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Catabolism of osmotically-active amino acids in two groups of osmoconformers, bivalve molluscs and elasmobranchs.

by

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ABSTRACT

Catabolism of intracellular free amino acids accompanies hypoosomotic stress in bivalve molluscs and elasmobranchs. The catabolic pathways utilized have been established for only a few amino acids. This study examines the role of glutamate dehydrogenase (GDH) in the deamination of osmotically active amino acids in bivalve tissues. The oxidative pathway for sarcosine and the mechanism responsible for reduction of sarcosine levels during hypoosmotic stress in skates was also examined.

The role of GDH in amino acid catabolism in the mantle of *Mya arenaria* was studied using coupled mitochondria and *in vitro* enzyme activity measurements. Although the activities of the mitochondrial transaminases, GOT and GPT, are greater than GDH (deaminating), the relative activities of these enzymes *in vivo* may be highly dependent on compartment pH. The *in vitro* activity of GDH was sufficient to account for all NADH produced in intact mitochondria oxidizing glutamate. Glutamate oxidation between pH 6.7 and 8.0 is limited by the electron transport system. Stimulation of mitochondrial glutamate oxidation by an uncoupler (carbonyl m-chlorophenyl-hydrazone) and lack of inhibiton by an aminotransferase inhibitor (aminooxyacetic acid) suggest that in spite of low activities compared to vertebrates,

GDH activities could be physiologically significant in amino acid metabolism of *M. arenaria* mantle.

Liver mitochondria of the little skate, *Raja erinacea*, oxidize sarcosine at low rates relative to other amino acids. After transport into the mitochondria, sarcosine is catabolized through the flavin-linked sarcosine oxidase with the resultant glycine oxidized in the glycine cleavage system (GCS). Oxidation of sarcosine by skate liver mitochondria and hepatocytes were inversely correlated with the tonicity of the assay medium. Based on the effects of tonicity on the mitochondrial oxidation of serine and glycine, tonicity did not affect sarcosine oxidation through the GCS. As tonicity was shown to affect glycine transport, it is likely that tonicity affects sarcosine oxidation through the mitochondrial transporter for sarcosine. This mechanism may aid in the reduction of sarcosine levels in skate liver and other tissues during hypoosmotic stress.

RESUME

Le catabolisme des acides aminées intracellulaires accompagne le stress hypoosmotique dans les mollusques bivalves et les élasmobranches. Le catabolisme utilisé a seulement été établi pour quelques acides aminées. Cette étude examine le role de la glutamate-déhydrogénase (GDH) dans la désamination des acides aminées qui sont actives osmotiquement dans les tissues des bivalves. L'oxydation de la sarcosine et le méchanisme responsable pour la réduction des niveaux de sarcosine durant le stress hypoosmotique a aussi été examiné.

Le role de la GDH dans le catabolisme des acides aminées dans le manteau de *Mya arenaria* a été étudié en utilisant des mitochondries couplés et des mesures d'activités enzymatiques *in vitro*. Même si les activités des transaminases mitochondriaux, GOT et GPT, sont plus grandes que celles de GDH (désaminant), les activités relatives de ces enzymes *in vivo* sont peut-être dépendantes du pH du compartiment. L'activité, *in vitro*, de GDH était suffisante pour tenir compte de tout le NADH produit dans les mitochondries intactes qui oxidaient le glutamate. L'oxidation du glutamate entre les pH 6.7 et 8.0 est limité par la chaine de transfert d'électrons. La stimulation de l'oxidation mitochondrial

du glutamate par un découpleur (carbonyl m-chlorophenyl-hydrazone) et le manque d'inhibiton par un inhibiteur d'aminotransférases (acide aminooxyacétic) suggèrent que même si son activité est inférieur comparé aux vertébrés, l'activité de GDH peut être physiologiquement signifiante au niveau du métabolisme des acides aminées du manteau de *Mya arenaria*.

Les mitochondries hépatiques de la petie raie, *Raja erinacea*, oxide la sarcosine à des vitesses basses relatif au transport dans la mitochondrie, la sarcosine-oxydase qui est lié à la flavine et la glycine qui en résulte est oxidée dans le système de rupture de la glycine (GCS). L'oxidation de la sarcosine par les mitochondries hépatiques de la raie et les hépatocytes sont correlés inversément avec la tonicité du medium d'analyse. Basé sur les effets de la tonicité sur la glycine, la tonicité n'affectait pas l'oxidation de la sarcosine par le GCS. Comme la tonicité affecte le transport de la glycine tel que démontré, il est vraisemblable que la tonicité affecte l'oxidation de la sarcosine via le transporteur mitochondrial de la sarcosine. Ce méchanisme pourrait aider à la réduction des niveaux de sarcosine dans le foie de la raie et dans d'autres tissues durant le stress hypoosmotique.

ABBREVIATIONS

ADP.....adenosine diphosphate

ADH.....alanine dehydrogenase

AOA.....aminooxyacetate

ATP.....adenosine triphosphate

BALA.....beta-alanine

BSA.....bovine serum albumin, esentially fatty acid free

CCCP.....carbonyl m-chlorophenylhydrazone

GCS.....glycine cleavage system

GDH.....glutamate dehydrogenase

GPT.....glutamate-pyruvate transaminase

GOT.....glutamate-oxaloacetate transaminase

HEPES.....N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

MDH.....malate dehydrogenase

MeTHF.....$^{5}N,^{10}N$-methylene tetrahydrofolate

MeTHFDH...MeTHF dehydrogenase

NAD^{+}.....nicotinamide adenine dinucleotide (oxidized)

NADH.....nicotinamide adenine dinucleotide (reduced)

RCR.....respiratory control ratio

SO.....sarcosine oxidase

TAPS.....tris(hydroxymethyl)methylaminopropanesulphonic acid

THF.....tetrahydrofolate

TMAO.....trimethylamine oxide

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CHAPTER 1: INTRODUCTION

Animals inhabiting estuarine waters can experience wide fluctuations in environmental salinity. Osmoregulators control the movement of water across peripheral tissues so that variations in ambient salinity do not cause changes in the extra- or intracellular osmolarity. Osmoconformers, however, have little ability to prevent the movement of water across external surfaces. Consequently, the osmolarity of the extracellular fluid varies in response to changes in ambient osmolarity. As the movement of water between the extra- and intracellular compartments is also unregulated, detrimental deviations from normal cell volume would occur in response to anisosmotic environments in lieu of compensatory mechanisms. However, osmoconformers are capable of cell volume regulation by manipulating intracellular osmolyte concentrations (Pierce, 1971).

Cell volume regulation has been studied extensively in two groups of osmoconformers, bivalve molluscs and elasmobranch fishes. In both groups organic molecules are important intracellular osmolytes, responsible for approximately 40% of the total tissue osmolarity in bivalves (Gilles, 1972; Virkar and Webb, 1972) and up to 75% in elasmobranchs (Robertson, 1975). The remainder of the intracellular osmolarity is due primarily to inorganic ions

(Pierce, 1971; Robertson, 1975). In animals acclimated to dilute seawater, changes in the concentration of organic solutes account for a large component of the change in tissue osmolarity. Bivalves differ from elasmobranchs however in the choice of organic solute. In bivalves, amino acids, particularly glycine, alanine and proline are the most abundant osmotically active organic solutes (Gilles, 1972; Virkar and Webb, 1972). The concentration of taurine is also high, however taurine levels change only after long-term exposure to anisosmotic media (Livingstone et al., 1979). In elasmobranchs, urea is the most abundant osmolyte (Boyd et al., 1977). Amino acids are also important solutes in skates, although the specific amino acids used are different from those employed by bivalves. Beta-alanine (BALA) and sarcosine (N-methylglycine) are the two most abundant amino acid osmolytes in skate tissues (Boyd et al., 1977). Trimethylamine oxide (TMAO) is also present in high concentrations in skate tissues (Goldstein and Forster, 1971). Although betaine (trimethylglycine) is abundant in dogfish tissues (Robertson, 1975), the concentration in skate tissues has not been determined.

Maintenance of cell volume during hypoosmotic stress demands a reduction in the number of intracellular solute molecules. Renal excretion appears to be the mechanism for the reduction of urea concentrations in skates exposed to dilute seawater (Goldstein and Forster, 1971). However,

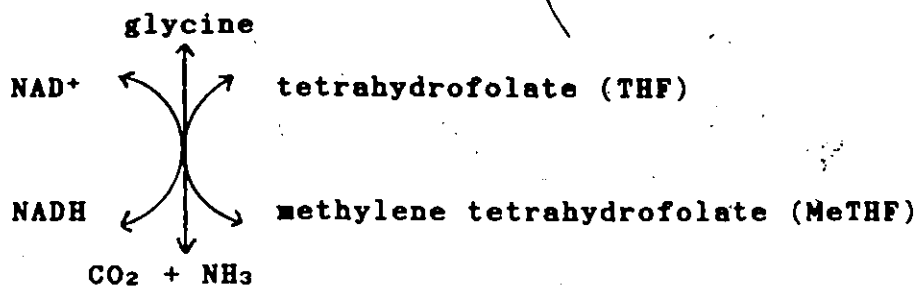
excretion of intact amino acids under these conditions appears to be low in skates (King et al., 1980) and bivalves (Bishop, 1976; Livingstone et al., 1979). The only exception is taurine which is excreted in both skates (King et al., 1980) and bivalves (Allen and Garrett, 1972) during hypoosmotic stress. Several studies have shown that bivalves exposed to hypoosmotic media increase the production of ammonia, concommitant with a reduction in the amino acid concentration of the tissues (Strange and Crowe, 1979 - *Modiolus demissus*; Livingstone et al., 1979 - *Mytilus edulis*; Henry and Mangum, 1980 -*Rangia cuneata*). This suggests that at least part of the amino acid pool is deaminated under these conditions. In the study by Strange and Crowe (1979), this response occurred within 3 hours of exposure to dilute seawater. After the amino acids are deaminated, the remaining carbon skeletons or their metabolic products must be excreted or incorporated into larger molecules in order to achieve a reduction in intracellular osmolarity. While the physiological response of the amino acid pool is well established in both skates and bivalves, the biochemical pathways for the deamination and catabolism of amino acids are less well understood.

I. BIVALVES

While it is clear tha in hypoosmotically stressed bivalves, at least part of the amino acid pool is being deaminated (Strange and Crowe, 1979; Livingstone et al., 1979; Henry and Mangum 1980), the biochemical pathways by which this deamination occurs have not been established. The purine nucleotide cycle (Bishop, 1976) and the serine cycle (Gilles, 1969) were found to be of little importance in affecting a reduction in amino acid levels during hypoosmotic stress (Bishop et al., 1981). L-Amino acid oxidase is not involved in the deamination of important amino acid osmolytes (Burcham et al., 1980). A role for glutamate dehydrogenase (GDH) in the deamination of amino acids in bivalve tissues has not been established. The biochemical pathway for deamination and catabolism of glycine, the most important osmolyte in bivalves, has only recently been identified (Ellis et al., 1985).

Catabolism of glycine in bivalves, as in rat liver, proceeds through the mitochondrial glycine cleavage system (1) (Ellis et al., 1985):

(1)



In rat mitochondria, the methylene group of MeTHF derived from the C(2) carbon of glycine can be released as CO_2 or formaldehyde, or transferred to a receptor molecule by various enzymes including serine hydroxymethyltransferase (SHMT)(2):

(2) glycine + MeTHF $\longleftrightarrow$ L-serine + THF

The kinetics of SHMT from rat liver favour production of glycine (Nakano et al., 1968); however, in bivalve tissues the levels of glycine are considerably greater than serine (Gilles, 1972; Virkar and Webb, 1972). Glycine flux through the GCS of *Modiolus demissus* gill mitochondria was shown to increase in response to assay medium dilution (Ellis et al., 1985). Unfortunately, it was not established whether the increased flux was due to stimulation of the GCS or the mitochondrial transporter for glycine, which could limit glycine availability to the GCS.

While the potential physiological significance of enhanced oxidation of glycine under hypoosmotic stress is obvious, more information regarding the GCS pathway is

required. In particular, what is the fate of the C(2) carbon of the glycine molecule? Ellis et al. (1985) found that the rate of production of single carbon units (as CO_2 and formaldehyde) from $^{14}C(1)$-glycine and $^{14}C(2)$-glycine was approximately equal under isosmotic conditions in isolated gill tissue. Under severe hypoosmotic stress, the release of C(1) carbon, primarily as CO_2, greatly exceeded the rate of C(2) carbon release (as CO_2 and formaldehyde). The carbon from MeTHF was probably transferred to a second glycine molecule to form serine (reaction 2). Under these conditions however, the amount of radioactivity in the serine fraction did not increase. As a large increase in radioactivity was observed in the protein- and amino acid-free water soluble fraction, it is possible that the increased radioactivity was asssociated with glycogen. Gluconeogenesis with subsequent glycogen synthesis would allow regeneration of the MeTHF while reducing the intracellular osmolarity.

i. Glutamate dehydrogenase in bivalves

The enzyme or enzymes responsible for the deamination of osmotically-active amino acids other than glycine has not been established in bivalves. In most animal tissues, GDH is responsible for the deamination of most amino acids, including alanine, proline, aspartate and serine (Lehninger, 1976). Bivalve GDH activities (e.g., Reiss et al., 1977) are typically two orders of magnitude lower than those in rat or

pig liver (deRosa and Swick, 1975). The low activities of GDH (deaminating) prompted Reiss et al. (1977) to suggest that this enzyme would have little significance in amino acid catabolism under hypoosmotic stress. It was also noted that, if mitochondrial NAD^+/NADH ratios were the same as in rat mitochondria, ammonia fixation, not deamination would be favoured. Although the maximal activities of GDH in the aminating direction were 5- to 10-fold greater than the deaminating direction, Reiss et al. (1977) concluded that GDH activity in both directions was too low to be physiologically significant. Alanine dehydrogenase (ADH) was suggested to be the ammonia-fixing enzyme, given the low GDH activities and the pattern of amino acid accumulation (Livingstone et al., 1979; Henry et al., 1980; Zurburg and deZwaan, 1981). This enzyme has not been demonstrated in any bivalve tissue (Bishop et al., 1983). However, Zurburg and deZwaan (1981) cautioned against the use of *in vitro* enzyme data to evaluate the physiological significance of GDH in bivalve tissues. The exclusive mitochondrial localization of GDH permits the use of a coupled, intact mitochondrial preparation, a system more closely resembling the *in vivo* situation, to assess the role of GDH in these deaminating processes.

Glutamate oxidation has been studied in coupled, intact mitochondria isolated from mammals (LaNoue et al., 1983), fish (Campbell et al., 1983) and several bivalves including *Mytilus edulis* hepatopancreas (Ballantyne and

Moon, 1985), *Mercenaria mercenaria* ventricle (Ballantyne and Storey, 1983) and hepatopancreas (Ballantyne and Storey, 1984), and *Crassostrea virginica* gill (Burcham et al., 1983). Although every bivalve tissue examined to date oxidizes glutamate at high rates relative to other substrates, this is not conclusive evidence of a role for GDH.

The first step in the mitochondrial catabolism of glutamate is the production of 2-oxoglutarate. This reaction can be catalyzed by reaction (3) GDH, or an aminotransferase such as glutamate-pyruvate aminotransferase (GPT) (4) or glutamate-oxaloacetate aminotransferase (GOT) (5).

(3) L-glutamate + NAD^+ ⇕ GDH → 2-oxoglutarate + NH_3 + NADH + H^+

(4) L-glutamate + pyruvate ⇕ GPT → 2-oxoglutarate + L-alanine

(5) L-glutamate + oxaloacetate ⇕ GOT → 2-oxoglutarate + L-aspartate

Burcham et al. (1983) suggested that the high rates of oxygen consumption observed with mitochondria given glutamate

indicated a significant role for GDH in glutamate catabolism. The aminotransferase catalyzed reactions do not in themselves result in oxygen consumption. However, the 2-oxoglutarate produced by the aminotransferase (or by GDH) is further catabolized within the Krebs cycle, resulting in the production of NADH (or other reducing equivalents) and oxygen consumption in the electron transport system, as the reducing equivalent is in turn oxidized. Clearly, the observation that the rate of oxygen consumption is high in mitochondria given glutamate may not be indicative of an active GDH enzyme.

ii. Experiments on the role of GDH in *Mya arenaria*

The relative importance of GDH, GOT and GPT in mitochondrial glutamate oxidation has not been evaluated in any bivalve. In this study, intact mitochondria from the mantle of the euryhaline bivalve, *Mya arenaria*, are used to examine the role of GDH. Several approaches are used to examine the relative importance of GDH and the aminotransferases in the initial step of glutamate catabolism. The *in vitro* activities of the competing enzymes are examined. The *in vitro* NADH-producing capacity of GDH is compared to the rate of production of reducing equivalents by intact mitochondria oxidizing glutamate. An indication of the importance of GDH in glutamate metabolism of *Mya arenaria* mantle may also be gained by studying the response of the

mitochondria to an uncoupler of oxidative phosphorylation. Similar studies on rat mitochondria have suggested that the effect of uncouplers on mitochondrial glutamate oxidation is related to the relative importance of GDH to that tissue. Rat heart mitochondria have relatively low GDH levels and demostrate reduced oxygen consumption in the presence of an uncoupler, whereas oxygen uptake by rat liver mitochondria, which have high GDH activities, is stimulated by uncouplers (LaNoue et al., 1974). Phosphate has been shown to counteract the effects of the uncoupler on rat heart mitochondria oxidizing glutamate (LaNoue et al., 1974). The effect of pH is also examined, as pH regulates glutamate oxidation in mammalian kidney (Tannen and Ross, 1979) and liver (LaNoue et al., 1983). Intracellular pH of *Mytilus edulis* is known to vary in response to environmental stressors (Walsh et al., 1984).

II. SKATES

In skate tissues, BALA and sarcosine are the most abundant amino acid osmolytes. As in bivalve tissues, amino acid catabolism accompanies hypoosmotic stress (Goldstein, 1981). Oxidation of sarcosine has not been well studied as radiolabelled sarcosine is not currently available. The metabolism of BALA has been studied extensively by Goldstein and co-workers (see below).

The concentration of BALA is high in the wing muscle (40.7 umoles (g tissue)$^{-1}$) and erythrocyte (50.6 umoles (g tissue)$^{-1}$) but low in the liver (0.97 umoles (g tissue)$^{-1}$) and other tissues (Boyd et al., 1977; King et al., 1980). Six hours after transfer to dilute seawater, plasma BALA levels increased 2-fold with no detectable excretion of BALA (Leech and Goldstein, 1983). After 7 days exposure to dilute seawater, BALA levels dropped in wing muscle and erythrocyte (Boyd et al., 1977), but not in the liver (King et al., 1980). The stimulus for the expulsion of BALA and other amino acids from skate tissues has not been established. The release of amino acids from isolated bivalve tissues has been shown to be related to membrane potential, tissue ATP content and the levels of divalent cations in the incubation medium (Pierce and Greenberg, 1976).

Although it is clear that there is an increased oxidation of BALA *in vivo* in response to hypoosmotic stress, oxidation of BALA by liver and kidney slices *in vitro* does not increase with assay medium dilution. A stimulation of oxidation was observed in liver slices of animals acclimated to dilute seawater (King et al., 1980); it was later suggested, however, that this was an artifact of the preparation (Shuttleworth and Goldstein, 1984). As the liver slices from the dilute seawater accclimated animals were apparently incubated in full strength saline, it was proposed that the associated cell shrinkage stimulated transport by

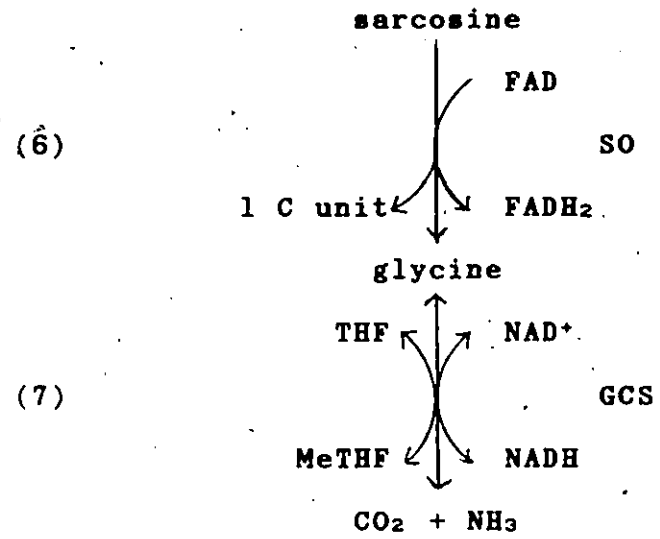
increasing the availability of transporter sites. If the hyperosmotic-induced shrinkage of cells was responsible for the observed stimulation of oxidation, a reduction in oxidation may be expected in response to incubation of liver slices in a hypoosmotic medium. However, the oxidation of BALA by slices from animals acclimated to full strength saline was unaffected by assay medium dilution (King et al., 1980). It is possible that cell swelling under these conditions was restricted by the tissue structure.

Shuttleworth and Goldstein (1984) demonstrated that the kinetics of the BALA transporter of skate hepatocytes were not affected by acclimation osmolarity. As the apparent Michaelis constant (Km_{app}) for transport was similar to the actual plasma concentrations of BALA, it was suggested that the rate of transport would be very sensitive to plasma BALA levels. Acclimation osmolarity also had no effect on the maximal activities of the enzymes catabolizing BALA- BALA transaminase and malonate semialdehyde dehydrogenase (Leech and Goldstein, 1983). The Km_{app}(BALA) for the transaminase was found to be similar to the levels of BALA found in the liver. It was suggested that flux through the oxidative pathway would be very sensitive to liver concentrations of BALA; however, the intracellular localization of the oxidative pathway was not considered. It has been established that the oxidative pathway for BALA occurs mitochondrially in skates (Moyes et al., 1986).

Consequently, the comparison of Km_{app}(BALA) for the transaminase to the BALA concentration in the liver is not appropriate unless it can be demonstrated that the mitochondrial and cytosolic concentrations are similar.

The following mechanism for reduction of BALA levels in the whole animal was proposed by Shuttleworth and Goldstein (1984). Under hypoosmotic stress, BALA is expelled from wing muscle and other tissues unable to oxidize it, causing an elevation of the plasma concentration. Transport of BALA into the liver is enhanced due to increased substrate availability to the transporter. Hepatic oxidation is stimulated, due to increased substrate availability for the transaminase. It has not been established that either the transaminase or transport into the cell are rate-limiting in the oxidation of BALA.

King et al. (1980) studied sarcosine metabolism using radioactive choline, a precursor of sarcosine, and suggested that the liver is the primary site of sarcosine oxidation in skates. In mammals, sarcosine is transported into the mitochondria and oxidized through the mitochondrial enzymes sarcosine oxidase (SO) (6) and the glycine cleavage system (GCS) (7):



The mitochondrial transporter for sarcosine has not been identified. Although sarcosine is a neutral amino acid, methylation of the primary amino group may prevent its binding to the neutral amino acid carrier (Cybulski and Fisher, 1977). Sarcosine oxidase is a membrane bound flavin-linked enzyme in mammals (Hoskins and Mackenzie, 1961). The GCS is a membrane-bound multi-enzyme complex consisting of the pyridoxal phosphate-requiring enzyme glycine decarboxylase, a lipoic acid-containing aminomethyltransferase, a $^{5}N,^{10}N$-methylenetetra- hydrofolate synthesizing protein, and the flavoprotein enzyme, dihydrolipoyl dehydrogenase (reviewed by Hampson et al.,1983). The mitochondrial localization of this pathway permitted the study of sarcosine catabolism polarographically.

i. Experiments on the oxidation of sarcosine and BALA

Preliminary studies demonstrated that the oxidation of sarcosine and BALA by skate liver mitochondria occurs by the same pathway as in mammalian mitochondria. The low rates of oxidation relative to other amino acids prompted the investigation of the effects of assay medium osmolarity on sarcosine and BALA oxidation. It was found that the mitochondrial rate of oxidation of sarcosine but not BALA was very sensitive to the osmolarity of an assay medium composed primarily of urea, TMAO and sucrose. Studies were done to establish whether the observed effect on sarcosine oxidation was due to osmolarity *per se*, osmolarity- induced mitochondrial volume changes (i.e., medium tonicity), or a direct effect of one of the solutes. Studies were also done to establish the sensitive component of the pathway (transport, SO or GCS).

Ballantyne and Storey (1985) found that the oxidation of proline by *Mercenaria mercenaria* ventricle mitochondria was strongly stimulated by assay medium dilution. A slight stimulation of glutamate oxidation was observed in dogfish liver mitochondria under severe osmotic stress (Lewiston et al., 1979), although the integrity of the mitochondria under these conditions was not established. Atsmon and Davis (1967) suggested that a stimulation of succinate oxidation observed with assay medium dilution was due either to increased transport of substrate into the mitochondria, or to an

improved access of substrate to the appropriate dehydrogenases, possibly through minor temporary structural alterations in the inner mitochondrial membrane. In the sarcosine oxidative pathway, each component (transporter, SO and GCS) is associated with the inner membrane such that osmotically-induced membrane alterations could be affecting oxidation. Volume changes could also affect sarcosine oxidation by concentrating or diluting some impermeable perturbing solute or enzyme modulator within the mitochondrial matrix.

The effect of assay medium osmolarity on oxidation of sarcosine could also be due to a specific effect of one of the solutes used to vary osmolarity. Urea is a perturbing solute, in that a high concentration can affect the kinetics of certain enzymes (Yancey et al., 1982). It has been shown that urea is freely permeable to the mitochondria (Ballantyne and Moon, in prep.) and could, therefore, be affecting the mitochondial enzymes directly. As the mitochondrial permeability to TMAO has not been established, any direct effects on the sarcosine oxidation process may be restricted to interactions with the sarcosine transporter.

The oxidation of serine and glycine was examined to establish the sensitive component of sarcosine oxidation. As sarcosine is oxidized through glycine and GCS, the effects of osmolarity on the GCS can be examined directly using exogenous glycine. As this introduces the step of glycine transport,

parallel studies were done using serine, which generates glycine intramitochondrially through SHMT. The fate of the MeTHF produced in GCS (reaction 1) and SHMT (reaction 2) is a complicating factor in this study. If the MeTHF group is released as CO_2, through the MeTHF dehydrogenase (MeTHFDH) initiated pathway, two reducing equivalents are produced which contribute to oxygen consumption by intact mitochondria. These are not produced if the carbon is transferred or released as formaldehyde. A comparison of $^{14}CO_2$ production from $^{14}C(1)$- and $^{14}C(2)$-glycine was required to determine the activity of the MeTHFDH pathway.

CHAPTER 2: MATERIALS AND METHODS

I. ROLE OF GDH IN BIVALVES

Animals

Mantle tissue from soft-shelled clams, *Mya arenaria*, was chosen for this experiment. This tissue may be responsible for the deamination of amino acids expelled from other tissues in hypoosmotically stressed bivalves (Bartberger and Pierce, 1976). The mantle has a high mitochondrial content and is not a gametogenic tissue in *Mya arenaria* ensuring a homogeneous mitochondrial preparation. Animals, 4 - 8 cm shell length, were dug in Brandy Cove, St. Andrews, New Brunswick and held in continuously flowing seawater (935 mOsmoles kg^{-1}) at the Huntsman Marine Laboratory. Water temperatures over the course of the experiment dropped from 14.5°C in September to 7.5°C in November (1984). Uncoupler experiments were performed in June 1985 (water temperature 10.0°C). Animals were held up to 4 weeks before use.

Isolation of clam mitochondria

Mantle tissue from 8 - 15 clams was rapidly excised, blotted dry and placed in 10 ml of ice-cold isolation medium consisting of 400 mM sucrose, 100 mM KCl, 6 mM EGTA (ethyleneglycol-bis-(2-aminoethyl ether) N,N,N',N'-tetraacetic acid), 3 mM EDTA (disodium ethylenediamine tetraacetate), 70 mM HEPES (N-2-hydroxyethylpiperazine-

N-2-ethanesulfonic acid) (pH 7.6 at 20°C), and 1% esssentially fatty acid free bovine serum albumin (BSA). The tissue was homogenized with three passes of a loosely-fitting Potter-Elvehjem tissue grinder. The homogenate was centrifuged for 10 min at 1,150 x g. The resultant supernatant was centrifuged for 10 min at 10,500 x g. The resultant pellet was resuspended in 1-1.5 ml of ice-cold isolation medium unless stated otherwise. Mitochondrial protein was 5-10 mg ml^{-1}.

Mitochondrial oxidation

A small volume of mitochondrial suspension (0.078 - 0.115 ml) was added to 9 volumes of a reaction mixture consisting of 560 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4 , 70 mM HEPES (pH variable), and 1% BSA. The pH of the mixture was measured at the end of the assay. Temperature of the cuvette was held constant at 15°C using a Haake refrigerated water circulator. Oxygen concentration of the assay mixture was monitored using a Clarke type electrode. Respiratory rates were determined as defined by Chance and Williams (1956). State 2 is the rate of oxygen consumption prior to the addition of ADP. State 3 is the rate in the presence of ADP and state 4 is the rate of oxygen consumption after the ADP is exhausted. The respiratory control ratio (RCR) is the ratio of state 3 to state 4 (Estabrook, 1967). ADP and glutamate were at saturating concentrations of 0.1 and 40 mM, respectively.

The effects of the uncoupler of oxidative phosphorylation, CCCP (carbonyl m-chlorophenylhydrazone) on the oxidation of glutamate was examined in the presence and absence of phosphate at pH 7.4. In one experiment, mitochondria incubated in the standard reaction mixture were given ADP, allowed to return to state 4, and then given CCCP. Uncoupler was also given to mitochondria incubated in the standard assay mixture with phosphate and ADP omitted. In both uncoupler experiments, low concentrations of CCCP stimulated respiration. Small volumes (1 - 2 ul) of 10 mM CCCP in 95 % ethanol were added at 5 min intervals until the maximum stimulation of oxygen consumption was achieved. Equal volumes of ethanol only had no effect on these mitochondria.

Mitochondria in state 3 were given aminooxyacetic acid (AOA - 5 mM final concentration) to examine the effects of aminotransferase inhibition on the rate of glutamate oxidation. AOA is permeable to rat mitochondria (Meijer et al., 1972), but the exact intramitochondrial concentration achieved is unknown. The concentration used in this study is approximately 100-fold greater than the I_{50} found for GOT and GPT of *Modiolus demissus* heart (Greenwalt and Bishop, 1980). Although preincubation with AOA is the usual application, preliminary experiments established that complete inhibition of GOT and GPT from *M. arenaria* mantle mitochondria was achieved using 0.1 mM AOA, 10 min after its addition to a complete assay mixture. Higher concentrations

(1.0 mM) inhibited these enzymes immediately. Since the reduction in oxygen consumption by intact mitochondria was immediate and linear until returning to state 4, it can be assumed that AOA completely inhibited the enzyme *in situ*.

Electron transport rate

The maximum rate of electron transport was determined in mitochondria broken by a combination of osmotic swelling and sonic disruption. The mitochondrial pellet was resuspended in ice-cold medium of low osmolarity (10 mM HEPES, pH 7.6 at 20°C) and sonicated while chilled, using a Branson Sonifier (model 350) microtip at level 4 for 4 sec. Oxygen uptake was measured as with intact mitochondria, using 0.2 mM NADH (saturating) as substrate. Mitochondria prepared in this way gave higher oxygen uptake than if broken by Polytron treatment, repeated freezing and thawing cycles, treatment with toluene (Wadano et al., 1984) or detergents (CHAPS - (3((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) or Triton X-100), or exposure to either osmotic shock or sonication alone.

Enzyme assays

Mitochondrial pellets were prepared as described above, resuspended in ice-cold 100 mM TAPS (tris (hydroxymethyl) methylaminopropane sulfonic acid) (pH 7.6 at 20°C) and broken using a Brinkman polytron (10 sec burst at setting 7). In an experiment measuring the activity of GDH in the absence of free nucleotides, this solution was dialyzed

16 hr at 5°C in 50 volumes of 100 mM TAPS (pH 7.6 at 20°C) with 3 changes of solution and no loss of activity at pH 8.5 in the presence of 1 mM ADP. All enzymes were assayed at 15°C by monitoring changes in absorbance at 340 nm with an LKB Ultraspec 4050 equipped with a refrigerated cell holder. The final volume of the assay mixture was 1.0 ml. Assay pH was measured after the reaction. The substrate concentrations employed were found to be saturating. Where coupling enzymes were used, no contaminating transaminase activity could be detected in assays with only the sample omitted.

Glutamate dehydrogenase (E.C. 1.4.1.3): forward (deaminating) 0.3 mM NAD^+, 40 mM glutamate, 1.0 mM ADP in 100 mM TAPS (pH variable); reverse (aminating) 0.15 mM NADH, 20 mM 2-oxoglutarate, 100 mM ammonium acetate, 1.0 mM ADP in 100 mM TAPS (pH variable). Assays were also done in the forward direction to determine the activity in the absence of ADP. In this case dialyzed enzyme was used, ADP was omitted from the reaction mixture and 2.0 mM NAD^+ was required to saturate the enzyme.

Glutamate-oxaloacetate transaminase (E.C. 2.6.1.1): 10 mM 2-oxoglutarate, 25 mM L-aspartate, 0.3 mM NADH, 0.05 mM pyridoxal-5-phosphate, 0.5 units malate dehydrogenase in 100 mM TAPS (pH variable).

Glutamate-pyruvate transaminase (E.C. 2.6.1.2): 10 mM 2-oxoglutarate, 100 mM L-alanine, 0.3 mM NADH, 0.05 mM

pyridoxal-5-phosphate, 0.5 units lactate dehydrogenase in 100 mM TAPS (pH variable).

II. AMINO ACID OXIDATION IN SKATES

Animals

Little skates, *Raja erinacea*, were captured locally by bottom trawl and were distinguished from the closely related species *R. ocellata* by tooth-row count, using the criteria of McEachran and Musick (1973). Animals (20 to 50 cm total length; both sexes) were held in continuously flowing seawater (920 mOsmoles kg^{-1}) at seasonal temperatures (8-14°C) for up to 2 weeks before use.

Isolation of mitochondria

Skates were double pithed and liver tissue (2 - 4 g) from a single animal was rapidly excised, chopped, blotted dry and placed in 10 ml of ice-cold isolation medium containing 800 mM sucrose and 1% BSA in 30 mM HEPES (pH 7.2 at 20°C). The tissue was homogenized with three passes of a Potter-Elvehjem tissue grinder with a loosely-fitting teflon pestle. The homogenate was centrifuged for 10 min at 600 x g. The supernatant was collected by Pasteur pipet from between the pellet, consisting mainly of red blood cells, and the surface layer, consisting of unbroken liver cells and lipid, and re-centrifuged for 10 min at 11,000 x g. The fluffy light-coloured layer of mitochondria was separated from a

dense dark-coloured portion of the pellet and resuspended in 12 ml of ice-cold isolation medium. This solution was centrifuged for 10 min at 11,000 x g and the resultant pellet resuspended in 1-2 ml of the same medium. Mitochondrial protein concentration was 8-30 mg ml^{-1}. This isolation procedure yields mitochondria, with high respiratory control ratios (e.g., RCR with isocitrate = 9.9; Moyes et al., 1986).

Mitochondrial oxidation

One volume of mitochondrial suspension (0.078 - 0.115 ml) was added to 9 volumes of reaction medium. The standard reaction medium consisted of 370 mM urea, 185 mM TMAO, 46.25 mM sucrose, 140 mM KCl, 10 mM KH_2PO_4, 1% BSA and 30 mM HEPES (pH 7.2 at 20°C). The urea concentration used here approximates the physiological concentration (King et al., 1980). A 2:1 ratio of urea:methylamines (e.g., TMAO, betaine, sarcosine) is thought to be important in preventing the adverse effects of urea (Yancey et al., 1982). TMAO was used exclusively, as it was not oxidized by these mitochondria and, therefore, did not elevate the endogenous rate of oxidation. The osmolarity of the assay mixture was varied by changing the concentration of urea, TMAO and sucrose, keeping the ratio of concentrations constant at 8:4:1. Unless stated otherwise, the concentration of KCl was kept constant. Temperature of the cuvette was held at 10°C using a Haake refrigerated water circulator. Oxygen consumption of the assay mixture was monitored using a Clarke-type electrode.

Respiratory states 3 (ADP and substrate present) and 4 (after ADP is exhausted) were determined (Chance and Williams, 1956). Endogenous rates of oxidation were determined in the presence of ADP but no substrate. Where sparkers were used, these were included in the assay for the endogenous rate. The oxidation rate is the state 3 rate minus the endogenous rate. Respiratory control ratios (RCR) were calculated as outlined by Estabrook (1967). Substrate concentrations employed were found to be saturating unless stated otherwise.

Inhibitor studies

The effects of rotenone (5 uM), malonate (5 mM) and sodium arsenite (5 mM) on the mitochondrial oxidation of several substrates were examined. Rotenone inhibits NADH-linked enzymes, but not flavin-linked enzymes. Malonate, through its effect on succinate dehydrogenase, inhibits oxidation of Krebs cycle intermediates and other substrates oxidized through this pathway. Arsenite inhibits lipoamide-dependent dehydrogenases such as the GCS. Mitochondrial preparations were given ADP, to determine the endogenous rate, then given substrate, to determine the state 3 rate. While in state 3, the inhibitor was added to the cuvette to determine the inhibited rate. In each case, the response to the inhibitor was immediate and linear and could not be increased by increasing the concentration of inhibitor. Parallel assays were done to determine the inhibitor-sensitive portion of the endogenous rate. Inhibition is expressed as

per cent of the control rate and is adjusted for effects of the inhibitor on the endogenous rate.

Oxidation of ^{14}C- amino acids by intact mitochondria

Mitochondria (0.1 ml) were added to a 25 ml serum vial containing 0.7 ml of reaction medium which included an ADP regenerating system consisting of 20 units hexokinase and 10 mM glucose. The serum vial was then capped with a rubber stopper fitted with a well containing a glass fiber filter disc (Whatman GF/A). After a 10 min preincubation at 10°C, 0.2 ml of reaction medium containing ADP (0.2 mM final concentration), cold amino acid (5 mM glycine; 10 mM serine) and ^{14}C amino acid (0.1 uCi ^{14}C-glycine; 0.02 uCi ^{14}C-serine) were injected through the cap. The vials were shaken continuously and maintained at 10°C for 60 min. Immediately prior to termination, hyamine hydroxide (150 ul) was injected through the cap into the well to collect CO_2. The reaction was terminated by injection of 150 ul of 35% perchloric acid. After a further 90 min of shaking at 10°C, the filter discs were removed and counted directly in 5 ml of a scintilation cocktail containing 400 ml toluene, 100 ml ethanol (95%), 1.0 g PPO and 0.05 g POPOP (Mommsen et al., 1983). Background radioactivity was estimated by adding perchloric acid to the mitochondrial incubate prior to addition of the ^{14}C amino acid. In vials without mitochondria, radioactivity was at background. All assays were performed in duplicate.

Hepatocyte preparation

Whole livers were removed from double pithed skates and placed on an iced watch glass. Polyethylene cannulas (PE 50) were inserted into each of two branchs of the hepatic portal vein. The following solutions were used in the hepatocyte isolation; solution 1 - 350 mM urea, 281 mM NaCl, 6 mM KCl, 0.5 mM $MgSO_4$, 2.5 mM $MgCl_2$, 1 mM NaH_2PO_4, 8 mM $NaHCO_3$, 5 mM HEPES; solution 2 - as solution 1 with 1mM $CaCl_2$ and 1% BSA; solution 3 - 241 mM NaCl, 80 mM TMAO with the remaining components as in solution 2. All solutions were bubbled with 99.5% O_2:0.5% CO_2 prior to addition of $CaCl_2$, bicarbonate and BSA, then the pH was adjusted to 7.6 at 20°C. The liver was perfused (3.0 ml min^{-1} through each cannula) with solution 1 until the blood was cleared (less than 20 min), then perfused with solution 1 containing Type IV collagenase (100 units ml^{-1}) for a further 10 - 20 min. The liver was chopped with razor blades and passed through nylon filters (400 um followed by 150 um) washing each filter with solution 1. The filtrate was centrifuged in 40 ml polycarbonate tubes at 170 x g for 90 sec. The cells were washed twice with solution 1, once with solution 2, then washed once and resuspended in solution 3.

Effects of osmolarity on hepatocytes

A small volume of the hepatocyte suspension was added to 9 volumes of skate saline of variable osmolarity but

constant pH (7.6 at 20°C). Osmolarity was varied by changing concentrations of all components of solution 3 except phosphate, HEPES and bicarbonate. Oxygen uptake of the solution was measured at 10°C using the apparatus described previously for the mitochondrial experiments. Stimulation of respiration in response to the addition of sarcosine was determined in the absence and presence of rotenone. Hepatocyte integrity at each osmolarity was assessed by measuring leakage of malate dehydrogenase (MDH). Activity of MDH was greater than other commonly used marker enzymes (LDH, GPT, GOT). After incubation of the hepatocytes at their test osmolarity, the incubate was centrifuged at 15,000 x g and MDH activity determined (Moon, 1981) in the pellet and supernatant. Hepatocyte volume was determined at three osmolarities using microhematocrit tubes spun for 10 min.

Protein determination

The protein concentration of clam and skate mitochondrial suspensions was measured using a modified biuret procedure, adding 10% deoxycholate (pH 13) to solubilize mitochondrial protein (Gornall et al., 1949). BSA was used as the standard. A biuret assay was also done on the medium used to resuspend the mitochondrial pellet. This value was subtracted from that of the mitochondrial suspension to determine mitochondrial protein.

Chemicals

All biochemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo) or Boehringer-Mannheim, Canada. Other chemicals and scintilation cocktails were purchased from Fisher Scientific Co., Canada. Radioisotopes and hyamine hydroxide were purchased from New England Nuclear Co. (Lachine, Que).

CHAPTER 3; THE ROLE OF BIVALVE GDH

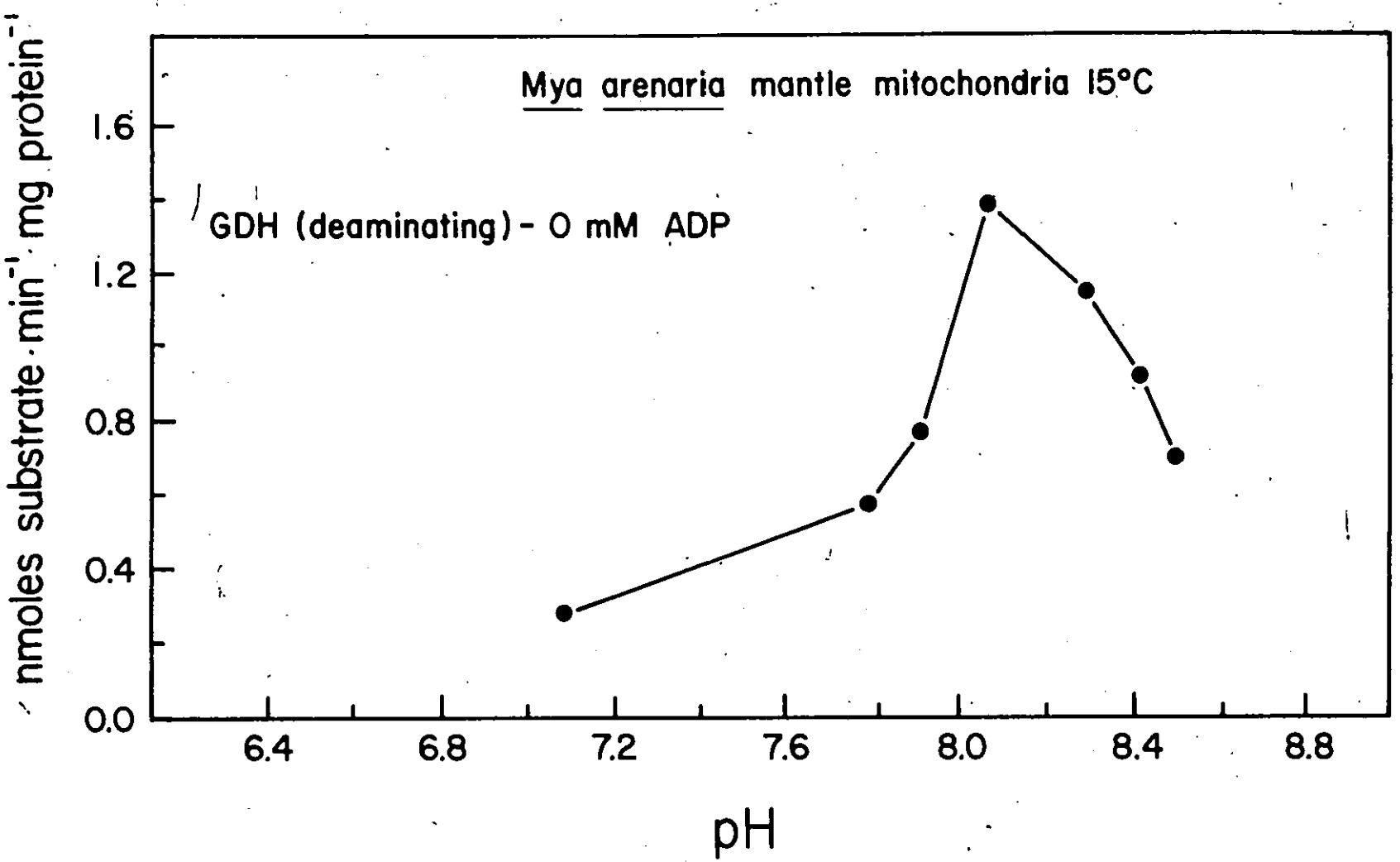
The concentrations of intracellular free amino acids in bivalve molluscs decrease in response to hypoosmotic stress (Gilles, 1972; Virkar and Webb, 1972). As excretion of intact amino acids is low (Livingstone et al., 1979) and an increase in ammoniagenesis is observed under these conditions (Strange and Crowe, 1979), it is clear that at least part of the amino acid pool is deaminated during hypoosmotic stress. The biochemical pathways important in the deamination of amino acids have not been identified for any osmotically active amino acid except glycine (Ellis et al., 1985). While GDH functions in this role in most animal tissues (Lehninger, 1976) its activity in bivalve tissues is extremely low relative to mammals (deRosa and Swick, 1975) and cephalopod molluscs (Storey et al., 1978). This study examines the capacity of GDH in mantle mitochondria of the bivalve mollusc *Mya arenaria* to ascertain the role of this enzyme in amino acid deamination in this tissue.

RESULTS

The pH profile for GDH (deaminating) in the absence of ADP is presented in Figure 1. The maximal enzyme activity was 1.4 nmoles NADH min^{-1} (mg protein)$^{-1}$ at pH 8.05. Addition of ADP caused a shift in the pH optimum from 8.1 to 8.6 and at their respective pH optima, the activated enzyme

Figure 1: The activity of GDH (deaminating) from *Mya arenearia* mantle mitochondria in the absence of ADP, in relation to assay pH.

Assay conditions are described in Materials and Methods. The concentration of all substrates was saturating. Values at each pH are means of 2 determinations.


Mya arenaria mantle mitochondria 15°C
GDH (deaminating) - 0 mM ADP
nmoles substrate·min⁻¹·mg protein⁻¹
1.6
1.2
0.8
0.4
0.0
6.4
6.8
7.2
7.6
8.0
8.4
8.8
pH

had 7-fold greater activity (Fig. 2). The pH optimum for the activated enzyme (+ADP) in the reverse direction (aminating) was 0.5 units more acidic than the forward direction (deaminating). Activity in the aminating direction was greater than in the deaminating direction at all pH values tested. At the pH optimum for the reverse direction (8.1), the activity was about 10-times greater. The activity was 3.8-times greater at the pH optimum for the deaminating direction (8.6) and 5.2-times greater at their respective pH optima.

The activities of the mitochondrial aminotransferases are also presented in Figure 2. The pH optimum for GOT (6.9) was much lower than that of GPT (7.7) or of GDH in either direction. GOT had about twice the activity of GPT and 4-times the activity of GDH (deaminating), at their respective pH optima.

The pH optima for the RCR and for the state 3 rate of mantle mitochondria oxidizing glutamate were between 7.0 and 7.4 (Fig. 3). The maximum state 3 rate achieved was 5.8 nmoles O min^{-1} (mg protein)$^{-1}$ at pH 7.2. State 4 rates were low and constant except for an increase at the lowest pH (Fig. 3).

The addition of CCCP to mitochondria incubated in the presence of phosphate stimulated the state 4 respiration by 2.7-fold (Table 1). In the absence of phosphate, CCCP stimulated the state 2 rate of oxygen consumption by 3.9-fold.

Figure 2: The activity of GDH (deaminating), GDH (aminating), GOT and GPT from *Mya arenaria* mantle mitochondria in relation to assay pH.

Assay conditions are described in Materials and Methods. The concentration of all substrates was saturating. Assays for both directions of GDH were done in the presence of 1.0 mM ADP. Values for GDH are means $\pm$ SE for 3 determinations. Values for GOT and GPT are means of 2 determinations. Assays were done in duplicate at each pH.

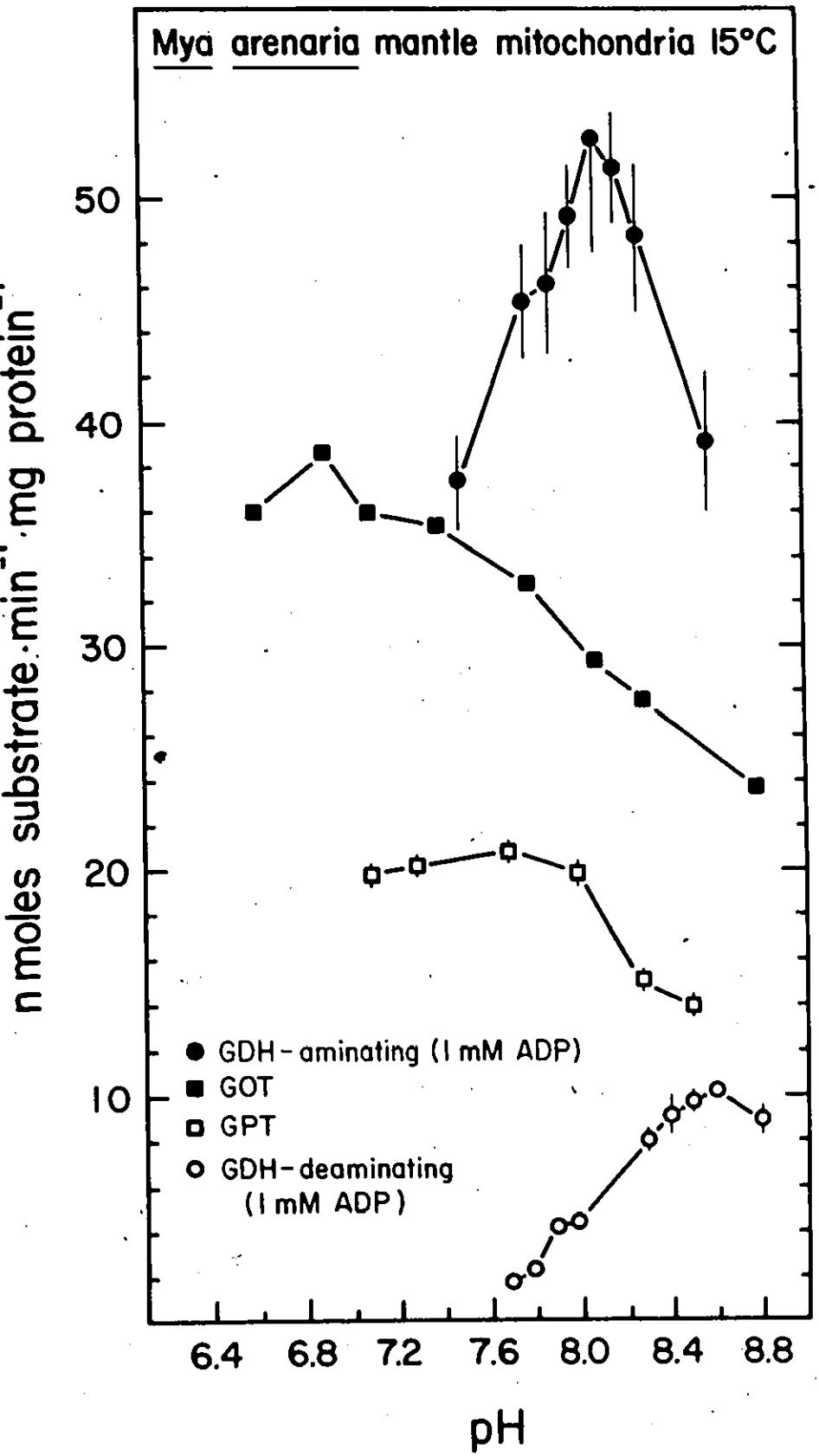
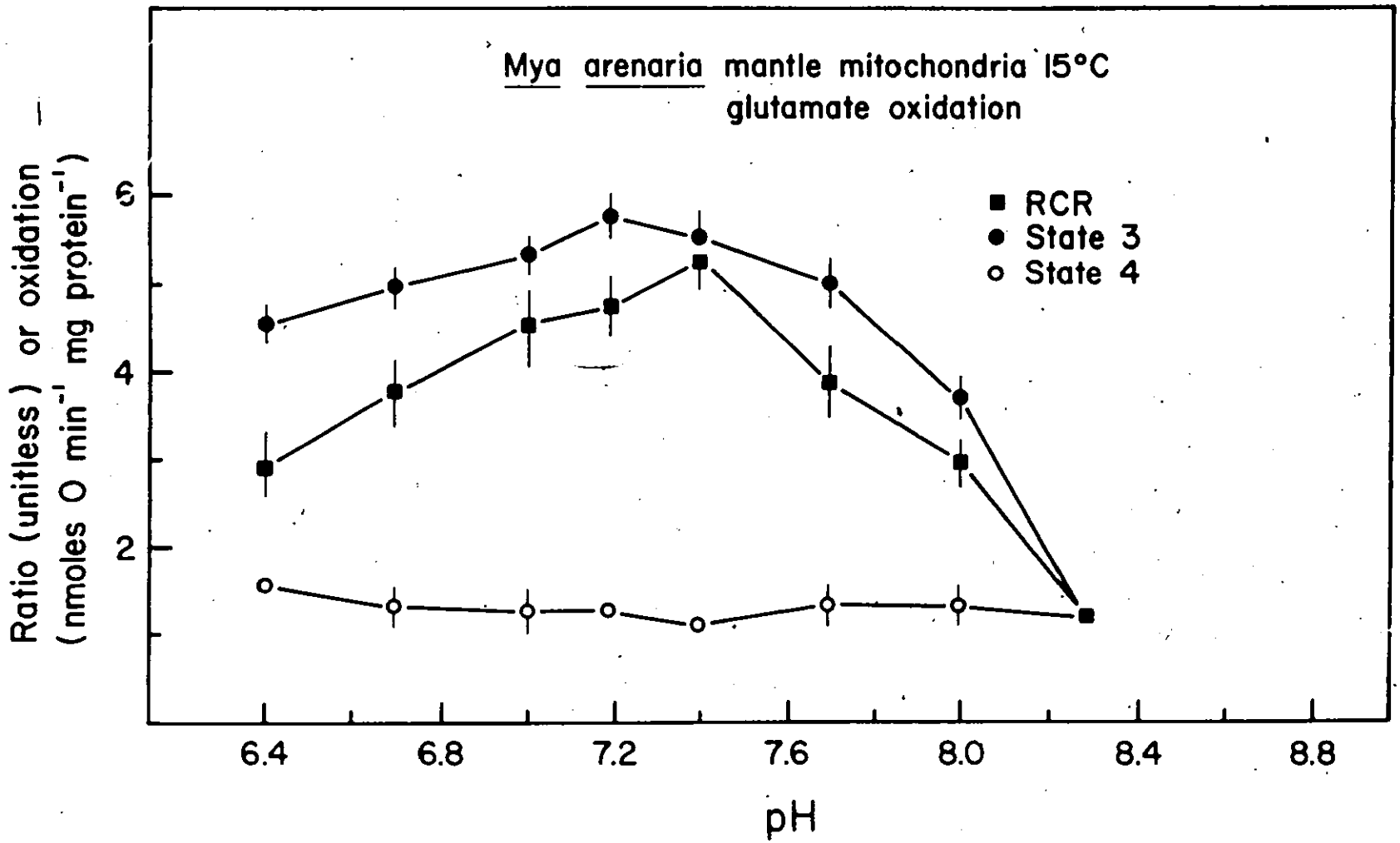

Mya arenaria mantle mitochondria 15°C
n moles substrate·min⁻¹·mg protein⁻¹
50
40
30
20
10
6.4
6.8
7.2
7.6
8.0
8.4
8.8
pH
GDH - aminating (1 mM ADP)
GOT
GPT
GDH - deaminating (1 mM ADP)

Figure 3: The RCR, state 3 and state 4 rates of glutamate oxidation for mitochondria from *Mya arenaria* mantle, in relation to pH.

Assay conditions are described in Materials and Methods. The glutamate concentration was 40 mM; ADP was 0.1 mM. Values are means ± SE for 4-8 determinations. Ordinate values are both rates and RCR (unitless).


Mya arenaria mantle mitochondria 15°C
glutamate oxidation
RCR
State 3
State 4
Ratio (unitless) or oxidation
(nmoles O min⁻¹ mg protein⁻¹)
6
4
2
6.4
6.8
7.2
7.6
8.0
8.4
8.8
pH

Glutamate oxidation by intact mitochondria was insensitive to AOA in state 3 at pH 7.4 or 8.2 (Table 2). At the lowest pH (6.7), there was a decrease in oxygen consumption (45.4% ± 4.1 SE) that was immediate and linear until all ADP was exhausted. This value is not significantly different from the inhibition obtained in preliminary experiments where AOA was added 10 min prior to the addition of glutamate.

The rate of electron transport is given in Figure 4. The NADH oxidation rate for sonicated mitochondria at any pH was no greater than any state 3 rate over the pH range 6.4 to 7.7. Above pH 7.7, where state 3 rates decreased, the electron transport rate remained high resulting in a broad pH profile.

DISCUSSION

The activities of glutamate dehydrogenase in *M. arenaria* mantle are low in comparison to vertebrate tissues, as previously reported for other bivalves (Bishop et al., 1983). Although the whole tissue levels of GPT and GOT are high in *M. arenaria* (Dupaul and Webb, 1974), the mitochondrial activities are similar to GDH (deaminating) (Fig. 2). Burcham et al. (1983) made a similar observation for the mitochondrial enzymes of oyster. However, in their study, GDH activity was measured at a single pH and only in the glutamate forming direction, which has been shown to be

TABLE 1

Glutamate oxidation in *Mya arenaria* mantle mitochondria as affected by CCCP.

Conditions	Rate +CCCP/Rate -CCCP*
10 mM phosphate	2.7 (0.4)
no phosphate	3.9 (0.7)

* mean (SE); n=4

TABLE 2

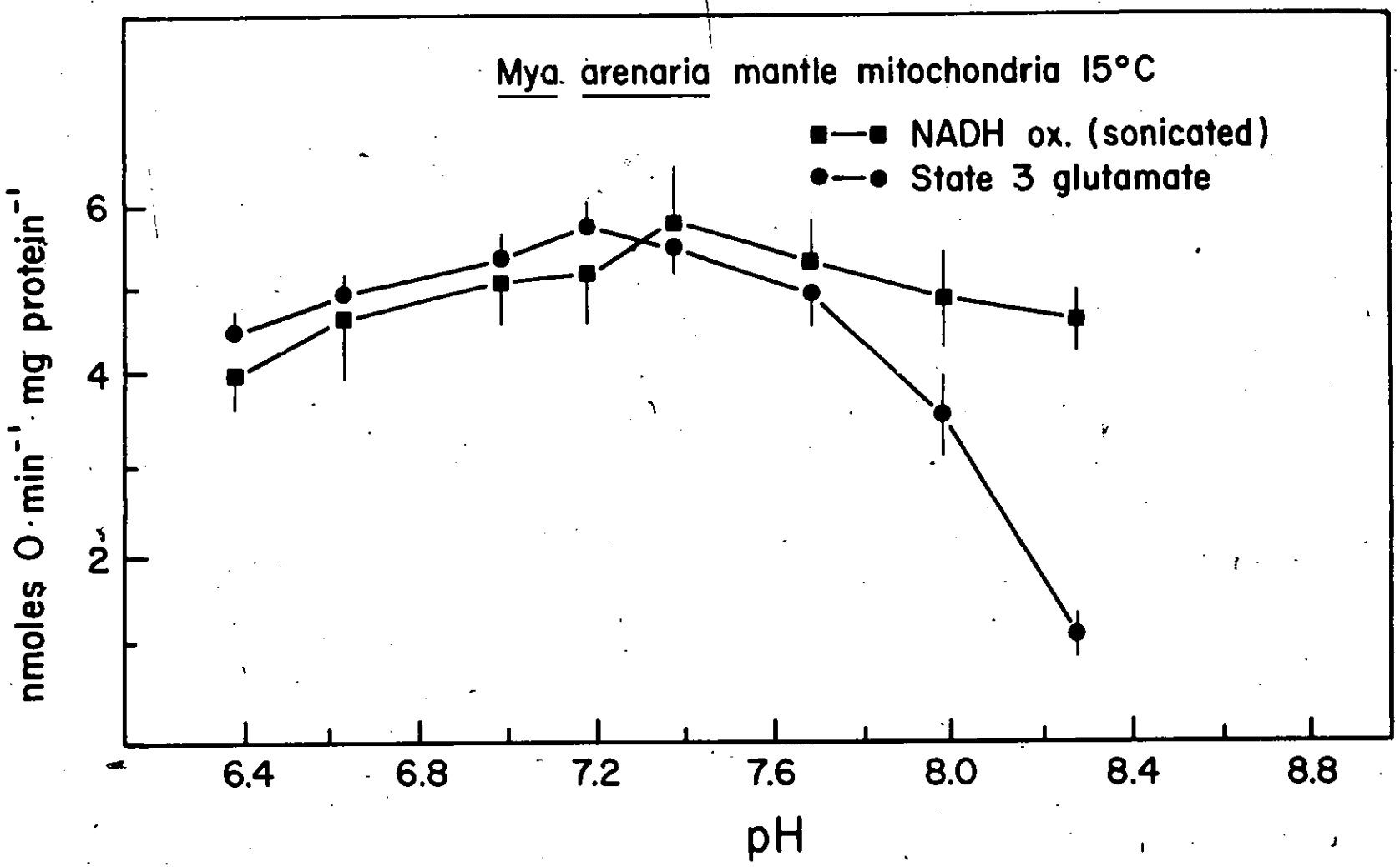
Glutamate oxidation in mitochondria of *Mya arenaria* mantle as affected by 5 mM aminooxyacetic acid (AOA).

	REACTION pH		
RATE	6.7	7.4	8.2
State 3*	5.38 (.26)	5.23 (.35)	2.87 (.09)
State 3 + AOA	3.49 (.23)	5.12 (.37)	3.08 (.14)
% change	-45.4 (4.1)	-3.7 (4.2)	+5.2 (4.9)

* nmoles O min^{-1} (mg protein)$^{-1}$; mean (SE), n=5

Figure 4: The maximum rate of the electron transport system in broken mitochondria from *Mya arenaria* mantle, in relation to pH.

Assay methods are described in Materials and Methods. The maximum rate of electron transport was estimated by measuring the rate of oxidation of a saturating concentration of NADH (0.2 mM) in mitochondria broken by a combination of osmotic shock and sonic disruption. Values are means ± SE for 5 determinations.


Mya. arenaria mantle mitochondria 15°C
NADH ox. (sonicated)
State 3 glutamate
nmoles O·min⁻¹·mg protein⁻¹
6
4
2
6.4
6.8
7.2
7.6
8.0
8.4
8.8
pH

favoured in *M. arenaria* when assayed using saturating substrate concentrations. Based on relative activities of GDH and the aminotransferases in this study, a physiological role for GDH in glutamate catabolism can be postulated.

It is difficult to make valid conclusions regarding the role of GDH *in vivo* based only on enzyme levels determined in *in vitro* assays, where all conditions are optimized. For instance, the effects of ADP on GDH (deaminating) are dramatic *in vitro* (a 15-fold stimulation and a 0.5 unit shift in pH optimum), but it is not known whether ADP concentration is of any regulatory significance *in vivo*. Burcham et al. (1983) suggested that the enzyme would be very responsive to ADP concentrations. However, Reiss et al. (1977) compared GDH K_a(ADP) values to whole tissue ADP levels (Wijsman, 1976) and concluded that ADP concentrations were adequate to maintain GDH fully activated *in vivo*. In rat liver, mitochondria ADP levels are considerably higher than cytoplasmic levels (Soboll et al., 1976). Although ADP concentrations in the mitochondria may be higher than those which would be required to fully activate GDH, Addink and Veenhof (1975) have shown that ATP concentrations can affect the ADP·activation of GDH from *Mytilus edulis*. Mitochondrial substrate concentrations and the physiological significance of ADP in relation to other purine nucleotide regulators of GDH activity must be known before accurate conclusions about the role of GDH can be made

based only on *in vitro* enzyme levels. Many of the problems associated with the interpretation of *in vitro* levels can be avoided by examining glutamate oxidation in well-coupled, intact mitochondria, a preparation more like the *in vivo* situation.

Comparison of the state 3 oxygen consumption rates (rate in the presence of ADP) of intact mitochondria (Fig. 3) to the *in vitro* activity of GDH (Fig. 2) confirms that the enzyme could have a physiologically important role in glutamate metabolism. Under steady state conditions, oxygen consumption of 1 nmole O min^{-1} (mg protein)$^{-1}$ occurs in response to the mitochondrial production of 1 nmole NADH (or other reducing equivalent) min^{-1} (mg protein)$^{-1}$, with subsequent oxidation in the electron transport system. Intact mitochondria oxidizing glutamate can produce reducing equivalents in several reactions. If an aminotransferase catalyzes the conversion of glutamate to 2-oxoglutarate, reducing equivalents are produced only by the subsequent oxidation of the 2-oxoglutarate in the Krebs cycle (see Chapter 1). An additional NADH is produced if the 2-oxoglutarate is produced through GDH rather than an aminotransferase. Intact mitochondria in this experiment produce a maximum of 6 nmoles reducing eqiuvalents min^{-1} (mg protein)$^{-1}$ (Fig. 3). GDH alone is capable of producing up to 10 nmoles NADH min^{-1} (mg protein)$^{-1}$ (Fig. 2). The actual maximal GDH actvity corresponding to any

particular reaction pH is not known because the transmembrane pH gradient is unknown. GDH capacity in intact mitochondria can be estimated by examining the effects of AOA on glutamate oxidation. AOA is an aminotransferase inhibitor that is permeable to the mitochondrial membrane (Meijer et al., 1972).

The addition of AOA to mitochondria inhibits aminotransferase activities, forcing all glutamate to be oxidized through GDH. At pH 7.4, AOA had no effect on the state 3 oxidation rate (Table 2). This pH is in the range of the optima for the RCR and the state 3 rate (7.0 - 7.4) and close to the intracellular pH measured in whole mantle tissue from *Mytilus edulis* (7.15) (Walsh et al., 1984). At this pH (7.4) there is clearly enough GDH activity to maintain the state 3 rate of glutamate oxidation when the aminotransferases are inhibited. In fact, glutamate oxidation in intact mitochondria at this pH is limited not by GDH, but by the electron transport system (Fig. 4). The rate of NADH oxidation by broken mitochondria is no greater than the state 3 rate at pH 7.4, with or without AOA. Only at the lower pH (6.7) was there insufficient GDH activity to sustain the state 3 rate when the aminotransferases were inhibited (Table 2). This is not surprising given the narrow pH profile and alkaline pH optimum for the enzyme *in vitro* (Fig. 2). Glutamate oxidation in intact mitochondria in the absence of AOA appears to be limited by the electron transport system. This does not appear to be the case at pH 8.3, where state 3

rates are well below the rate of electron transport. At a high intracellular pH, glutamate transport may be rate limiting.

The effects of uncouplers on mitochondria are complex. Although they reduce the pH gradient and affect the redox state (LaNoue et al., 1983), they also reduce the membrane potential (LaNoue and Schoolwerth, 1979). The reduction of the membrane potential in uncoupler-treated rat mitochondria reduces aspartate efflux on the glutamate/aspartate antiport causing aspartate levels to rise, reducing the activity of GOT in the glutamate transaminating direction. In mitochondria with high GDH levels, such as rat liver, inhibition of GOT does not reduce glutamate oxidation. Mitochondria of rat heart, unlike liver, have relatively low GDH activities and demonstrate reduced glutamate oxidation when GOT is inhibited indirectly by uncouplers (LaNoue et al., 1974). In the present study, CCCP stimulated the state 4 oxidation rate in the presence of 10 mM phosphate (Table 1). LaNoue et al. (1974) however, have shown that 10 mM phosphate can protect the glutamate/aspartate exchanger against inhibition by uncouplers. In this study, CCCP also stimulated oxidation of glutamate in mitochondria in state 2 incubated in the absence of phosphate (Table 1). The response of *Mya arenaria* mantle mitochondria to the uncoupler is analagous to the situation in rat liver mitochondria, which have relatively high GDH levels.

In summary, based on comparison of state 3 rates to *in vitro* enzyme activities and the response of intact mitochondria to AOA, *M. arenaria* mantle GDH activities are adequate to catalyze all glutamate oxidation over a wide pH range. This does not imply that all glutamate is catabolized through GDH under any given set of metabolic conditions, including hypoosmotic stress,. Given the proper intracellular milieu, however, the potential exists for all glutamate catabolism to occur through GDH. Under conditions such as hypoosmotic stress, adenylate nucleotide concentrations or redox balance may change, changing the flux through GDH. Mitochondrial pH may also be involved in the partitioning of glutamate oxidation through GDH and the aminotransferases. Activation of GDH by ADP causes a shift in the pH optimum, an observation previously unreported for bivalve GDH. Also, pH would affect the relative activities of the enzymes competing for mitochondrial glutamate. A high mitochondrial pH would favour GDH (deaminating), but reduce the activity of GOT, GPT and GDH (aminating). GDH probably plays a critical role in adjusting the amino acid concentrations in response to osmotic stress. The effect of exposure to anisosmotic environments on intracellular or intramitochondrial pH has not been examined. Under hyperosmotic stress, biochemical pathways resembling those involved in anaerobic metabolism are involved in elevating the intracellular osmolarity (Zurburg and deZwaan, 1981). If anaerobically-induced increases in ADP/ATP ratios

reported in *Mytilus edulis* (Addink and Veenhof, 1975; Wijsman, 1976) occur with hyperosmotic stress, GDH may be stimulated (Zurburg and deZwaan, 1981). However, the *in vivo* role of ADP and other purine nucleotides has not been established. If a decrease in internal pH accompanies hyperosmotic stress as in true anaerobic metabolism (Walsh et al., 1984), the effects on GDH would favour ammonia fixation and would reduce the catabolism of glutamate and other amino acids.

CHAPTER 4: SKATE AMINO ACID OXIDATION

Sarcosine and BALA are the two most abundant amino acid osmolytes in skate tissues, each at a concentration of approximately 40 mM in wing muscle. Exposing skates to 50% seawater leads to a 50% reduction in the levels of these osmolytes (Boyd et al., 1977). Under these conditions, no excretion or *in situ* oxidation of either BALA or sarcosine occurs (King et al., 1980). It has been suggested that enhanced hepatic oxidation is responsible for the reduction in the whole animal levels of BALA and sarcosine (Goldstein, 1981). In the case of BALA, enhanced oxidation may be due to increased plasma concentrations, as BALA is expelled from the wing muscle and other tissues which are unable to oxidize it (Goldstein, 1981). The mechanism responsible for stimulating sarcosine oxidation has not been identified and is examined in this study. Preliminary studies demonstrated that the oxidation of sarcosine, but not BALA, in liver mitochondria is very sensitive to assay medium osmolarity. In this study, the mechanism by which osmolarity affects the mitochondrial oxidation of sarcosine is examined. Also, the sensitive component of the oxidative pathway is identified.

RESULTS

Table 3 summarizes the oxidation rates of amino acids by isolated skate liver mitochondria. The preferred amino acid substrate was glutamate (in the presence of pyruvate) based on both RCR and oxidation rate. All other amino acids examined were oxidized at rates less than 4 nmoles O min^{-1} (mg protein)$^{-1}$. Of the amino acids thought to be transported on the mitochondrial neutral amino acid carrier (Cybulski and Fisher, 1977), the following were oxidized at detectable rates; L-alanine, L-serine, BALA, glycine and L-proline. Although the other amino acids thought to be transported on this carrier were not oxidized at detectable levels (Table 3), they may have been transported without subsequent oxidation. Both of the important osmotically-active amino acids were oxidized, although sarcosine was oxidized at about half the rate of BALA.

The effects of osmolarity on the rate of oxidation of BALA are presented in Figure 5. With KCl in the reaction medium kept constant at 140 mM, and osmolarity varied in the standard manner (urea, TMAO and sucrose varied but kept in constant proportion), the oxidation of BALA was found to be insensitive to osmolarity over the range of 380 - 1270 mOsmoles l^{-1}.

Oxidation of sarcosine by isolated mitochondria was strongly affected by assay medium osmolarity (Fig. 6). It was stimulated by assay medium dilution and inhibited by assay

TABLE 3

Oxidation of amino acids by liver mitochondria of *Raja erinacea**

SUBSTRATE	mM	n	STATE 3 **	STATE 4 **	R.C.R	OXIDATION **
glutamate	20	14	12.50 (1.16)	1.58 (0.09)	7.97 (0.60)	10.49 (0.94)
glutamine	3	7	4.63 (1.13)	1.30 (0.10)	3.59 (0.81)	2.37 (0.90)
GABA	2.5	6	6.98 (0.65)	2.14 (0.28)	3.45 (0.34)	3.09 (0.47)
aspartate	10	6	3.65 (0.58)	1.40 (0.13)	2.68 (0.42)	1.15 (0.49)
alanine	10	5	5.96 (0.97)	1.52 (0.17)	3.90 (0.38)	2.80 (0.69)
proline	5	6	4.81 (0.91)	1.44 (0.12)	3.49 (0.80)	2.28 (1.14)
BALA	10	4	7.30 (1.73)	1.91 (0.09)	3.78 (0.82)	2.99 (0.97)
serine	10	7	5.48 (0.68)	1.54 (0.13)	3.51 (0.24)	2.90 (0.76)
sarcosine	10	5	6.40 (1.80)	1.92 (0.13)	3.27 (0.82)	1.47 (0.55)
DMG	2.5	5	5.31 (0.94)	2.11 (0.13)	2.59 (0.45)	3.80 (0.25)

abbreviations: GABA- γ-aminobutyric acid; BALA-β-alanine; DMG- dimethylglycine
* medium osmolarity = 960 mOsmoles l^{-1}: the following amino acids and derivatives were not oxidized at detectable rates; L-threonine, L-methionine, L-valine, L-leucine, L-isoleucine, L-asparagine, L-lysine, L-arginine, L-ornithine, L-citrulline, glyoxylate, trimethylamine.
** nmoles O min^{-1} (mg protein)$^{-1}$; mean (SE)

Figure 5: Oxidation of BALA by *Raja erinacea* liver mitochondria in relation to assay medium osmolarity.

Assay methods are described in the Materials and Methods. BALA oxidation was determined polarographically using 10 mM BALA. The KCl concentration in the reaction medium was kept constant at 140 mM. Osmolarity was varied by altering the concentration of urea, TMAO and sucrose, keeping the ratio of these components constant at 8:4:1. Values are means ± SE of 6 determinations.

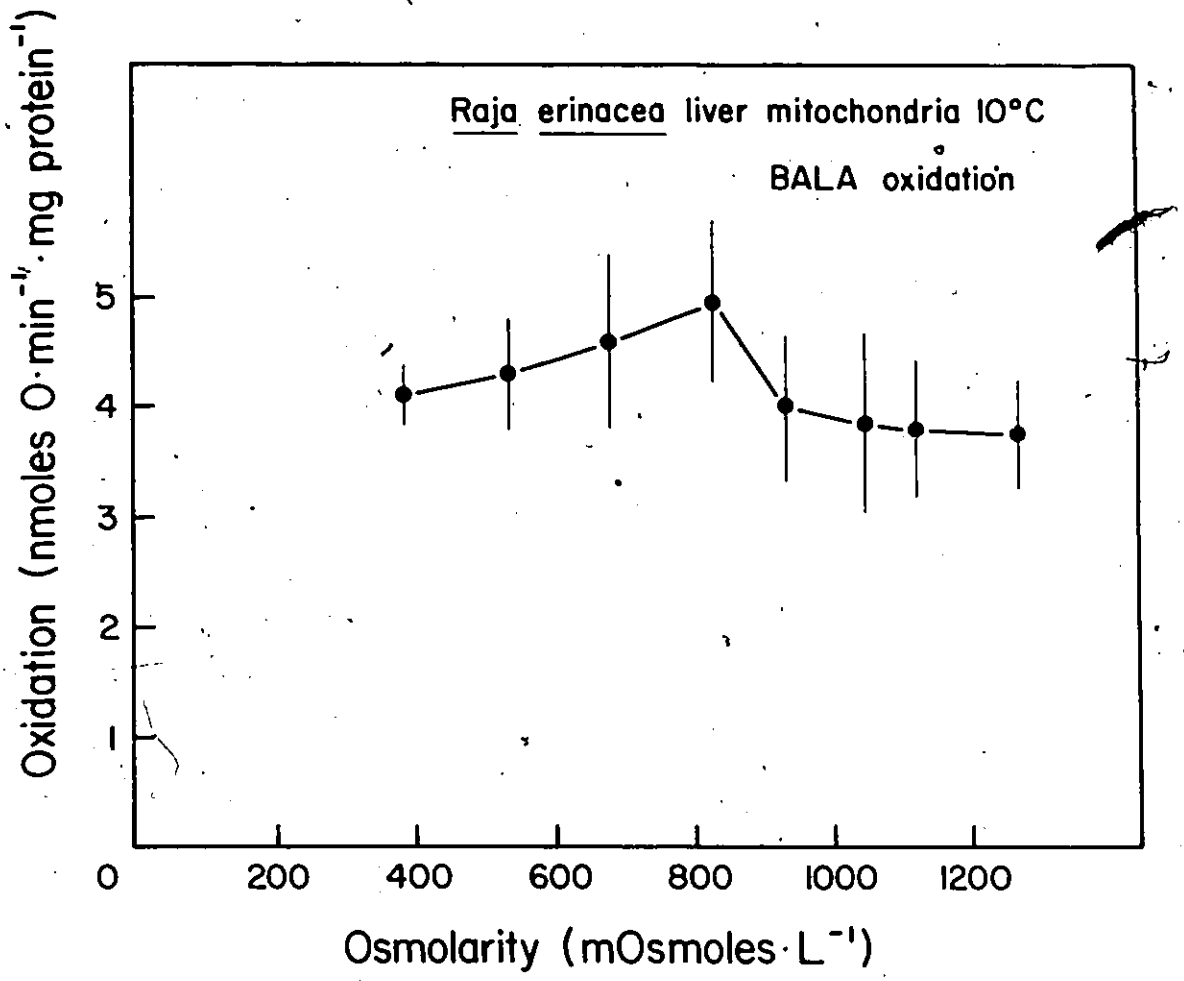
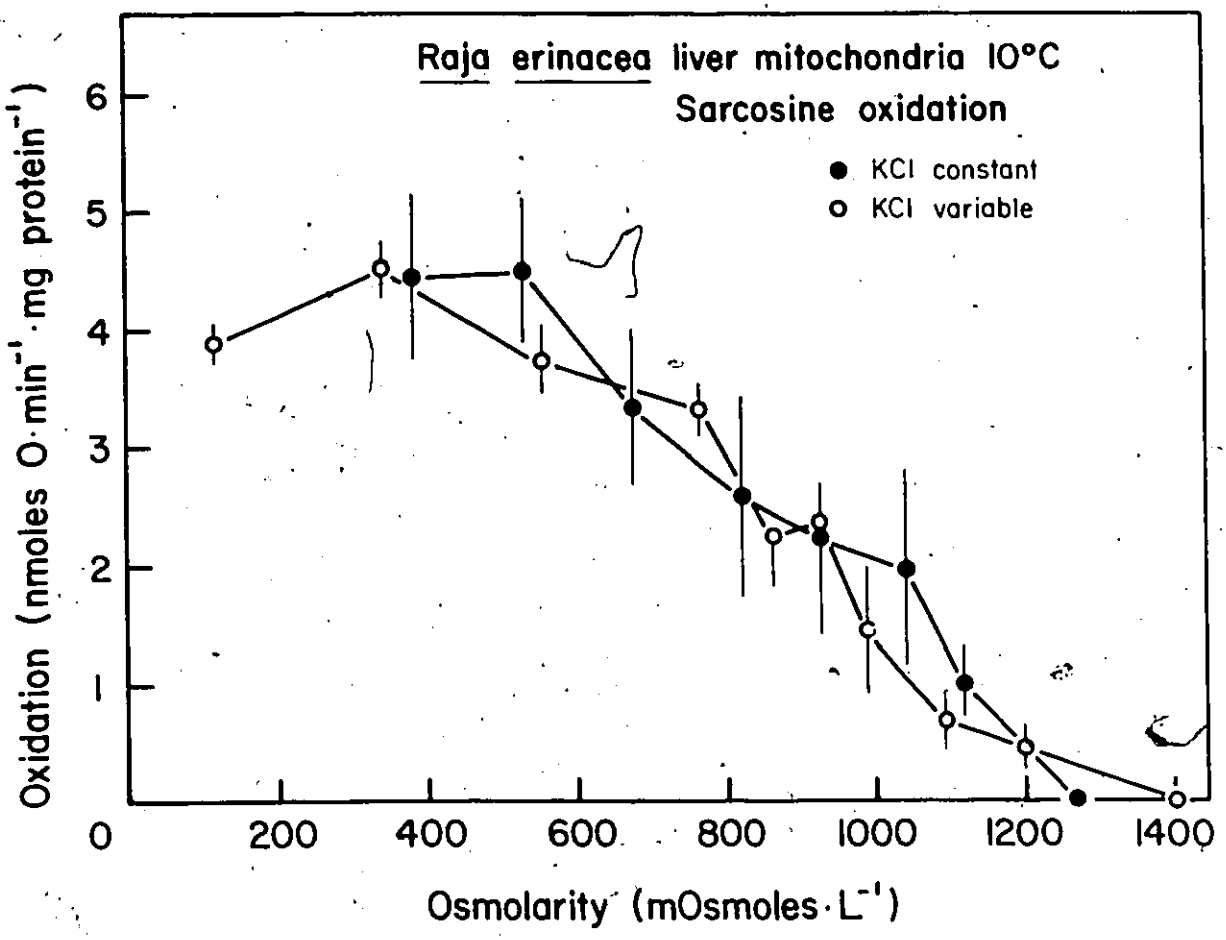

Raja erinacea liver mitochondria 10°C
BALA oxidation
Oxidation (nmoles O·min⁻¹·mg protein⁻¹)
0
1
2
3
4
5
200
400
600
800
1000
1200
Osmolarity (mOsmoles·L⁻¹)

Figure 6: Oxidation of sarcosine by *Raja erinacea* liver mitochondria in relation to assay medium osmolarity with constant or variable KCl concentrations.

Assay methods are described in the Materials and Methods. Sarcosine oxidation was determined polarographically using 10 mM sarcosine. The concentration of KCl in the reaction medium was kept constant at 140 mM or varied, but kept in constant proportion to urea, TMAO and sucrose. In both cases osmolarity was varied by altering the concentration of urea, TMAO and sucrose, keeping a constant ratio of 8:4:1. Values are means ± SE of 6 determinations.


Raja erinacea liver mitochondria 10°C
Sarcosine oxidation
KCl constant
KCl variable
Oxidation (nmoles O·min⁻¹·mg protein⁻¹)
Osmolarity (mOsmoles·L⁻¹)
0
1
2
3
4
5
6
200
400
600
800
1000
1200
1400

medium concentration. Decreasing assay medium osmolarity from isosmotic (930 mOsmoles l^{-1}) to 540 or 380 mOsmoles l^{-1} resulted in a 2-fold stimulation of oxidation. Increasing osmolarity from isosmotic to 1270 mOsmoles l^{-1} reduced oxidation to below detectable levels. When the concentration of KCl was varied as well as urea, TMAO and sucrose, the effects of osmolarity were similar (Fig. 6).

Medium osmolarity also affected the apparent Michaelis constant for the oxidation process (Table 4). If reaction medium KCl was kept constant at 140 mM, the Km_{app} was increased approximately 2-fold by reducing the osmolarity from 930 to 540 mOsmoles l^{-1}. A similar increase in Km_{app} occurred if KCl was also varied.

The oxidation of sarcosine by isolated hepatocytes is presented in Figure 7A. As was observed in intact mitochondria, the rate of sarcosine oxidation was inversely proportional to the osmolarity of the assay medium. Sarcosine oxidation increased by approximately 30% in response to a 45% reduction in osmolarity. A 40% reduction in oxidation occurred in response to a 45% increase in osmolarity. Osmolarity did not profoundly affect the leakage of MDH, except at the lowest osmolarity tested (Fig. 7B). Trypan blue was excluded from the cells at all experimental osmolarities. Hepatocyte volume increased in the dilute incubation medium and decreased in the concentrated incubation medium (Table 5).

An experiment was done to determine whether this

TABLE 4

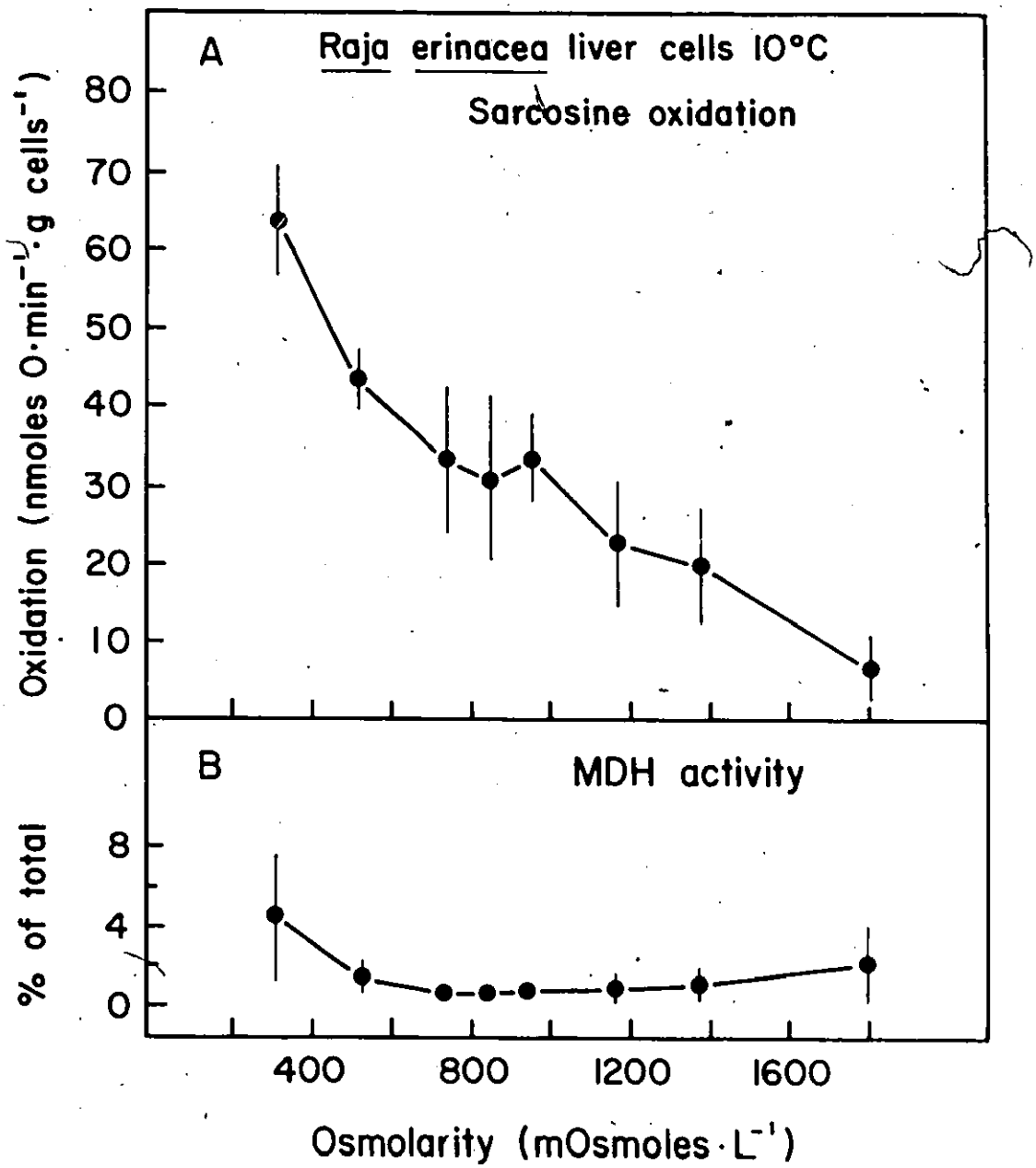
The apparent Michaelis constant of sarcosine oxidation by *Raja erinacea* liver mitochondria in relation to assay medium osmolarity and KCl concentration.

Osmolarity mOsmoles l^{-1}	KCl mM	N	Apparent Michaelis constant* mM
940	140.0	6	0.87 (.18)
940	37.5	4	0.49 (.09)
350	37.5	4	1.50 (.26)
535	140.0	4	1.66 (.19)

* mean (SE)

Figure 7: Sarcosine oxidation and MDH leakage in relation to assay medium osmolarity, in hepatocytes isolated from *Raja erinacea*.

The composition of the incubation medium is described in the Materials and Methods. Osmolarity of the assay medium was varied by changing the concentration of all components except bicarbonate, HEPES and BSA. Fig 7A: Sarcosine oxidation was determined polarographically using 10 mM sarcosine. Assay conditions are described in the Materials and Methods. Values are means ± SE of 6 determinations. Fig. 7B: Activities of MDH are expressed as per cent of the activity present in the pellet (mean ± SE) for 3 determinations. Assay condtions are described in the Materials and Methods.


A
Raja erinacea liver cells 10°C
Sarcosine oxidation
Oxidation (nmoles O·min⁻¹·g cells⁻¹)
80
70
60
50
40
30
20
10
0
B
MDH activity
% of total
8
4
0
400
800
1200
1600
Osmolarity (mOsmoles·L⁻¹)

effect on sarcosine oxidation was due to a direct effect of urea, TMAO or sucrose (i.e., the variable components of the assay medium). Control assays were performed using the standard medium containing 100 mM urea, 50 mM TMAO and 12.5 mM sucrose. The effects of TMAO and urea on sarcosine oxidation were examined using special media with either TMAO or both TMAO and urea omitted, and the sucrose concentration elevated to maintain osmolarity constant at 540 mOsmoles l^{-1}. Table 6 summarizes the results of these experiments. When only TMAO was replaced with sucrose, a 14.7% reduction in the rate of sarcosine oxidation was observed. A 53.7% reduction in the rate of sarcosine oxidation was observed when both TMAO and urea were replaced with sucrose.

The effect of osmolarity on sarcosine oxidation in the presence of rotenone is presented in Figure 8. Sarcosine oxidation, in the presence of rotenone, proceeds through SO, but catabolism of the resulting glycine in the GCS is inhibited. Sarcosine oxidation was reduced by rotenone at each osmolarity, although the effect of osmolarity was the same as in uninhibited mitochondria. A 2-fold stimulation in sarcosine oxidation occurred in response to a 50% reduction in assay osmolarity. Increasing osmolarity resulted in a reduction in sarcosine oxidation although it remained above detectable levels, even at the highest osmolarity.

The effects of osmolarity on the oxidation of serine and glycine were examined in an attempt to establish the

TABLE 5

Raja erinacea hepatocyte volume in relation to assay medium osmolarity.

Osmolarity mOsmoles·l^{-1}	hepatocyte volume ul·g^{-1}*
529	1.47 (.07)
953	1.04 (.02)
1383	0.78 (.04)

* mean (SE); n=7

TABLE 6

Oxidation of sarcosine by *Raja erinacea* liver mitochondria in media with either TMAO or urea and TMAO omitted*

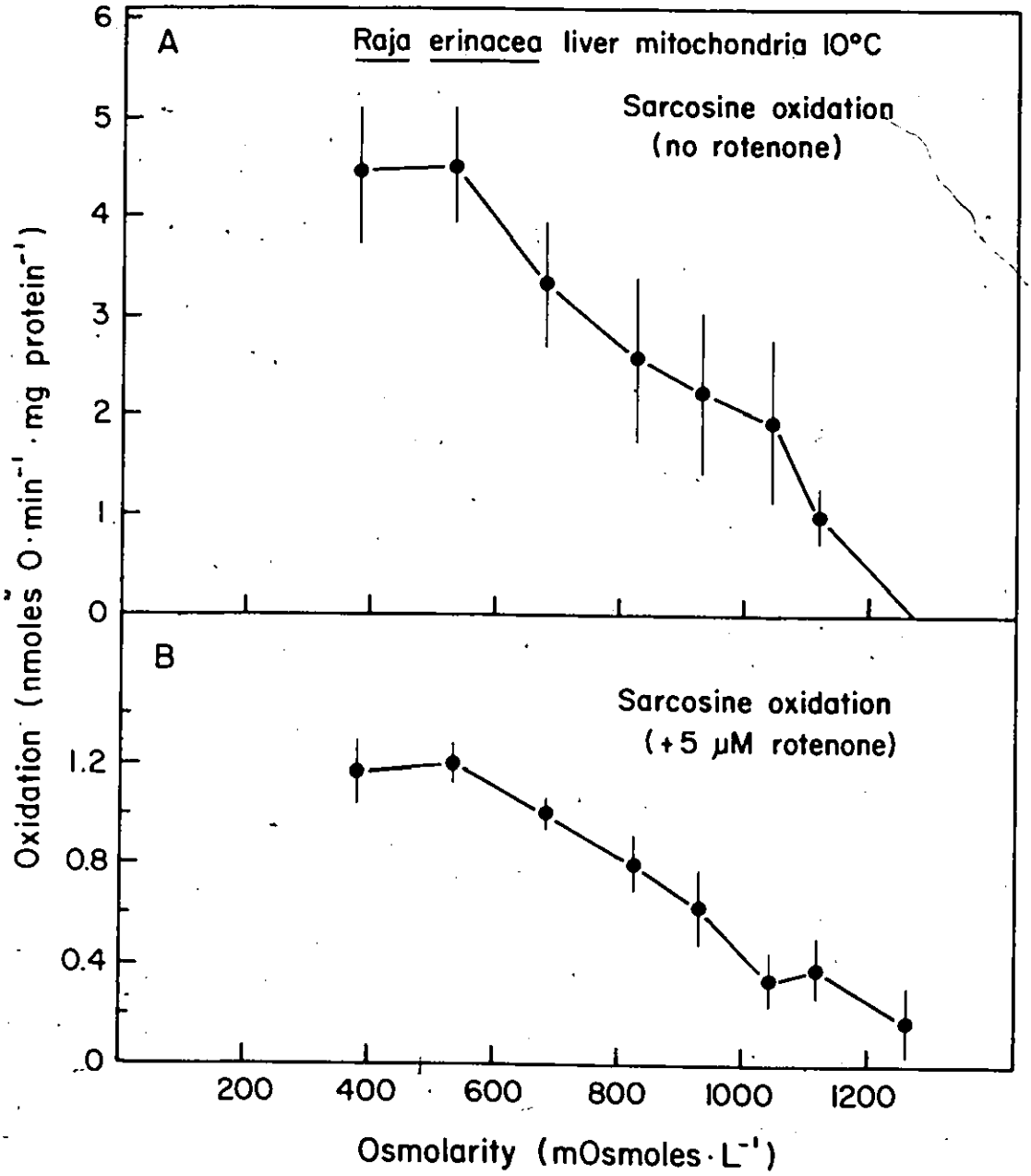
TMAO (mM)	UREA (mM)	Experimental rate/Control rate**
0	100	0.85 (0.04)
0	0	0.47 (0.05)

* osmolarity of both experimental media was maintained at the control osmolarity (540 mOsmoles l^{-1}) by elevating sucrose.

** mean (SE) for n=5; control rate was the rate in a medium with the standard ratio of 8:4:1 urea:TMAO:sucrose.

Figure 8: Sarcosine oxidation by *Raja erinacea* liver mitochondria in relation to osmolarity in the presence and absence of rotenone.

Osmolarity was varied as described in Fig. 6 keeping the KCl concentration in the reaction medium constant at 140 mM. Assay conditions are described in the Materials and Methods. Fig 8A: The rate of sarcosine oxidation in the absence of rotenone was determined polarographically as described in Fig. 6. Fig. 8B: Oxidation of sarcosine in inhibited mitochondria was also determined polarographically. Mitochondria were preincubated with rotenone (5 uM) for 5 min prior to the addition of 10 mM sarcosine. Values are means ± SE of 5 determinations.


A
Raja erinacea liver mitochondria 10°C
Sarcosine oxidation
(no rotenone)
B
Sarcosine oxidation
(+5 μM rotenone)
Oxidation (nmoles O·min⁻¹·mg protein⁻¹)
Osmolarity (mOsmoles·L⁻¹)
0
1
2
3
4
5
6
0.4
0.8
1.2
200
400
600
800
1000
1200

component of the sarcosine oxidative pathway which is sensitive to the osmolarity changes. Oxidation of serine was not inhibited by malonate, but it was completely inhibited by sodium arsenite, demonstrating that serine catabolism occurs through the GCS in these mitochondria. The effects of osmolarity on the oxidation of serine, determined polarographically, are shown in Figure 9. Although the rates are variable, osmolarity does not appear to affect serine oxidation. Serine oxidation measured radiometrically (Fig. 10A) did demonstrate a slight stimulation with a reduction in osmolarity and a slight decrease with increased osmolarity. An equivalent reduction in assay osmolarity (50%) increased sarcosine oxidation by 100% (Fig. 6) and serine oxidation by only 35% (Fig. 10A). Increasing osmolarity by 50% resulted in a 20% reduction in serine oxidation (Fig. 10A) whereas sarcosine oxidation was reduced to below detectable limits (Fig. 6).

Oxidation of glycine was determined radiometrically as it was not always detectable polarographically. Arsenite completely inhibited production of $^{14}CO_2$ from glycine, confirming that glycine oxidation occurs through the glycine cleavage system. Production of $^{14}CO_2$ from $^{14}C(U)$-glycine was stimulated at low osmolarities. As was the case with sarcosine, a 2-fold increase in oxidation of glycine occurred in response to a reduction of osmolarity from isosmotic to 540 mOsmoles l^{-1} (Fig. 10B). A decrease in

Figure 9: Oxidation of serine in mitochondria from the liver of *Raja erinacea* determined polarographically in relation to assay medium osmolarity.

Assay conditions are described in Materials and Methods. Osmolarity was varied as described in Fig. 6 with KCl kept constant. The serine concentration was 10 mM. Values are means ± SE for 6 determinations.

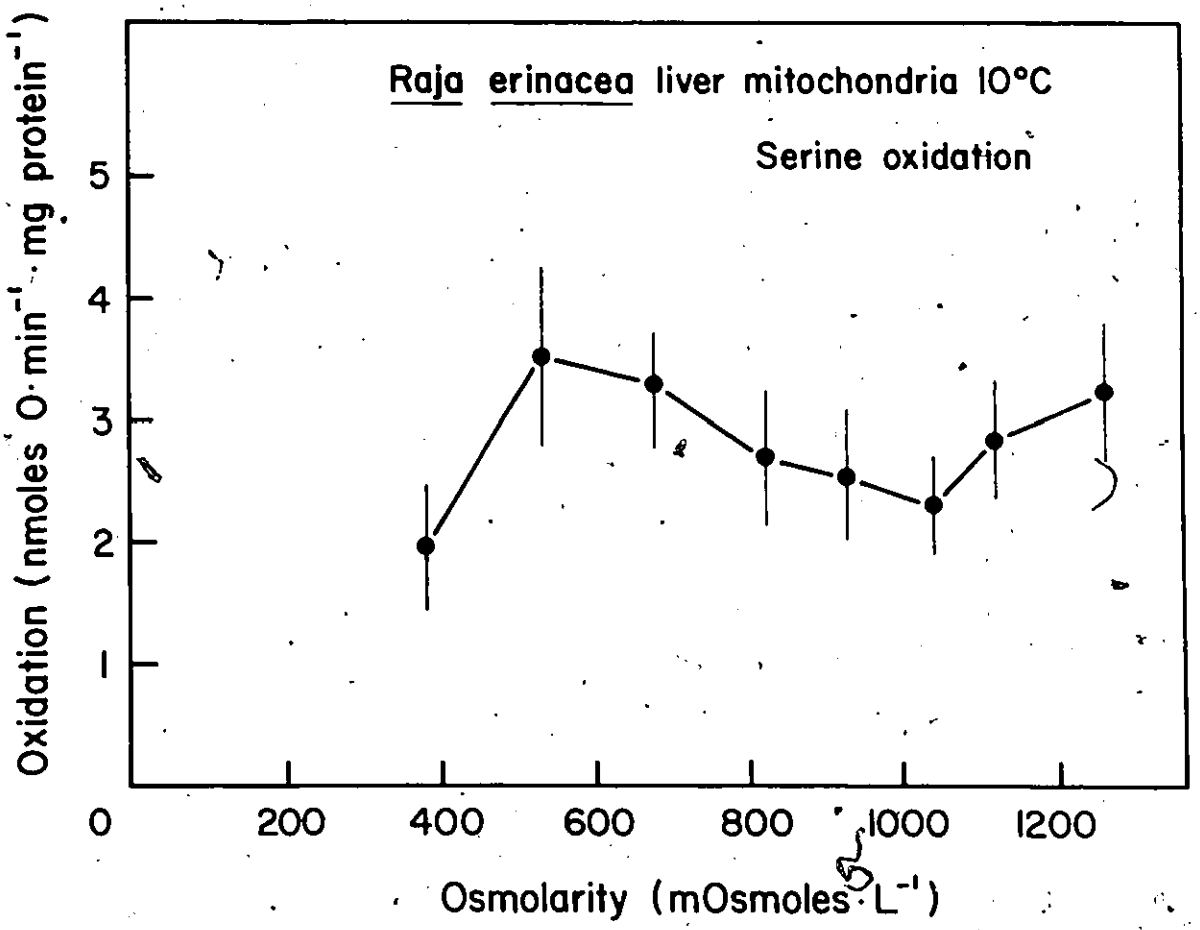
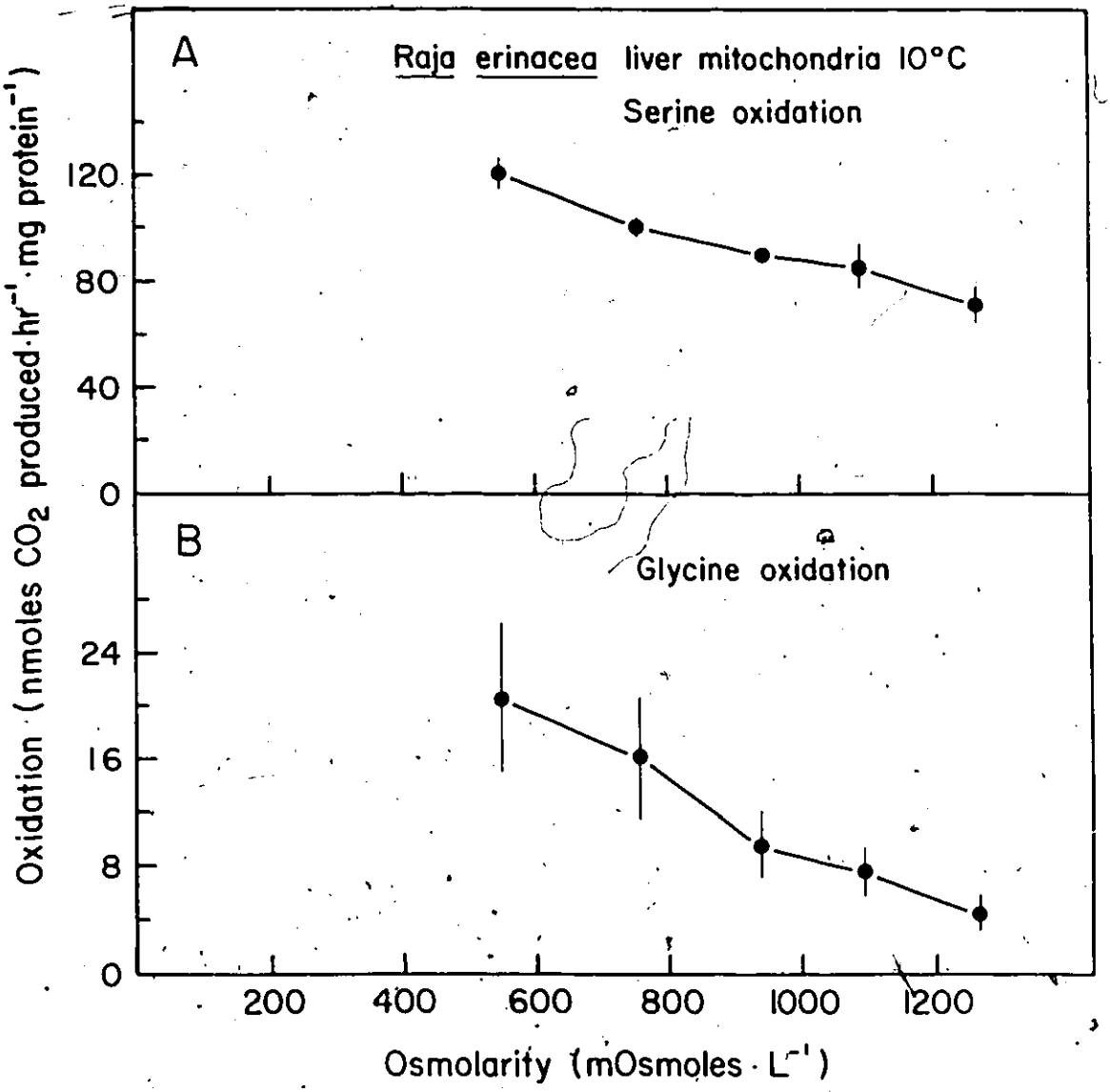

Raja erinacea liver mitochondria 10°C
Serine oxidation
Oxidation (nmoles O·min⁻¹·mg protein⁻¹)
5
4
3
2
1
0
200
400
600
800
1000
1200
Osmolarity (mOsmoles·L⁻¹)

Figure 10: Oxidation of serine and glycine in mitochondria from *Raja erinacea* liver determined radiometrically in relation to assay medium osmolarity.

Assay conditions are described in the Materials and Methods. Osmolarity was varied as described in Fig. 6. Mitochondria were incubated for 1 hr in state 3. Values are means ± SE of 5 determinations. Fig. 10A: Mitochondria were given 10 mM cold serine and 0.02 uCi $^{14}C(U)$-serine. Fig. 10B: Mitochondria were given 5 mM cold glycine and 0.1 uCi $^{14}C(U)$-glycine.


A
Raja erinacea liver mitochondria 10°C
Serine oxidation
120
80
40
0
B
Glycine oxidation
24
16
8
0
200
400
600
800
1000
1200
Oxidation (nmoles CO_2 produced·hr^{-1}·mg protein^{-1})
Osmolarity (mOsmoles·L^{-1})

the rate of glycine oxidation was observed with hyperosmotic media, although the relative decrease was not as pronounced as with sarcosine. Where oxidation of sarcosine decreased by 100% in response to a 50% increase in assay osmolarity, glycine oxidation decreased by only 50%. The response to media of variable tonicity but constant osmolarity is summarized in Table 7. As was the case with sarcosine, the maximum rate of oxidation of glycine occurred in the standard media containing urea and TMAO. Replacement of both urea and TMAO with sucrose caused marked reductions in the rate of glycine oxidation, particularly at the highest osmolarity tested.

The relative rates of $^{14}CO_2$ production from $^{14}C(1)$- and $^{14}C(2)$-glycine (Table 8) reveal the existence of an active MeTHFDH pathway in these mitochondria. Production of CO_2 from the C(2) position of the glycine molecule is 75 to 80% of that from the C(1) position and this ratio was not affected by osmolarity. It is not known whether the balance of the MeTHF is regenerated through SHMT or a formaldehyde generating pathway.

DISCUSSION

The absence of an osmolarity effect on the oxidation of BALA is not surprising, given the previous work of Goldstein and co-workers (see below). The maximal activities of the BALA oxidizing enzymes were not affected by osmolarity

TABLE 7

Oxidation of glycine by *Raja erinacea* liver mitochondria in media with either TMAO or both TMAO and urea omitted*.

TMAO (mM)	Urea (mM)	Osmolarity mOsmoles·l^{-1}	Experimental rate/Control rate**
0	100	540	0.76 (0.09)
0	0	540	0.53 (0.09)
0	100	920	0.28 (0.05)
0	0	920	0.05 (0.01)
0	100	1260	0.14 (0.02)
0	0	1260	0.10 (0.02)

mean (SE); n=6

* osmolarity of each experimental medium was maintained at the control osmolarity (540, 920 or 1260 mOsmoles·l^{-1}) by elevating sucrose.

** control rate was the rate in a medium with the standard ratio of 8:4:1 urea:TMAO:sucrose

TABLE 8

The ratio of $^{14}CO_2$ production from $^{14}C(1)$- and $^{14}C(2)$-glycine in *Raja erinacea* liver mitochondria at three osmolarities.

Osmolarity mOsmoles l^{-1}	$\frac{^{14}C(2)\text{-}CO_2}{^{14}C(1)\text{-}CO_2}$*
380	0.77 (0.07)
940	0.80 (0.06)
1270	0.75 (0.02)

* mean (SE); n=3

(Leech and Golstein, 1983). Shuttleworth and Goldstein (1985) demonstrated that no stimulation of transport into the cell occurred in hepatocytes of animals acclimated to low salinity. In fact, Shuttleworth and Goldstein (1985) re-evaluated the results of an earlier study (King et al., 1980) and suggested that a stimulation of transport had occurred in liver slices acutely exposed to a hyperosmotic media, clearly not an adaptive response. The mitochondrial studies presented here do not contradict the model proposed by Shuttleworth and Goldstein (1985) to account for the reduction in the BALA levels observed with hypoosmotic stress. The rate of mitochondrial oxidation of BALA *in vivo* may indeed reflect the availability of substrate as ultimately determined by its efflux from muscle and subsequent increases in its plasma concentration. If assay osmolarity affected the rate of transport into the mitochondria, this was not detected using the present methodology. The possibility that osmolarity affected the affinity of the mitochondrial transporter for BALA was not investigated in this study.

The rate of oxidation of sarcosine by liver mitochondria and hepatocytes was strongly affected by osmolarity. Medium dilution enhanced the rate of oxidation, however, as medium dilution involved proportionate reductions in the concentrations of urea, TMAO and sucrose, the nature of the effector was not clear. As urea and TMAO are known to be perturbing solutes (Yancey et al. 1982), the possibility was

examined that the effect of assay medium dilution on sarcosine oxidation by both mitochondria and hepatocytes was a direct effect of urea or TMAO. In experiments where the TMAO of a hypoosmotic medium was replaced with sucrose without altering osmolarity, a reduction in sarcosine oxidation was observed (Table 6). Clearly, the osmotic response of these mitochondria in the standard medium was not due to changes in TMAO. As TMAO has been reported to counteract the deleterious biochemical effects of urea (Yancey et al., 1982), this experiment could be taken as evidence for a urea-sensitive pathway. However, when both urea and TMAO were replaced with sucrose, a much more pronounced reduction in oxidation was observed (Table 6). If urea was exerting a direct inhibitory effect on sarcosine oxidation, its omission should have stimulated oxidation.

Instead of a direct effect of a solute, it is apparent that the mitochondrial response to assay medium dilution is dependent on the permeability of the solutes involved. Urea is freely permeable to skate mitochondria, whereas sucrose is impermeable (Ballantyne and Moon, in prep.). Although the media used in this experiment were of equal osmolarities, they were clearly of different tonicities. Incubation of mitochondria in a hypoosmotic medium where the major solute is permeable (e.g., urea) causes a greater degree of swelling than incubation in media dominated by impermeable solutes (e.g., sucrose). The permeability of TMAO to the

mitochondrial membrane is currently unknown. Sarcosine oxidation was reduced when assay medium TMAO was replaced with sucrose (Table 6), suggesting that TMAO is permeable to the mitochondrial membrane. If TMAO was impermeable and was replaced with sucrose (another impermeable solute), no effect on oxidation would have been expected.

Mitochondrial volume changes in response to anisotonic media are well established (Tedeschi and Harris, 1955). Temporary modifications in the configuration of the mitochondrial membrane in anisotonic media are also well-documented (Tedeschi and Harris, 1955; Klein and Neff, 1960; Tedeschi, 1961; Zimmer et al.,1972). Atsmon and Davis (1967) suggested that these changes were responsible for the inhibition of succinate oxidation in rat mitochondria incubated in hyperosmotic media. Similar changes may be responsible for the effect on sarcosine oxidation observed in this study. The sensitive component in the mitochondrial oxidation of sarcosine is clearly not the electron transport system, phosphate or ADP transporters. Perturbation of any of these processess would have affected the polarographic determination of the mitochondrial oxidation of BALA as well as sarcosine. Therefore, medium tonicity must be affecting either transport into the mitochondria or the catabolic enzymes- SO or GCS. The sarcosine transporter, SO (Hoskins and Mackenzie, 1961) and GCS (Hampson et al., 1983) are each associated with the inner mitochondrial membrane and could be

sensitive to membrane alterations. As Burcham et al. (1985) reported that glycine oxidation in GCS of bivalve gill mitochondria was strongly stimulated by assay medium dilution, the effects of tonicity on the GCS of skate mitochondria were examined.

Sarcosine oxidation in the presence of rotenone proceeds as far as sarcosine oxidase, as rotenone inhibits all NAD-linked enzymes, including the GCS (see chapter 1). The effects of osmolarity on sarcosine oxidation suggest that the GCS is not involved in the effects on sarcosine oxidation. Although the absolute rates of oxygen consumption are lower than in the uninhibited mitochondria, the effect of medium tonicity remains (Fig. 8). As the resulting glycine is not metabolized when the GCS is inhibited, these results also suggests that either SO is insensitve to glycine concentrations, or that glycine efflux under these conditions occurs at a rate sufficient to prevent endproduct inhibition.

The investigation of serine oxidation is complicated by side reactions involving MeTHF metabolism which could lead to substantial errors in interpretation of both polarographic and radiometric studies. Preliminary experiments were required to establish that the values determined for serine oxidation were *valid* estimates of flux through the *entire* pathway, including the GCS. Arsenite, but not malonate, completely inhibited serine oxidation, confirming

that the oxidation of serine proceeds through the GCS. The results of the $^{14}C(1)$- and $^{14}C(2)$-glycine studies (Table 8) demonstrate that the pathway for producing CO_2 from MeTHF is present and active under these conditions. As the rate of CO_2 production from MeTHF was unaffected by osmolarity, any osmotic effects on the rate of production of CO_2 from serine can be assumed to be effects on flux through SHMT and the GCS. The hypothesis that the GCS is not the sensitive component of the sarcosine oxidative pathway is supported by the effects of osmolarity on serine oxdation. Results from the polarographic studies (Fig. 9) are variable, but no obvious trend towards increased oxidation at low osmolarities occurs. The radiometric studies (Fig. 10A) are less variable and reveal a slight stimulation of oxidation at low osmolarities, but not nearly as pronounced as with sarcosine oxidation.

Although the rates of both serine oxidation and rotenone-insensitve sarcosine oxidation suggest that osmolarity does not affect the GCS, the oxidation of glycine was strongly affected by osmolarity. As both serine and glycine are oxidized through the GCS, the relative rates of oxidation (Fig. 10A cf Fig. 10B) suggest that transport of glycine is rate limiting to its oxidation. Therefore, the increased oxidation of glycine at low osmolarities reflects a stimulation of the mitochondrial transporter, while high osmolarities reduce the rate of transport. Based on the

response to media of constant osmolarity, but composed of solutes of variable permeability (Table 7), transport of glycine is dependent on medium tonicity, as is the oxidation of sarcosine.

The tonicity-sensitive component of the sarcosine oxidative pathway is clearly not the GCS. The effects on the oxidation of glycine demonstrate that mitochondrial volume changes induced by anisotonic media are capable of stimulating or inhibiting the transport of at least glycine. Complete characterization of the neutral amino acid carrier has not been done, although it has been suggested that serine, glycine and several other amino acids are transported on a single neutral amino acid transporter in rat liver (Cybulski and Fisher, 1977). If serine shares the tonicity-sensitive transporter for glycine in skate mitochondria, the lack of a pronounced effect of medium tonicity on serine oxidation suggests that even in tonicity-inhibited mitochondria, transport can occur at a rate greater than is required to saturate the oxidative enzymes. The minor effect that was observed in the radiometric determination of serine oxidation may have been due to a reduction in the steady state concentrations of serine intramitochondrially, affecting flux through SHMT.

The mitochondrial transporter for sarcosine has not been established. Cybulski and Fisher (1977) found that sarcosine was unable to protect against p-mercurobenzoate

inactivation of the transporter for glycine, serine and other neutral amino acids, suggesting sarcosine does not share the glycine transporter in rat liver mitochondria. It was proposed that methylation of the primary amine group of sarcosine prevented its binding to the carrier. As with sarcosine, both BALA and proline were unable to prevent p-mercurobenzoate inactivation of the neutral amino acid transporter, suggesting different transporters. However, in the reciprocal experiment, neutral amino acids did protect against p-mercurobenzoate inactivation of BALA and proline transport, implying that these amino acids are transported on the neutral amino acid carrier. As the reciprocal experiment was not done with sarcosine, it is possible that sarcosine is in fact, transported on the same carrier as glycine in rat liver. The effects of tonicity on the oxidation of sarcosine and on the transport of glycine suggest that these amino acids either share a common transporter or that their separate transporters respond similarly to tonicity. The possibility that tonicity may be affecting SO directly can not be eliminated.

Regardless of the nature of the sensitive component of the sarcosine oxidative pathway, the mitochondrial response to medium dilution may have important consequences *in vivo*. Enhanced oxidation of this important osmolyte in a hypoosmotically stressed animal may contribute to the cell volume regulatory response of the liver. It is clear that the

cell membrane does not prevent the effects of tonicity on the mitochondria, as oxidation of sarcosine by intact skate hepatocytes was affected by osmolarity in the same manner as the isolated mitochondria. The mechanism responsible for decreasing sarcosine levels from 44 to 16 mmoles kg^{-1} in the mitochondria-poor wing muscle (Boyd et al., 1977) has not been established. If sarcosine is expelled from the wing muscle under hypoosmotic stress, as is BALA, tonicity-mediated stimulation of oxidation by liver mitochondria is probably responsible for reducing sarcosine levels in the whole animal.

CHAPTER 5: GENERAL DISCUSSION

Osmoconforming animals that experience changes in ambient osmolarity, regulate cell volume by adjusting the concentration of certain intracellular solutes. Amino acids (particularly glycine, alanine, proline, glutamate, betaine, BALA and sarcosine) are employed as intracellular osmolytes in both bivalve molluscs and elasmobranch fishes, although the specific amino acids used are different. It is not known why either group employs a particular amino acid over another as an osmolyte. Certain generalizations can be made, however, concerning the metabolism of the amino acids used in cell volume regulation of osmoconformers. First, the excretion of amino acids as a mechanism for the reduction of intracellular osmolarity would not be expected to occur in response to hypoosmotic stress. This strategy would be energetically expensive, particularly in intertidal bivalves which can be exposed to a fluctuating salinity regime twice daily. Excretion of amino acids has, in fact, been shown to be low in both bivalves (Livingstone et al., 1979) and skates (King et al., 1980). Second, to achieve the high steady state concentrations of amino acids, it would be advantagous to maintain the rates of both *in situ* oxidation and efflux from the cell at low levels while the animal is in an isosmotic environment. Third, there must be a mechanism for reducing the levels of amino acids in tissues of animals

during exposure to hypoosmotic media, either through *in situ* oxidation or expulsion into the extracellular fluid. Fourth, there must be some mechanism to enhance the rate of catabolism of the expelled amino acid osmolytes in response to environmental dilution.

Oxidation of BALA and sarcosine has been reported to be low in the wing muscle of *R. erinacea* (King et al., 1980) where the concentrations of these amino acids is approximately 40 mM (Boyd et al., 1980). The lack of oxidation by wing muscle is probably related to the low mitochondrial content, as oxidation of both BALA and sarcosine occurs in the mitochondria (Table 3). In tissues with high mitochondrial content such as liver, the concentration of BALA (sarcosine has not been measured in skate liver) is much lower than wing muscle (King et al., 1980). Efflux of amino acids is much lower in skate wing muscle in animals under isosmotic conditions than in animals exposed to dilute media (Goldstein, 1981). This is also the situation in isolated bivalve heart tissue (Pierce and Greenberg, 1972; 1973; 1976).

Expulsion of amino acids into the blood appears to be the mechanism by which the concentration of BALA is reduced in wing muscle of skates exposed to dilute media (Goldstein, 1981). Heart tissue of the bivalve *Modiolus modiolus* also expels amino acids with little *in situ* oxidation when incubated in dilute saline (Pierce and Greenberg 1972; 1973; 1976). Subsequent oxidation of the expelled amino acids

occurs in both bivalves and skates. The increased ammonia production and excretion observed in bivalves concommitant with the reduction in tissue amino acid levels is indicative of enhanced amino acid oxidation (Strange and Crowe, 1979). In skates exposed to environmental dilution, the increase in plasma BALA concentration (King et al., 1980) is only temporary (see Boyd et al., 1977 for acclimation levels), suggesting that subsequent oxidation of BALA occurs. Goldstein (1981) proposed that the liver was responsible for oxidation of amino acids expelled from the wing muscle. The mantle (Bartberger and Pierce, 1976) or gill (Zurburg and deZwaan, 1981) may be the homologous tissue in bivalves.

At least two mechanisms responsible for enhancing oxidation of amino acids in response to hyposmotic media have been identified. BALA oxidation in skate liver appears to be stimulated through a mass action effect. Schoolwerth and Goldstein (1985) proposed that the increased plasma levels of BALA enhance transport into the cell, and oxidation is stimulated due to an increased availability of BALA to the catabolic enzymes. The intracellular compartmentalization of the BALA catabolizing enzymes was not considered. The mitochondrial oxidation of BALA was not affected by osmolarity in the present study (Fig. 5), supporting the model proposed by Schoolwerth and Goldstein (1985).

The present study suggests that an entirely different mechanism is responsible for the stimulation of sarcosine

oxidation in skates exposed to dilute media. Stimulation of sarcosine oxidation occurred in *R. erinacea* liver mitochondria incubated in dilute assay media (Fig. 6). The effects on mitochondrial oxidation appear to be related to volume changes associated with the tonicity of the medium (Table 6). Oxidation of sarcosine by hepatocytes responds similarly to assay medium tonicity (Fig. 7A), suggesting that this mechanism may be of physiological significance in reducing the levels of sarcosine in the whole animal during hypoosmotic stress. As tonicity affects the oxidation of glycine through effects on the rate of transport, it is possible that the effects on sarcosine oxidation are also effects on the transporter. *Mercenaria mercenaria* heart mitochondria oxidizing proline (Ballantyne and Storey, 1985) and *Modiolus* gill mitochondria oxidizing glycine (Ellis et al., 1985), respond to osmolarity in the same manner as skate liver mitochondria oxidizing sarcosine and glycine. It has been suggested that the enhanced oxidation of proline in bivalve mitochondria is due to effects on the transporter (Ballantyne and Storey, 1985). The enhanced oxidation of glycine in bivalve mitochondria in response to assay medium dilution (Ellis et al., 1985) may also be due to effects on the transporter, as in skate liver mitochondria (this study). It is not known whether this mechanism operates on the transport of other amino acid osmolytes. If the effect of tonicity on the amino acid transporters was a general

phenomenon, it would most dramatically affect the rate of oxidation when transport is the rate limiting step of the pathway. Enhanced transport into the mitochondria would be of little physiological significance if the capacity of subsequent oxidative enzymes was exceeded.

All of the osmotically important amino acids in bivalve tissues, except glycine, are typically deaminated through GDH (glutamate, proline, alanine, aspartate) (Lehninger, 1976). Wickes and Morgan (1976) demonstrated that the activities of this enzyme increased in bivalve populations exposed to low ambient salinities. In this situation, increased GDH activities are probably responsible for maintaining steady state amino acid concentration at lower levels. The possibility that GDH activities increase in populations exposed to fluctuating salinity regimes has not been studied. It is not known whether enhanced oxidation of these amino acids in bivalves is achieved through a mass action effect, as with BALA in skates, or through a stimulation of transport, as with glycine and possibly sarcosine in skates. Which ever mechanism operates, GDH activity probably does not limit the oxidation of these osmolytes in bivalves. The results of the studies on GDH in bivalve tissues confirm that this enzyme has a high capacity for glutamate oxidation *in situ*. The *in vitro* GDH activity is low in comparison to the enzyme in mammals, however this low activity simply reflects the low metabolic

rate of bivalves. Flux through GDH alone is capable of producing reducing equivalents at rates great enough to saturate the mantle mitochondria electron transport system (Fig. 4). It is not suggested that GDH proceeds at its maximum velocity *in vivo*. In fact, under isosmotic conditions, it would be advantagous to maintain GDH (deaminating) activities low, thus reducing the oxidation of amino acid osmolytes and allowing their intracellular accumulation.

Glutamate oxidation probably proceeds through both GDH and the aminotransferases under most conditions. In tissues exposed to dilute media, an enhanced flux through GDH (deaminating) is probably required. The effects of pH on the activity of GDH (deaminating), GDH (aminating) and the competing transaminases suggests that mitochondrial pH could be of regulatory significance. A high intracellular pH would favour deamination of glutamate. The effects of osmolarity on intracellular and intramitochondrial pH have not been examined in any animal. ADP and other purine nucleotide regulators may be important in affecting increased flux through GDH (deaminating). Whole tissue levels of ADP drop (and ATP rise) in response to hypoosmotic stress in the clam *Corbicula japonica* (Matsushima et al., 1984). Mitochondrial ADP/ATP ratios are considerably greater than cytosolic ratios in rat liver (Soboll et al. 1976). It is not known how ambient salinity affects the mitochondrial adenine nucleotide

concentrations in bivalves. Stimulation of GDH activity in response to reduced inorganic ion levels has been shown in polycheates (Batrel and Le Gal, 1984), crustaceans (Gilles, 1974) and sea anenomes (Male and Storey, 1983). The effect of inorganic ions on GDH (deaminating) from bivalves has not been examined.

In this study, aspects of amino acid metabolism in relation to hypoosmotic stress were examined in two groups of osmoconformers, bivalve molluscs and elasmobranchs. Oxidation of sarcosine by skate liver mitochondria was found to be very sensitive to assay medium dilution and concentration. Tonicity was shown to affect mitochondrial sarcosine oxidation, probably through effects on the mitochondrial transporter. A physiologically significant role for GDH (deaminating) in the oxidation of amino acids in bivalve tissues was established. Although these osmoconforming animals are phylogenetically unrelated and utilize different amino acids as osmolytes, regulatory strategies in amino acid metabolism in relation to cell volume regulation are highly conserved.

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