer. Samples were incubated in 1% SDS at 80⁰C for 20 minutes. Proteins were detected by silver staining. Subunit molecular weight for the proteins was determined from a plot of R_f versus molecular weight using the standard proteins: ribonuclease A (13,800) , chymotrypsinogen(24,000) ovalbumin (45,000) and bovine glutamate dehydrogenase (54,000). $R_f = (\text{distance protein migrates}) / (\text{distance to bromothymol blue dye front})$. Molecular weight is expressed in thousands. Solid line drawn by the method of least squares.

Fig. 7 pH profiles for Eurosta solidaginis glutamate dehydrogenase with respect to the four coenzyme-linked activities. Specific activity is expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. Optimal assay conditions for GDH were: (A) 50mM Imidazole buffer pH 7.5, 300mM NH_4Cl , 25mM α -ketoglutarate, 0.15mM NADH and 0.5mM ADP for the NADH-linked activity ($\square$) ; 50mM imidazole-HCl buffer, pH 6.75, 100mM NH_4Cl , 5mM α -ketoglutarate, 0.20mM NADPH, and 0.1mM ADP for the NADPH-linked activity ($\bullet$) ; (B) 50mM Tris-HCl buffer pH 8.0, 25mM L-glutamate, 2mM NAD and 1mM ADP for the NAD-linked activity ($\circ$) ; 50mM Tris-HCl buffer pH 8.0, 25mM L-glutamate, 2mM NADP and 0.5mM ADP for the NADP-linked activity ($\diamond$).

Table 2 Kinetic Constants for the NADH-linked Reaction of Eurosta solidaginis Glutamate Dehydrogenase:
Effects of Activators and Inhibitors

Addition	NH ₄ Cl		L-Ketoglutarate		NADH	
	S _{0.5}	n	S _{0.5}	n	S _{0.5}	n
Control	210 ± 10	3.5 ± 0.05	2.7 ± 0.2	1.2 ± 0.1	0.032 ± 0.001	1.5 ± 0.1
ADP, 0.5mM	52 ± 2	1.6 ± 0.1	2.3 ± 0.1	1.3 ± 0.1	0.027 ± 0.001	1.5 ± 0.1
ATP, 2mM	400 ± 13	7.3 ± 0.5	2.3 ± 0.2	1.1 ± 0.1	0.030 ± 0.003	1.7 ± 0.1
GTP, 20μM	440 ± 26	7.6 ± 0.8	2.8 ± 0.03	1.3 ± 0.1	0.028 ± 0.003	1.7 ± 0.2
ADP, 0.5mM plus ATP, 2mM	300 ± 16	5.1 ± 0.6				
ADP, 0.5mM plus GTP, 20μM	370 ± 18	6.5 ± 0.6				

S_{0.5} values are in mM ± s.e.m., from determinations on at least 3 different enzyme preparations. Kinetic constants were determined from Hill plots using V_{max} measured at substrate concentrations: 25mM L-ketoglutarate, 0.15mM NADH and 450mM (control), 300mM (plus ADP) or 600mM (all other additions) NH₄Cl. Assays were performed in 50mM imidazole buffer, pH 7.5 at 25°C.

Table 3 Kinetic Constants for the NADPH-linked Reaction of *Eurosta solidaginis* Glutamate Dehydrogenase:
Effect of Activators and Inhibitors

Addition	NH ₄ Cl		α -Ketoglutarate		NADPH	
	S _{0.5}	n	S _{0.5}	n	S _{0.5}	n
Control	61 ± 2.0	2.8 ± 0.1	0.56 ± 0.06	1.1 ± 0.1	0.060 ± 0.003	1.7 ± 0.2
ADP, 0.1mM	15 ± 1.0	1.4 ± 0.1	0.51 ± 0.03	1.1 ± 0.1	0.062 ± 0.003	1.3 ± 0.1
ATP, 2mM	12 ± 1.0	1.1 ± 0.2	0.54 ± 0.06	1.2 ± 0.02	0.063 ± 0.001	1.3 ± 0.2
GTP, 2μM	110 ± 8.0	7.8 ± 0.9	1.3 ± 0.1	1.3 ± 0.1	0.074 ± 0.003	1.8 ± 0.1
ADP, 0.1mM plus GTP, 2μM	19 ± 0.03	1.1 ± 0.2				
ATP, 2mM plus GTP, 2μM	11 ± 2.0	1.1 ± 0.01				

S_{0.5} values are in mM ± s.e.m. from determinations on at least 3 different enzyme preparations. Kinetic constants were derived from Hill plots using V_{max} measured at the following substrate concentrations: 0.20mM NADPH, 5mM α -ketoglutarate (7.5mM for GTP alone), and 100mM (ADP or ATP), 180mM (GTP) or 120mM (control + all others) NH₄Cl. Assays were performed in 50mM imidazole buffer pH 6.75 at 25°C.

for NADH is, however, about 2 fold lower than the affinity constant for NADPH. Ammonium ion kinetics are strongly sigmoidal in the presence of either coenzyme with values for n of about 3.

B. ADP effects: ADP activated both the NADH and the NADPH linked reactions. For the NADH reaction, maximal activation was 143% of control activity and occurred at 0.5 mM ADP. Maximal activation of the NADPH linked reaction was 138% at 0.05 mM ADP. ADP effects on enzyme velocity for the E. solidaginis enzyme are considerably less than those noted for the enzyme from other sources, activation by ADP being about 4 fold for bovine liver GDH (Goldin and Frieden, 1971) and as high as 100 fold for squid muscle GDH (Storey et al., 1978). Half maximal activation by ADP occurred at 0.1 and 0.01 mM ADP for the NADH and NADPH linked reactions, respectively. For the NADPH reaction, ADP activation occurred at levels well within the physiological concentration range of ADP in E. solidaginis (Storey et al., 1981b).

C. Modifier effects on substrate affinities: Table 2 shows the effects of metabolite modulators on substrate affinities of the NADH linked reaction. The activator, ADP, and the inhibitors, ATP and GTP, had no significant effects on the $S_{0.5}$ or n for either α -ketoglutarate or NADH. All three modulators strongly affected ammonium ion kinetics. The addition of 0.5 mM ADP decreased $S_{0.5}$ for NH_4^+ by 4 fold and reduced n by one-half (producing a more hyperbolic substrate saturation curve). The inhibitors, ATP and GTP, however, increased the $S_{0.5}$ for NH_4^+ by 2 fold (over control values) and also doubled the n value. ADP added along with either ATP or GTP produced a partial reversal of ATP and GTP inhibitions lowering both $S_{0.5}$ for NH_4^+ and n . The effects of the various modulators and combinations of modulators on the NH_4^+ ion kinetics

of the NADH-linked reaction are shown diagrammatically in the v versus s curves of Figure 8.

The $S_{0.5}$ for NH_4^+ was also affected by assay pH, decreasing as pH increased from 6.0 to 8.0 but then increasing as pH rose farther. $S_{0.5}$ for NH_4^+ was 170, 130, 90, 50, 40 and 60 mM at pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively for the NADH linked enzyme assayed in the presence of 0.5 mM ADP as shown in figure 9. A similar effect of pH on ammonium ion affinity was reported for the Drosophila larva enzyme by Bond and Sang (1968) but has not been reported for other GDH's. Monovalent cations had very little effect on the kinetics of GDH at physiological concentrations as can be seen in figure 10, with only a slight activation noted for low concentrations of lithium.

Metabolite modulator effects on the NADPH linked reaction of GDH were also primarily confined to effects on NH_4^+ kinetics (Table 3) although GTP inhibition of the enzyme increased $S_{0.5}$ for both α -ketoglutarate and NADPH as well. As for the NADH linked reaction, ADP decreased the $S_{0.5}$ for NH_4^+ of the NADPH reaction by 4 fold and reduced n by one-half. ATP effects on the NADPH linked reaction are opposite to those of the metabolite on the NADH reaction. ATP activates the NADPH reaction, decreasing $S_{0.5}$ by 5 fold and strongly reducing n to produce hyperbolic kinetics. The major effect of GTP inhibition on the NADPH linked reaction was a 2 fold increase in $S_{0.5}$ for NH_4^+ with a corresponding increase in n similar to the effects of GTP on the NADH linked enzyme. Unlike the NADH linked reaction, however, GTP inhibition of the enzyme was completely overridden by the addition of ADP and also by addition of ATP, $S_{0.5}$ for NH_4^+ being reduced to the level of that in the presence of ADP alone. The effect of the various modulators and

Fig. 8 Effect of modifiers on the $S_{0.5}$ of NH_4Cl for the NADH-linked activity of glutamate dehydrogenase of Eurosta solidaginis. Specific activity is expressed in $\mu\text{mol/min/mg}$ protein while concentration is in mM . Assay conditions are as outlined in table 2. Symbols are; (O), control; (●), ADP; (+), ATP; (◇), GTP; (□), ADP plus ATP.

Fig. 9 Effect of pH on the $S_{0.5}$ of NH_4Cl for glutamate dehydrogenase of Eurosta solidaginis in the NADH-linked reaction. $S_{0.5}$ values are the average of determinations on at least 3 different enzyme preparations and were derived from Hill plots with V_{max} measured at the following concentrations 25mM α -ketoglutarate, 0.15mM NADH and 0.5mM ADP and NH_4Cl being the substrate varied to obtain V_{max} .

Fig. 10 Effect of monovalent cation concentrations on the activity of glutamate dehydrogenase of Eurosta solidaginis. Specific activity is expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein under the optimal assay conditions for the NADH-linked activity; 300mM NH_4Cl , 0.15mM NADH, 25mM α -ketoglutarate, and 0.5mM ADP. Concentration is expressed in mM for NaCl ($\square$), KCl ($\bullet$), and LiCl ($\circ$).

combination of modulators on the NH_4^+ ion kinetics of the NADPH-linked reaction are shown diagrammatically in the v versus s curves of figure 11.

The forward reaction:

A. Substrate affinities and ADP effects: Activity of E. solidaginis GDH in the forward (glutamate oxidizing) direction is completely dependent upon the presence of ADP with no enzyme activity detected in the absence of this activator. Maximal enzyme activity occurred with the addition of 1.0 or 0.5 mM ADP for the NAD and NADP linked reactions, respectively. The kinetic constants for glutamate, NAD and NADP are shown in Tables 4 and 5. Unlike the reverse reaction, the NAD and NADP linked reactions showed similar kinetic constants for both glutamate and coenzyme. The $S_{0.5}$ for glutamate was 3.1 (for the NAD reaction) and 3.8 mM (for NADP) similar to the K_m for glutamate for the Drosophila larval enzyme and well within in vivo levels of glutamate in E. solidaginis (3 to 6 $\mu\text{mol/g}$ wet weight (Storey et al., 1981b)).

B. Effect of modifiers: The NAD linked reaction was inhibited by ATP and GTP, both of which decreased enzyme velocity by approximately 50% over control (ADP activated) activity. However, $S_{0.5}$ for both glutamate and NAD were also reduced in the presence of ATP or GTP (Table 4) showing that the inhibitors act in an uncompetitive manner. ATP produced a 3 fold decrease in $S_{0.5}$ for glutamate and a 7 fold decrease in $S_{0.5}$ for NAD. GTP inhibition had somewhat greater effects on $S_{0.5}$ for NAD, decreasing coenzyme affinity by 12 fold.

ATP and GTP had little effect on glutamate affinity of the NADP linked reaction (Table 5) but both decreased the $S_{0.5}$ for NADP by about 3 fold. Inhibition, again, was of the uncompetitive type.

Fig. 11 Effect of modifiers on the $S_{0.5}$ of NH_4Cl for the NADPH-linked activity of glutamate dehydrogenase of Eurosta solidaginis. Specific activity is expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein while concentration is in mM. Assay conditions are as outlined in table 3. Symbols are; (O), control; (●), ADP; (+), ATP; (□), GTP; (◇), ATP plus GTP.

Table 4 Kinetic Constants for the NAD-linked Reaction of Eurosta solidaginis Glutamate Dehydrogenase:
Effect of Modifiers

Addition	Glutamate		NAD	
	$S_{0.5}$	n	$S_{0.5}$	n
ADP, 1mM	3.1 ± 0.2	1.0 ± 0.03	0.22 ± 0.02	1.1 ± 0.1
ADP, 1mM plus ATP 0.1mM	0.90 ± 0.01	1.2 ± 0.2	0.031 ± 0.002	1.0 ± 0.1
ADP, 1mM plus GTP, 1.5uM	1.5 ± 0.1	1.6 ± 0.2	0.018 ± 0.001	1.1 ± 0.2

$S_{0.5}$ values are in mM $\pm$ s.e.m. and are the average of determinations on at least 3 different enzyme preparations. Kinetic constants were derived from Hill plots with V_{max} measured at the following substrate concentrations: ADP alone, 25mM glutamate, 2mM NAD; ADP + ATP, 12.5mM glutamate, 0.6mM NAD; and ADP + GTP, 20mM glutamate, 0.4mM NAD. Assays were performed in 50mM Tris-HCl buffer, pH 8.0 at 25°C.

Table 5 Kinetic Constants for the NADP-linked Reaction of Eurosta solidaginis Glutamate Dehydrogenase:
Effect of Modifiers

Addition	Glutamate		NADP	
	$S_{0.5}$	n	$S_{0.5}$	n
ADP, 0.5mM	3.8+ 0.3	1.4+ 0.1	0.20+ 0.02	1.2+ 0.04
ADP, 0.5mM plus ATP, 0.5mM	3.3+ 0.2	1.5+ 0.2	0.058+ 0.003	1.0+ 0.1
ADP, 0.5mM plus GTP, 4uM	2.9+ 0.4	1.4+ 0.3	0.061+ 0.013	1.1+ 0.02

$S_{0.5}$ values are in mM + s.e.m. and are the average of determinations on at least 3 different enzyme preparations. Kinetic constants were derived from Hill plots with V_{max} measured at the following substrate concentrations: ADP alone, 25mM glutamate, 2mM NADP, all others, 20mM glutamate, 1mM NADP. Assays were performed in 50mM Tris-HCl buffer, pH 8.0 at 25°C.

Inhibitor constants: Table 6 shows the inhibitor constants for ATP and GTP for the forward and reverse reactions of E. solidaginis GDH. In the reverse direction, the inhibitor constant for GTP was much lower for the NADPH linked activity than for the NADH linked activity suggesting stronger control by the guanylates on NADPH linked GDH activity. Reversal of GTP inhibition by ADP increased the I_{50} for GTP 2 fold for the NADH linked reaction and 7.5 fold for the NADPH linked reaction. GTP inhibition of the forward reaction was significantly more potent than that of the reverse reaction with I_{50} 's about 10 fold lower than the corresponding I_{50} 's for the reverse reaction.

ATP effects on the NAD and NADH reactions followed a similar pattern. For the NADH linked activity, the I_{50} for ATP (2.5 mM) is similar to intracellular ATP levels in E. solidaginis (Storey et al., 1981b) but in the presence of ADP, I_{50} increases significantly. The I_{50} for ATP in the forward direction is about 50 fold lower than the corresponding I_{50} for the reverse reaction indicating a much tighter control of the forward, glutamate-oxidizing, reaction by high cellular energy levels, a factor which may be very important in regulating the rates of the forward versus reverse reactions in vivo.

Temperature effects on GDH: Figure 12 (a & b) shows Arrhenius plots for E. solidaginis GDH. The plots are linear over the range 25° to 0°C. For both the NADH and NADPH reactions, in the absence of modifiers, calculated activation energy was 12,000 and 15,000 cal/mol respectively as seen in tables 7 and 8. The Q_{10} values are about 2. ADP, while activating enzyme activity, did not affect the slope of the Arrhenius plot. GTP, however, sharply increased the slope of the line suggesting stronger inhibition of GDH by GTP as

Table 6 Inhibitor Constants (I_{50}) for ATP and GTP for Eurosta solidaginis Glutamate Dehydrogenase

Addition	Coenzyme Linked Reaction				
	NADH 25°C	5°C	25°C	NADPH 5°C	NAD 25°C
ATP	2.5± 0.3	0.88± 0.15	-	-	n.a.
ATP + saturating ADP	6.2± 0.4	2.5± 0.1	-	-	0.11± 0.01
GTP	19± 1.0	4.4± 0.5	1.7± 0.2	0.81± 0.08	n.a.
GTP + saturating ADP	38± 0.4	9.9± 0.9	13± 1.0	27± 2.0	1.4± 0.1
					4.3± 0.8

I_{50} 's were determined by the method of Job et al. (1978) at the following substrate concentrations: for NADH: 0.15mM NADH, 450mM NH_4Cl (300mM in the presence of 0.5mM ADP), and 25mM (25°C) or 40mM (5°C) α -ketoglutarate; for NADPH: 0.2mM NADPH, 120mM NH_4Cl (100mM with 0.1mM ADP), and 5mM (25°C) or 15mM (5°C) α -ketoglutarate; for NAD: 2.0mM NAD, 25mM L-glutamate and 1mM ADP; for NADP: 2.0mM NADP, 25mM L-glutamate and 0.5mM ADP. Values are means ± s.e.m. for at least 3 determinations on separate preparations. n.a.- no activity in the absence of ADP.

Fig.12 Arrhenius plots for Eurosta solidaginis glutamate dehydrogenase. $1/T$ is expressed as $\times 10^{-3} (^{\circ}K)$.

Assays were measured under optimal conditions for (A) the NADH-linked and (B) the NADPH-linked reactions at $25^{\circ}C$ as outlined in tables 7 and 8. The temperature range for the Arrhenius plots was -5° to $25^{\circ}C$.

Symbols are: ($\bullet$), control(minus ADP); ($\square$), plus ADP; ($\circ$), plus GTP; and (+), plus ADP and GTP.

Table 7 Activation Energy from Arrhenius Plots for Glutamate Dehydrogenase of Eurosta solidaginis
in the NADH-linked Reaction

Condition	Slope Value (deg K)	Activation Energy (kcal/mol)
Control	-2700 $\pm$ 180	12
ADP (0.5mM)	-2300 $\pm$ 80	11
ATP (2.5mM)	-4700 $\pm$ 220	22
GTP (20uM)	-6000 $\pm$ 240	27
ADP (0.5mM) + ATP (6.0mM)	-2400 $\pm$ 220	11
ADP (0.5mM) + GTP (40uM)	-4800 $\pm$ 380	22
Sorbitol (250mM)	-2500 $\pm$ 280	11
Glycerol (500mM)	-2600 $\pm$ 380	12
Sorbitol (250mM) + Glycerol (500mM)	-2600 $\pm$ 190	12

Assays were measured under optimal conditions for the NADH-linked reaction at 25°C: 0.15mM NADH, 25 mM α -ketoglutarate, 450 mM (controls, polyols) 300 mM (ADP) or 600 mM (all other conditions) NH_4Cl in 50 mM imidazole buffer, pH 7.5. Slope values were derived from a plot of log V vs 1/K. Temp. range -5 to 25°C.

Table 8 Activation Energy from Arrhenius Plots for Glutamate Dehydrogenase of Eurosta solidaginis
in the NADPH-linked Reaction.

Condition	Slope Value (deg K)	Activation Energy (kcal/mol)
Control	-3200	15
ADP (0.1mM)	-2900+ 100	13
ATP (2.5mM)	-3500+ 120	16
GTP (2uM)	-4700+ 590	22
ADP (0.1mM) + ATP (6.0mM)	-3400+ 270	16
ADP (0.1mM) + GTP (10uM)	-2500+ 200	11
Sorbitol (250mM) + Glycerol (500mM)	-3000+ 180	14

Assays were measured under optimal conditions for the NADPH-linked reaction at 25°C: 0.20mM NADPH, 5 mM α-KG, (7.5 mM for GTP alone), and 100 mM (ADP or ATP), 180 mM (GTP) or 120 mM (control + all others) NH₄Cl in 50 mMimidazole buffer, pH 7.5. Slope values were derived from a plot of log V vs. 1/K from different preparations of enzyme. Temperature range -5 to 25°C.

temperature decreases. In the presence of both GTP and ADP, the slope of the Arrhenius plot returned to control levels for the NADPH reaction but was only partially reversed for the NADH reaction. The addition of glycerol (0.5 M) or sorbitol (0.25 M) or both, the polyols accumulated by *E. solidaginis* during overwintering, had no effect on the slope of the Arrhenius plot.

Temperature effects on GDH kinetic constants were compared at 25° and 5°C to determine whether temperature affected enzyme properties. $S_{0.5}$ for NADH and NADPH as well as for NH_4^+ (with either coenzyme and + ADP) were found to be the same at both temperatures. Enzyme affinity for α -ketoglutarate, however, decreased significantly at the lower temperature as indicated by the data in tables 9 and 10. For the NADH linked enzyme, $S_{0.5}$ for α -ketoglutarate increased from 2.7 mM at 25°C (Table 9) to 4.1 ± 0.3 mM at 5°C. $S_{0.5}$ for α -ketoglutarate for the NADPH linked enzyme also increased with decreasing temperature from 0.56 mM at 25°C (Table 10) to 1.5 ± 0.1 mM at 5°C. Enzyme affinity for α -ketoglutarate at either temperature was unaltered by the addition of ADP.

The major difference in GDH kinetics at 5° versus 25°C was in the effects of inhibitors, both ATP and GTP showing more potent inhibition of the enzyme at low temperature. Inhibitor constants for ATP and GTP at 5°C are shown in Table 6 along with those for 25°C. For ATP, I_{50} decreased about 3 fold at 5° compared to 25°C. I_{50} 's for GTP were also reduced with a greater reduction in I_{50} (4 fold) seen for GTP effects on the NADH linked enzyme than for the NADPH linked enzyme (2 fold). This decrease in I_{50} with decreasing temperature produces the effect of GTP on the Arrhenius plot seen in Figure 12; at constant GTP concentration, enzyme activity is

Table 9 Kinetic Constants for the NADH-linked Reaction of Eurosta solidaginis Glutamate Dehydrogenase:
Temperature Effect at 5°C

Condition	NH ₄ Cl		α -Ketoglutarate		NADH	
	S _{0.5}	n	S _{0.5}	n	S _{0.5}	n
Control	210 ± 6	2.7 ± 0.2	4.1 ± 0.3	1.3 ± 0.1	0.031 ± 0.002	1.6 ± 0.2
ADP, 0.5mM	60 ± 2	1.5 ± 0.1	3.9 ± 0.2	1.2 ± 0.1	0.033 ± 0.002	1.5 ± 0.2

S_{0.5} values are in mM + s.e.m. from determinations on at least 3 different enzyme preparations. Kinetic constants were determined from Hill plots using V_{max} measured at substrate concentrations: 0.15mM NADH, 40mM α -Ketoglutarate, and 450mM NH₄Cl(control), or 300mM NH₄Cl (ADP). Assays were performed in 50mM imidazole buffer, pH 7.5 at 5°C.

Table 10 Kinetic Constants for the NADPH-linked Reaction of Eurosta solidaginis Glutamate Dehydrogenase:
Temperature Effect at 5°C

Condition	NH ₄ Cl		α-Ketoglutarate		NADPH	
	S _{0.5}	n	S _{0.5}	n	S _{0.5}	n
Control	55± 7	2.3± 0.4	1.5± 0.1	1.2± 0.1	0.062± 0.008	1.3± 0.1
ADP, 0.1mM	17± 0.7	1.2± 0.1	1.5± 0.1	1.3± 0.1	0.061 ± 0.008	1.5± 0.2

S_{0.5} values are in mM± s.e.m. from determinations on at least 3 different enzyme preparations. Kinetic constants were determined from Hill plots using V_{max} measured at substrate concentrations: 0.20mM NADPH, 15mM α-Ketoglutarate, and 120mM NH₄Cl (control) or 120mM NH₄Cl (ADP). Assays were performed in 50mM imidazole buffer, pH 6.75 at 5°C.

inhibited to a greater percentage as temperature decreases. ADP raises the I_{50} for both GTP and ATP at both temperatures. The effect is an approximately 2 to 2.5 fold increase in I_{50} for NADH linked enzyme at 5°C, similar to the effect at 25°C. GTP/ADP interactions for the NADPH linked enzyme were differentially effected by temperature, however. At 25°C, ADP reversal of GTP inhibition resulted in a 7.5 fold rise in I_{50} to 13 μ M while at 5°C ADP effects raised the I_{50} for GTP to 27 μ M, a 34 fold increase. This effect could be important in allowing GDH function (and proline synthesis) at low temperatures.

DISCUSSION

Glutamate dehydrogenase from E. solidaginis larvae, like most animal GDH's, a) is a hexamer of molecular weight 350,000 daltons, b) occurs in a single form with no evidence for isozymes and no alterations of enzyme form during low temperature acclimation (Storey et al., 1981c), c) displays a dual nucleotide specificity utilizing both NAD(H) and NADP(H), and d) is a regulatory enzyme showing allosteric effects by the modulators, ADP, ATP and GTP. Some kinetic properties of insect larval GDH were previously studied by Bond and Sang (1968) using an unpurified and highly unstable ($t_{1/2}$ = 10 h) GDH preparation from Drosophila larvae. The present study, utilizing the purified and stable GDH preparation from E. solidaginis, provides a more complete kinetic analysis of the enzyme. The two enzymes share some common properties including a strong effect of pH on enzyme affinity for ammonium ion, similar activity ratios, NAD/NADP and NADH/NADPH, and similar enzyme affinities for substrates.

While strongly controlled by allosteric modulations, GDH from different sources can also be poised for differential function in

oxidative deamination (NH_4^+ excretion, oxidation of amino acids for energy production) or reductive animation (amino acid synthesis, nitrogen storage) through adjustments in the relative rates of the NAD(H) versus NADP(H) reactions (Smith et al., 1975) and the relative rates of the forward (NAD(P) utilizing) versus reverse (NAD(P)H utilizing) reactions. The activity ratio forward:reverse for E. solidaginis GDH was 1:10 for the NAD(H) reaction compared to 1:15 for bovine liver, 1:6 for insect flight muscle and 1:1 for squid mantle muscle GDH (Storey et al., 1978). GDH function in the latter two tissues is involved in glutamate (derived from proline) oxidation as a fuel for muscle work and appears to be poised for this function via a relatively high activity of the forward reaction compared to the reverse. Compared to the adult flight muscle GDH, larval GDH has an activity ratio favouring the reverse reaction, similar to that of bovine liver GDH. This perhaps suggests that the larval enzyme, is more strongly poised than the adult flight muscle enzyme for a role in glutamate synthesis than in glutamate oxidation. Our observations of the accumulation of proline, by de novo synthesis of the amino acid, during low temperatures acclimation of E. solidaginis, shows that a major function of E. solidaginis GDH is probably the synthesis of amino acids.

The relative rates of the NAD versus NADP and NADH versus NADPH reactions also vary among GDH from different sources. The activity ratio NADH/NADPH is 3.5 (at pH 6.75) for E. solidaginis GDH identical to that for the Drosophila larva enzyme (Bond and Sang, 1968). GDH from adult insect flight muscle, however, is highly NAD(H) specific (Bursell, 1975; Mills and Cochran, 1963), Bursell (1975) reporting an NADPH activity less than 2% of the NADH

activity. GDH from squid mantle muscle and from various lower vertebrate species also shows very low activity with NADPH but the bovine liver enzyme utilizes the two coenzymes at almost equal rates (Storey *et al.*, 1978; Smith *et al.*, 1975). In mammalian systems, the NADPH linked activity of GDH is believed to be associated with glutamate synthesis *in vivo*; studies with perfused liver or isolated liver cells suggest that the primary source of reducing power for the reductive amination of α -ketoglutarate, stimulated by ammonium ion, is NADPH (Sies *et al.*, 1974, 1975; Tischler *et al.*, 1977). Extending this analogy to insect larval versus adult flight muscle GDH, it could be suggested that the relatively high NADPH linked activity shown by larval GDH underlies a major role in amino acid synthesis for the larval enzyme, a function which appears to be NADPH linked.

The kinetic properties of the reverse reaction of *E. solidaginis* GDH also suggest that the NADPH linked reaction may be favoured *in vivo*. $S_{0.5}$ for α -ketoglutarate is 5 fold lower for the NADPH linked enzyme than for the NADH linked reaction and at 0.56 mM is perhaps within the physiological concentration range of this compound *in vivo*. $S_{0.5}$ for NH_4^+ is similarly 3.5 fold lower for the NADPH linked enzyme and, particularly for the ADP-activated NADPH linked enzyme, is probably within physiological ammonium ion concentrations. GDHs with dual nucleotide specificities probably preferentially utilize the NADPH linked function for glutamate biosynthesis. Enzymes which are highly specific for NAD(H), however, perhaps permit NADH linked glutamate synthesis through modifications lowering the affinity constants for α -ketoglutarate ($S_{0.5}$ for α -ketoglutarate for the NADH linked enzyme is 2.7, 0.8 and 0.35 mM for *E. solidaginis*, insect flight muscle and squid

mantle muscle GDH, respectively (Bursell, 1975; Storey et al., 1978)).

In the forward direction, enzyme affinities for glutamate and coenzyme were similar for both the NAD and NADP linked reactions. However, the activity ratio, NAD/NADP was higher at all pH's than the ratio NADH/NADPH for the reverse reaction. This may suggest that the forward reaction is preferentially NAD linked. Modifier effects on the forward reaction also suggest this result (see below). However, while enzyme kinetic properties may suggest a coenzyme preference by GDH, it must be remembered that coenzyme utilization by GDH in vivo is probably largely governed by the mitochondrial availability of NAD versus NADP or NADH versus NADPH while the direction of GDH function (forward versus reverse) is partially determined by mitochondrial redox ratios.

The effects of metabolite modulators on E. solidaginis GDH are similar to those reported for GDH from other sources: ADP activates the enzyme, GTP inhibits and ATP is an inhibitor of the NAD(H) linked reaction but an activator of the NADP(H) reaction. Like GDH from a variety of invertebrate sources (Bursell, 1975; Storey et al., 1978), enzyme activity in the forward direction is virtually undetectable in the absence of ADP. In the reverse direction, ADP activates enzyme activity but the effects of ADP on enzyme velocity for E. solidaginis GDH (about 140% activation at optimal substrate levels) are much lower than the effects of ADP on the insect flight muscle enzyme (a 20 fold activation) (Bursell, 1975) and more closely resemble the 4 fold activation by ADP reported for bovine liver GDH. ADP had strong effects on enzyme affinity constants, however. In the reverse direction, ADP produced a 4 fold decrease in $S_{0.5}$ for ammonium ion for both the NADH and NADPH. For the NADPH

linked reaction, this effect occurred not only at ADP levels (0.1 mM) which are physiological (Storey et al., 1981b) but also lowered enzyme affinity for NH_4^+ into a range which may be physiological.

GTP inhibition of GDH is extremely potent. I_{50} 's for the forward reaction occurred in the range 1 to 4 μM while I_{50} 's for the reverse reaction were about 10 fold higher. ATP inhibition of the NAD(H) linked reactions is similarly much stronger (I_{50} about 50 times lower) for the forward direction. Oxidation of glutamate (or other amino acids which are catabolized via glutamate) is, therefore, strongly controlled by cellular energy status, mobilization of amino acid stores as metabolic fuels occurring only in low energy states. Glutamate synthesis (for amino acid and protein production), however, should occur during high energy, growth states consistent with the lower inhibitory effects of GTP and ATP on the reverse reaction and the activating effects of ATP on NADPH linked glutamate synthesis.

Temperature change had minimal effects on E. solidaginis GDH. No abnormal effect of low temperature on enzyme activity was seen: Arrhenius plots were linear and Q_{10} was 2. Enzyme function in this cold hardy larva appears to be controlled and co-ordinated over a wide temperature range, a feature which is well suited for maintaining metabolic control during large daily and seasonal ambient temperature changes. The production of proline (a possible cryoprotectant) in the larvae during low temperature acclimation (between 15° and -5°C) (Storey et al., 1981b) may be favoured by low temperature alterations in ADP/GTP modifier interactions on E. solidaginis GDH. GTP inhibition of NADPH linked glutamate synthesis is much more strongly reversed by low (physiological) levels of ADP at low temperature (5°C) than at high temperature (25°C). This

effect could be responsible for increased synthesis of glutamate leading to proline accumulation as acclimation temperature decreases.

CHAPTER TWO
PURIFICATION AND PROPERTIES OF GLUTAMATE DEHYDROGENASE
FROM THE INTERTIDAL SEA ANEMONE, ANTHOPEURA XANTHOGRAMMICA

INTRODUCTION

One of the key adaptive properties of marine invertebrates is their ability to cope with changes in salinity of the surrounding seawater. Marine species fall into two classes of adaptation with respect to osmotic stress; osmoregulators and osmoconformers. Osmoconformers are osmotically dependent, such that their body fluid concentrations change with alterations in the salinity of the medium. Osmoregulators maintain their body fluids at a concentration different from that of the medium over part of their tolerance range (Livingstone et al., 1979). Osmoregulators are usually isotonic to the medium at high salinity, but hypertonic at low salinity.

Cell volume control can be established by the active regulation of various intracellular osmotic effectors, so that the intracellular fluid is in osmotic equilibrium with the extracellular fluid. The commonly studied intracellular osmolytes used by vertebrates are the inorganic ions Na^+ , K^+ , and Cl^- . The total tissue concentration of these inorganic ions in marine invertebrates is much lower than that of the surrounding extracellular fluid (Gilles, 1979). As a result, in marine invertebrates this deficit is compensated for by organic substances, such as small molecular weight nitrogenous compounds, chiefly free amino acids, and also betaine and trimethylamine oxide (Duchateau-Bosson and Florkin, 1961). Therefore, the intracellular osmotic pressure in marine invertebrates is balanced by both inorganic ions and organics. The involvement of organics during salinity stress helps to keep the inorganics at a level at which the membrane potential will remain stable. Also large amounts of free amino acids are not as harmful to enzyme function as large

concentrations of inorganics such as K^+ , Na^+ , or Cl^- (Bowlus and Somero, 1979).

Alterations in the external salinity are met by adjustments in the free amino acid pool. Hypoosmotic stress leads to a rapid deamination of amino acids and release of ammonium, whereas hyperosmotic stress leads to a rapid increase in the intracellular free amino acid pool (Gilles, 1979). The amino acids which are most often found to be involved in this increase in the free amino acid pool are alanine, proline, glycine and taurine.

There are several theories as to how the free amino acid pool increases during hyperosmotic stress, but none have been demonstrated conclusively (Allen, 1961; Florkin and Schoffeniels, 1969; Bedford and Anderson, 1972). One theory is that amino acids arise from protein catabolism, whereas a second theory favours the de novo synthesis of these amino acids. De novo synthesis would arise from the production of short chain keto acids from glycogen, followed by amination or transamination to produce amino acids. For a net increase in amino acids to occur there must be an ammonium fixing step. The ammonium fixation could be achieved via the purine nucleotide cycle or by the action of the enzyme alanine dehydrogenase ($pyruvate^- + NH_4^+ + H^+ + NADH \rightarrow alanine + NAD^+$), but as of yet neither of these pathways have been found to be significant in marine invertebrates. Another possibility is the coupling of the enzymes glutamine synthetase ($glutamate^- + NH_4^+ + ATP \rightarrow glutamine + ADP + Pi$) and glutamate synthase ($glutamine + \alpha\text{-ketoglutarate}^{2-} + H^+ + NADPH \rightarrow 2\ glutamate^- + NAD^+$), but this has only been found in microorganisms and plants to date (Miller and Stadtman, 1972). The combination of these two enzymes gives the same net reaction as glutamate dehydrogenase, which is also a possible nitrogen fixing

step. The role of GDH in osmoregulation is not supported in general, however, due to the very low GDH activities reported in most marine invertebrates (Sudgen and Newsholme, 1975; Addink and Veenhof, 1975). A role for GDH in osmoregulation has been suggested for some marine invertebrates containing significant amounts of GDH, such as the crab. It is believed that the metabolic adjustment to high salinity may be triggered by the modification in the intracellular inorganic ion content that occurs during osmotic stress. GDH from the crab is activated by these ions, but it is not clear as to whether it is the anions or the cations which are causing the effects (Schoffeniels, 1964; Chaplin et al., 1965).

GDH is present in substantial activity in sea anemones, but unlike other animal GDHs it is NADP dependent and apparently is not effected by the usual modulators, ADP, ATP and GTP (Hoffman et al., 1978). GDH from Anthorpleura xanthogrammica, a solitary intertidal species, was studied to look at the possible role of the enzyme in the regulation of amino acid pools during osmotic stress. Like the GDH from other anemones, this one was also found to be NADP specific and non-regulatory. In addition the enzyme was shown to have the capability of utilizing glutamine as an alternative substrate for ammonium ion. Activation of the enzyme by salts, resulted from alterations in the enzyme's kinetic parameters such as, the $S_{0.5}$ and V_{max} . Studies in which anemones were adapted to 100% vs 150% sea water, showed a 45% increase in inorganic ion content at high salinity and a 27% increase in the free amino acid pool, with the amino acids glutamate, alanine and glycine showing the major increase.

Materials and Methods

Animals and chemicals: Sea anemones, *A. xanthogrammica*, were obtained from Pacific Bio-Marine Laboratories Inc. All biochemicals were purchased from Boehringer Mannheim Corp., molecular weight marker proteins were from Sigma Chemical Company, Sepharose CL-6B was from Pharmacia Fine Chemicals, Affigel Blue was from Biorad Laboratories, and Matrix Red A was from Amicon Corp.

Purification of GDH: All buffers were adjusted to pH at 25°C and columns for purification and molecular weight data were run at room temperature (25°C). Sea anemone tissue (15 grams per preparation) including the basal foot muscle and the body wall muscle was homogenized in 2 vol. ice cold 20 mM Imidazole-HCl buffer pH 7.0 using a Polytron PT 10-35 homogenizer at full speed for 3 bursts of 10 sec. each. The homogenate was centrifuged at 27,000 g for 30 min at 4°C. The supernatant was removed and layered onto an Affigel Blue column (5 x 1.5 cm) equilibrated in 10 mM Imidazole-HCl pH 7.5. The column was washed with buffer until the A_{280} dropped to a minimum value (approx. 100 ml). GDH was eluted from the column with 25 mls of the equilibration buffer containing 30 mM glutamate and 0.4 mM NADP. Peak fractions were pooled and dialysed against 3 mM Imidazole HCl pH 7.5 twice for one hr. The dialysed sample was then layered onto a Matrix Red A column (5 x 1.5 cm) equilibrated in 10 mM Imidazole-HCl pH 7.5. The column was washed until A_{280} dropped to a minimum value (25 ml). GDH was eluted from the column using a 0 to 2 M gradient of KCl in buffer run at 40 mls/hr. Peak fractions were pooled and the final purified sample was stable in salt for at least 1 week. This purification scheme is essentially that of Walsh (1981) with minor modifications.

Molecular weight determination: Molecular weight

determinations on both crude and purified preparations were performed using a column (50 x 1.5 cm) of Sepharose CL-6B with 20 mM Imidazole-HCl buffer pH 7.0 containing 400 mM KCl as the equilibration/elution buffer. One ml fractions were collected and assayed for GDH activity. Molecular weight was determined from a plot of K_{av} versus $\log M_r$ for standard proteins: thyroglobulin ($M_r = 669,000$) ferritin ($M_r = 440,000$), catalase ($M_r = 232,000$) and aldolase ($M_r = 158,000$).

SDS polyacrylamide gel electrophoresis: Electrophoresis using 7.5% slab gels was carried out at 50 mA constant current for 4 hours at 4°C using Tris-glycine (46 mM/35 mM) pH 8.5 containing 1% SDS as the electrode buffer. Prior to running, protein samples were preincubated with 1% SDS at 80°C for 20 min. Proteins were detected using the silver stain of Switzer et al. (1979). Subunit molecular weight was determined from a plot of R_f versus molecular weight of protein standards, ribonuclease A (13,800) chymotrypsinogen (24,000), ovalbumin (45,000) and bovine liver glutamate dehydrogenase (54,000).

Protein assay: Protein concentration was measured by the method of Bradford (1976) using the prepared reagent from Bio-Rad Laboratories and bovine gamma globulin as the standard.

Enzyme assay and kinetics: GDH activity was monitored by following NADP(H) utilization at 340 nm using a Gilford recording spectrophotometer with water jacketed cell holder for temperature control. Optimal assay conditions (final volume = 1 ml) for GDH in the forward direction were: 50 mM Tris-HCl buffer, pH 8.5, 30 mM L-glutamate and 0.25 mM NADP. In the reverse direction, optimal conditions were: 50 mM imidazole-HCl buffer, pH 7.0, 300 mM ammonium acetate, 5 mM α -ketoglutarate and 0.10 mM NADPH for the

ammonium-linked activity and 50 mM Imidazole-HCl buffer pH 8.0, 100 mM L-glutamine, 5 mM α -ketoglutarate and 0.10 mM NADPH for the glutamine-linked activity. One unit (U) of enzyme activity is defined as the amount of enzyme utilizing 1 μ mol NADPH per min at 25°C, with ammonium acetate as substrate. Ammonium acetate was used rather than ammonium chloride to avoid the effects of chloride ion to be investigated later.

Kinetic constants, $S_{0.5}$ and n were determined from Hill Plots using experimentally measured V_{max} values determined at saturating substrate and cofactor concentrations.

Salinity stress experiments: Sea anemones all originally at 100% sea water were separated into two test groups. One group was placed in 100% sea water (968 mOsm), whereas the second group was placed in 1.5X sea water (1435 mOsm), for a period of two weeks. The tanks were aerated at 17-20°C. A third test group at 50% sea water did not survive the stress. After the two week period samples of sea anemone muscle were excised, blotted, frozen in liquid nitrogen and used for experiments. This means that both intracellular and extracellular (body fluids) liquids were utilized in our experimental protocol.

For amino acid analysis frozen tissue samples were ground to a powder under liquid nitrogen using a mortar and pestle. The resulting powder was homogenized in 3 volumes of ice cold sulphosalicylic acid (0.5% w-v) using a Tekmar tissuemizer. After centrifugation at 27,000 g for 20 minutes the protein precipitate was discarded and the supernatant was collected and analyzed for amino acids using a Beckman 119BL amino acid analyzer.

Frozen tissue samples for inorganic ion determinations were first dried in an oven at 65°C for 48 hours. The dried tissue was then charred to an ash in a muffle furnace at 550°C for 48 hours.

One ml of concentrated nitric acid was added to dissolve the ash and then this solution was brought up to 4 mls with water. Phosphate ions were measured using a Technicon Auto Analyzer II. Samples were diluted 50 fold and measured in mg% compared to known phosphate standards. Chloride ions were analyzed using a Buchler-Cotlove Chloridometer. The concentrated samples were measured in milliequivalents per litre. The cation concentrations of sodium, potassium, calcium, and magnesium were determined using a Jarrel-Ash Atomic Absorption-Flame Emission Spectrophotometer. Flame emission was carried out for sodium and potassium at 587 nm and 766 nm respectively, while atomic absorption was performed on calcium and magnesium at 423 nm and 285 nm respectively. The unknown samples were compared to standards measured in milliequivalents per litre. For potassium and magnesium analysis, samples were diluted 50 fold.

Results

Purification and physical properties: The maximal activity of GDH in A. xanthogrammica was 4.8 ± 0.3 units/g wet weight of muscle. The purification of the enzyme is summarized in Table 11. The final preparation was purified 430 fold to a final specific activity of 61.1 U/mg protein compared to specific activities of 40 U/mg for ox liver and brain GDH (McCarthy et al., 1980) and 43-50 U/mg for commercial bovine liver GDH from Sigma Chemical Co.. The elution profile for the Matrix Red A column is shown in Figure 13 (enzyme eluted at about 400 mM). The enzyme was judged to be pure by non-SDS and SDS polyacrylamide gel electrophoresis (Plates 1 and 2 - See expl.). The apparent MW for A. xanthogrammica GDH (Figure 14) was determined to be $325,000 \pm 5,000$ ($n = 5$) from a plot of $\bar{M}_{av}$ vs log MW. These plots for the sepharose gels tend to be slightly curved (See Pharmacia booklet 'Gel Filtration. Theory and

Table 11 Purification of Glutamate Dehydrogenase from the Sea Anemone, Anthopleura xanthogrammica

Step	Total Activity (units)	Total Protein (mg)	Yield	Purification	Specific Activity (units/mg protein)
Crude homogenate	72	507	-	✓	0.142
Affinity Chromatography (Affigel Blue)	36	1.73	50	146	20.8
Affinity Chromatography (Matrix Red A)	18	0.29	25	430	61.1

Activities were measured under the following conditions; 0.10mM NADPH, 5mM α -ketoglutarate, 300mM ammonium acetate, in 50mM imidazole buffer pH 7.0 at 25°C. Proteins were measured using the Bio-Rad protein assay. GDH was eluted from the blue column with a solution containing 30mM glutamate and 0.4mM NADP. GDH was eluted from the red column by a KCl gradient 0-2M.

Fig. 13 Purification of glutamate dehydrogenase from Anthopleura
xanthogrammica via affinity chromatography using a
Matrix Red A column eluted with a 0-2M KCl gradient.
A dialysed sample obtained from substrate elution of an
Affigel Blue affinity column was layered onto a Matrix
Red A column(5ml) equilibrated in 10mM imidazole-HCl
buffer pH 7.5 and eluted by KCl in the same buffer.
GDH activity ($\square$) is expressed as a percentage of the
peak tube activity, KCl gradient ($\bullet$) is expressed in mM
and the fraction size was 1.8 ml.

Plate 1 Polyacrylamide gel electrophoresis and SDS gel electrophoresis of purified glutamate dehydrogenase from Anthopleura xanthogrammica. Gels, PAGE (A), SDS (B), were run at a constant current of 40mA at 4°C for 4 hours with tris-glycine as the electrode buffer.

Gel Explanation: The photographs (Plates 1 and 2) show staining artifacts such as, surface staining and mock banding at the dye front both of which were occasionally observed. Plate 1 also shows a minor, fast migrating protein contaminant of purified GDH that only occurred in a small fraction of the purification procedures used. Normally gels of purified GDH showed no contaminants.

A

B

Plate 2 SDS gel electrophoresis of purified glutamate dehydrogenase from Eurosta solidaginis and Anthopleura xanthogrammica. This plate indicates the subunit molecular weight of the purified GDHs in relation to bovine liver GDH. The gel was run at constant current (40mA) for 4 hours at 4°C with tris-glycine as the electrode buffer. Eurosta GDH (A&B), Anthopleura GDH (C&D), bovine GDH (E), and molecular weight standards (F), (ovalbumin, chymotrypsinogen, and ribonuclease A).

A B C D E F

Fig. 14 Molecular weight determination of glutamate dehydrogenase from Anthopleura xanthogrammica by gel filtration with Sepharose CL-6B. Molecular weight determinations were performed using a column (50 x 1.5 cm.) of Sepharose CL-6B with 20mM imidazole-HCl buffer pH 7.0 as equilibration/ elution buffer. One ml fractions were collected and assayed for GDH activity while standard proteins were measured by A_{280} . Molecular weight was determined from a plot of K_{av} versus M_r for standard proteins; thyroglobulin ($M_r=669,000$) ferritin ($M_r= 440,000$) catalase ($M_r=232,000$) and aldolase ($M_r= 158,000$). $K_{av}=(V_e-V_o)/(V_t-V_o)$ where V_e is the elution volume of the solute, V_o is the void volume of the column and V_t is the total volume of the column.

Practice'). Two distinctly different subunit sizes of approximately equal concentration were detected by SDS gel electrophoresis corresponding to 46,000 and 49,000 (N = 5) as shown in Figure 15. The enzyme, like all other animal GDHs, was a hexamer, but unlike other animal GDHs, appeared to consist of two different subunits.

Substrate and coenzyme specificity: The enzyme was NADP(H) specific and did not utilize NAD in the forward direction or NADH in the reverse direction. In the reverse direction, the enzyme GDH could utilize glutamine as an alternative substrate for ammonium ion. With glutamine as substrate the enzyme was also NADPH specific.

pH optima and activity ratios: Figure 16 shows pH profiles for A. xanthogrammica GDH. In the reverse direction the enzyme has pH optima of 7.0 and 8.0 for the NH_4^+ -linked and glutamine-linked activities respectively, while in the forward direction the optimum is 8.5 with glutamate as substrate. In the reverse direction the activity ratio, $\text{NH}_4^+/\text{glutamine}$, was 11 at pH 7.0 but dropped to 2 at pH 8.0, the optimum for the glutamine-linked activity. The ratio of enzyme activity in the forward versus reverse direction (at the respective pH optima) was approximately 1:10 for the glutamate versus ammonium-linked activities.

The reverse reaction: A. Substrate affinities: Tables 12 and 13 show the substrate affinities ($S_{0.5}$) and Hill coefficients (n) for A. xanthogrammica GDH for the ammonium and glutamine-linked activities respectively. Enzyme affinity for α -ketoglutarate is higher ($S_{0.5}$ 2 fold lower) for the ammonium-linked reaction than the glutamine-linked reaction. α -ketoglutarate kinetics are

Fig. 15 Subunit molecular weight determination of glutamate dehydrogenase from Anthopleura xanthogrammica by SDS gel electrophoresis. Electrophoresis using 7.5% acrylamide and 1% SDS slab gels was carried out at 40mA constant current for 4 hours at 4°C. using Tris-glycine (46mM/35mM) pH 8.5 containing 1% SDS as the electrode buffer. Samples were incubated in 1% sds at 80°C for 20 minutes. Protein was detected by silver staining. Subunit molecular weight was determined from a plot of R_f versus molecular weight for the protein standards; ribonuclease A (13,800), chymotrypsinogen (24,000), ovalbumin (45,000), and bovine liver glutamate dehydrogenase (54,000). $R_f = (\text{distance protein migrates}) / (\text{distance to bromothymol blue dye front})$. Molecular weight is expressed in thousands. Solid line is drawn by the method of least squares.

marine invertebrates subjected to hyperosmotic stress, from virtually no changes to the case of the bivalve Gryphea angulata which shows a dramatic increase in the inorganic ion contribution to the osmolarity (214 to 517 mOsm) (Schoffeniels and Gilles, 1972). A. xanthogrammica inorganics increased as salinity rose with large changes in sodium ion, and chloride ion, but virtually no change in potassium ion. Volume readjustment after shrinkage usually results in an increase in the permeability to sodium ions much larger than that of potassium ions (Schmidt-Nielson, 1975), which was also observed for the sea anemone.

These increases in intracellular salt ions are immediate and serve as the simplest and most important signal for the need to adjust the amino acid pool to meet the hyperosmotic stress. This signal would directly activate glutamate dehydrogenase. In other cells glutamate dehydrogenase regulation via an environmental stress is thought to be mediated by allosteric effectors. The possibility of this direct "salt signal" may explain the lack of effect on the enzyme of the usual allosteric modulators. In any case, the fact that the product of the glutamate dehydrogenase reaction, glutamate, increases greatly during hyperosmotic stress indicates the important role played by GDH in the de novo synthesis of the amino acids in vivo. Alanine also increased substantially and this increase likely occurs through the coupling of GDH with the enzyme glutamate-pyruvate transaminase (glutamate + pyruvate $\rightarrow$ α -ketoglutarate + alanine). Ammonium ion, also necessary for the de novo synthesis of these amino acids, increases upon increasing salinity, and this change is thought to be achieved by ammonium uptake from the medium.

In summary, the glutamate dehydrogenase purified from A.

Fig. 16 pH profiles of glutamate dehydrogenase of Anthopleura xanthogrammica with respect to substrates utilized. Specific activity is expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. Optimal assay conditions for GDH were: (A) 50mM imidazole-HCl buffer pH 7.0, 300mM ammonium acetate, 5mM α -ketoglutarate and 0.1mM NADPH for the ammonium-linked activity in the reverse direction ($\square$); 50mM imidazole-HCl buffer pH 8.0, 100mM glutamine, 5mM α -ketoglutarate, and 0.1mM NADPH for the glutamine-linked activity ($\bullet$) in the reverse direction; (B) 50mM Tris-HCl buffer, pH 8.5 30mM glutamate and 0.25mM NADP the forward direction ($\circ$).

Table 12 Kinetic Constants for the Ammonium-linked Reverse Reaction of Anthopleura xanthogrammica

Glutamate Dehydrogenase: Effect of Salts

Addition	Ammonium Acetate		α -Ketoglutarate		NADPH	
	$S_{0.5}$	n	$S_{0.5}$	n	$S_{0.5}$	n
Control	61 $\pm$ 4	1.2 $\pm$ 0.2	0.59 $\pm$ 0.06	1.3 $\pm$ 0.04	0.0091 $\pm$ 0.0011	0.9 $\pm$ 0.1
KCl, 150mM	17 $\pm$ 2	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1		
NaCl, 150mM	18 $\pm$ 2	1.4 $\pm$ 0.1	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1		
K ₂ SO ₄ , 150mM	11 $\pm$ 2	1.1 $\pm$ 0.1	1.4 $\pm$ 0.2	1.2 $\pm$ 0.1		
Phosphate, 150mM	21 $\pm$ 1	1.0 $\pm$ 0.1	0.81 $\pm$ 0.10	1.2 $\pm$ 0.0		

$S_{0.5}$ values are in mM $\pm$ s.e.m., from determinations on at least 3 different enzyme preparations. Kinetic constants were derived from Hill plots using V_{max} measured at substrate concentrations: 5mM α -ketoglutarate, 0.10mM NADPH, 300mM Ammonium Acetate (control), and 10mM α -ketoglutarate, 0.10mM NADPH and 300mM Ammonium Acetate (salts). Assays were performed in 50mM imidazole buffer, pH 7.0 at 25°C. Phosphate was prepared from equimolar amounts of K₂HPO₄ and KH₂PO₄ and pH to 7.0.

Table 13 Kinetic Constants for the Glutamine-linked Reverse Reaction of Anthopleura xanthogrammica

Glutamate Dehydrogenase: Effect of Salts

Addition	L-Glutamine		α -ketoglutarate		NADPH	
	$S_{0.5}$	n	$S_{0.5}$	n	$S_{0.5}$	n
Control	5.1 ± 1.0	0.8 ± 0.1	1.2 ± 0.2	5.3 ± 0.6	0.0027 ± 0.0004	1.1 ± 0.1
KCl, 150mM	5.6 ± 1.1	1.0 ± 0.1	3.0 ± 0.5	3.3 ± 0.3		
NaCl, 150mM	5.5 ± 1.1	1.0 ± 0.1	3.2 ± 0.7	3.5 ± 0.4		
K ₂ SO ₄ , 150mM	7.3 ± 0.3	0.9 ± 0.1	4.2 ± 0.7	3.5 ± 0.4		
Phosphate, 150mM	6.5 ± 1.2	1.0 ± 0.04	1.6 ± 0.2	2.3 ± 0.3		

$S_{0.5}$ values are in mM $\pm$ s.e.m., from determinations on at least 3 different enzyme preparations. Kinetic constants were derived from Hill plots using V_{max} measured at substrate concentrations: 5mM α -ketoglutarate, 0.10mM NADPH, 100mM glutamine (control), and 10mM α -ketoglutarate, 0.10mM NADPH, and 100mM glutamine(salts). Assays were performed in 50mM imidazole buffer, pH 8.0 at 25°C. Phosphate was prepared from equimolar amounts of K₂HPO₄ and KH₂PO₄ and pH to 8.0.

strongly sigmoidal in the presence of glutamine with a value for n of 5 while α -ketoglutarate kinetics are hyperbolic in the presence of ammonium ion. The $S_{0.5}$ for NADPH is about 3 fold higher for the ammonium-linked reaction compared to the glutamine-linked activity. Enzyme affinity for glutamine was significantly higher ($S_{0.5}$ 10 fold lower) than for ammonium ion.

B. Salt effects: All of the four salts tested had similar effects on the ammonium-linked activity. Salt lowered the $S_{0.5}$ of ammonium ion three fold to about 20 mM for KCl, NaCl and phosphate and six fold to 10 mM for K_2SO_4 . The $S_{0.5}$ for α -ketoglutarate on the other hand, was increased with the addition of salt by about two fold to 1.3 mM in the case of KCl, NaCl and K_2SO_4 . The $S_{0.5}$ for α -ketoglutarate in the presence of phosphate was intermediate between the other salts and the control value. Salt did not effect the $S_{0.5}$ for NADPH. Similar effects resulted when salts were added to the glutamine-linked assay mixture. As in the case of the ammonium-linked activity salts did not alter the $S_{0.5}$ of NADPH. Once again salt increased the $S_{0.5}$ for α -ketoglutarate about 3 fold to 3-4 mM for KCl, NaCl, and K_2SO_4 while the $S_{0.5}$ for α -ketoglutarate in the presence of phosphate was slightly greater than the control value. Salts appear to reduce the sigmoidal nature of the α -ketoglutarate kinetics as the n value drops from 5 to about 2 or 3. Salt had little effect on the $S_{0.5}$ of the substrate glutamine unlike that of ammonium ion. $S_{0.5}$'s for glutamine in the presence of monovalent salts KCl and NaCl were similar to the control value while the $S_{0.5}$ of glutamine increased 1.4 fold in the presence of the polyvalent salts potassium phosphate and potassium sulphate. Salts had differential effects on the V_{max} values for the reverse direction depending on whether ammonium ion

or glutamine was the substrate as can be noted in Table 14. The activity is almost doubled in the presence of salt when glutamine is the substrate, however when ammonium ion is the substrate salts do not effect the $V'_{\max}$ appreciably.

The Forward reaction: Table 15 shows the substrate affinities ($S_{0.5}$) and Hill coefficients (n) for *A. xanthogrammica* GDH for the forward reaction, under various conditions. Both the substrate glutamate and the cofactor NADP exhibit hyperbolic kinetics as indicated by the n values of approximately 1.0. With the addition of salts the $S_{0.5}$ of NADP remained virtually unchanged for the monovalent salts KCl and NaCl, however the value increased 3-4 fold in the presence of the polyvalent salts potassium phosphate and potassium sulphate. The $S_{0.5}$ values for glutamate increased with the addition of KCl, NaCl, and K_2SO_4 but this increase was less than 2 fold in all cases. In the presence of phosphate the $S_{0.5}$ for glutamate remains unchanged from the control value. As can be seen from Table 14 the addition of salts increased the $V_{\max}$ values from 50-80% for the forward reaction.

Modifier effects: Allosteric effectors, tested over the concentration range shown in brackets, including leucine (1-50 mM), ADP (0.1-10 mM), ATP (0.1-10 mM) and GTP (1 μ M-1 mM) had no effect on the enzyme activity in either the reverse (glutamine or ammonium ion as substrate) or forward direction.

Salinity stress experiments: Hyperosmotic stress did not alter GDH activity in the sea anemones. Enzyme activity in the control group was 4.3 ± 0.3 ($n = 3$) units per gram wet weight while the activity for the 1.5X sea water group was 4.5 ± 1.0 ($n = 3$) units per gram wet weight.

Acclimation to high salinity increased the free amino acid

Table 14 Effect of Salts on the $V_{\max}$ Values of the Forward and the Reverse-linked Reactions of Anthopleura xanthogrammica Glutamate Dehydrogenase

Salt	Relative Activity	
	Reverse	Forward
	L-Glutamine-linked	L-Glutamate-linked
Control	100	100
KCl, 150mM	190+ 8	115+ 4
NaCl, 150mM	203+ 10	116+ 7
K ₂ SO ₄ , 150mM	190+ 12	100+ 10
Phosphate, 150mM	172+ 8	108+ 2
		155+ 5
		158+ 8
		179+ 6
		149+ 5

Assays for the glutamine-linked reaction were at substrate concentrations: 0.10mM NADPH, 100mM glutamine and 10mM α -ketoglutarate. For the ammonium-linked reaction the assay conditions were: 0.10mM NADPH, 300mM ammonium acetate, and 10mM α -ketoglutarate. For the glutamate-linked reaction the following assay conditions were used: 50mM glutamate and 0.25mM NADP. The assays were performed in 50mM imidazole pH 7.0 (ammonium), 50mM imidazole pH 8.0 (glutamine) and 50mM Tris pH 8.5 (glutamate) at 25°C. Phosphate was prepared from equimolar amounts of K₂HPO₄ and KH₂PO₄ and pH according to the pH optimum of the reaction.

Table 15 Kinetic Constants for the Glutamate-linked Forward Reaction of Anthopleura xanthogrammica

Glutamate Dehydrogenase: Effect of Salts

Addition	L-Glutamate		NADP	
	$S_{0.5}$	n	$S_{0.5}$	n
Control.	4.7+ 0.5	1.0+ 0.1	4.6+ 0.3	1.1+ 0.1
KCl, 150mM	6.8+ 0.3	1.1+ 0.1	5.2+ 0.7	0.9+ 0.03
NaCl, 150mM	7.1+ 0.4	1.1+ 0.1	5.8+ 0.8	1.1+ 0.03
K ₂ SO ₄ , 150mM	8.5+ 0.6	1.1+ 0.1	15.5+ 1.4	1.0+ 0.1
Phosphate, 150mM	5.1+ 0.1	1.1+ 0.03	12.3+ 1.0	1.3+ 1

$S_{0.5}$ values are in mM $\pm$ s.e.m. for glutamate and μ M $\pm$ s.e.m. for NADP, from determinations on at least 3 different enzyme preparations. Kinetic constants were derived from Hill plots using V_{max} measured at substrate concentrations: 30mM glutamate; and 0.25mM NADP (control) and 50mM glutamate and 0.25mM NADP (salts). Assays were performed in 50mM Tris-HCl buffer, pH 8.5 at 25°C. Phosphate was prepared from equimolar amounts of K₂HPO₄ and KH₂PO₄ and pH to 8.5.

pool in the animals. Total pool conc. increased by 27 percent, from 108 (at 100% sea water) to 137 (at 1.5X sea water) $\mu\text{mol/g}$ wet weight (Table 16). At both salinities taurine was the major amino acid component accounting for 90% and 80% of the total pool at 100% versus 1.5X sea water respectively. Taurine content increased by 10% upon adaptation to the higher salinity. Content of most amino acids increased at the higher salinity, in particular levels of glutamate, glycine and alanine (the major amino acids aside from taurine) increased by 6.1, 3.8, and 7.6 fold respectively.

Table 17 shows the effect of increasing the salinity from 100 to 1.5X sea water on the tissue inorganic ion concentrations. Pool conc. of the ions measured increased from 466 to 677 $\mu\text{mol/g}$ wet weight, a 45% increase. All ions tested, with the exception of potassium and phosphate, increased significantly. The increases for chloride, sodium and magnesium were 2.2, 1.7, and 1.6 fold respectively.

Discussion

Glutamate dehydrogenase from A. xanthogrammica, like most GDHs was a hexamer of molecular weight 325,000 daltons. However unlike other animal GDHs which show dual nucleotide specificity, the A. xanthogrammica enzyme was specific for NADP. GDH from A. xanthogrammica was not regulated by the effectors ADP, ATP, and GTP, whereas other animal GDHs show allosteric effects by these modulators. GDHs of sea anemones previously studied also show this coenzyme specificity and lack of modulator effects (Walsh, 1981; Bishop et al., 1978). GDH from A. xanthogrammica was found to utilize glutamine as an alternative substrate for ammonium ion in the reverse direction, and this activity may have a physiological function in amino acid biosynthesis in vivo. GDH from A.

Table 16 Amino Acid Concentrations in the Tissues of the
Sea Anemone, Anthopleura xanthogrammica adapted
to 100% and 1.5X Sea Water

Amino Acid	Sea Water Concentration	
	100%	1.5X
Taurine	100 $\pm$ 8.7	110 $\pm$ 4.1
Aspartate	0.20 $\pm$ 0.02	0.34 $\pm$ 0.12
Hydroxyproline	0.36 $\pm$ 0.06	0.46 $\pm$ 0.09
Threonine	0.32 $\pm$ 0.06	0.56 $\pm$ 0.13
Serine	0.28 $\pm$ 0.04	0.37 $\pm$ 0.08
Glutamate	0.77 $\pm$ 0.19	4.6 $\pm$ 0.77
Glutamine	0.09 $\pm$ 0.01	0.12 $\pm$ 0.01
α -NH ₂ adipic	0.16 $\pm$ 0.05	0.35 $\pm$ 0.02
Proline	0.17 $\pm$ 0.04	0.49 $\pm$ 0.15
Glycine	1.5 $\pm$ 0.41	5.7 $\pm$ 1.3
Alanine	0.82 $\pm$ 0.14	6.2 $\pm$ 1.4
Valine	0.33 $\pm$ 0.06	0.38 $\pm$ 0.07
Cysteine	0.22 $\pm$ 0.05	0.23 $\pm$ 0.02
Methionine	0.07 $\pm$ 0.01	0.14 $\pm$ 0.02
Cystathione	0.05 $\pm$ 0.01	0.15 $\pm$ 0.03
Isoleucine	0.12 $\pm$ 0.03	0.16 $\pm$ 0.02
Leucine	0.08 $\pm$ 0.02	0.20 $\pm$ 0.04
Tyrosine	0.17 $\pm$ 0.04	0.49 $\pm$ 0.08
Phenylalanine	0.04 $\pm$ 0.01	0.14 $\pm$ 0.05

continued .....

Table 16 (Cont.) Amino Acid Concentrations in the Tissues
of the Sea Anemone, Anthopleura xanthogrammica
adapted to 100% and 1.5X Sea Water

Amino Acid	Sea Water Concentration	
	100%	1.5X
Ammonium	0.91 $\pm$ 0.09	3.5 $\pm$ 0.36
Ornithine	0.03 $\pm$ 0.01	0.07 $\pm$ 0.01
Lysine	0.23 $\pm$ 0.05	0.86 $\pm$ 0.19
1-Methyl Histidine	0.07 $\pm$ 0.01	0.04 $\pm$ 0.01
Histidine	0.07 $\pm$ 0.03	0.08 $\pm$ 0.01
Arginine	0.70 $\pm$ 0.15	0.86 $\pm$ 0.14
Total	108	137

Concentrations are in $\mu\text{mol/g}$ wet weight $\pm$ s.e.m. from determinations on at least $n=3$ samples.

Table 17 Inorganic Ion Concentrations in the Tissues of
the Sea Anemone, Anthopleura xanthogrammica
adapted to 100% and 1.5X Sea Water

Inorganic Ion	Sea Water Concentration	
	100%	1.5X
Phosphate	37 $\pm$ 2	32 $\pm$ 1
Chloride	17 $\pm$ 3	38 $\pm$ 2
Sodium	180 $\pm$ 4	310 $\pm$ 6
Potassium	160 $\pm$ 8	180 $\pm$ 6
Calcium	9.9 $\pm$ 0.4	14.5 $\pm$ 0.8
Magnesium	64 $\pm$ 2	101 $\pm$ 9
Total	466	677

Concentration are in $\mu\text{mol/g}$ wet weight $\pm$ s.e.m. from determinations on n= 3 samples.

xanthogrammica appeared to contain two different molecular weight subunit forms unlike other GDHs which all have six identical subunits.

GDH from different organisms can be poised for function in either reductive amination or oxidative deamination (oxidation of amino acids for energy production) through adjustments in the relative rates of the forward versus reverse reactions. The activity ratios forward:reverse are 1:15 for bovine liver (Smith et al., 1975), 1:10 for E. solidaginis (Chapter 1), 1:6 for insect flight muscle (Bursell, 1975) and 1:1 for squid mantle muscle GDH (Storey et al., 1978). In the latter two tissues GDH appears to be primarily involved in glutamate oxidation hence the relatively high activity of the forward reaction, while the former two tissues have activity ratios favouring the reverse reaction suggesting a primary role in glutamate synthesis. The ratio for A. xanthogrammica GDH was 1:10 suggesting that it too has a primary function in glutamate synthesis and nitrogen storage.

In the reverse direction the enzyme was capable of utilizing glutamine as an alternative substrate to ammonium ion. Purification of GDH to homogeneity showed that this activity could not be attributed to a separate enzyme, glutamate synthase. (found in bacteria (Miller and Stadtman, 1972)), but was rather the result of a GDH capable of utilizing glutamine. This property has been shown for dual specific GDHs such as the bovine liver enzyme where the glutamine activity is up to 40% of the ammonium activity (Smith et al., 1975). Like the bovine enzyme, the sea anemone GDH has a more basic pH optimum with glutamine as substrate compared to ammonium ion. The activity ratio ammonium ion/glutamine was 11:1 at pH 7.0, but dropped to 2:1 at pH 8.0, the pH optimum when glutamine was

utilized, thus it is possible that substrate utilization of this GDH may be linked to intracellular pH fluctuations. A possible function of this glutamine linked GDH activity is as an alternative means for the net incorporation of ammonium ions into amino acids. When linked with the enzyme glutamine synthetase the net reaction is the fixation of ammonium:

For the bacterial system in which the K_m for ammonium ion with glutamine synthetase and glutamate dehydrogenase is 0.2 mM and 3 mM respectively, this alternative pathway to GDH has been shown to occur since ammonium ion substrate affinity is 10 fold higher (Miller and Stadtman, 1972). It is possible that under certain in vivo conditions this alternative pathway could be better suited for glutamate production in sea anemones as well.

In the absence of salts the affinity for the substrates, either ammonium ion or glutamine, are very low with $S_{0.5}$ values 10 fold higher than the in vivo concentrations as given in Table 16. Although the glutamine $S_{0.5}$ value was 10 fold lower than the $S_{0.5}$ for ammonium ion, its concentration in vivo was also 10 fold lower. α -Ketoglutarate kinetics are sigmoidal when glutamine was the substrate, whereas they were hyperbolic if ammonium ion was used, and this difference could arise from steric hindrance caused by the

bulkier glutamine substrate at the active site.

A. xanthogrammica GDH was not regulated by the allosteric effectors, ADP, ATP, and GTP normally associated with GDH function, but rather control seemed to be linked to salt ions. Anions and cations have little effect on many animal GDHs as was evident in the case of E. solidaginis GDH, however effects have been shown for some marine invertebrates. Although it is not completely clear as to whether the anions or cations are responsible for GDH activation observed in these marine invertebrates, the evidence seems to be weighted in favour of the anions (Schoffeniels, 1964; Chaplin et al., 1965). For A. xanthogrammica GDH it was found that salt ions highly activated the enzyme. In the case of the glutamine linked activity the $V_{\max}$ activity was increased, while for the ammonium linked reaction $S_{0.5}$ of ammonium ion was decreased. In the forward direction salts may have an inhibitory action, since the substrate affinities for both NADP and glutamate decrease even though $V_{\max}$ increases. Substrate affinities are likely to be more important than $V_{\max}$ activities for in vivo considerations. The data on the effects of salts on A. xanthogrammica GDH seem to indicate that it may well be the anions which cause the activation of GDH. The salts KCl and NaCl which have the same anions but different cations gave almost identical results. However, when the salts having same cations but different anions are compared, the results on GDH kinetics are noticeably different. In addition, the substitution of ammonium chloride for ammonium acetate in the assay mixture was found to cause an activation of the enzyme again indicating that it is likely that different anions affect GDH activity differentially.

Many marine invertebrates have been shown to contain considerable amounts of non-essential amino acids such as alanine,

glycine, glutamate, aspartate, proline and taurine which can all play a role in intracellular volume regulation (Severin et al., 1972). In A. xanthogrammica, taurine accounts for over 90% of the total free amino acid pool with glutamate, alanine and glycine representing the majority of the rest of the pool. A similar amino acid pool make up was found for another sea anemone, Metridium sinely, with taurine, alanine, glycine and glutamate representing 61, 5, 3 and 7% of the total pool by weight respectively (Severin et al., 1972). The size of the total free amino acid pool in coelenterates is about 20% of the pool conc. of many other marine invertebrates such as the gastropods and bivalves (300 mg% for sea anemones vs 1500 mg% for bivalves) (Severin et al., 1972). In most marine animals the organic component can contribute up to 60% of the osmolarity with inorganic ions taking up the remainder. However, coelenterates differ with a much smaller free amino acid pool similar to that of fresh water animals. In this study the organic composition accounted for only about 15-20% of the total osmotically active substances at both 100% and 1.5X salinity (see Tables 16 and 17).

Increasing the salinity of the external environment 50%, from 100% to 1.5X sea water, resulted in a 27% increase in the free amino acid pool and a 45% increase in the inorganic ion pool of the sea anemone. This represents a 41% increase in the total osmotically active substances, therefore it would appear as if the sea anemones are osmoregulating rather than osmoconforming. Rapid increases in the intracellular salts will stimulate glutamate dehydrogenase which probably plays a key role in the de novo synthesis of amino acids seen during hyperosmotic stress. The change in the intracellular inorganic pool varies drastically for

marine invertebrates subjected to hyperosmotic stress, from virtually no changes to the case of the bivalve Gryphea angulata which shows a dramatic increase in the inorganic ion contribution to the osmolarity (214 to 517 mOsm) (Schoffeniels and Gilles, 1972). A. xanthogrammica inorganics increased as salinity rose with large changes in sodium ion, and chloride ion, but virtually no change in potassium ion. Volume readjustment after shrinkage usually results in an increase in the permeability to sodium ions much larger than that of potassium ions (Schmidt-Nielson, 1975), which was also observed for the sea anemone.

These increases in intracellular salt ions are immediate and serve as the simplest and most important signal for the need to adjust the amino acid pool to meet the hyperosmotic stress. This signal would directly activate glutamate dehydrogenase. In other cells glutamate dehydrogenase regulation via an environmental stress is thought to be mediated by allosteric effectors. The possibility of this direct "salt signal" may explain the lack of effect on the enzyme of the usual allosteric modulators. In any case, the fact that the product of the glutamate dehydrogenase reaction, glutamate, increases greatly during hyperosmotic stress indicates the important role played by GDH in the de novo synthesis of the amino acids in vivo. Alanine also increased substantially and this increase likely occurs through the coupling of GDH with the enzyme glutamate-pyruvate transaminase (glutamate + pyruvate $\rightarrow$ α -ketoglutarate + alanine). Ammonium ion, also necessary for the de novo synthesis of these amino acids, increases upon increasing salinity, and this change is thought to be achieved by ammonium uptake from the medium.

In summary, the glutamate dehydrogenase purified from A.

xanthogrammica is NADP specific and appears to be involved in the de novo synthesis of amino acids, particularly glutamate and alanine which arises when the sea anemones are subjected to hyperosmotic stress. This function is enhanced by activation of GDH by salt ions.

CHAPTER THREE

REGULATION OF COENZYME UTILIZATION BY BOVINE LIVER

GLUTAMATE DEHYDROGENASE: INVESTIGATIONS USING

THIONICOTINAMIDE ANALOGUES OF NAD AND NADP IN A

DUAL WAVELENGTH ASSAY

INTRODUCTION

Bovine liver glutamate dehydrogenase, like many other mammal GDHs reacts in vitro with either nicotinamide nucleotide, NAD(H) or NADP(H), with comparable facility. A number of studies have used indirect means to suggest a coenzyme preference for GDH function in vivo. Studies using perfused liver or isolated liver cells suggest that the primary source of reducing power for the reductive amination of α -ketoglutarate, stimulated by high ammonium concentrations, is NADPH (Sies et.al., 1974; Tischler et.al., 1977). Equivalent experiments, probing the coenzyme preference for the reverse direction, oxidative deamination of glutamate, have proven too difficult to date due to the problem of interferences by such enzymes as glutamine synthetase and the glutamate utilizing transaminases.

Inferences about the coenzyme preference of bovine liver GDH have been drawn from the nucleotide specific GDHs of non-animal sources. NAD-specific GDHs are primarily involved in the oxidation of exogenous glutamate, while NADP-specific enzymes are involved in the biosynthesis of glutamate and can be induced in the presence of NH_4^+ (Smith et.al., 1975). Thus the circumstantial evidence would suggest that the forward, glutamate oxidizing, reaction of bovine liver GDH may be preferentially NAD linked while the reverse direction is preferentially NADPH linked. Such coenzyme preferences could have important implications for the regulation of GDH in vivo where, of course, both coenzymes coexist. More concrete evidence is needed in order to determine these preferences especially in the direction of oxidative deamination of glutamate.

The nature of the spectrophotometric assay prevents the investigation of coenzyme preference NAD versus NADP, when the

enzyme is faced with both nucleotides simultaneously. Both coenzymes have absorbance maximum at 340 nm upon reduction and are thus indistinguishable.

Recently, the coenzyme preference of the dual nucleotide specific glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides was examined by making use of the thionicotinamide analogues of NAD and NADP, shown in Figure 17, (Levy and Daouk, 1979). These analogues, S-NAD and S-NADP, which have an absorbance maximum at 400 nm and exhibit an isobestic point at 340 nm upon reduction, behave sufficiently like the natural coenzymes to allow their use in enzyme kinetic studies. Thus, the simultaneous reduction of coenzyme pairs NAD and S-NADP or S-NAD and NADP, can be monitored using a spectrophotometer capable of automatic switching between two preset wavelengths, 340 nm and 400 nm. The technique was first applied to bovine liver GDH in a study by Alex and Bell (1980), which placed particular emphasis on the investigation of the phenomenon of negative co-operativity displayed by the enzyme.

The study described in this chapter made use of the dual wavelength assay to investigate the nucleotide preference of bovine liver glutamate dehydrogenase and the differential effects of enzyme inhibitors and activators on the NAD versus NADP linked activity of the enzyme. The data suggest that NADP is the preferred coenzyme for the oxidative deamination of glutamate by bovine liver GDH under the conditions of increasing glutamate concentrations, a preference which is promoted by the actions of metabolic modulators.

Fig.17 Thioderivatives of NAD and NADP.

S-NAD

S-NADP

MATERIALS AND METHODS

Bovine liver GDH (in 50% glycerol with 10 mM potassium phosphate buffer, pH 7.0), thionicotinamide adenine dinucleotide (S-NAD) and thionicotinamide adenine dinucleotide phosphate (S-NADP) were purchased from Boehringer Mannheim Corp. All other biochemicals were from Sigma Chemical Co. For use, GDH was diluted 20-fold in 50 mM Tris-HCl buffer, pH 8.0 containing 50% glycerol.

Conventional and dual wavelength assays were conducted using a Pye Unicam SP 8-100 recording spectrophotometer with a program controller accessory allowing automatic switching between two preset wavelengths, 340 and 400 nm. All assays were performed at 25°C in 50 mM Tris-HCl buffer, pH 8.0 in a final volume of 1 ml and initiated by addition of enzyme. Reaction rates, expressed as $\mu\text{mol/min/mg}$ protein, were calculated from the molar absorptivities of the reduced forms of the natural coenzymes, 6.22×10^6 at 340 nm, or of the thionicotinamide analogues, 11.9×10^6 for S-NAD and 11.7×10^6 for S-NADP at 400 nm (Stein *et.al.*, 1963). Kinetic constants were derived from direct linear plots (Cornish-Bowden, 1979) and Hanes plots.

The dual wavelength assay relies upon the fact that there is very little overlap in the absorbance spectra of the reduced, natural coenzymes and their reduced thioderivatives. In preliminary tests of single coenzyme assays, we verified that no significant absorbance change occurred at 400 nm during the reduction of the natural coenzymes or conversely at 340 nm during the reduction of the thioderivatives.

RESULTS

The applicability of the dual wavelength assay for the study of GDH coenzyme preference rests on the assumption that the

thionicotinamide derivatives behave in a kinetically similar manner to their parent compounds. Tables 18 to 20, showing results from assays using each of the coenzymes singly, demonstrate that this is in fact the case. Enzyme apparent affinity for each coenzyme and its corresponding thioderivative was found to be similar although the maximum velocity observed with the thioderivatives was considerably reduced (to about ~~30~~ 50% of the corresponding rate with NAD or NADP) (Table 18). It is well known that GDH shows complex, non-linear kinetics with NAD(H) (Goldin and Frieden, 1971; Smith et.al., 1975). In our experiments this was noted for both NAD and S-NAD and was also demonstrated by Alex and Bell (1980). In both our single and dual nucleotide assays, therefore, NAD concentration ranges were restricted (0.05 - 0.50 mM) to remain in the range of linear NAD kinetics. Mechanistic conclusions about nucleotide binding, cannot be drawn from this study; however, the prime interest of this study is the physiological function of GDH and the competition between coenzymes in vivo at physiological concentrations of the two coenzymes.

The apparent K_m for glutamate was also essentially unaltered by the substitution of the thioderivatives for NAD or NADP (Table 19). In addition, the effect of metabolite modulators of GDH activity was the same in the presence of either the natural coenzymes or the thioderivatives (Table 20). Thus ADP and leucine were activators of both the S-NAD and S-NADP linked reactions as well as of the NAD and NADP linked reactions; however, the magnitude of the activator effects was not quite as great in the presence of the thioderivatives as with the natural coenzymes. GTP was a strong inhibitor of enzyme activity with any of the four coenzymes. ATP effects were of particular importance in

Table 18 Comparison of Kinetic Constants of Natural Coenzymes and Thionicotinamide Analogues at Subsatürating Glutamate Concentrations

Coenzyme	Glutamate Concentration (mM)					
	20		5		1	
	$K_m(\text{app})$	$V_{\text{max}}(\text{app})$	$K_m(\text{app})$	$V_{\text{max}}(\text{app})$	$K_m(\text{app})$	$V_{\text{max}}(\text{app})$
NAD	0.19	2.38	0.22	1.62	0.24	1.12
S-NAD	0.13	0.80	0.22	0.76	0.29	0.58
NADP	0.14	1.20	0.20	0.98	0.33	0.62
S-NADP	0.16	0.56	0.19	0.42	0.26	0.32

Assays were carried out in 50mM Tris-HCl pH 8.0 at 25°C. Reaction rates were measured at 340nm for NAD and NADP and at 400nm for S-NAD and S-NADP. K_m 's for coenzymes were determined over coenzyme concentration ranges 0.05-0.50mM for NAD, 0.05-0.40mM for NADP and 0.05-0.35mM for S-NAD and S-NADP. $K_m(\text{app})$'s are expressed as mM while $V_{\text{max}}(\text{app})$ are expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. The results are the means for at least 2 trials and are reproducible to within $\pm 10\%$.

Table 19 Effect of Natural Coenzymes and Thionicotinamide Analogues on Glutamate Kinetic Constants

Coenzyme	Concentration(mM)	Glutamate $K_m(\text{app})$	$V_{\text{max}}(\text{app})$
NAD	0.60	1.74	2.38
	0.15	2.13	1.06
S-NAD	0.25	1.61	0.72
	0.05	2.02	0.27
NADP	0.40	2.33	0.98
	0.10	2.93	0.52
S-NADP	0.25	2.21	0.31
	0.05	2.58	0.13

Assays were carried out in 50mM Tris-HCl pH 8.0 at 25°C. Reaction rates were measured at 340nm for NAD and NADP and at 400nm for S-NAD and S-NADP. Glutamate K_m 's were determined over a glutamate concentration range of 1-8mM. $K_m(\text{app})$'s are expressed as mM while $V_{\text{max}}(\text{app})$'s are expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. The results are the means for two trials and are reproducible to within $\pm 10\%$.

Table 20 Effect of Inhibitors and Activators on GDH Activity in Single Nucleotide Assays with the Natural Coenzymes or the Thioderivatives

Addition	Enzyme Activity ($\mu\text{mol/min/mg protein}$)		
	NAD	S-NAD	S-NADP
Control	3.28 (100)	1.93 (100)	0.98 (100)
ADP (0.1mM)	4.44 (135)	2.12 (110)	2.25 (230)
Leucine (5mM)	5.02 (153)	2.32 (120)	2.57 (262)
GTP (2 μM)	0.68 (21)	0.64 (33)	0.29 (30)
ATP (5 mM)	2.44 (75)	1.70 (88)	1.51 (154)
			0.73 (133)

Assays were carried out in 50mM Tris-HCl buffer, pH 8.0 with 50mM glutamate and 0.25mM coenzyme. Absorbance changes were measured at 340nm for NAD and NADP and at 400nm for S-NAD and S-NADP. Numbers in brackets are relative velocities compared to control activities set at 100.

demonstrating that the thioderivatives behave in an analogous manner to their natural coenzymes. ATP was an inhibitor of the NAD linked GDH reaction and also of the S-NAD reaction while both the NADP and the S-NADP linked reactions were activated by ATP. Based on these findings it was concluded that the thioderivatives behaved kinetically in an analogous manner to the natural coenzymes such that experiments utilizing the thioderivatives to study NAD versus NADP preference of GDH would be valid.

Table 21 shows the results of dual wavelength experiments pairing S-NAD and NADP or NAD and S-NADP (at equal, 0.25 mM concentrations of each coenzyme) under conditions of increasing glutamate concentrations. In Table 22 the same measurements were made using single coenzyme assays to provide a control. Activity ratios, $v_{(S-NAD)}/v_{(NADP)}$ and $v_{(NAD)}/v_{(S-NADP)}$ were calculated in both cases. In the control, single coenzyme assays, the activity ratios for both coenzyme pairs remained constant with increasing glutamate concentrations. In the dual nucleotide assays, however, activity ratios decreased in response to increasing glutamate levels. The dual nucleotide assay resulted in differential effects on the rates of the NAD versus NADP linked reactions. As glutamate concentration increased, enzyme velocity with NADP or S-NADP as the coenzyme increased proportionally faster than did the rates with NAD or S-NAD. In addition, an examination of the NADP or S-NADP reaction rates over the 0.1 to 50 mM glutamate concentration range reveals very similar percentage increases in rate in both the single or dual nucleotide situations. Parenthetically it should be noted that absolute reaction velocities for all 4 coenzymes were lower in all cases in the dual nucleotide versus the single nucleotide reactions. For NAD and S-NAD, however, the percentage

Table 21 Effect of Glutamate Concentration on the Reduction of Nucleotide Pairs, S-NAD and NADP or NAD and S-NADP, by GDH in the Dual Wavelength Assays

Coenzyme Pair	Glutamate Concentration (mM)										
	0.1	0.2	0.5	1.0	2.0	5.0	10.0	20.0	30.0	40.0	50.0
$v(S-NAD)$	0.078	0.100	0.114	0.121	0.135	0.171	0.185	0.171	0.171	0.164	0.164
$v(NADP)$	0.109	0.150	0.204	0.232	0.272	0.354	0.409	0.436	0.477	0.531	0.586
$v(S-NAD)/v(NADP)$	0.72	0.67	0.56	0.52	0.50	0.48	0.45	0.39	0.36	0.31	0.28
$v(NAD)$	0.204	0.214	0.360	0.642	0.836	0.992	1.07	1.07	1.03	1.07	1.07
$v(S-NADP)$	0.008	0.009	0.017	0.034	0.052	0.064	0.073	0.079	0.084	0.092	0.101
$v(NAD)/v(S-NADP)$	25.5	23.8	21.2	18.9	16.1	15.5	14.7	13.5	12.3	11.6	10.6

Assays were carried out in 50mM Tris-HCl buffer, pH 8.0 at 25°C. Coenzyme concentration for all four coenzymes was 0.25mM. Reaction rates were measured simultaneously at 340nm and 400nm and v was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

Table 22 Effect of Glutamate Concentration on the Reduction of Natural Coenzymes and their Thioderivatives
in Single Nucleotide Assays

Coenzyme	0.1	0.5	2.0	5.0	10.0	30.0	50.0
$v(S-NAD)$	0.257	0.630	1.41	1.72	1.97	2.17	2.20
$v(NADP)$	0.097	0.244	0.450	0.553	0.649	0.746	0.836
$v(S-NAD)/v(NADP)$	2.7	2.6	3.1	3.1	3.0	2.9	2.6
$v(NAD)$	0.257	0.900	1.64	1.95	2.20	2.41	2.64
$v(S-NADP)$	0.063	0.216	0.414	0.513	0.589	0.635	0.666
$v(NAD)/v(S-NADP)$	4.1	4.2	4.0	3.8	3.7	3.8	4.0

Assays were carried out in 50mM Tris-HCl buffer, pH 8.0 at 25°C. Coenzyme concentration for all four coenzymes was 0.25mM. Reaction rates were measured at 340nm and 400nm for the natural coenzymes and their thioderivatives respectively. v was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

increases in reaction rates with increasing glutamate levels were strongly depressed in the dual nucleotide versus the single nucleotide situation. The data indicate therefore, that in a dual nucleotide situation, the NADP reaction of GDH is favoured and the NAD reaction depressed with rising glutamate concentrations promoting the NADP reaction. If these in vitro results are transferable to the situation in vivo, this could imply that in situations of increased glutamate oxidation in tissues, it is the NADP linked reaction which is stimulated and promoted to respond to this metabolic demand.

The effects of metabolite modulators on the activity of GDH in the dual nucleotide assay is shown in Tables 23 & 24 for the S-NAD and NADP pair and in Tables 25 & 26 for NAD and S-NADP pair. The activity ratios, $v_{(S-NAD)}/v_{(NADP)}$ and $v_{(NAD)}/v_{(S-NADP)}$, for assays in the presence of modifiers were in all cases lower than the corresponding control ratios, a result which indicates a differential effect of enzyme modifiers on the NAD versus NADP rates. In all cases, the net effect is in fact to promote the NADP linked reaction over the NAD linked one. In addition, the differential effect of rising glutamate concentrations in promoting the NADP linked reaction over the NAD linked reaction is still apparent in the presence of enzyme activators or inhibitors.

ADP is a well known activator of both the NAD and the NADP linked GDH reactions (Goldin and Frieden, 1971; Smith et.al., 1975). When both coenzymes were present simultaneously in the dual wavelength assay, however, ADP preferentially activated the NADP or S-NADP reactions. The observed activation was 130-150% over the control activity (at 50 mM glutamate) (Tables 23 & 25), somewhat less than the activation produced by ADP in single nucleotide

Table 23 Effect of the Activators Leucine and ADP on the S-NAD versus NADP Activities of GDH in the Dual Coenzyme Assay

Condition	Glutamate Concentration(mM)						
	0.1	0.5	2.0	5.0	10.0	30.0	50.0
Control $v(S-NAD)$	0.078	0.114	0.135	0.171	0.185	0.171	0.164
$v(NADP)$	0.109	0.204	0.272	0.354	0.409	0.477	0.586
$v(S-NAD)/v(NADP)$	0.72	0.56	0.50	0.48	0.45	0.36	0.28
Leucine (5mM) $v(S-NAD)$	0.082	0.128	0.167	0.199	0.207	0.199	0.171
$v(NADP)$	0.204	0.381	0.545	0.681	0.763	0.845	0.899
$v(S-NAD)/v(NADP)$	0.40	0.34	0.31	0.29	0.27	0.24	0.19
ADP (0.1mM) $v(S-NAD)$	0.057	0.103	0.146	0.174	0.185	0.167	0.157
$v(NADP)$	0.150	0.286	0.436	0.559	0.627	0.695	0.749
$v(S-NAD)/v(NADP)$	0.38	0.36	0.33	0.31	0.30	0.24	0.21

Assays were carried out in 50mM Tris-HCl pH 8.0 at 25°C. The coenzyme concentrations were both 0.25mM. Reaction rates measured at 340nm and 400nm, v in $\mu\text{mol}/\text{min}/\text{mg}$ protein, values means for $n=3$, reproducible within + 10%

Table 24 Effect of GTP and ATP on the S-NAD versus NADP Activities of GDH in the Dual Coenzyme Assay

Condition	Glutamate Concentration(mM)					
	0.1	0.5	2.0	5.0	10.0	30.0 50.0
Control	$v_{(S-NAD)}$	0.078	0.114	0.135	0.171	0.185 0.171 0.164
	$v_{(NADP)}$	0.109	0.204	0.272	0.354	0.409 0.477 0.586
	$v_{(S-NAD)}/v_{(NADP)}$	0.72	0.56	0.50	0.48	0.45 0.36 0.28
GTP (2 μ M)	$v_{(S-NAD)}$	0.028	0.040	0.048	0.050	0.060 0.048 0.043
	$v_{(NADP)}$	0.057	0.084	0.109	0.125	0.161 0.172 0.174
	$v_{(S-NAD)}/v_{(NADP)}$	0.49	0.48	0.44	0.40	0.37 0.28 0.25
ATP	$v_{(S-NAD)}$	0.050	0.085	0.128	0.163	0.171 0.185 0.185
	$v_{(NADP)}$	0.123	0.232	0.381	0.531	0.627 0.708 0.763
	$v_{(S-NAD)}/v_{(NADP)}$	0.41	0.37	0.34	0.31	0.27 0.26 0.24

Assays were carried out in 50mM Tris-HCl pH 8.0 at 25°C. Reaction rates were measured simultaneously at 340nm and 400nm and v was expressed as μ mol/min/mg protein. The values given are the means for $n=3$ trials and are reproducible within $\pm 10\%$.

Table 25 Effect of the Activators Leucine and ADP on the NAD versus S-NADP Activities of GDH in the Dual Wavelength Coenzyme Assay

Condition	Glutamate Concentration (mM)					
	0.1	0.5	2.0	5.0	10.0	30.0 50.0
Control						
$v(\text{NAD})$	0.204	0.360	0.836	0.992	1.07	1.03 1.07
$v(\text{S-NADP})$	0.008	0.017	0.052	0.064	0.073	0.084 0.101
$v(\text{NAD})/v(\text{S-NADP})$	25.5	21.2	16.1	15.5	14.7	12.3 10.6
Leucine (5mM)						
$v(\text{NAD})$	0.174	0.341	0.672	0.814	0.925	1.04 1.03
$v(\text{S-NADP})$	0.015	0.032	0.087	0.116	0.144	0.173 0.204
$v(\text{NAD})/v(\text{S-NADP})$	11.6	10.7	7.7	7.0	6.4	6.0 5.0
ADP (0.1mM)						
$v(\text{NAD})$	0.204	0.412	0.715	0.890	0.962	1.01 1.05
$v(\text{S-NADP})$	0.016	0.038	0.070	0.094	0.108	0.138 0.163
$v(\text{NAD})/v(\text{S-NADP})$	12.8	10.8	10.2	9.5	8.9	7.3 6.4

Assays were carried out in 50mM Tris-HCl pH 8.0 at 25°C. The coenzyme concentrations were both 0.25mM. Reaction rates are measured simultaneously at 340nm and 400nm and v was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. The values given are the means for $n=3$ trials and are reproducible within $\pm 10\%$.

Table 26 Effect of GTP and ATP on the NAD versus S-NADP Activities of GDH in the Dual Wavelength Coenzyme Assay

Condition	Glutamate Concentration(mM)					
	0.1	0.5	2.0	5.0	10.0	30.0 50.0
Control						
$v(NAD)^*$	0.204	0.360	0.836	0.992	1.07	1.03 1.07
$v(S-NADP)$	0.008	0.017	0.052	0.064	0.073	0.084 0.101
$v(NAD)/v(S-NADP)$	25.5	21.2	16.1	15.5	14.7	12.3 10.6
GTP (2uM)						
$v(NAD)$	0.093	0.148	0.219	0.259	0.290	0.311 0.336
$v(S-NADP)$	0.011	0.019	0.029	0.039	0.049	0.057 0.067
$v(NAD)/v(S-NADP)$	8.5	7.8	7.6	6.6	5.9	5.5 5.0
ATP (5mM)						
$v(NAD)$	0.236	0.286	0.677	0.831	0.907	0.831 0.749
$v(S-NADP)$	0.016	0.026	0.072	0.091	0.104	0.128 0.137
$v(NAD)/v(S-NADP)$	14.8	11.0	9.4	9.1	8.7	6.5 5.5

Assays were carried out in 50mM Tris-HCl pH 8.0 at 25°C. The coenzyme concentrations were both 0.25mM. Reaction rates were measured simultaneously at 340nm and 400nm and v was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. The values given are the means for $n=3$ trials and are reproducible within $\pm 10\%$.

assays (Table 20). The NAD or S-NAD activities, however, showed no activation by ADP. Again, this finding has implications for the control of enzyme activity in vivo. Increased levels of ADP, a signal of low energy status, would activate and promote the NADP linked oxidation of glutamate.

An equivalent effect of the activator, leucine, was found. Leucine activated both the NAD and the NADP linked reactions in single coenzyme assays (Table 20). In the dual wavelength assay, however, leucine produces an almost 2-fold activation of the NADP or S-NADP linked reactions but had no effect on the NAD linked reaction (in the NAD/S-NADP couple) (Table 25) and produced only low activation of the S-NAD reaction (Table 23).

GTP is an inhibitor of both the NAD and the NADP linked reactions of GDH (Goldin and Frieden, 1971; Smith et al., 1975) (Table 20) and similarly, in the dual wavelength assays, GTP acts as an inhibitor of enzyme activity with all four coenzymes. In the dual wavelength assays, the percentage inhibition by GTP of enzyme activity with each of the four coenzymes (activity with 50 mM glutamate and 2 μ M GTP was 31, 26, 30 and 66% of control activity for NAD, S-NAD, NADP and S-NADP, respectively) was very similar to the inhibitions observed in single nucleotide assays indicating no differential effect of GTP on coenzyme reduction in the presence of dual coenzymes. However, GTP was in all cases a relatively stronger inhibitor of the NAD linked reaction such that in the dual coenzyme assay, the activity ratios, $v_{(S-NAD)}/v_{(NADP)}$ and $v_{(NAD)}/v_{(S-NADP)}$, were decreased in the presence of GTP as compared to the control situation (Tables 24 & 26). The net effect is, again, to promote the NADP linked reaction.

The effects of ATP on GDH activity, an inhibition of the NAD

reaction and an activation of the NADP reaction, are also apparent in the dual wavelength assay. The combined effects of ATP resulted in a decrease in the activity ratios, $v_{(S-NAD)}/v_{(NADP)}$ and $v_{(NAD)}/v_{(S-NADP)}$, effectively promoting the NADP linked reaction (Tables 24 & 26). ATP was a rather weak inhibitor of the S-NAD reaction in the dual coenzyme system; at high glutamate concentrations, ATP inhibition was essentially lost.

DISCUSSION

Levy and Daouk (1979) were the first investigators to recognize the potential of the thionicotinamide derivatives of NAD and NADP for use in studying coenzyme utilization by dual nucleotide specific dehydrogenases. They demonstrated that S-NAD and S-NADP behaved in a kinetically similar manner to their respective parent compounds and then used the dual nucleotide assay, pairing S-NAD and NADP or NAD and S-NADP, to study the simultaneous reduction of each coenzyme pair by glucose-6-phosphate dehydrogenase from L. mesenteroides (Levy and Daouk, 1979; Levy et.al., 1979). Alex and Bell (1980) applied the dual wavelength assay to a study of bovine liver GDH. They demonstrated that enzyme apparent affinity for the thioderivatives and their parent compounds was very similar, showed that each thionicotinamide analogue was an effective competitive inhibitor with respect to its parent compound, and then applied the dual wavelength assay to a study of the phenomenon of negative co-operativity. These results, like those of Alex and Bell (1980) demonstrated that GDH affinity for each of the thioderivatives is similar to the affinity for the natural coenzyme although lower maximal velocities are found with the thioderivatives in all cases. Additionally it was shown that the thioderivatives mimic their parent compounds with respect to

metabolite modulator effects both in type of effect and in degree of activation or inhibition seen. Thus ADP and leucine activated both the S-NAD and S-NADP linked reactions just as they activated the NAD and NADP reactions while GTP was an inhibitor of enzyme activity with any of the four coenzymes. The S-NAD linked reaction mimics the NAD reaction in showing inhibition by ATP while both the NADP and S-NADP linked reactions are activated by ATP. These results demonstrated that the thioderivatives of NAD and NADP are effective alternative substrates for GDH and validate their use in the dual wavelength assay for examining the coenzyme preference of GDH.

Glutamate dehydrogenase is an extremely important enzyme locus in metabolism. In the direction of oxidative deamination the enzyme functions in the generation of NH_4^+ for the urea cycle and in the oxidation of amino acids and protein reserves as an energy source; in the reverse direction the enzyme has a key role in amino acid biosynthesis and in nitrogen storage (glutamine production) (Goldin and Frieden, 1971; McGiven and Chappell, 1975). The complex regulatory controls displayed by GDH (metabolite effects by guanidine and adenine nucleotides, leucine, NAD(H), reversible polymerizations of subunits) probably are all related to the need to closely regulate enzyme activity to allow GDH to respond to a wide variety of metabolic demands. Yet another level at which GDH could be regulated is in the choice of nicotinamide nucleotide utilized by the reaction. Does the enzyme in vivo respond primarily to the NAD/NADH signal, to the NADP/NADPH signal, to both, or to one or the other depending upon other regulatory factors? Various investigators, working with isolated liver cells, have suggested that the reductive amination of α -ketoglutarate, stimulated by

ammonia, utilizes NADPH as the primary source of reducing power (Sies et al., 1974; Tischler et al., 1977). But a direct investigation of the coenzyme preference of the isolated enzyme, when given the choice of both coenzymes, has never previously been possible. In the present study the dual wavelength assay was used to probe coenzyme preference by GDH at low, physiological concentrations of coenzyme. The prime interest was the utilization of coenzyme by GDH in vivo, in a situation where both NAD and NADP are present simultaneously and in which substrate and effector concentrations can readily be altered by changing physiological states. The results seem to indicate that NADP is the preferred coenzyme for GDH oxidation of glutamate.

In the dual wavelength assay, rising glutamate concentrations quite clearly resulted in a promotion of the NADP or S-NADP reactions over the NAD or S-NAD ones demonstrating that high glutamate favours the utilization of NADP as the coenzyme for GDH function. Such an effect could not have been uncovered by conventional assays. A similar experiment by Levy and Daouk (1979) demonstrated the opposite behaviour by glucose-6-phosphate dehydrogenase, rising glucose-6-phosphate concentrations favouring the NAD linked reaction. Situations of elevated glutamate concentrations leading to increased glutamate oxidation can occur in vivo as a result of hormonal action, metabolic acidosis, or protein breakdown during starvation. During metabolic acidosis flux through GDH in renal mitochondria is markedly accelerated by the provision of increased amounts of glutamate via the action of glutaminase (Schoolwerth et al., 1978). The results presented here would suggest that in vivo such an increased flux through GDH brought on by elevated intramitochondrial levels of glutamate would

preferentially stimulate the NADP linked reaction. Conversely, the rate of glutamate oxidation should be responsive to the NADP/NADPH ratio. Indeed, liver GDH may, in fact, be primarily responsive to the intramitochondrial NADP/NADPH ratio in both directions.

The NADP linked reaction of GDH is also promoted by the effects of metabolite modulators. While the effects of individual modulators were varied, their net results in the dual wavelength assay were always to favour the NADP over the NAD linked reaction. Thus ADP and leucine activated the NADP or S-NADP reactions to a much greater degree than the NAD or S-NAD reactions; in fact, in the dual wavelength assay little or no activation of the NAD or S-NAD reactions was apparent with either ADP or leucine. GTP inhibition resulted in a favouring of the NADP and S-NADP reactions because of a lower inhibitory effect of GTP on the reaction utilizing these nucleotides. And finally, ATP effects, an inhibition of NAD and S-NAD reactions and an activation of NADP and S-NADP reactions, produced a net stimulation of the NADP linked reaction.

In summary, then, the dual wavelength assay is a powerful new tool for use in studying dehydrogenases displaying dual nucleotide specificities. The assay allows an investigation of enzyme activity and enzyme regulation under conditions which closely resemble the situation in vivo, i.e., in the presence of both NAD and NADP simultaneously. Application of the assay to GDH in the present study provides the first direct in vitro kinetic evidence that the oxidative deamination of glutamate by GDH may be primarily an NADP linked function.

DISCUSSION AND CONCLUSIONS

Glutamate dehydrogenase is an extremely important control enzyme in the cell in vivo interrelating carbohydrate and amino acid metabolism through both its catabolic (glutamate oxidation) and biosynthetic (glutamate synthesis) functions (Smith et al., 1975). The enzyme is tightly regulated by allosteric effectors and, as shown in this thesis, by the choice of coenzyme utilized.

A limited amount of work has been carried out on glutamate dehydrogenase with respect to coenzyme utilization and the in vivo significance of the dual coenzyme specificity expressed by many GDHs in vitro is not well understood. It has been postulated that the catabolic direction of the enzyme is NAD linked in vivo while the biosynthetic direction of the enzyme is NADPH linked in vivo (Smith et al., 1975). My studies indicate that this may not be true. The dual specific GDH from bovine liver seemed to show a preference for the NADP reaction in in vitro experiments simulating the in vivo situation. The results of this thesis, extrapolated to the cell itself, indicate that the bovine enzyme which is primarily involved in glutamate biosynthesis in vivo would respond to the NADPH/NADP pool rather than to both the NADPH/NADP and NADH/NAD pools. Thus the activity seen with NAD or NADH, although high in vitro, may have little significance in vivo. Eurosta solidaginis and Anthopleura xanthogrammica GDH, like the bovine liver enzyme, appeared to be primarily involved in a biosynthetic function in vivo. The sea anemone GDH was found to be solely NADP specific and would therefore only respond to the NADPH/NADP pool in vivo. Experiments on the dual specific GDH from the gall fly larvae (Chapter 1) indicate that the enzyme likely prefers NADPH in vivo in the biosynthetic direction. From these studies it is indicated that GDHs involved in biosynthetic functions in vivo are

likely responding to the NADPH/NADP pool in the cell. GDHs which have been shown to be involved in oxidative functions in vivo, however, such as the squid mantle muscle GDH (Storey et al., 1978) or insect flight muscle GDH (Bursell, 1975) are highly NAD specific. In these tissues GDH would respond to the NADH/NAD pool in the cell with enzyme function therefore linked to energy production via the oxidative functions of the Kreb cycle and oxidative phosphorylation. Future experiments to test this coenzyme preference of GDH in vivo could include study of a dual specific GDH which is primarily involved in the catabolic direction in vivo using thioderivatives to determine which coenzyme is being utilized in either direction. A good tissue for this study would be the rat kidney where GDH is functionally linked to glutaminase for the oxidative deamination of glutamine through glutamate to α -ketoglutarate (Schoolwerth and LaNoue, 1980).

Regulation of GDH by allosteric effectors has been studied in great detail for the bovine liver enzyme and in this thesis the regulatory property of allosteric effectors was studied with respect to the function of GDH in gall fly larvae, sea anemone and also bovine liver. Allosteric effects of leucine, ADP, ATP, and GTP on the bovine liver GDH indicated that these effectors enhanced the preference of the enzyme for NADP in in vitro studies. The effects of these activators and inhibitors further support the idea that the bovine enzyme is responding to the NADPH/NADP pool rather than the NADH/NAD pool in the cell. The effectors activated and inhibited with respect to both cofactors in single coenzyme assays but when the dual coenzyme assays were incorporated they only affected the NADP cofactor. Like the bovine liver enzyme, the GDH from E. solidaginis is primarily linked to the biosynthetic

function. The effects of allosteric modulators ADP, ATP, and GTP appeared to promote the use of the cofactor NADPH in the biosynthetic direction. This GDH functions at low temperature to synthesize glutamate necessary for proline biosynthesis. This function was enhanced by allosteric effector interactions which occur at low but not high temperatures. For example the activator ADP produced a greater reversal of the inhibitory action of GTP at low temperatures. It was interesting to note that this interaction of temperature and allosteric effectors occurs when NADPH was cofactor and not when NADH was cofactor. The action of allosteric effectors, therefore, supports the idea that the E. solidaginis GDH is responding to NADPH levels in the cell with regard to coenzyme utilization. Like the bovine liver and E. solidaginis GDHs, the enzyme from A. xanthogrammica is also probably linked to a biosynthetic function. However the sea anemone enzyme was not regulated by the usual allosteric effectors. Instead this NADP specific GDH was regulated by salt ion effects. This GDH functions during hyperosmotic stress to synthesize glutamate thus fixing ammonium ion for biosynthesis of other amino acids such as alanine. Function of GDH in organic osmolyte production is important for cell volume regulation. Salt ions activate this biosynthetic function by altering the kinetic parameters of the enzyme.

In summary, regulation of the enzyme glutamate dehydrogenase by the choice of coenzyme utilization and by the effects of allosteric modulators is an extremely important property for the determination of the enzyme's function in vivo under various metabolic conditions.

REFERENCES

- Addink, A. and Veenhof, P. 1975. Glutamate dehydrogenase from Mytilus edulis. Proc. 9th Eur. Biol. Symp.: 109-119.
- Alex, S. and Bell, J.E. 1980. Dual nucleotide specificity of bovine glutamate dehydrogenase. Biochem. J. 191: 299-304.
- Allen, K. 1961. The effect of salinity on the amino acid concentration in Rangia cuneata (Pelecypoda). Biol. Bull., Woods Hole 121: 419-424.
- Bedford, W.B. and Anderson, J.W. 1972. The physiological response of estuarine clam Rangia cuneata (Gray) to salinity I: Osmoregulation. Physiol. Zool. 45: 255-260.
- Bishop, S.H., Klotz, A., Drolet, L.L., Smullin, D.H. and Hoffman, R.J. 1978. NADP specific glutamate dehydrogenase in Metridium senile. Comp. Biochem. Physiol. vol. 61B: 185-187.
- Bond, P.A. and Sang, J.H. 1968. Glutamate dehydrogenase from Drosophila larvae. J. Insect Physiol. 14: 341-359.
- Bowlus, R. and Somero, G. 1979. Solute compatibility with enzyme function and structure: Rationales for the selection of osmotic agents and end products of anaerobic metabolism in marine invertebrates. J. Exp. Zool. 208: 137-152.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.
- Braunstein, A. 1957. Les voies principales de l'assimilation et dissimulation de l'azote chez les animaux. Adv. Enzymol. 19: 335-389.
- Bursell, E. 1975. Glutamate dehydrogenase from the sarcosomes of the tsetse fly and blowfly. Insect Biochem. 5: 289-297.
- Chaplin, E.A., Huggins, A.K., and Munday, K.A. 1965. Ionic effects on glutamate dehydrogenase activity from beef liver, lobster muscle and crab muscle. Comp. Biochem. Biophys. 16: 49-62.
- Chen, S.S. and Engel, P.C. 1975. Equilibrium protection studies of the interaction of bovine glutamate dehydrogenase with purine nucleotide effectors. Febs. Letts. 58: 202-205.
- Cornish-Bowden, A. 1979. Estimation of kinetic constants. In Fundamentals of enzyme kinetics. Butterworths, London. pp. 200-210.
- Dieter, H., Koberstein, R. and Sund, H. 1981. The interaction of ADP, GTP and NADPH in complexes with glutamate dehydrogenase. Eur. J. Biochem. 115: 217-226.
- Duchateau-Bosson, G. and Florkin, M. 1961. Change in intracellular concentration of free amino acids as a factor of euryhalinity in the crayfish Astacus astacus. Comp. Biochem. Physiol. 3: 245-249.

- Engels, P.C. and Dalziel, K. 1970. Kinetic studies of glutamate dehydrogenase. The reduction amination of 2-oxoglutarate. *Biochem. J.* 118: 409-419.
- Fisher, H. 1973. Glutamate dehydrogenase ligand complexes and their relationship to the mechanism of the reaction. *Adv. Enzymol.* 39: 369-417.
- Florkin, M. and Schoffenials, E. 1969. In *Molecular approaches to ecology*. Academic Press, New York. pp. 1-232.
- Frieden, C. 1963. L-glutamate dehydrogenase. In *The enzymes*. P. Boyer, H. Lardy and K. Myrback eds. Academic Press, New York. pp. 3-24.
- Gilles, R. 1979. In *Mechanisms of osmoregulation in animals*. Maintenance of cell volume. Wiley and Sons, Toronto. Chapters 3 & 4.
- Goldin, B.R. and Frieden, C. 1971. Glutamate dehydrogenases. *Curr. Top. Cell. Regul.* 4: 77-117.
- Hemmings, B.A. 1978. Evidence for the degradation of NADP-dependent glutamate dehydrogenase of *Candida utilis* during rapid enzyme inactivation. *J. Bacteriol.* 133: 867-877.
- Hemmings, B.A. 1981. Reactivation of the phospho-form of NAD-dependent glutamate dehydrogenase by a yeast protein phosphatase. *Eur. J. Biochem.* 116: 47-50.
- Hoffmann, R.J., Bishop, S.H. and Sassaman, C. 1978. Glutamate dehydrogenase from coelenterates NADP-specific. *J. Exp. Zool.* 203: 165-170.
- Job, D., Cochet, C., Dhien, A. and Chambaz, E.M. 1978. A rapid method for screening inhibitor effects: Determination of I and its standard deviation. *Anal. Biochem.* 84: 68-77.
- Kimura, K., Miyakawa, A., Imai, T. and Sasakawa, T. 1977. Glutamate dehydrogenase from *Bacillus subtilus* PCI 219. *J. Biochem.* 81: 467-476.
- Levy, H.R. and Daouk, G.H. 1979. Simultaneous analysis of NAD and NADP-linked activities of dual nucleotide-specific dehydrogenases. *J. Biol. Chem.* 254: 4843-4847.
- Levy, H.R., Daouk, G.H., and Katopes, M.A. 1979. Regulation of coenzyme utilization by the dual nucleotide specific glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroids*. *Arch. Biochem. Biophys.* 198: 406-413.
- Livingstone, D.R., Widdows, J. and Fieth, P. 1979. Aspects of nitrogen metabolism of the common mussel *Mytilus edulis*:

Adaptation to abrupt and fluctuating changes in salinity.
Marine Bio. 53: 41-55.

Mansingh, A. 1967. Changes in the free amino acids of the hemolymph of Antheraea pernyi during induction and termination of diapause. J. Insect Physiol. 13: 1645-1655.

McCarthy, A.D., Walker, J.M. and Tipton, K.F. 1980. Purification of glutamate dehydrogenase from ox brain and liver. Biochem. J. 191: 605-611.

McGiven, J.D. and Chappell, J.B. 1975. On the metabolic function of glutamate dehydrogenase in rat liver. Febs. Letts. 52: 1-7.

Melzi D'Eril, G. and Dalziel, K. 1973. The behaviour of regulatory enzymes. Biochem. Soc. Monogr.: 33-46.

Miller, R.E., and Stadtman, E.R. 1972. Glutamate synthase from Escherichia coli. J. Biol. Chem. 247: 7407-7419.

Mills, R.R. and Cochran, D.G. 1963. Purification and properties of glutamic acid metabolizing enzymes from cockroach muscle mitochondria. Biochem. Biophys. Acta 73: 213-221.

Palaiologos, G. and Felig, P. 1976. Kinetic studies of glutamate dehydrogenase from rat diaphragm mitochondria. Fed. Proc. 35: 299.

Schmidt-Nielsen, B. 1975. Comparative physiology of cellular ion and volume regulation. J. Exp. Zool. 194: 107-220.

Schoolwerth, A.C., Nazar, B.L. and LaNoue, K.F. 1978. Glutamate dehydrogenase activation and ammonium formation by rat kidney mitochondria. A. Biol.Chem. 253: 6177-6183.

Schoolwerth, A.C. and LaNoue, K.F. 1980. The rôle of micro-compartmentation in the regulation of glutamate metabolism by rat kidney mitochondria. J. Biol. Chem. 255: 3403-3411.

Schoffeniels, E. 1964. Effect of inorganic ions on the activity of L-glutamic acid dehydrogenase. Life Sciences 3: 845-850.

Schoffeniels, E. and Gilles, R. 1972. Ionoregulation and osmoregulation in mollusca. Chemical Zool. 7: 393-420.

Severin, S.E., Boldyrev, A.A. and Lebedev, A.V. 1972. Nitrogenous extractive compounds of muscle tissue in invertebrates. Comp. Biochem. Physiol. 43B: 369-381.

Shaw, J. 1958. Osmoregulation in the muscle fibres of Carcinus maenas. J. Exp. Biol. 35: 920-929.

Sies, H., Haussinger, D. and Grosskopf, M. 1974. Mitochondrial nicotinamide nucleotide systems: Ammonium chloride responses and associated transactions in hemoglobin-free perfused rat liver. Hoppe-Seyler's Z. Physiol. Chem. 355: 305-320.

- Sies, H., Summer, K.H. and Bucher, T. 1975. A process requiring mitochondrial NADPH: Urea formation from ammonia. *Febs. Letts.* 54: 274-278.
- Smith, E.L., Austen, B.M., Blumenthal, K.M. and Nyc. J.F. 1975. Glutamate dehydrogenases. In *The enzymes*. Boyer, P.D. ed. Academic Press, New York. Vol. 11, pp. 293-367.
- Somero, G.N. 1978. Temperature adaptation of enzymes: Biological optimization through structure-function comprises. *Ann. Rev. Ecol. Syst.* 9: 1-29.
- Stein, A.M., Lee, J.K., Anderson, C.D. and Anderson, B.M. 1963. The thionicotinamide analogs of DPN and TPN I. Preparation and analysis. *Biochemistry* 2: 1015-1017.
- Storey, K.B., Fields, J.H.A. and Hochachka, P.W. 1978. Purification and properties of glutamate dehydrogenase from the mantle muscle of the squid *Loligo paeleii*. Role of the enzyme in energy production from amino acids. *J. Exp. Zool.* 205: 111-118.
- Storey, K.B. and Storey, J.M. 1981. Biochemical strategies of overwintering in gall fly larvae *Eurosta solidaginis*: Effect of low temperature acclimation on the activities of enzymes of intermediary metabolism. *J. Comp. Physiol.* 144: 191-199.
- Storey, K.B., Baust, J.G. and Buescher, P. 1981a. Determination of water "Bound" by soluble subcellular components during low temperature acclimation in the gall fly larva, *Eurosta solidaginis*. *Cryobiology* 18: 315-321.
- Storey, K.B., Baust, J.G. and Storey, J.M. 1981b. Intermediary metabolism during low temperature acclimation in the overwintering gall fly larvae *Eurosta solidaginis*. *J. Comp. Physiol.* 144: 183-190.
- Storey, K.B., Park, I.A.R. and Storey, J.M. 1981c. Isozyme composition and low temperature acclimation in the overwintering gall fly larvae *Eurosta solidaginis*. *Cryo Letters* 2: 179-284.
- Sugden, P. and Newsholme, E. 1975. Activities of citrate synthase, NAD and NADP-linked isocitrate dehydrogenase, glutamate dehydrogenase, and alanine aminotransferases in nervous tissues from vertebrates and invertebrates. *Biochem. J.* 150: 105-111.
- Switzer, R.C., Merril, C.R. and Shifran, S. 1979. A highly sensitive stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* 98: 231-237.
- Tischler, M.E., Hecht, P. and Williamson, J.R. 1977. Effect of ammonia on mitochondrial and cytosolic NADH and NADPH systems in isolated rat liver cells. *Febs. Letts.* 76: 99-104.

Uhler, L.D. 1949. Biology and ecology of the goldenrod gall fly, Eurosta solidaginis. Fitch. Cornell Univ. Agric. Exp. Sta. Memoir 300.

Walsh, P. 1981. Purification and characterization of glutamate dehydrogenase from three species of sea anemones. Adaptation to temperature within and among species from different thermal environments. Marine Biology Letters 2: 289-299.

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