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AMINO ACID CATABOLISM BY RIBBED MUSSEL (MODIOLUS DEMISSUS) GILL TISSUE: STUDIES ON ISOLATED MITOCHONDRIA AND THE L-AMINO ACID OXIDASE

Iowa State University PH.D. 1983

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Amino acid catabolism by ribbed mussel (Modiolus demissus) gill tissue: Studies on isolated mitochondria and the L-amino acid oxidase

by

James M. Burcham

A Dissertation Submitted to the
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Iowa State University
Ames, Iowa

1983

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
°C	degrees, Celsius
g	grams
xg	times the force of gravity
h	hour(s)
M	molar
min	minute(s)
ml	milliliter(s)
mg	milligram(s)
n	nano-
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
μ	micro-

GENERAL INTRODUCTION

All living cells have some volume regulatory capacity. This ability may function over only a very narrow range of osmotic pressure as in mammalian cells (Hendil and Hoffman, 1974) or a very wide range (freshwater to full seawater), such as the cells of oligohaline invertebrates (Gainey and Greenberg, 1977). In fact, for many if not most species the mechanisms by which organisms regulate cellular hydration are probably similar (Pierce, 1982). Many species which are normally subjected to high osmotic stress including bacteria, algae, and plants (LeRudulier and Valentine, 1982; Yancey et al., 1982) accumulate specific amino acids and amines as intracellular osmolytes to prevent water loss or salt gain during hyperosmotic stress and tend to regulate the concentrations of these compounds as part of the cell volume regulation process. There appear to be two major processes or components which regulate the amino acid concentrations in osmotically stressed species: 1) a plasma membrane component which restricts passage of specific inorganic and organic ions outward and provides transport of organic compounds inward at high extracellular osmotic pressure, and controlled leakage of specific ions at low extracellular osmotic pressure (Pierce, 1982); 2) a metabolic process that determines which amino acids or amines accumulate regulates the final intracellular concentrations of these compounds (Gilles, 1979).

Euryhaline marine bivalves serve as good model systems for studying the membrane and metabolic processes which regulate the concentration of amino acids responsible for cellular osmotic control. The blue

mussel, Mytilus edulis, and the ribbed mussel, Modiolus demissus, are the most studied species of this group of organisms. These two euryhaline marine bivalves can tolerate a wide range of salinities (100 mOsmoles to 1400 mOsmoles) and do so by specifically regulating the intracellular concentration of taurine, glycine, alanine, and in some tissues also proline (Bishop, 1976; Livingstone et al., 1979).

Studies by this laboratory have been concentrated toward understanding the metabolic component regulating the levels of intracellular free amino acids in tissues of the ribbed mussel, Modiolus demissus. The work reported here is an extension from that of Greenwalt (1981) who established the probable pathways for amino acid interconversions in ribbed mussel gill tissue using a series of radiocarbon tracer experiments with isolated salt stressed gill tissue. These studies pointed to the mitochondria as having a central role in adjusting the levels of amino acids in ribbed mussels subjected to hyperosmotic stress.

The studies reported in this text are divided into three sections. The first stems from initial work on gill tissue mitochondria and deals with the presence and general properties of an L-amino acid oxidase which may be mitochondrial and which is also present in all ribbed mussel tissues. The second section focuses on the development of a method for the preparation of coupled mitochondria from ribbed mussel gill tissue for subsequent experiments on the role of the mitochondria in amino acid metabolism. Lastly, the third section deals specifically with the metabolism of proline, arginine, ornithine, and glutamate in respiring mitochondria and closely complements the radiotracer studies by Greenwalt (1981).

PART I. THE L-AMINO ACID OXIDASE FROM RIBBED MUSSEL GILL TISSUE

Summary

Gill tissue from Modiolus demissus has a general L-amino acid oxidase (EC 1.4.3.2) associated with proteinaceous particles sedimenting at 15,000xg. The oxidase is most active between pH 4.5 and pH 5 in citrate buffer with L-α-amino acids having three or more carbons, with no hydroxyl or methyl substitutions for hydrogens on the β-carbons, and no charged groups on the γ-carbon. The apparent K_ms for L-leucine and L-ornithine were identical (2.5mM). Glycine, taurine, L-proline, aminooxyacetic acid, L-cycloserine, and EDTA would not act as substrates or inhibitors. The enzyme is reasonably active at pH 4.8 (6-8 μmoles O_2 consumed/g wet tissue/h at 25°C) and may have a role in the catabolism of some of the amino acids which do not accumulate during hyperosmotic stress.

Introduction

The tissues of euryhaline bivalve molluscs have high intracellular free amino acid concentrations which increase or decrease with corresponding changes in the salinity of the bathing medium (Bishop, 1976; Gilles, 1979). The changing amino acid levels aid cell volume control during salinity stress in osmoconforming species.

With the euryhaline ribbed mussel, Modiolus demissus, these amino acids are probably derived from intermediates in the glycolytic pathways,

tricarboxylic acid cycle or amino acid metabolic pathways (Baginski and Pierce, 1975, 1977, 1978; Bishop, 1976). Although the specific catabolic pathways are uncertain, the amino group is removed from the amino acids and excreted as ammonia (Bartberger and Pierce, 1976). Recent studies with the transaminase inhibitors, aminooxyacetic acid and L-cycloserine, have indicated that transaminase linked pathways have a major role in both the accumulation and catabolism of these amino acids (Bishop et al., 1981; Greenwalt and Bishop, 1980; Greenwalt et al., 1978). However, the catabolism of some of the amino acids which do not accumulate during hyperosmotic stress is not blocked by these inhibitors.

The results of these studies are suggestive of direct, transaminase independent, deamination of some of the amino acids. While direct deamination of glutamate can be attributed to glutamate dehydrogenase (Reiss et al., 1977), studies on direct deamination by the more nonspecific L-amino acid oxidases have been neglected in recent years because of the presumed low activity and limited distribution of this enzyme among animal species. In molluscs, however, two types of L-amino acid oxidases have been described: the L-amino acid:2-oxidoreductase (deaminating)(EC 1.4.3.2) (Blaschko and Hope, 1956; Hope and Horncastle, 1967) and the L-amino acid monooxygenase (decarboxylating), EC 1.13.12.(?) (Olomucki et al., 1960; Roche et al., 1959). This report describes the general properties of the L-amino acid oxidase from M. demissus gill tissue with special reference to the type of enzyme, specificity, pH optimum, and the action of inhibitors used in evaluating in vivo metabolic conversions.

Materials and Methods

Reagents and animals

Amino acids, Triton X-100 and X-114, ortho-aminobenzaldehyde, dithiothreitol, α-keto acids, catalase, 2,2'-dithiobis(5-nitro-pyridine), iodoacetic acid, dithiodinitrobenzoate, phenylmethanesulfonyl fluoride, aminooxyacetic acid, N-ethylmaleimide, and digitonin were purchased from Sigma Chemical Company. Hexadecyl-trimethylammonium bromide and 2,4-dinitrophenylhydrazine were purchased from Eastman Organic Chemicals. 2,5-Diphenyloxazole, 1,4-bis[2-(5-phenyloxazolyl)]benzene, [U-^{14}C]-L-arginine and [U-^{14}C]-L-leucine were obtained from New England Nuclear, Inc. All other chemicals were purchased from Fisher Scientific Company. Modiolus demissus was collected in Little Sippewisset Marsh, Cape Cod, Massachusetts, and maintained in 450mOsmoles artificial sea water (Instant Ocean) at 15°C.

Enzyme assay

Rates were measured using the Yellow Springs Oxygen Monitor (Model 53) at 25°C. The standard reaction mixture contained either 100 μmoles potassium phosphate (pH 7.0) or 200 μmoles sodium citrate (pH 4.8), 10 μmoles of amino acid substrate, and either 0.3 ml (pH 4.8 reactions) or 0.5 ml (pH 7.0 reactions) of the enzyme preparation in a total volume of 2.3 ml or 2.5 ml, respectively. The reaction was initiated by substrate addition and the difference in O_2 consumption with and without substrate was used to calculate reaction rate. Oxygen consumption by the enzyme preparation in the absence of substrate was identical to the O_2 consumption by substrate in the absence of enzyme indicating

that this small usage was due to the electrode. Protein was measured by the method of Lowry et al. (1951).

Reaction products

The reaction product with L-Orn as a substrate was identified by derivative formation using ortho-aminobenzaldehyde and 2,4-dinitrophenylhydrazine (Meister, 1954). The 2,4-dinitrophenylhydrazone derivative of the product from the L-Leu reaction was prepared and identified by the method of Struck and Sizer (1960). Absorption spectra were recorded on a Beckman 3600 recording spectrophotometer.

Liquid scintillation counting was performed using a Beckman LS-250 liquid scintillation counter. The scintillation counting fluid (15 ml/vial) consisted of 1250 ml Triton X-114, 1875 ml xylene, 15.63 g 2,5-diphenyloxazole, and 0.63 g 1,4-bis[2-(5-phenyloxazolyl)]benzene. Released ^{14}C-CO_2 and ammonia were trapped and measured as described previously (Cooley et al., 1976).

Results

Enzyme preparation

Gill tissue from Modiolus demissus was homogenized in 10 ml/g wet tissue of 50 mM potassium phosphate (pH 7.0) plus 1 mM EDTA using a polytron tissue homogenizer (Ultra Turrax, Tekmar Co., Cincinnati, OH). After centrifugation at 15,000 xg for 20 minutes, the pellet was rehomogenized in the same volume of buffer and recentrifuged. This pellet was resuspended in 2 ml of buffer per g of original tissue using a

ground glass homogenizer. The suspension was dialyzed at 2°C against 30 volumes of 20 mM potassium phosphate (pH 7.0) plus 1 mM EDTA for 48 hours changing the dialysis buffer every 24 hours. The dialyzed preparation was then centrifuged at 15,000 xg for 20 minutes. The tan-yellow colored upper layer of the pellet was carefully separated from the light gray bottom layer with a spatula and the upper layer resuspended in 50 mM potassium phosphate (pH 7.0) 1 mM EDTA. This last centrifugation step increased the specific activity 2-4 fold over the dialysis suspension. The final preparation was resuspended by homogenization at a concentration of 10 mg protein/ml. O_2 consumption in the standard reaction mixture (pH 4.8) containing 0.78 mM NaN_3 and 4.3 mM L-Orn at 25°C was 6-8 μmoles/g wet tissue/hr with the crude homogenate and 0.013 μmoles/min/mg protein with the final washed, particulate enzyme preparation used in the studies described below. The specific activity of this washed enzyme was essentially ten-fold greater than the specific activity of the original crude particulate preparation.

Attempts to solubilize the particulate enzyme at this stage of preparation were unsuccessful. No solubilization was achieved (1) by homogenization with 1% digitonin, 0.1% hexadecyltrimethylammonium bromide, or 1% Triton X-100, (2) by suspension in 6M urea with homogenization, centrifugation, and dialysis of the supernatant and pellet fractions against the phosphate buffer and (3) by homogenization of an acetone powder (Blaschko and Hope, 1956) of the final preparation in phosphate buffer (pH 7.0). With each solubilizing method attempted, all of the original activity remained in the pellet after centrifugation at 10,000 xg for 10 min.

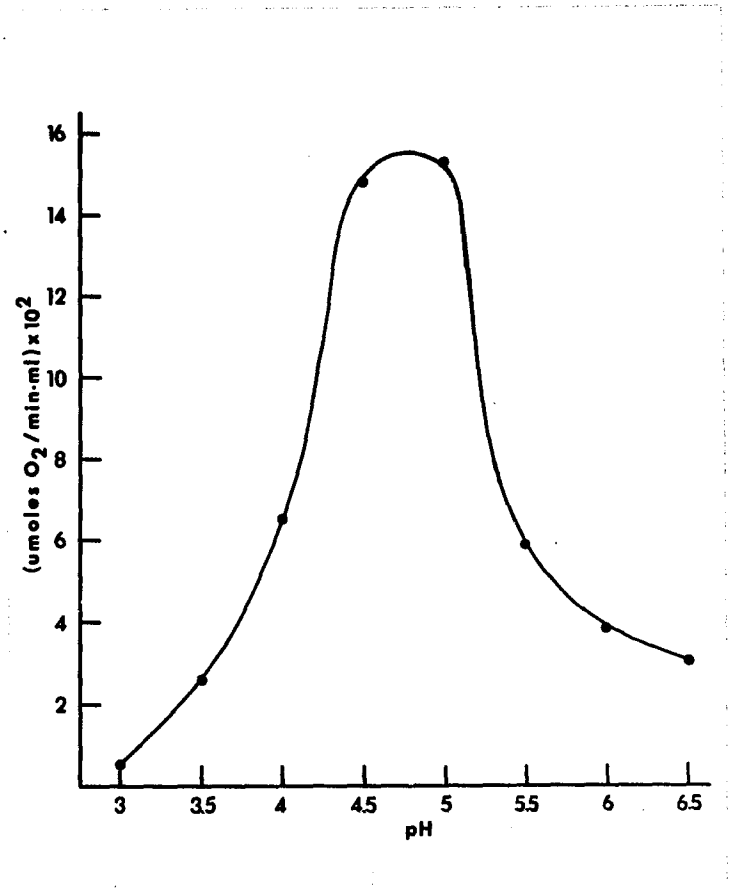
Initial experiments with the washed particulate enzyme preparation indicated that both L-Leu and L-Orn stimulated O_2 consumption at both pH 4.8 and pH 7.0 using the standard assay system. Enzyme heated to 60°C for 5 min was inactive. The amount of O_2 consumed in the standard reaction mixture increased linearly with time after addition of the substrates and the rate of O_2 consumption increased linearly with increased enzyme concentration. The rate of O_2 consumption with both L-Leu and L-Orn was pH dependent and optimal at pH 4.8 (Figure 1). Addition of catalase to preparations containing enzyme and L-Orn, L-lys, L-Cys(SH), or L-Leu at pH 4.8 and at pH 7 reduced the rate of O_2 consumption to about 50%. When 0.78 mM NaN_3 was added to these catalase containing preparations, the rate of O_2 consumption was restored to its original rate. These results indicated that the washed particulate enzyme preparation was reasonably free of catalase activity. The addition of catalase or NaN_3 did not alter optimal pH, H_2O_2 was a reaction product, and the enzyme was probably deaminating.

Reaction products

To confirm this hypothesis, an analysis of the reaction products with L-Orn and L-Leu was undertaken. Samples were incubated with a large amount of enzyme (10-20 mg) and substrate (25 μmoles) for one to two h, then trichloroacetic acid added (5% solution) and the reaction products identified from the deprotenized reaction mixture. Control preparations containing no substrate or no enzyme were also analyzed and no product was found. Addition of catalase or NaN_3 to the reaction mixture did not alter the identity of the reaction products from L-Orn and L-Leu.

Figure 1. Effect of pH on the rate of oxygen consumption by the L-amino acid oxidase from M. demissus gill tissue

The reaction mixture contained 10 μmoles L-Orn, 3.4 mg of washed enzyme protein, and 200 μmoles of sodium citrate at the indicated pH in 2.5 ml.


16
14
12
10
8
6
4
2
(umoles O_2/min-ml) x 10^2
3
3.5
4
4.5
5
5.5
6
6.5
pH

With L-Orn, the reaction product reacted with 2,4-dinitrophenylhydrazine in methanolic-HCl to form a yellow crystalline material. This material gave no red color in 1N NaOH and had the absorption spectrum of the aldo-keto form of the 2,4-dinitrophenylhydrazone of Δ^1-pyrroline-2-carboxylate in 0.25 N HCl (Meister, 1954). The reaction product with L-Orn also formed a yellow color with ortho-aminobenzaldehyde and had the absorption spectrum of the Δ^1-pyrroline-2-carboxylate derivative of ortho-aminobenzaldehyde, and NH_3 was also formed.

The products formed from L-Leu were NH_3 and a 2,4-dinitrophenylhydrazine reactive material which was crystallized from methanolic-HCl. This hydrazone formed a red-brown color in 1 N NaOH and had an absorption spectrum identical to the 2,4-dinitrophenylhydrazone of a α-keto-isocaproic acid in 2 N HCl (Struck and Sizer, 1960).

Samples of deproteinized reaction mixtures with L-arg and with L-Orn as substrates were heated in 1 N HCl for 1 h at 100°C then chromatographed on paper (Olomucki et al., 1960) and sprayed with the Sakaguchi reagent or ninhydrin reagent, respectively. No γ-amino-butyrate was produced from L-Orn and no γ-guanidinobutyrate was produced from L-arg. However, H_2O_2 oxidation of pH neutralized L-Orn reaction product produced nearly a complete conversion to a compound having the same R_f as γ-aminobutyric acid, and alkaline reduction of the L-Orn reaction product yielded pure L-proline (by amino acid analysis). Additionally, incubations (pH 4.8) with [U-^{14}C]-L-Orn (3.4×10^4 dpm/μmole) which resulted in the formation of 4.5 μmoles of Δ^1-pyrroline-2-carboxylate produced only 0.018 μmoles of CO_2. Incubations (pH 4.8) of an appropriate amount of enzyme with [U^{14}-C]-L-Leu (1.1×10^6 dpm/μmole) produced

4.1 μmoles of α-keto-isocaproic acid and only 0.0068 μmoles of CO_2. The 2,4-dinitrophenylhydrazone of ^{14}C-α-keto-isocaproic acid was separated and identified by paper chromatography (Struck and Sizer, 1960) on Whatman #1 using n-butanol:ethanol:H_2O (50:40: 10) as a developing solvent.

The enzyme in M. demissus is similar to the deaminating L-amino acid oxidase described for M. edulis (Hope and Horncastle, 1967), turkey liver (Boulanger et al., 1957), and chicken liver (Struck and Sizer, 1960). There was no evidence for decarboxylation of the type found with the L-amino acid monooxygenases. The small amount of ^{14}C-CO_2 produced from ^{14}C-L-Orn and ^{14}C-L-Leu may be the result of an oxidative decarboxylation of the keto acid product in the presence of H_2O_2 (Chen and Koeppe, 1970; Hope et al., 1967; Meister, 1960). In this regard, the claim for the decarboxylating monooxygenase activity (forms γ-amino-butyrate and γ-guanidinobutyrate from L-Orn and L-arg, respectively) in the pulmonate snail Lymnea stagnalis (Olomucki et al., 1960) and in the bivalve Cardium tuberculatum (Roche et al., 1959) should be reinvestigated. In both of these cases, catalase reduced the apparent oxygen consumption indicating that H_2O_2 is a product. However, H_2O_2 is not a product with true monooxygenases (Pho et al., 1966; Takeda et al., 1969). This aspect is of some interest because the L-lysine monooxygenase from Pseudomonas has a deaminating oxidase activity with L-Orn and can exhibit both the decarboxylating monooxygenase activity and the deaminating oxidase activity with L-lys depending upon the conditions in the reaction mixture (Flashner and Massey, 1974a,b; Nakazawa et al., 1972; Yamamoto et al., 1972; Yamauchi et al., 1975).

Table 1. Specificity of L-amino acid oxidase from M. demissus gill tissue

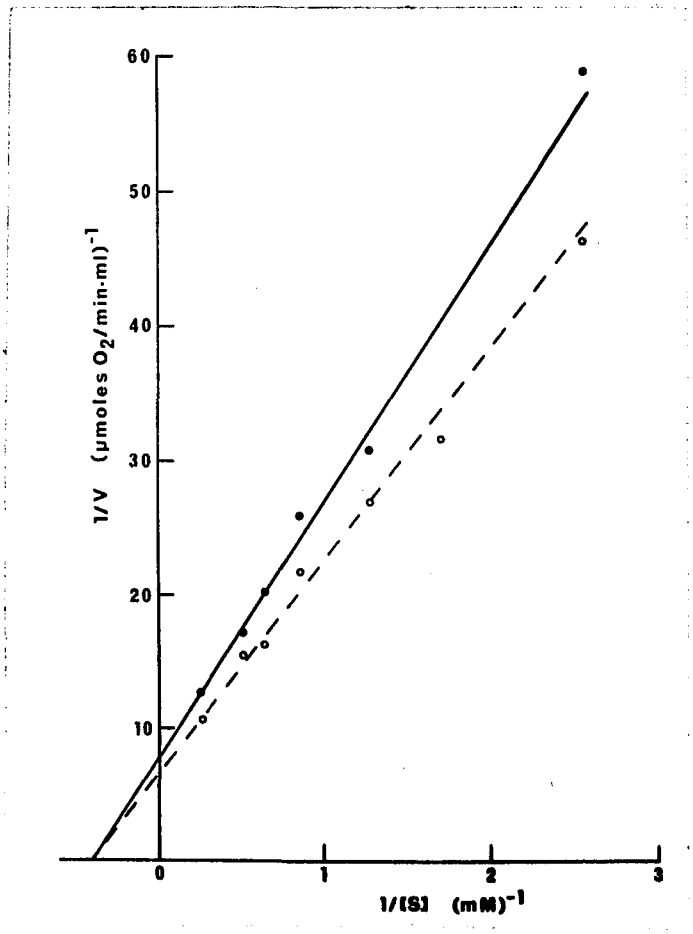
Amino acid substrate[b]	Relative rate of oxygen consumption[a]	
	pH 4.8	pH 7
L-alanine	11	3.4
L-arginine	66	4.2
L-asparagine	35	3.6
L-citrulline	106	8.8
L-cysteine	100	-
L,L-cystine	63	-
L-glutamate	17	0
L-glutamine	15	2.2
L-histidine	80	-
L-homoarginine	141	10.8
L-homocitrulline	120	-
L-homoserine	100	-
L-leucine	84	2.1
L-lysine	98	6.0
L-methionine	105	7.7
L-ornithine	100[a]	10.0
L-phenylalanine	102	-
L-tryptophan	114	-
L-tyrosine	68	-

[a]Each 2.3 ml reaction mixture contained enzyme (see text), 200 μmoles of buffer and 10 μmoles of amino acid. Incubations were at 25° and the reaction initiated by addition of the substrate after equilibration. The buffers were sodium citrate at pH 4.8 and sodium phosphate at pH 7.0.

[b]The rates are calculated relative to the rate with L-ornithine at pH 4.8. Other substrates tested which did not stimulate oxygen consumption were D-aspartate, D-leucine, D-ornithine, β-alanine, L-cycloserine, aminooxyacetic acid, γ-aminobutyric acid, 2-aminoethylphosphonic acid, taurine, glycine, 2,4-(DL) diaminobutyric acid, L-aspartate, L-isoleucine, L-proline, L-serine, L-threonine, L-valine, L-argininic acid, and dithiothreitol.

Figure 2. Effect of L-Orn (-o-o-) and L-Leu (-●-●-) concentrations on the rate of oxygen consumption by the L-amino acid oxidase from M. demissus gill tissue

The reaction mixture contained the indicated amount of L-Orn or L-Leu, 3.4 mg of washed enzyme protein, and 200 μmoles of sodium citrate at pH 4.8 in 2.5 ml.


60
50
40
30
20
10
0
1
2
3
1/V (μmoles O_2/min-ml)$^{-1}$
1/[S] (mM)$^{-1}$

Specificity

Only L-amino acids served as substrates (Table 1); activities with D-orn, D-leu, and D-asp were below detectable levels (< 1% of the rate with L-Orn at pH 4.8 or at pH 7.0). The specificity data (Table 1) and the nearly identical apparent K_ms (2.5 mM) for L-Leu and L-Orn (Figure 2) suggest a space filling role for R-groups extending beyond the γ-carbon of the primary L-amino acids. Methyl, hydroxyl or other substitutions for hydrogen atoms on the β-carbon (i.e. L-ser, L-thr, L-val, L-ile) or the presence of a charged group on the γ carbon (i.e. L-asparate or α, γ-diaminobutyrate as compared with L-homoserine, L-asparagine or L-glutamate) rendered the L-amino acid essentially inactive as a substrate. This specificity was similar to that reported for Mytilus edulis (Blaschko and Hope, 1956), Cardium tuberculatum (Roche et al., 1959), chicken liver (Struck and Sizer, 1960), turkey liver (Boulanger and Osteux, 1956), and Neurospora crassa (Bender and Krebs, 1950).

Inhibitors

The rate of O_2 consumption with L-Orn or L-Leu as a substrate in the standard reaction mixture was not inhibited by addition of the following compounds at a concentration of 2 mM: the transaminase inhibitors aminooxyacetic acid and L-cycloserine; the sodium salts of the dehydrogenase or oxidase inhibitors, benzoate, nitroprusside, cyanide, and arsenite; the reactive serine reagent, phenylmethanesulfonylfluoride; and the sulfhydryl reactive reagents, dithiodinitrobenzoate, N-ethylmaleimide, 2,2-dithiobis (5-nitropyridine), $CdCl_2$, and dithiothreitol. Iodoacetate at 2 mM caused a 30% inhibition. This

lack of strong inhibition by the sulfhydryl and other reagents has also been noted for the enzyme from Mytilus (Blaschko and Hope, 1956), Cardium (Roche et al., 1959) and chicken liver (Struck and Sizer, 1960).

Roche et al. (1959) found some inhibition of the oxidase from Cardium by amino acids which did not serve as substrates but accumulated as putitive osmotically active metabolites and has suggested a possible regulatory role for these metabolites. With the M. demissus oxidase, addition of 500 mM NaCl or KCl or of 100 mM gly, taurine, and L-pro which accumulate during hyperosmotic stress (Baginski and Pierce, 1975) did not inhibit O_2 consumption with L-Orn or L-Leu in the standard reaction mixture. However, a 30% and 46% inhibition was observed with γ-aminobutyrate and β-ala, respectively, at 4.3 mM with 4.3 mM L-Orn as a substrate in the standard reaction mixture. A small but significant accumulation (approximately 2 mM) of β-ala has been observed during short term hyperosmotic stress (Greenwalt and Bishop, 1980). The change in rates at various β-ala and L-Orn concentrations indicated a complex pattern of inhibition which is under continuing investigation. In any case, it would appear that except for β-ala and L-ala which are poor substrates for this L-amino acid oxidase, the nonsubstrate amino acids which accumulate would not appear to have a major role in the regulation of this enzyme.

Distribution

The levels of L-amino acid oxidase were highest in hepatopancreas and gill tissues (Table 2). The activity in heart was low compared to

Table 2. Activity of the L-amino acid oxidase in various tissues of M. demissus

Tissue	Activity (units[a]/g wet tissue) with L-Orn	with L-Leu
Gill	0.093	0.076
Heart	0.078	0.019
Hepatopancreas	0.153	0.105
Mantle	0.041	0.025
Adductor muscle	0.027	0.017

Enzyme preparation: after homogenization of the tissue in 10 ml of 50 mM potassium phosphate 1 mM EDTA (pH 7) buffer per g wet tissue, the particulate matter was collected by centrifugation, resuspended and dialyzed (see methods), then centrifuged and the pellet resuspended in 2 ml of buffer per g of original tissue. Activity was determined (see methods) with 25 mM substrate, resuspended enzyme, and 0.78 M NaN_3 at 25°C in citrate buffer at pH 4.8.

[a]Units represent that amount of enzyme which stimulated utilization of one μmole of O_2 per minute.

gill and showed a low percentage activity with L-Leu compared to L-Orn. These differences in the L-amino acid oxidase activities between heart and other tissues may indicate some variability in substrate specificity among tissues and is under investigation.

Discussion

A most interesting aspect of this investigation is the finding that this deaminating L-amino acid oxidase is most active between pH 4.5 and 5 (Figure 1). Roche et al. (1952) found considerable activity at pH 5 with the Mytilus enzyme but reported a shift in its pH optimum to pH 7 on treatment with NaN_3. All subsequent studies with the Mytilus enzyme and a survey for the activity in other bivalve species (Blaschko and Hope, 1956; Hope and Horncastle, 1967) were performed at pH 7.1 where the M. demissus oxidase shows only 10% of its optimal activity. In M. demissus, the activity of this L-amino acid oxidase at its optimal pH is similar to the mitochondrial glutamate dehydrogenase activity at its optimal pH (Reiss et al., 1977). The oxidases from chicken liver (Struck and Sizer, 1960), Cardium (Roche et al., 1959), and Lymnea (Olomucki et al., 1960) are optimally active above pH 7.4.

The enzymes from Mytilus (Blaschko and Hope, 1956) and chicken liver (Struck and Sizer, 1960) are also particulate (mitochondrial?) and seem to be of comparable solubility and general specificity to this bivalve gill enzyme. The particulate nature and low pH optimum of this enzyme seems indicative of a lysosome associated activity, eventhough the conditions of preparation did not suggest lysosomal latency. The lack of solubilization of these enzymes by hypoosmotic shock or by detergent or acetone extraction indicates that they are probably not soluble components within membrane bound organelles. However, these enzymes could be located on high density particles in the cytosol or associated within membraneous structures such as lysosomes or mitochon-

dria. Particles have been detected in the tissues of Mytilus (Humphreys, 1962; Zaba et al., 1978) but no function has been assigned to these structures. In this regard, the lysosomes, which contain an aminopeptidase (Young et al., 1979) and other catabolic enzymes, become activated when mussels (Mytilus) are subjected to various environmental stresses (Moore, 1976; Moore et al., 1979; Thompson et al., 1978). The possibility that the activity of the lysosomal proteases and this L-amino acid oxidase may be under coordinate control to regulate aspects of protein turnover and amino acid catabolism during hypo- or hyperosmotic stress in euryhaline mussels is under investigation.

PART II. PREPARATION AND SOME RESPIRATORY PROPERTIES OF COUPLED MITOCHONDRIA FROM RIBBED MUSSEL (MODIOLUS DEMISSUS) GILL TISSUE

Summary

Preliminary experiments with 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), and sodium azide (NaN_3) indicated that most of the oxygen consumption by ribbed mussel gill tissue was the result of mitochondrial respiration. A procedure utilizing isoosmotic sucrose, EGTA, defatted serum albumin, and HEPES as the isolation medium was devised for the preparation of fully coupled ribbed mussel gill mitochondria. Optimal rates of respiration and respiratory coupling required substrate, ADP, inorganic phosphate and a fairly high KCl concentration (90 mM) in the assay medium. Glutamate, proline, malate, and succinate stimulated oxygen consumption with high respiratory control indices and P/O of 3, 3, 3, and 2, respectively. Pyruvate was a weak stimulator of mitochondrial respiration and showed a low respiratory control index with a low P/O. Preparation of gill mitochondria in isoosmotic solutions containing high KCl concentrations (150 mM) yielded mitochondria with state 2 respiration, slow partially uncoupled, ATP synthesis during state 3 respiration and no state 4 respiration. D-mannitol was not used in the mitochondrial isolation or assay medium because of the probable presence of a D-mannitol oxidase in these gill mitochondria.

Introduction

Mitochondria isolated from the tissues of bivalve and gastropod species (Newell, 1967; Vorhaben et al., 1980; Zaba et al., 1978; Akberali and Earnshaw, 1982; Zaba, 1983) tend to show no respiratory control or very low respiratory control indices (RCI) (Estabrook, 1967). The mitochondrial respiratory states have been defined by Chance and Williams (1956) as a means of determining the competency of mitochondria following isolation from various tissues. For instance, state 2 respiration is the oxygen consumption rate after addition of substrate but not ADP and inorganic phosphate; state 3 respiration is the oxygen consumption rate in the presence of substrate, ADP and inorganic phosphate; and state 4 respiration is the oxygen consumption rate in the presence of substrate after the conversion of all added ADP to ATP. So called "normal mitochondria" should exhibit very low rates of oxygen consumption in states 2 and 4 and a high rate of oxygen consumption in state 3. Mitochondria which fail to show high respiratory control indices (ratio of respiratory rate in state 3 to state 4) or have strong ADP independent respiration are usually judged to be either damaged in preparation or very unusual.

Mitochondria with unusual respiratory characteristics and missing respiratory components have been found in some helminth parasites with high rates of anaerobic glycolysis, high anaerobic tolerance and low rates of oxygen consumption (van den Bergh et al., 1980; Köhler and Bachmann, 1980; Rodrick et al., 1982; Saz, 1981). Although many bivalve and gastropod molluscs show high degrees of anaerobic tolerance (Hammen, 1976), high rates of anaerobic glycolysis (de Zwaan, 1977; Hochachka,

1981), possible high tolerances to some of the respiratory chain inhibitors (Mattisson, 1961a; Zaba, 1983), and in some cases, abilities to accumulate unusual end-products (succinate, propionate, acetate, etc.) of anaerobic glycolysis that are identical to those accumulated by some helminths (see Saz, 1981; Kluytmans et al., 1975, 1977, 1978; Zurburg and Kluytmans, 1980), none of these studies give direct evidence for unusual respiratory chains in the mitochondria of tissues from these molluscs. In fact, recent studies (Jamieson and de Rome, 1979) on respiratory rates of isolated bivalve hearts (Tapes walingi) in the presence and absence of inhibitors of the mitochondrial respiratory chain are in agreement with the concept that, as with mammalian liver tissue (Jacobus et al., 1982), almost all of the oxygen consumption by the clam heart was mitochondrial and that the clam heart mitochondria probably have a normal respiratory system that show normal respiratory control. Earlier studies on the properties of mitochondrial respiratory chain components of bivalve and gastropod tissues (Strittmatter and Strittmatter, 1961; Tappel, 1960; Pablo and Tappel, 1961; Mattisson, 1959, 1961a,b,c; Mattisson and Birch-Andersen, 1962; Black, 1962a,b) are in agreement with the concept of a "normal" respiratory system.

Most procedures for the isolation of mitochondria from the tissues of molluscs have employed media made isoosmotic with buffered sucrose solutions similar to the procedure described by Zaba et al. (1978). This procedure was modeled after the original mitochondrial isolation procedure for mammalian tissues (Schneider and Hogeboom, 1950; Hogeboom et al., 1948). However, other media utilizing isoosmotic mannitol with sucrose in a fashion similar to media used by some investigators for

the isolation and/or assay of mitochondria from helninths (Papa et al., 1970; Murfitt et al., 1976; Köhler, 1977; Köhler and Bachmann, 1980; Rodrick et al., 1982) blue crab gill (Chen and Lehninger, 1973) and mammalian tissues (Johnson and Lardy, 1967; Greenawalt, 1974) have been used with some molluscan tissues (Holwerda and de Zwaan, 1979; Vorhaben et al., 1980; de Zwaan et al., 1981; Zaba, 1983). Isoosmotic solutions containing KCl with sucrose have been used for the isolation of mitochondria from the tissues of a number of cephalopods (Mommsen and Hochachka, 1981; Ballantyne et al., 1981). Although the inclusion of D-mannitol in the isolation medium apparently has no deliterious effect on mitochondria isolated from the helminth parasites and mammalian tissues, a D-mannitol oxidase has been found in mitochondria from some molluscan tissues (Vorhaben et al., 1980) that compromises measurements of respiration by mitochondria isolated or incubated in this medium. Inclusion of KCl (150 mM) in the medium for isolation and measurements of respiration of squid heart mitochondria apparently had no adverse effect on respiration or the coupling of respiration to phosphorylation (Mommsen and Hochachka, 1981). Although some potassium ions may be required for efficient respiratory control with most mitochondria (Clark and Nicklas, 1970; Kernan, 1980), high concentrations of potassium ions with permeant anions such as chloride or nitrate can cause mitochondrial swelling and dysfunction (Brierley et al., 1977; Azzi and Azzone, 1967) particularly if the mitochondria are held in these solutions at low temperatures (Amoore and Bartley, 1958).

Previous studies from this laboratory on the metabolic component of the processes regulating high intracellular free amino acid levels

in the tissues of euryhaline bivalves indicated that mitochondrial glutamate dehydrogenase may be important in amino acid deamination and turnover and that the mitochondria may contain the other specific enzymes regulating turnover of the accumulated amino acids (Reiss et al., 1977; Burcham et al., 1980; Bishop et al., 1981).

This part describes a procedure for the preparation of mitochondria showing respiratory control from the gill tissue of a euryhaline bivalve (Modiolus demissus).

Materials and Methods

Ribbed mussels (Modiolus demissus) were purchased from Northeast Marine Environmental Laboratory (Monument Beach, MA) and maintained in artificial sea water (ASW) (Jungle Laboratories Corp., Sanford, Florida) at 450 mOsmoles and 15°C. Except where indicated all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Defatted bovine serum albumin (BSA) was prepared from fraction V (Sigma) by modifying the procedure of Chen (1967) in the following manner. BSA (35 g) was dissolved by stirring at room temperature into 350 ml of 50 mM KCl with the aid of 1.5 ml sec-butanol added dropwise to prevent foaming. After cooling to 0°C, the solution was acidified to pH 3 with slow addition of 3 N HCl, and 17.5 g, activated charcoal (HCl washed grade) was slowly mixed into the solution. After 1 h of gentle stirring with a large magnetic stir bar, the mixture was centrifuged at 20,000 xg for 20 min at 0°C. The supernatant fraction was adjusted to pH 7.5 with slow addition of 3 N KOH and dialyzed against 500 volumes of

distilled-deionized water. Residual charcoal was removed from the dialysate by filtering thru 0.45 μm membrane filters (Millipore Corp., Bedford, MA) and the solution was diluted to 5% BSA concentration before storage at -20°C.

Gill mitochondria were isolated in two different isolation buffers. Buffer A (420 mOsmoles) contained 0.4 M enzyme grade sucrose (Schwartz-Mann, Orangeberg, NY), 20 mM potassium HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.5, 1 mM potassium EGTA (ethyleneglycol-bis(β-aminoethyl ether)N, N'-tetraacetic acid) pH 7.5, and 0.5% defatted BSA. Buffer B (450 mOsmoles) contained 0.13 M sucrose, 0.15 M KCl, 20 mM potassium HEPES (pH 7.5), 1 mM potassium EGTA (pH 7.5), and 0.5% defatted BSA.

The procedures for isolating the mitochondria were performed at 0°C in the following manner. Gill tissue was collected, drained of sea water, suspended in 10 ml buffer/g tissue of either buffer A or buffer B depending upon the experiment, and then homogenized twice at the 40-45 power setting for 10 seconds using an Ultra Turrax Model T45/N (Tekmar Co., Cincinnati, OH). Additional buffer was added with stirring to double the volume, and this homogenate was filtered through one layer of Miracloth (Calbiochem, Irvine, CA). The filtrate was homogenized in a glass-teflon homogenizer (5×10^{-3} inch clearance) at 100 RPM with one complete passage of the pestle. This homogenate was centrifuged at 1500 xg for 8 min. The supernatant fluid was collected then re-centrifuged at 9000 xg for 15 min. Fresh isolation buffer (10 ml) was carefully added to the 9000 xg pellet and any loosely packed material on the top surface of the pellet was carefully suspended by gentle

swirling and discarded. The pellet was resuspended in fresh isolation buffer by careful irrigation with a Pasteur pipette, taking care to leave behind the hard packed bright yellow halo on the bottom of the tube. The mitochondrial fraction was washed twice by resuspension in 4 ml of isolation buffer per g of tissue initially used followed by centrifugation at 9000 xg for 15 min. The processes of removing loose and hard packed material from the pellet surfaces were duplicated after each centrifugation. The final pellet was treated in like fashion and suspended in isolation buffer at a concentration of approximately 5 mg mitochondrial protein/ml.

The medium for measuring oxygen consumption of isolated mitochondria consisted of 0.05 ml substrate, 0.05 ml KH_2PO_4 (0.2 M), 1 ml of mitochondria isolated and suspended in either buffer A or buffer B and 1.4 ml of either buffer A or buffer B (depending upon the experiment). Buffer C consisted of 1 ml mitochondria isolated and suspended in buffer A, 1.4 ml buffer B, 0.05 ml substrate, and 0.05 ml of 0.2 M KH_2PO_4. In all cases, the final volume of the reaction was 2.5 ml. All substrates were prepared in buffer B and titrated to pH 7.5 with KOH. Stock solutions (2 mM) of rotenone and antimycin A were prepared in 95% ethanol.

Oxygen measurements were performed with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The electrode was calibrated using air-saturated deionized water and by the submitochondrial particle (pig heart) procedure described by Estabrook (1967). All incubations and calibrations were performed at 25 ± 0.1°C.

Oxygen consumption in isolated gill tissue was measured using a Gilson Differential Respirometer by the following procedure. Whole

gills were collected and maintained at 15°C in aerated ASW (450 mOsmoles) then cut between gill filaments into 1 cm strips, blotted on filter paper and weighed. The gill strips (100 mg) were placed in a 10 ml respirometer flask at 15°C containing 2 ml ASW. The center well contained 4 M KOH (0.2 ml) for CO_2 absorption. The flasks were attached to the respirometer and incubated at 25°C with shaking (100 cycles/min). Measurements were initiated after 30 min of temperature equilibration.

A modification of the glucose-hexokinase trap procedure was employed with ^{32}P-inorganic phosphate to measure ATP synthesis by isolated mitochondria. Specific conditions concerning the reaction mixture are described with the results. The reaction was terminated by precipitation of the proteins from the reaction mixture by addition of $HC1O_4$ and the protein removed by centrifugation. The supernatant fluid containing the soluble components was neutralized with KOH, and the $KC1O_4$ removed by centrifugation. Samples of the neutralized supernatant fluid were spotted on 20 × 46 cm sheets of Whatman 3 mm filter paper for paper chromatography. The chromatograms were developed by descending chromatography using isopropylether:90% formic acid (90:60, vol/vol) as the developing solvent (Hanes and Isherwood, 1949). In these assays, two areas of radioactivity corresponding to inorganic phosphate and glucose-6-phosphate were detected by autoradiography using nonscreen x-ray film. The amount of ^{32}P labelled glucose-6-phosphate was estimated by cutting the radioactive areas from the chromatograms, placing the cut-outs in vials containing Econofluor (NEN) and measuring the radioactivity in a Beckman LS 250 liquid scintillation counter.

Electron microscopy of unfixed mitochondria was accomplished by negative staining at pH 7 in 0.4% sodium phosphotungstate (PTA) and 0.05 to 0.1% bovine serum albumin (fraction V, Sigma). Intact mitochondria in buffer A or B, were diluted 1:20 in the PTA/BSA stain and held for 3 to 5 minutes at room temperature. The suspension was sprayed onto carboned collodion-filmed grids with a Vaponefrin-type nebulizer (Ted Pella Co., Tustin, CA) and examined immediately in a Philips EM-200 operated at 60 KV and with a 30-35 μm copper aperature. Contrasting of cristae was most readily achieved by diluting the mitochondria in H_2O, centrifuging at 1000 xg (10 min), and resuspending in the PTA/BSA stain. ATP synthase particles were most easily detected after mild disruption of the mitochondria in a glass-teflon homogenizer (Tri-R, Rockville Center, N.Y.). This procedure was similar to that described by Fernandez-Moran et al. (1964).

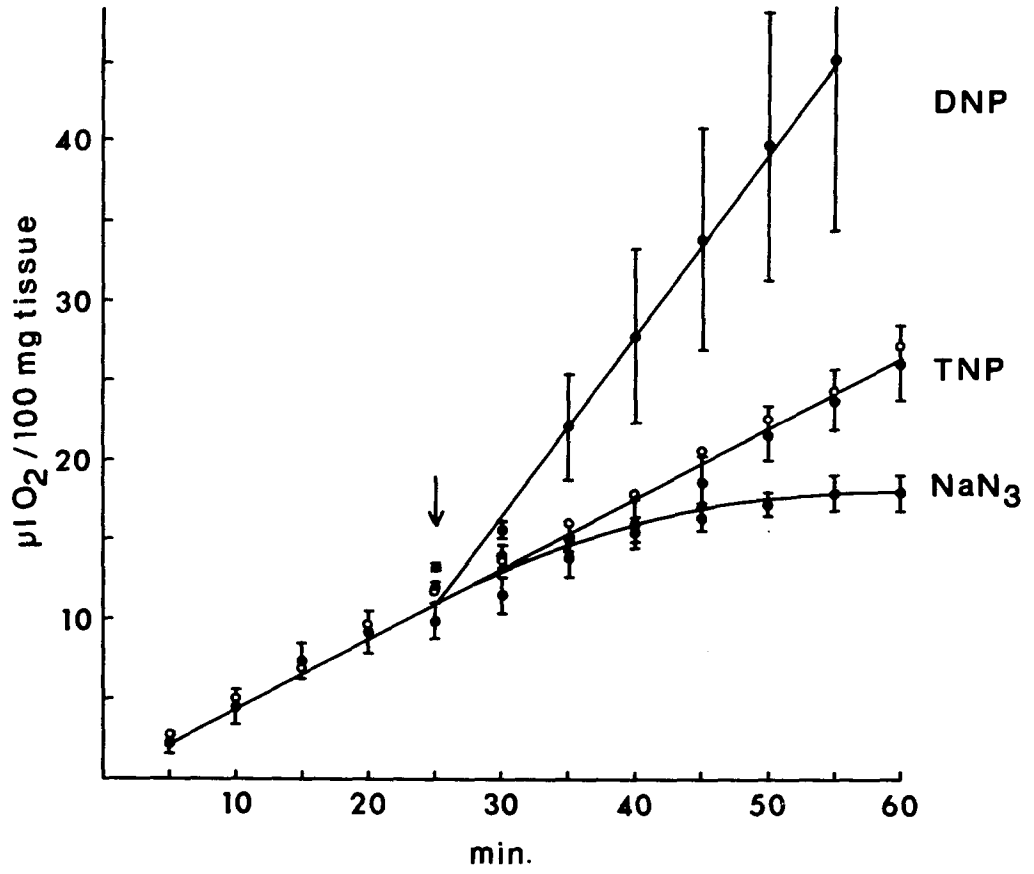
Osmotic pressure was measured by freezing point depression using an Advanced Instruments osmometer (Newton Highlands, MA). Protein was estimated using a biuret method (King, 1967) and standardized with sucrose-BSA solutions.

Results

In order to ascertain whether gill tissue mitochondria exhibited coupled respiration in vivo, oxygen consumption was measured on gill tissue incubated with ASW (450 mOsmoles) in the presence and absence of DNP, TNP, and NaN_3 (Figure 1). Under the conditions of the assay method, the controls consumed oxygen linearly for at least 60 min following a

Figure 1. Effect of nitophenols and NaN_3 on oxygen consumption by isolated gill tissue

Nitrophenols, TNP (2,4,6-trinitrophenol) and DNP (2,4-dinitrophenol), and NaN_3 were added to final concentrations of 0.5 mM, 0.5 mM and 2 mM, respectively. Compounds (-●-●-) were added (at the arrow) to separate flasks by mixing the bathing sea water with flask side arms containing an isoosmotic sea water solution of the compound. Controls (-o-o-) were also mixed with isoosmotic sea water (at the arrow) using volumes equal to the compound solutions. Error bars represent standard deviations (±) of means (points) where n = 3.


DNP
TNP
NaN_3
40
30
20
10
$\mu l\, O_2 / 100$ mg tissue
10
20
30
40
50
60
min.

30 min temperature equilibration period. During the course of the experiment, DNP, TNP, NaN_3, and ASW were added to the ASW bathing medium 25 min after the start of the experiment.

The uncoupler of oxidative phosphorylation, DNP, stimulated tissue oxygen consumption, and the cytochrome c oxidase inhibitor, NaN_3, blocked tissue oxygen consumption. The phenolic compound, TNP, which was used to control for possible membrane effects of DNP (Hanstein and Hatefi, 1974; Jamieson and de Rome, 1979; Kaila and Saarikoski, 1981), had no significant effect on the tissue oxygen consumption rate. These results support the conclusions that most of the tissue oxygen consumption is mitochondrial, that the mitochondria are probably tightly coupled in vivo, and that the mitochondria in these tissues are reasonably "normal" mitochondria with no obviously unusual respiratory properties. Therefore, specific experiments on the isolation of competent mitochondria from gill tissue were undertaken.

Initial experiments on the isolation of ribbed mussel gill mitochondria using isoosmotic isolation media containing d-mannitol, similar to procedures devised by Holwerda and de Zwaan (1979) and Zaba (1983) for the tissues of sea mussels (Mytilus edulis) yielded preparations of mitochondria that showed rapid rates of oxygen consumption (state 1 respiration) in the absence of added substrate, ADP, and inorganic phosphate (data not shown). Since these results indicated the probable presence of a D-mannitol oxidase similar to that described by Vorhaben et al. (1980) for mitochondria from the hepatopancreas of land snails, D-mannitol was not used in the preparation of mitochondria from ribbed mussel tissues.

Ribbed mussel gill mitochondria prepared and incubated in the KCl rich buffer B showed a small increase in the rate of oxygen consumption after adding 1 mM glutamate (state 2); this rate of oxygen consumption was stimulated when both ADP and inorganic phosphate were present (state 3). However, these mitochondria showed no transition to state 4 respiration suggestive of a possible lack of ATP synthesis during "state 3" respiration (Figure 2). In separate experiments, the gill mitochondria prepared in buffer B possessed a Mg^{+2} stimulated ATPase whose activity was blocked by oligomycin, a specific inhibitor of the F^1-ATPase (Burcham et al., 1982).

To determine if the increased oxygen consumption observed upon addition of ADP and inorganic phosphate (state 3) resulted in ATP synthesis, the mitochondria (prepared in buffer B) were incubated aerobically using ^{32}P-inorganic phosphate and a hexokinase-glucose trap to monitor ATP synthesis (Table 1). Glutamate and proline substantially increased the formation of ATP (glucose-6-phosphate) compared to controls containing no substrate, the inhibitors rotenone and antimycin A, and the uncoupler, DNP. These results demonstrated that mitochondria prepared in buffer B could synthesize ATP from ADP and inorganic phosphate and that this synthesis was coupled to the oxidation of proline and glutamate through the electron transport chain.

The apparent lack of state 4 respiration by mitochondria prepared in buffer B prompted an electron microscopic examination to determine whether or not the elementary particles and membranes of the mitochondria were intact. Examination of these negatively stained mitochondria, revealed apparently intact mitochondria with diameters ranging from 0.2

Figure 2. Oxygen consumption by gill mitochondria isolated and incubated in buffer B

Reaction mixture contained 3 mg mitochondrial protein, 1 mM glutamate, and 4 mM K_2HPO_4 in 2.5 ml buffer B. Arrow indicates place where state 3 respiration should end and state 4 respiration should begin assuming a P/O of 3. Additions: 5 nmoles rotenone (R) and 1 mM potassium-succinate.

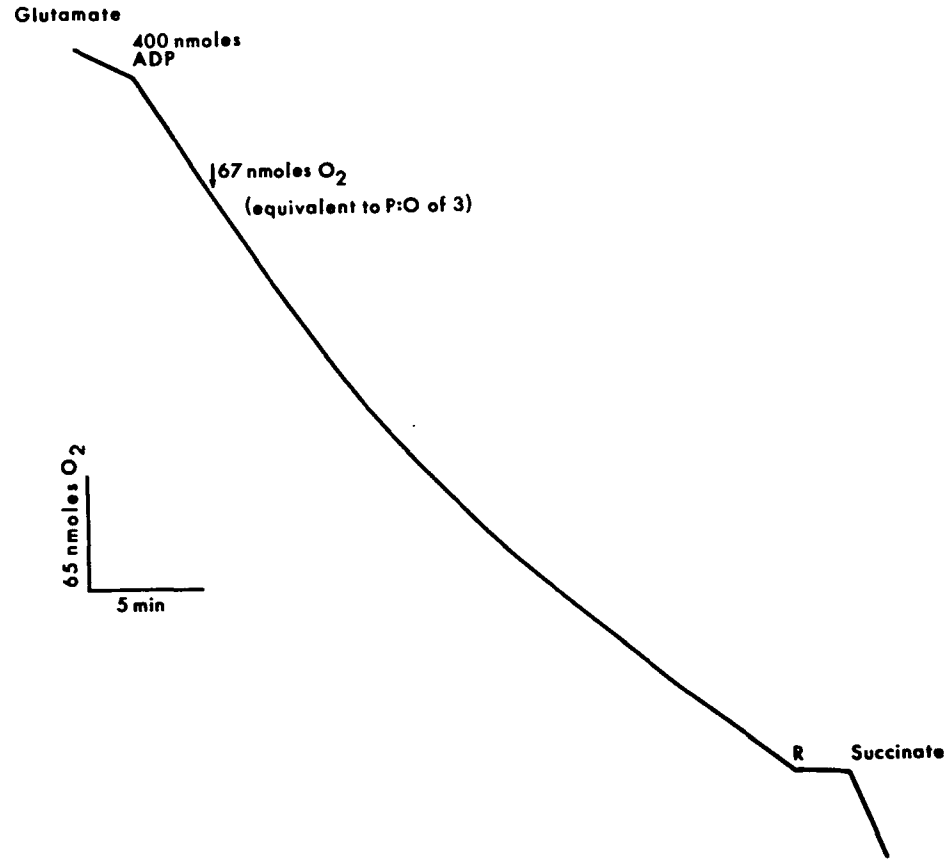

Glutamate
400 nmoles ADP
67 nmoles O_2
(equivalent to P:O of 3)
65 nmoles O_2
5 min
R
Succinate

Table 1. Synthesis of ATP by gill mitochondria prepared in buffer B

Substrate[a]	nmoles ATP/mg protein/30 min
None added	28.8
Glutamate	197.4
Proline	169.9
Proline + rotenone	30.0
Glutamate + rotenone	30.0
Glutamate + DNP	54.5
Proline + antimycin A	25.9

[a]Mitochondria (5 mg of protein) isolated and resuspended in buffer B were added to reaction mixtures of buffer B supplemented with the following: 5 mM glucose, 5 mM $MgSO_4$, 5 mM substrate, 2 mM ADP, 100 units of hexokinase (Type F-300, Sigma), and 2 mM (^{32}P)-K_2HPO_4 (specific radioactivity of 4.5×10^6 dpm/μmole) in a final volume of 1.1 ml. The reaction was initiated by addition of ADP containing the (^{32}P)-K_2HPO_4 and stopped after 30 min at 22°C by adding 0.1 ml of 3 N $HClO_4$. ATP synthesis was determined as glucose-6-phosphate production as described in Materials and Methods section. The inhibitors, rotenone and antimycin A, were added in ethanol (see Materials and Methods) at 1 nmole/mg of mitochondrial protein and DNP (2 mM) was added in H_2O to a final concentration of 50 μM. Data represent the means of two determinations.

to 1.5 μm. Although no cilia or bacteria were observed, most preparations had a small degree of contamination by a mucus-like material and small membrane bounded vesicles which were seen only at higher stain concentrations (0.6% PTA). The most common morphology of intact mito-

chondria is represented in Figure 3A. A clear distinction between the outer and inner mitochondrial membranes was uncommon. However, by washing the mitochondria in H_2O and quickly resuspending them in staining solution, the fine details of the both membranes were clearly seen (Figure 3B) as revealed by collapsing or flattening of the structures. Close examination showed that the cristae were decorated with many small particles (Figure 3C) characteristic of the "ball on a stick" conformation of the inner membrane bound ATP synthase complex (Fernandez-Moran et al., 1964; Soper et al., 1979).

These observations indicated that mitochondria prepared in the high KCl buffer (buffer B) appeared intact and from the data of Table 1 and Figure 2 also contained functional ATP synthetic enzymes that were, at least, partially coupled to the electron transport chain. The sustained state 3 respiration and lack of state 4 respiration seen following isolation (Figure 2) may be related to KCl loading during preparation.

The goal of preparing mitochondria showing respiratory control was realized using isoosmotic buffers lacking KCl. Mitochondria, isolated in buffer A, were assayed in a reaction mixture similar to the mixture used to assay mitochondria prepared in buffer B (Figure 4). These mitochondria exhibited a slow respiratory rate (state 2) in the presence of added substrate (glutamate). This rate increased with addition of ADP (state 3) then showed transition to a reduced respiratory rate (state 4) as the ADP was depleted.

Experiments by others (Mommsen and Hochachka, 1981) with mitochondria from cephalapod molluscs indicated that high KCl concentrations may be required for normal respiration by mitochondria from some molluscan

Figure 3. Electron micrographs of mitochondria prepared in buffer B, containing 150 mM KCl and other components (see Materials and Methods)

Magnifications: A) 90,000; B) 74,000; C) 200,000. The method of preparation is described in the text. Scale bars equal 0.1 μm.

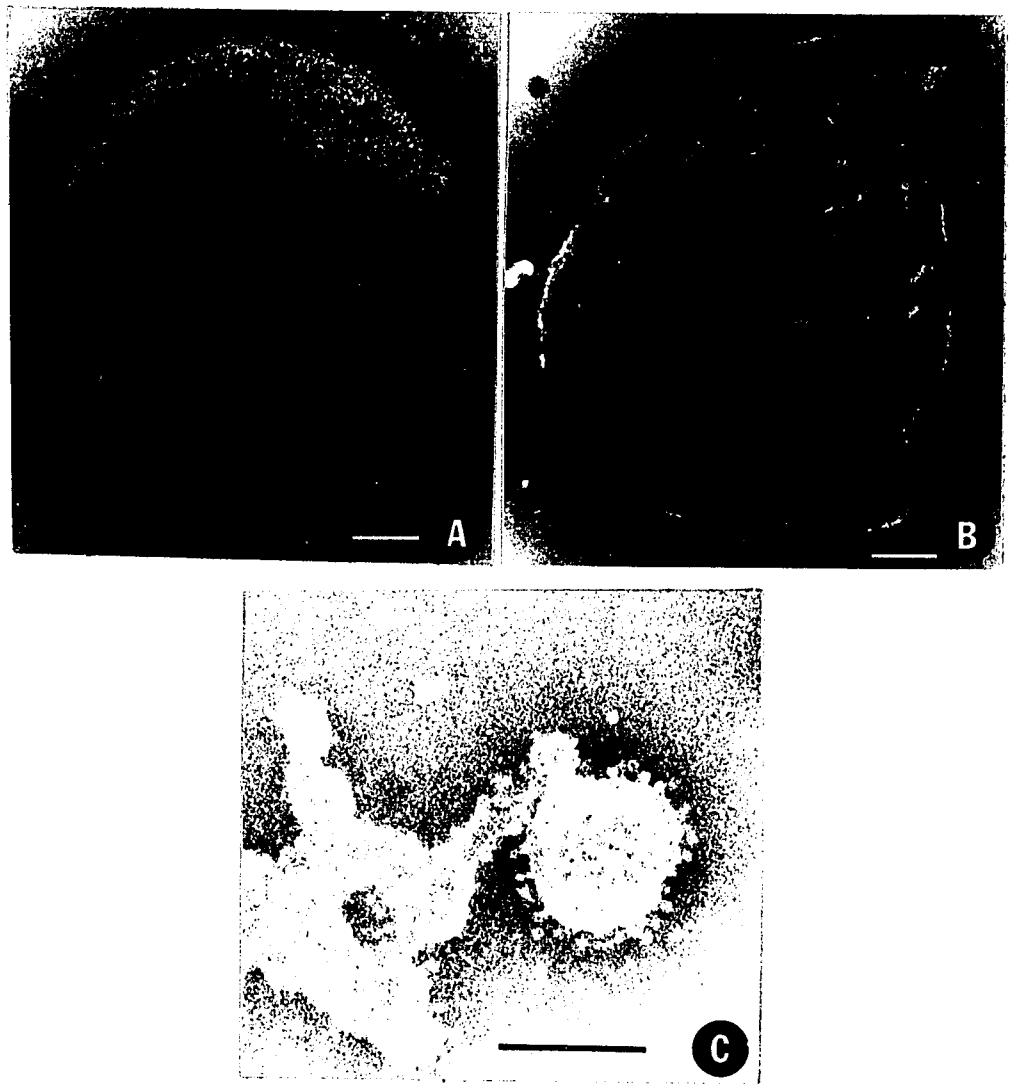

A
B
C

Table 2. Rates of oxygen consumption by gill mitochondria prepared in buffer A

Substrate[a]	nmole O_2/min/mg protein		coupling ratios	
	State 3	State 4	RCI	P/O
Glutamate[b]	3.2 ± 0.3	3.2 ± 0.3	1.0 ±0.1	0.0
Glutamate[c]	11.1 ± 0.6	2.5 ± 0.3	4.4 ± 0.1	3.0 ± 0.1
Glutamate[d]	12.7 ± 1.1	6.2 ± 0.5	2.0 ± 0.1	1.7 ± 0.1
Proline[c]	13.8 ± 0.6	3.0 ± 0.3	4.6 ± 0.1	3.0 ± 0.1
Pyruvate[c]	2.5 ± 0.0	2.5 ± 0.0	1 ± 0.0	0
Pyruvate plus malate[c]	7.8 ± 0.4	2.2 ± 0.0	3.6 ± 0.1	2.9 ± 0.1
Malate[c]	10.1 ± 0.2	2.6 ± 0.2	4.0 ± 0.1	3.0 ± 0.1
Malate plus pyruvate[c]	10.8 ± 0.4	2.6 ± 0.2	4.2 ± 0.2	3.0 ± 0.1
Succinate[c]	12.3 ± 0.9	5.1 ± 0.5	2.4 ± 0.1	2.0 ± 0.1

[a]The reaction mixture is described in Figure 4. Substrate levels were at 1 mM with 200 nmoles ADP and 4 mM K_2HPO_4 in 2.5 ml incubation medium. Data represent ± standard deviation of the mean where n = 3. Compositions of buffer A, B and C are described in the text.

[b]Mitochondria isolated in buffer A and incubated in buffer A (5 mM KCl).

[c]Mitochondria isolated in buffer A and incubated in buffer C (90 mM KCl).

[d]Mitochondria isolated in buffer A with resuspension and incubation in buffer B (150 mM KCl).

tissues. With glutamate as a substrate and with low KCl (5 mM) concentrations in the assay mixture, the respiratory rate and the RCl was reduced (Table 2). With high KCl concentrations (150 mM) in the assay mixture, the respiratory rate increased, but the RCI was reduced. A KCl

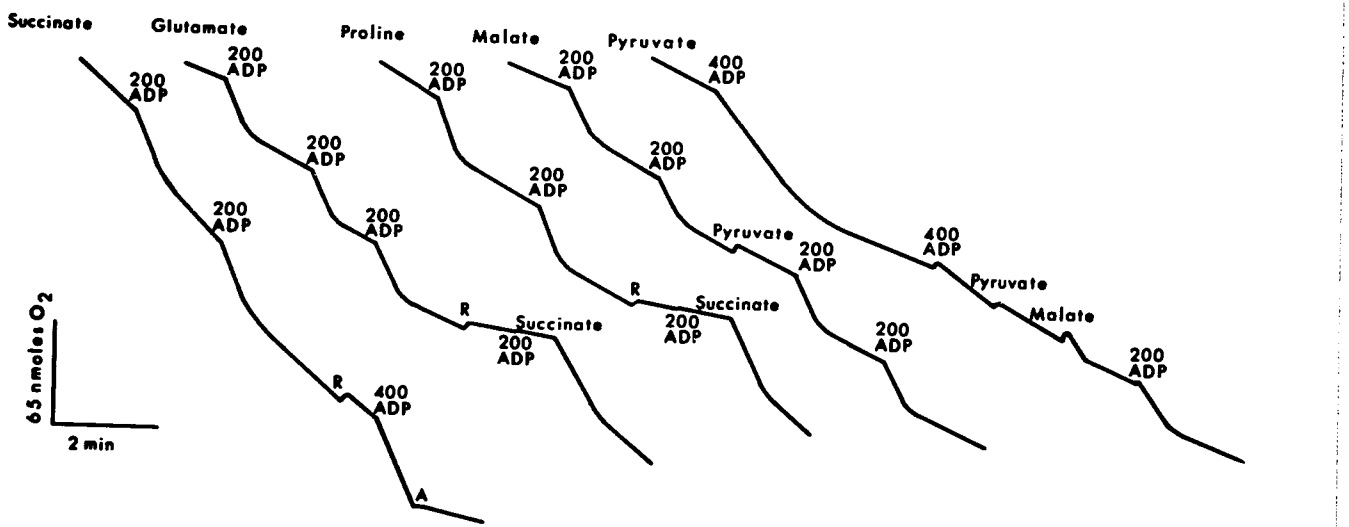
concentration of 90 mM in the assay mixture seemed optimal in terms of respiratory rate and RCI and was used in subsequent experiments.

Mitochondria prepared in buffer A had P/O ratios which were characteristic for the particular substrate (glutamate, proline, succinate, malate) and showed a high degree of respiratory control with most of these substrates (Table 2, Figure 4). Of the substrates tested, only pyruvate failed to generate state 3 respiration (Figure 4). The state 3 respiration initially seen with pyruvate (Figure 4) apparently was the result of endogenous substrate utilization by this preparation, because a second addition of ADP (Figure 4) did not duplicate the initial state 3 rate until another substrate (malate) was added to the reaction. Pyruvate lacked a "sparking" effect with malate (Figure 4), because pyruvate did not alter either the P:O ratios or the RC1's of the malate reaction (Table 2). These results demonstrate the possible absence of a rapid oxidation of exogenously supplied pyruvate by isolated mitochondria.

With glutamate as a substrate, the P/O ratio of about 3, plus inhibition by rotenone (Figure 4) and subsequent stimulation of respiration by succinate was indicative of respiratory coupling at the NADH oxidase step (Complex 1). With succinate as a substrate, the P/O of 2, inhibition by antimycin A, and lack of inhibition by rotenone indicated probable respiratory coupling through CoQ in complex 2. With proline as a substrate, the P/O ratio of about 3 and inhibition by rotenone were indicative of major respiratory coupling at the NADH oxidase step (complex 1) rather than through complex 2. In other studies, Δ^1-pyrroline-5-carboxylate (P-5-C) and glutamate have been identified as products of proline oxidation by ribbed mussel gill tissue (Bishop et al., 1981).

Figure 4. Oxygen consumption by gill mitochondria isolated in buffer A and incubated in buffer C

Reaction mixtures contained 3 mg mitochondrial protein, 1 mM substrates, and 4 mM K_2HPO_4 in 2.5 ml of buffer C. Numbers next to ADP represent nmoles of ADP added. Symbols denote additions of the following: A) 5 nmoles antimycin A; R) 5 nmoles rotenone.


Succinate
Glutamate
Proline
Malate
Pyruvate
200 ADP
400 ADP
R
Succinate
Pyruvate
Malate
65 nmoles O_2
2 min
A

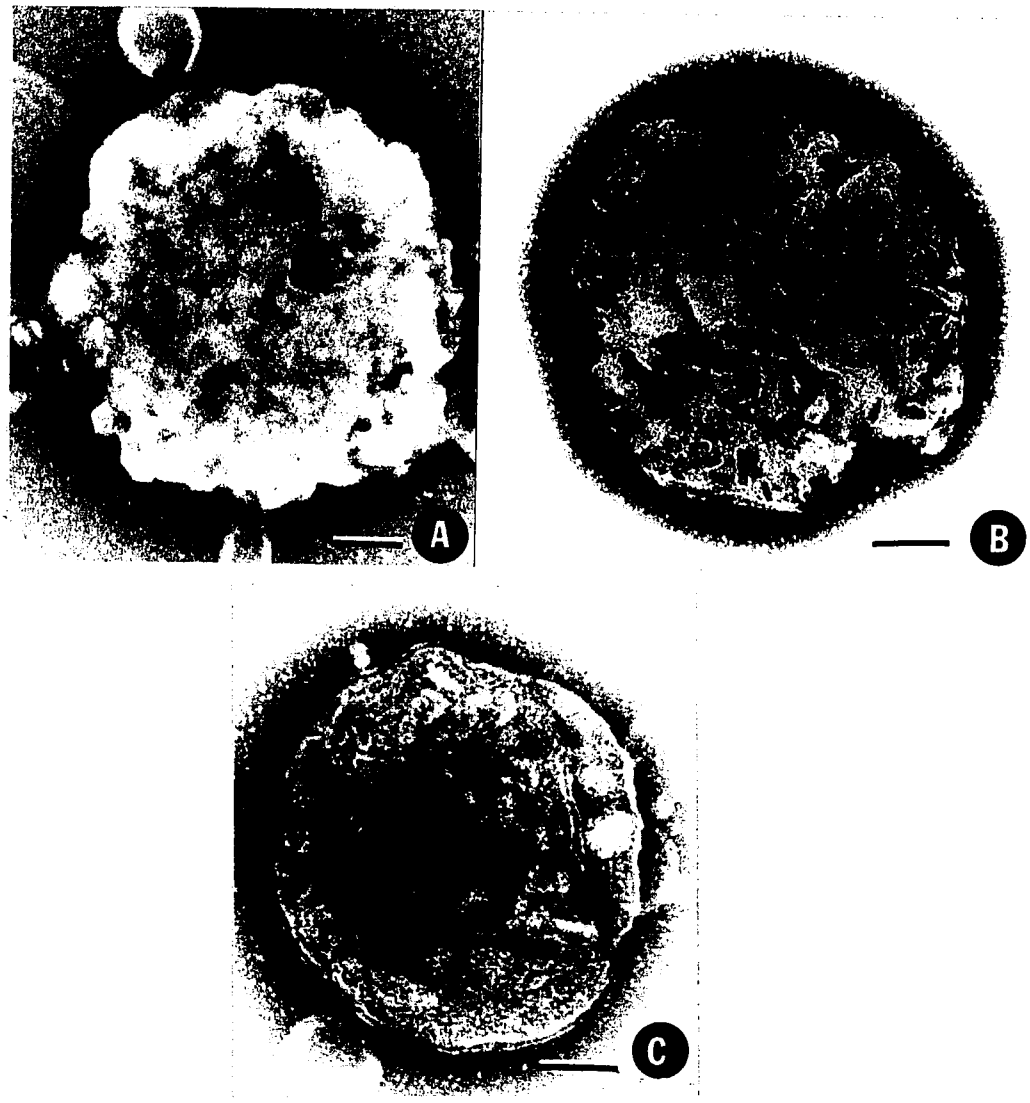
Gill mitochondria (Figure 5) prepared in buffer A (5 mM KCl) compared to those isolated in buffer B (150 mM KCl) exhibited similar overall ultrastructural characteristics to those prepared in buffer B. There were no obvious differences in either the extent or form of contaminating material when high stain concentrations (0.6% PTA) were used. Typically, these mitochondria were impermeable to stain and therefore, exhibited no staining of the inner membrane. Washing of mitochondria prepared in buffer A with H_2O prior to staining allowed partial penetration of PTA through the inner membrane and consequent staining of cristae. The higher sucrose concentration (0.4 M) of buffer A resulted in varying degrees of mitochondrial collapse. The more turgid mitochondria showed numerous convolutions of the inner membrane and a distinct space between the inner and outer membranes in some areas near the periphery (Figure 5B). At late stages of flattening, a clear delineation of membranes was seen (Figure 5C).

These observations (Figures 4, 5; Table 2) indicated that the majority of gill mitochondria prepared in buffer A were morphologically intact and fully functional in a physiological sense.

In the experiments reported here (Figures 2, 4; Tables 1, 2), serum albumin defatted by the procedure of Chen (1967) was added to adsorb endogenous fatty acids released during tissue homogenization and mitochondrial assay. In our hands, ribbed mussel gill mitochondria prepared and assayed with or without commercial preparations of "defatted" serum albumin showed partially uncoupled respiration.

Figure 5. Electron micrographs of mitochondria prepared in buffer A, containing 5 mM KCl and other components (see Materials and Methods)

Magnifications: A) 90,000; B) 85,000; C) 96,000. The method of preparation is described in the text. Scale bars equal 0.1 μm.


A
B
C

Discussion

Ribbed mussel gill tissue exhibits oxygen consumption that is tightly coupled to ATP synthesis and presumably cytochrome C oxidase as indicated by the effects of DNP and NaN_3, respectively (Figure 1). These findings also agree with those of Jamieson and de Rome (1979) and Barrow et al. (1980) on isolated hearts from the bivalve, Tapes watlingi, indicating that most of the observed tissue oxygen consumption was mitochondrial. Taken together, the above finds suggest that aerobic respiration in bivalve mitochondria is regulated by the availability of substrate, ADP, and inorganic phosphate as postulated for mammalian tissue mitochondria (Jacobus et al., 1982).

The ribbed mussel gill mitochondria prepared in the 420 mOsmole sucrose containing buffer A and assayed in an ioosmotic buffer containing 90 mM KCl with sucrose appeared to yield coupled mitochondria with normal P/O ratios for the substrates tested. The rapid ADP dependent metabolism of both proline and glutamate support previous studies on the presence of a physiologically important proline oxidase: P-5-C dehydrogenase system and glutamate dehydrogenase in these tissues (Bishop et al., 1981; Reiss et al., 1977). Mitochondria isolated from the tissues of sea mussels (Mytilus edulis) have been shown to have glutamate dehydrogenase (Addink and Veenhof, 1975) and enhanced ADP dependent oxygen consumption in the presence of added glutamate (Zaba et al., 1978; Akberali and Earnshaw, 1982) but no respiration in the presence of added proline (Zaba et al., 1978). On the other hand, mitochondria from cephalopod tissues showed normal respiratory control

and a rapid metabolism of both glutamate and proline (Mommsen and Hochachka, 1981). It would appear that, among molluscs, proline oxidase may be absent in sea mussel mitochondria but present in both the cephalopod and ribbed mussel mitochondria.

The P/O ratio of 3 and inhibition by rotenone with proline as a substrate during state 3 respiration are suggestive of respiratory coupling of the proline oxidase through the NADH dependent complex 1 and are in agreement with the data reported for L-proline metabolism by mitochondria from mammalian tissues (Meyer, 1977), insect tissues (Sactor and Childress, 1967; Bursell and Slack, 1976; Weeda et al., 1980) and cephalopod tissues (Mommsen and Hochachka, 1981). However, proline oxidase is considered to be a flavoprotein with respiratory coupling through complex 2 rather than complex 1 (see Adams and Frank, 1980). This apparent metabolic paradox has been rationalized by assuming a rapid metabolism of proline through the pathway, proline → P-5-C → glutamate → α-ketoglutarate and TCA cycle intermediates with strong inhibition of the proline oxidase by P-5-C that accumulates in the presence of a rotenone blockade of the P-5-C dehydrogenase step (see Sactor and Childress, 1967; Meyer, 1977). The coupling pattern for the proline oxidation in molluscs with proline oxidase would appear to be similar to the pattern in other species. Specific aspects of proline metabolism by tissues of the ribbed mussel are under investigation.

The lack of a clear cut, rapid respiratory response with pyruvate (Figure 4, Table 2) and the lack of a pyruvate "sparking" effect as described for cephalopod tissue mitochondria (Mommsen and Hochachka,

1981) may reflect some type of metabolic control that is related to the ability of the tissues of these bivalves to accumulate high concentrations of alanine in response to hyperosmotic stress (Baginski and Pierce, 1975, 1977; Greenwalt and Bishop, 1980; Bishop et al., 1981).

The probable presence of a D-mannitol oxidase in ribbed mussel gill mitochondria precluded use of D-mannitol in the isolation or assay medium for these gill mitochondria. Mitochondria from sea mussel tissues have been isolated in D-mannitol containing buffers for a number of studies on anaerobic mitochondrial metabolism (de Zwaan et al., 1981) and on some specific mitochondrial enzymes (Holwerda and de Zwaan, 1979). Subsequent studies on the aerobic metabolism of sea mussel tissue mitochondria prepared in these D-mannitol containing solutions (Zaba, 1983) indicated the presence of a state 1, cyanide, and salicylhydroxamate insensitive respiratory component similar to that described for similarly prepared nitochondria containing D-mannitol oxidase (Vorhaben et al., 1980). The conclusions drawn by Zaba (1983) concerning the presence of an alternate or modified electron transport system or cytochrome oxidase system in sea mussel tissue mitochondria needs re-evaluation.

The electron micrographs of the ribbed mussel gill mitochondria indicated reasonably normal mitochondria with intact elementary particles lining the inner membranes of the mitochondria when prepared in both the KCl and non-KCl containing media (Figures 3, 5). Thus, the presence or absence of state 4 respiration can not be explained by any clear differences in mitochondrial membrane morphology at this electron microscopic level.

The lack of state 4 respiration with coupled ribbed mussel gill mitochondria prepared and assayed in the KCl containing buffer B (Figure 2) as well as with the analogous high KCl (150 mM) media employed for Mytilus edulis mitochondrial preparations (Zaba et al., 1978; Akberali and Earnshaw, 1982) may reflect isotonic loading of the mitochondria with KCl during preparation in the cold as described by Amoore and Bartley (1958) followed by an energy dependent pumping (efflux) of potassium ion from the mitochondria during room temperature assay in the presence of inorganic phosphate, substrate and ADP (Gamble, 1957; Azzi and Azzone, 1967; Brierley et al., 1977). Mitochondria respiration during such ion pumping would give rise to an apparent ATPase artifact in the mitochondrial preparations. This explanation may also be applicable to ATP synthesis of buffer B preparations since the maximal observed ATP synthesis rates of buffer B prepared mitochondria (Table 1) were only 10 to 20% of the rates seen with mitochondria prepared in buffer A (Table 2). The data of Amoore and Bartley (1958) on cold dependent KCl loading of mitochondria suggest that there is a "threshold concentration" for KCl loading in the cold. Because the cephalopod tissue mitochondria prepared by Mommsen and Hochachka (1981) in KCl-sucrose containing buffers showed normal respiratory control, one must assume that KCl loading of these mitochondria may not have occurred under the conditions used by these investigators. The apparent requirement of ribbed mussel gill mitochondria for fairly high KCl concentrations to achieve high respiratory rates with high coupling ratios is curious but may reflect some similarities to the mitochondria from the cephalopod tissues.

PART III. METABOLISM OF ARGININE AND PROLINE IN *MODIOLUS DEMISSUS* GILL TISSUE

Summary

The presence of arginase, ornithine aminotransferase, P-5-C reductase, and proline oxidase was demonstrated in gill tissue from the ribbed mussel, *Modiolus demissus*. Ornithine aminotransferase and proline oxidase were found in mitochondrial fractions, and indirect evidence is presented for a mitochondrial P-5-C dehydrogenase. The proline oxidase lost its rotenone sensitivity after mechanical disruption while still retaining sensitivity to antimycin A. The apparent K_m's for partially purified arginase and ornithine aminotransferase were 7 mM for arginine and 4.8 mM and 2 mM for ornithine and 2-oxoglutarate, respectively. Amino acid analysis and radiotracer experiments indicated that at low concentrations proline is catabolized primarily to organic acids and CO_2, and that biosynthesis of proline results from arginine and ornithinine catabolism.

Introduction

Euryhaline bivalves such as ribbed mussels (*Modiolus demissus*) are of particular interest as models for studying the use of amino acids by invertebrate species as solutes for cellular osmotic regulation during hyper or hypoosmotic changes of the bathing medium. Together with Na^+, K^+, and Cl^- the free amino acid pool constitutes the major solute

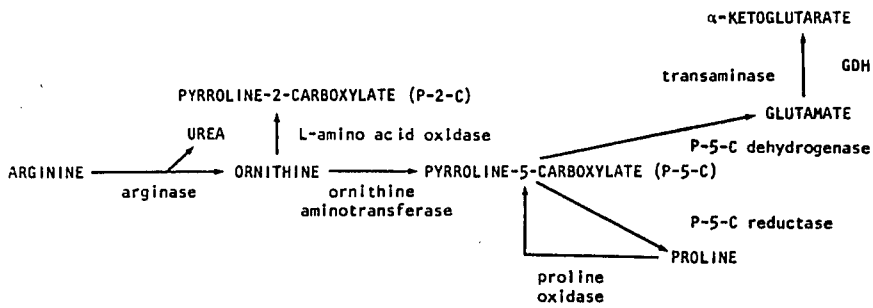
fraction responsible for the regulation of intracellular osmotic pressure and cell volume (see Pierce, 1982; Bishop et al., 1981). In ribbed mussel tissues, taurine, glycine, alanine, glutamate, and aspartate make up approximately 90% of the free organic solute which approaches 0.4 M when the animals are fully acclimated to high salinity environments (Baginski and Pierce, 1975, 1977; Greenwalt and Bishop, 1980; Bishop et al., 1981). During acclimation from low salinity to high salinity, each of these amino acids has a unique time course of concentration increase. This process is common to most osmo-conforming, halotolerant organisms including plants, microorganisms, algae, and marine invertebrate animals (Czonka, 1981; Gilles, 1979; Rains et al., 1980; LeRudulier and Valentine, 1982; Reynoso and De Gamboa, 1982). Isolated tissues such as ventricles and gills from the ribbed mussels respond to a hyperosmotic challenge in much the same fashion as whole animals by showing a rapid accumulation of alanine, followed by a slower accumulation of proline and glycine. Glutamate and asparate levels remain relatively constant during this process. As amino acid acclimation continues for several weeks, glycine and taurine levels rise then level off; alanine levels fall somewhat then level off; and proline levels decline to levels found prior to the hyperosmotic stress (Baginski and Pierce, 1975, 1977; Greenwalt, 1981).

This process of accumulation involves a membrane permeability component and a metabolic component. The membrane component acts to trap these amino acids in the cells during hyperosmotic shift and release them during hypoosmotic shift. The metabolic component directs the flow of carbon and nitrogen into the few amino acids that accumulate

and somehow regulates the amount that accumulates to adjust the intracellular osmotic pressure to that of the bathing medium (Bishop, 1976; Bishop et al., 1981; Pierce, 1982).

An interesting feature of this process was the change in proline concentration in isolated gills and ventricles from nearly undetectable levels to approximately 12 μmoles/g dry weight and 25 μmoles/g dry weight, respectively, in a 6 h period (Baginski and Pierce, 1975, 1977; Greenwalt, 1981; Greenwalt and Bishop, 1980; Bishop et al., 1981). In a series of [^{14}C]tracer experiments with ribbed mussel gill tissue, Greenwalt (1981) established that more than half of the proline accumulating during hyperosmotic shift arose from arginine and ornithine catabolism, and glutamate did not serve as a precursor for proline biosynthesis. In this sense, it appeared that proline accumulation resulted from an increased rate of proline synthesis from arginine and/or ornithine and of proline release from protein combined with some decrease in the rate of proline catabolism. The transaminase inhibitor, aminooxyacetic acid (AOA), caused a marked decrease in the rate of proline accumulation and blocked both ornithine aminotransferase and the conversion of arginine and ornithine to proline and glutamate indicating that ornithine aminotransferase was necessary for proline and glutamate synthesis from orginine and ornithine. On the other hand, AOA had little or no effect on either proline or glutamate catabolism suggesting that the major catabolic route for both these amino acids proceeded through a nontransaminase linked pathway. The general pathway proposed for the arginine-ornithine-proline-glutamate metabolism is described in Figure 1.

Figure 1. Metabolic pathways for arginine, proline, and their related metabolites


α-KETOGLUTARATE
transaminase
GDH
PYRROLINE-2-CARBOXYLATE (P-2-C)
GLUTAMATE
UREA
L-amino acid oxidase
P-5-C dehydrogenase
ARGININE
ORNITHINE
PYRROLINE-5-CARBOXYLATE (P-5-C)
arginase
ornithine
aminotransferase
P-5-C reductase
PROLINE
proline
oxidase

With the possible exception of taurine, the key metabolic enzymes acting to metabolize these amino acids seem to be localized in the mitochondria (Bishop et al., 1983). Therefore, an understanding of the metabolic interconversions for these amino acids should begin at the level of mitochondria particularly for proline and its related precursors or metabolites. The previous section (II) dealt with the preparation and analysis of ribbed mussel gill mitochondria that showed normal respiratory control. This section deals with changes in mitochondrial free amino acids during state 3 respiration of isolated mitochondria in the presence of proline and glutamate and some of the properties of the enzymes involved in proline and arginine metabolism in gill tissue.

Materials and Methods

Ribbed mussels (Modiolus demissus) were obtained from Northeast Marine Environmental Laboratory (Monument Beach, MA) and held in the laboratory at 15°C in tanks of aerated 450 mOsmole artificial seawater (Jungle Salts, Jungle Laboratories, Sanford, FL). Except where indicated all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All amino acids were the L-isomers. Enzyme grade sucrose was obtained from Schartz-Mann (Orangeburg, NY). Defatted serum albumin was prepared from fraction V (Sigma) using a modified procedure of Chen (see Section II).

Isolation of mitochondria

Gill tissue was homogenized in a buffered isolation medium (pH 7.5) containing 0.4 M sucrose, 20 mM potassium HEPES (4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid), 1 mM potassium EGTA (ethyleneglycol-bis(β-aminoethylether)N, N'-tetracetic acid) and 0.5% defatted BSA using an Ultraturrax Model T45/N (Tekmar, Cincinnati, OH). This isolation procedure essentially involves filtration of the homogenate through Miracloth (Calbiochemi, Irvine, CA), followed by differential centrifugation of the filtrate at 1500 xg for 8 min and 9000 xg for 15 min to separate the mitochondria in the 9000 xg pellet. The mitochondria were then washed and resuspended in the same isolation medium (see Section II for details).

Rat liver mitochondria were prepared from livers of 48 h starved Sprague-Dawley rats. Briefly, the livers were excised and minced with the Ultraturrax in buffered sucrose (10 ml buffer/g liver) containing 0.25 M sucrose, 20 mM potassium HEPES (pH 7.5), 1 mM potassium EGTA, and 0.5% defatted BSA. The tissue was further homogenized by two passages in a glass-Teflon homogenizer (0.005 inch clearance) operating at 100 RPM, and the mitochondria were isolated by the same differential centrifugation conditions used for gill mitochondria isolation.

In some experiments, isolated mitochondria from either gill or rat liver were disrupted by three freeze-thaw cycles over a three day period.

Oxygen consumption

Oxygen consumption by gill mitochondria was determined by mixing the resuspended mitochondria in respiration buffer (see below) then measuring change in oxygen content with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The electrode was

calibrated as previously described (Section II; Estabrook, 1967). All measurements were made at 25°C. Respiration was measured in a solution containing 1 ml of the resuspended mitochondria, 0.05 ml of 0.2 M KH_2PO_4, 0.05 ml of 50 mM substrate, and 1.4 ml of a solution containing 0.13 M sucrose, 150 mM KCl, 20 mM potassium HEPES (pH 7.5), 1 mM EGTA, and 5 mg/ml defatted BSA. The resulting reaction mixture (less ADP) contained 238 mM sucrose, 90 mM KCl, 20 mM potassium HEPES (pH 7.5), 1 mM EGTA, 5 mg/ml defatted BSA, 4 mM KH_2PO_4, 1 mM substrate, and mitochondria in 2.5 ml.

Oxygen consumption by rat liver mitochondria was measured in a medium containing 0.25 M sucrose, 20 mM potassium HEPES (pH 7.5), 1 mM potassium EGTA, 0.5% defatted BSA, and 0.3 ml mitochondria (resuspended in isolation buffer) in a 2.5 ml volume at 25°C.

Mitochondrial incubations for amino acid analysis experiments

Isolated mitochondria from gill tissue were incubated at 25°C in the respiration buffer (described above) using substrates at 0.5 mM concentration. In addition, the incubation mixture also contained 2.5 mM glucose, 5 units hexokinase, and 500 nmoles ADP in a final volume of 2.5 ml. The incubations were performed in 25 ml Erlenmeyer flasks mounted in a shaking water bath. The reactions were started by the addition of mitochondria and stopped with perchloric acid (0.3 N final concentration).

Enzyme assays

Enzyme solutions used for assaying ornithine aminotransferase (EC 2.6.1.13), Δ^1-L-pyrroline-5-carboxylate (P-5-C) reductase (EC 1.5.1.2)

arginase (EC 3.5.3.1), and proline oxidase were prepared in the following manner. Gill tissue (40 g) was homogenized in 400 ml of 0.88 M sucrose containing 50 mM potassium phosphate (pH 6.8) and 1 mM dithiothreitol (DTT) with a Douce homogenizer. The homogenate was centrifuged at 800 xg for 20 min and the supernatant fraction was collected and centrifuged at 10,000 xg for 20 min yielding a mitochondrial pellet and a high speed supernatant fraction. For proline oxidase measurements, the mitochondrial pellet was resuspended in 0.88 M sucrose containing 40 mM potassium phosphate (pH 7.0) and 1 mg/ml BSA.

Enzyme preparations for the other activities were as follows. The mitochondrial pellet was suspended in 150 ml of 0.1 M Tris.HCl (pH 8.1) at 5°C containing 4 μg/ml pyridoxal phosphate and 10 mg/ml digitonin. This mixture was stirred for 3 h at 5°C and then centrifuged (15 min at 10,000 xg). The supernatant fraction was adjusted to 70% saturation of $(NH_4)_2SO_4$ and after 30 min at 5°C was centrifuged (15,000 xg for 15 min). The pellet was suspended in 5 ml of 0.1 M potassium phosphate (pH 7.0) and dialyzed overnite against 500 ml of 20 mM potassium phosphate (pH 7.0) containing 4 μg/ml pyridoxal phosphate. The dialysate was centrifuged (10,000 xg for 15 min); the supernatant fraction used for ornithine aminotransferase.

The high speed supernatant fraction from the gill tissue homogenate was adjusted to 80% saturation of $(NH_4)_2SO_4$ and after 30 min at 0°C was centrifuged at 10,000 xg for 15 min. The precipitated protein was collected from the top surface of the solution and suspended in 5 ml of 50 mM potassium phosphate (pH 7.0) containing 1 mM DTT. This suspension was used as the source of P-5-C reductase.

The preparation of arginase activity for the kinetic study was as follows. Fifty ml of the high speed supernatant fraction (see above) was dialyzed against 2L of 10 mM Tris-HCl (pH 7.8) containing 0.1 mM $MnCl_2$ at 4°C for 12 h to remove the sucrose. The solution in the bag was removed and rapidly mixed with 500 ml of cold (-20°C) acetone, and the precipitated protein collected by rapid filtration with a Buchner funnel using Whatman #1 filter paper. The protein was removed from the filter, dried, and dissolved in 5 ml of 10 mM Tris-HCl (pH 7.8) containing 0.1 mM $MnCl_2$.

Ornithine aminotransferase was assayed colorimetrically using ortho-aminobenzaldehyde to determine the formation of P-5-C as described previously (Bishop et al., 1981) in a 1.0 ml reaction mixture containing 50 mM imidazole-HCl (pH 7.5), L-ornithine, 2-oxoglutarate, and enzyme.

The enzyme, P-5-C reductase, was assayed spectrophotometrically as the oxidation of NAD(P)H. The reaction mixture contained 100 μmoles potassium phosphate (pH 6.8), 2.4 μmoles P-5-C, 0.16 μmoles NAD(P)H, and enzyme in 2.0 ml volume at 22°C.

Arginase was assayed by determining the rate of urea formation using the colorimetric, 1-phenyl-1, 2-propanediole-2-oxime procedure described by Bishop and Campbell (1965). The reaction mixture contained 100 μmoles potassium glycinate (pH 9.5), the indicated amount of arginine.HCl at pH 9.5, 0.3 ml of enzyme solution (5 mg protein), and 0.5 μmoles of $MnCl_2$ in 1 ml. The reaction was initiated by addition of the enzyme and terminated after 20 min at 22°C by addition of 1 ml of 2N $HClO_4$. After centrifugation to remove the precipitated protein an appropriate sample of the supernatant fluid was taken for analysis of urea formation.

Proline oxidase was measured both by oxygen consumption and by P-5-C formation using particles from Dounce homogenizer disrupted mitochondria as the enzyme source. For oxygen consumption measurements, the resuspended mitochondrial particles (equivalent to 1 g gill tissue) were mixed with resuspension buffer (described above) to 2.5 ml, and oxygen consumption was measured at 15°C before and after addition of 10 mM L-proline. For P-5-C measurements, mitochondria were resuspended in 40 mM potassium phosphate (pH 7.5) buffer (4.0 ml/g original tissue) and re-homogenized with a Dounce homogenizer. The reaction mixture contained 50 μmoles potassium phosphate (pH 7.5), 0.8 mg beef heart cytochrome C, 1.0 ml mitochondria, and 25 μmoles L-proline in a volume of 2.0 ml. After 90 min at 22°C, the reaction was stopped with 1.0 ml of cold 10% trichloroacetic acid, and the amount of P-5-C formed was measured colorimetrically after adding 2.5 mg of ortho-aminobenzaldehyde.

Amino acid analysis and radiotracer analysis

Amino acid analysis was performed using a Glenco model MM-60 amino acid analyzer (Glenco Scientific, Houston, TX) with a lithium citrate buffer system for physiological fluids analysis (Greenwalt and Bishop, 1980). The instrument was calibrated with external standards and the data was collected by a Spectra-Physics Model System I computing integrator (Spectra-Physics, Santa Clara, CA).

Samples for amino acid analysis and radiometric analysis were prepared in the following manner. Perchloric acid deproteinized reactions were neutralized with KOH and applied to a 1.0 ml column of Dowex 50 (H^+ form). The column was washed with 2.0 ml water (organic acid frac-

tion) and next with 2.0 ml of 2N $(NH_4)OH$ (amino acid fraction). The ammonia wash was evaporated to dryness and the residue dissolved in 0.5 ml of 0.2 N lithium citrate (pH 2.2) prior to the injection into the amino acid analyzer.

Radioactive samples were treated identically to the amino acid analyzer samples except that the dried ammonia wash was dissolved in 0.1 ml of water, and 0.01 ml of this solution was spotted on silica gel (TLC) plates (nonheat treated). The plates were developed in the ascending direction for 10 h at 22°C using phenol:H_2O (75 g:25 g) as the solvent. In addition, the organic acid fraction was evaporated to dryness and dissolved in 2.0 ml water for liquid scintillation counting. Radioactive compounds on the TLC plates were detected by autoradiography using nonscreen x-ray film, scraped from the plates into scintillation vials, and quantitated by liquid scintillation counting using the previously described procedures (Bishop et al., 1981). Radioactivity as $[^{14}C]$ -CO_2 was determined by evolving the CO_2 from the reaction mixture in sealed 25 ml Erlenmeyer flasks then trapping the evolved CO_2 in 0.2 ml of 1 M Hyamine hydroxide (New England Nuclear) in a polyethylene cup suspended in the flask. The cup was removed, placed in 15 ml of scintillation counting fluid, and the radioactivity measured in a Beckman LS-250 Liquid Scintillation Counter. Procedures and composition of the counting fluid have been described previously (Cooley et al., 1976).

Results

Enzymes of arginine and proline metabolism

The observations by Greenwalt (Bishop et al., 1981; Greenwalt, 1981) that [^{14}C-U]-arginine produced ^{14}C-ornithine, ^{14}C-proline, ^{14}C-glutamate, and ^{14}C-alanine in hyperosmotically stressed gill segments indicated that arginine could serve as a precursor for proline biosynthesis. This pathway (Figure 1) requires the presence of arginase and ornithine aminotransferase. Arginase has been found in many vertebrate and invertebrate species and is considered nearly ubiquitous in the tissues of molluscs (Bishop et al., 1983). Measurements on Modiolus demissus tissues have indicated that this bivalve is no exception, and homogenates of whole gill tissue have approximated arginase activity at 30 μmoles/g wet tissue/h (Bishop et al., 1981). Table 1 demonstrates the presence of arginase in the supernatant fraction of the 10% gill homogenate (10 m/ buffer/g wet tissue) centrifuged at 10,000xg for 15 min.

At optimal pH (9.5), the arginase activity is fairly active in crude preparations, and although the results do not indicate a strict dependency on added Mn^{++}, Mn^{++} increases the total activity about 20%. This result with added Mn^{++} has been found for arginases from other molluscan tissues (Gaston and Campbell, 1966). Partially purifying the arginase activity by acetone precipitation produces enzyme preparations that are sufficiently concentrated for K_m determinations. Measurements on the partially purified arginase of Modiolus demissus give an apparent K_m value of 7 mM (Figure 2). Since the concentration of the free

Figure 2. Lineweaver-Burk plot of acetone precipitated arginase activity

Velocity is expressed as μmoles urea formed/min/mg protein ($x10^2$).

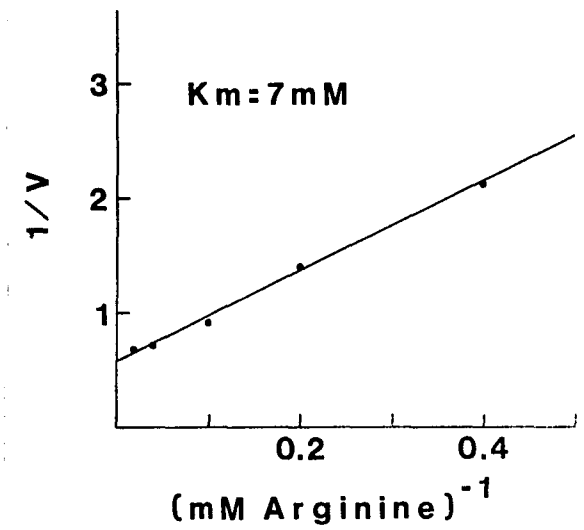

Km=7mM
1/V
3
2
1
0.2
0.4
(mM Arginine)$^{-1}$

Figure 3. The pH optimum of partially purified ornithine aminotransferase

Velocity is expressed as μmoles P-5-C formed/min/mg protein. Symbols denote imidazole (o-o) and Tris (x-x) buffers.

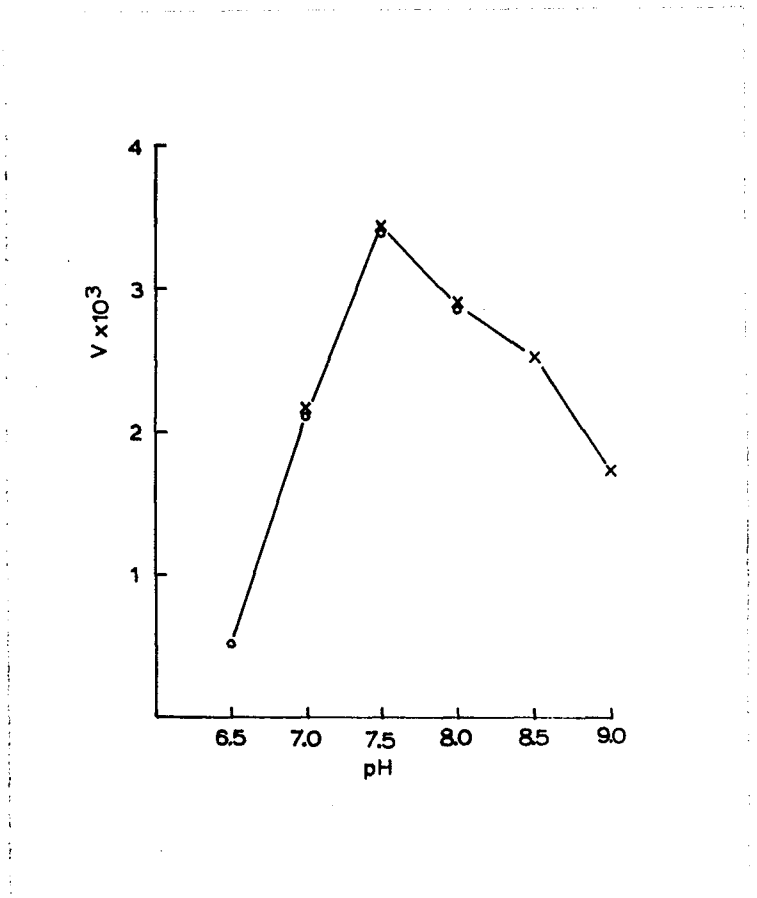

V x10³
4
3
2
1
6.5
7.0
7.5
8.0
8.5
9.0
pH

Table 1. Arginase activity in Modiolus demissus gill tissue. Heat killed enzyme was prepared by heating gill extract for 5 min at 100°C, and each reaction contained 0.5 ml of gill extract, 50 µmoles potassium arginate (pH 9.5), 0.5 µmoles $MnCl_2$, and 100 µmoles potassium glycinate (pH 9.5) in 1.0 ml

Reaction mixture	Incubation time (min)	moles urea formed
Complete	0	0.03
Complete (heat killed)	60	0.09
Complete	60	2.46
Less arginine	60	0.06
Less $MnCl_2$	60	2.04

arginine pool is 5 to 7 µmoles/g dry tissue or approximately 1 to 1.3 µmoles/g wet tissue, (Greenwalt, 1981), a K_m of 7 mM indicates that in vivo arginine is slowly metabolized and that the rate of metabolism would be influenced by changes in the relative concentration of arginine.

Ornithine aminotransferase is also a widely distributed enzyme whose reaction characteristics and mitochondrial location are very similar among animal species. The enzyme has been found in the tissues of some molluscs (Bishop et al., 1983), insects (Reddy and Campbell, 1969), fishes (Wekell and Brown, 1973), mammals (Peraino and Pitot, 1962), and numerous other organisms (Scher and Vogel, 1957). The enzyme from gill tissue of Modiolus demissus sediments with mitochondrial fractions when the tissue is homogenized gently. However, homogenations with Dounce

homogenizers release 50 to 80% of the total activity as a soluble enzyme that is not sedimented even after prolonged centrifugation at 40,000 xg.

Partial purification of Modiolus demissus ornithine aminotransferase was achieved by extracting the isolated gill mitochondria with digitonin (see Reiss et al., 1977) followed by ammonium sulfate precipitation of the solubilized protein. The activity present in such preparations did not show any unusual or unique parameters. It had a pH optimum of 7.5 in cationic buffers (Figure 3) with apparent K_m's of 4.8 mM (Figure 4A) and 2 mM (Figure 4B) for ornithine and 2-oxoglutarate, respectively. The activity of whole tissue homogenate approximates 3 μmoles/g wet tissue/h and 6 μmoles/g wet tissue/h in gill and heart tissue, respectively. In the standard assay mixture, the enzyme from both tissues shows 50% inhibition with 4×10^{-5} M aminooxyacetic acid (Greenwalt and Bishop, 1980; Bishop et al., 1981).

The compound, Δ^1pyrroline-5-carboxylate (P-5-C), is assumed to be the immediate precursor for proline biosynthesis in bacterial (Vogel and Davis, 1952) and animal species (Herzfeld et al., 1977) and is reduced to proline by P-5-C reductase, EC 1.5.1.2, (Peisach and Strecker, 1962).

The presence of P-5-C reductase in Modiolus demissus is demonstrated by data in Table 2. These results indicated that NADH is the preferred cofactor producing a net activity 5.9 fold higher than NADPH. This property has also been noted for P-5-C reductase from rat tissues (Herzfeld et al., 1977), baker's yeast (Matsuzawa and Ishiguro, 1980), blowfly (Aldrichina grahami) (Wadano et al., 1976), and calf liver (Peisach and Strecker, 1962). Estimates of P-5-C reductase from whole gill homogenates of Modiolus demissus range from 4.0 to 5.0 μmoles/g

Table 2. P-5-C reductase activity in homogenates of Modiolus demissus gill tissue. Net activity represents nmoles coenzyme oxidized/min after substracting the rate of coenzyme oxidation in the absence of P-5-C

Reaction mixture	Co-enzyme pyridine nucleotide	ml Enzyme	nmoles/min	Net nmoles/min
Complete	NADH	0	0	0
Complete	NADH	0.3	2.0	1.38
Less P-5-C	NADH	0.3	0.62	-
Complete	NADH	0.6	4.2	2.9
Less P-5-C	NADH	0.6	1.3	-
Complete	NADPH	0.6	1.13	0.49
Less P-5-C	NADPH	0.6	0.64	-
Complete	None	0.6	0	0

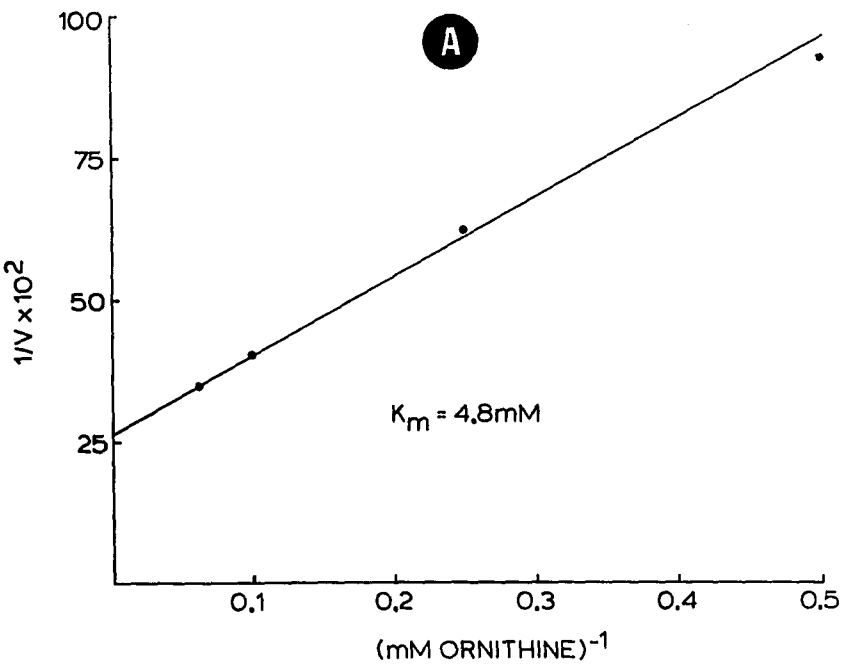
wet tissue/h which is a sufficient to account for the observed increases of proline in hyperosmotically stressed gill tissue (Bishop et al., 1981; Greenwalt, 1981).

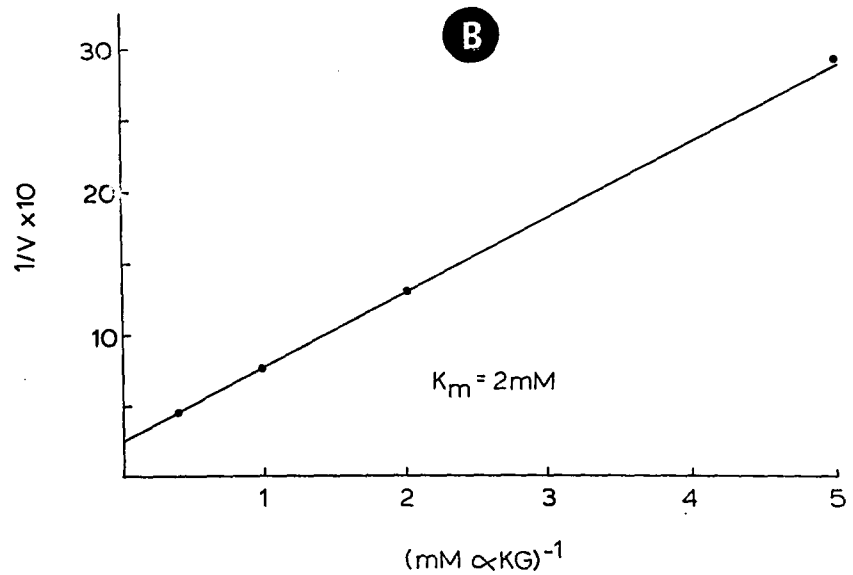
Greenwalt (1981) and Bishop et al. (1981) observed that [^{14}C-U]-glutamate does not give rise to either ^{14}C-proline or ^{14}C-ornithine when incubated with isolated gill pieces. In addition, they also observed that the incorporation of [^{14}C] from both arginine and ornithine into proline and glutamate by gill and heart tissue is blocked by the transaminase inhibitor, aminooxyacetic acid. In other experiments reported by Greenwalt (1981), both the Δ^1-pyrroline-2-carboxylate

Figure 4. Lineweaver-Burk plot of partially purified ornithine aminotransferase. Velocity is expressed as μmoles P-5-C formed/min/mg protein

A) Ornithine apparent Km at a saturating concentration of 2-oxoglutarate (20 mM)

B) 2-Oxoglutarate apparent Km at a saturating concentration of ornithine (30 mM)


A
100
75
50
25
1/V x 10²
Km = 4.8mM
0.1
0.2
0.3
0.4
0.5
(mM ORNITHINE)⁻¹


B
30
20
10
1/V x 10
Km = 2mM
1
2
3
4
5
(mM αKG)⁻¹

(P-2-C) (that arises from the action of the L-amino acid oxidase on ornithine) and P-5-C were shown to act as intermediate compounds in the synthesis of both proline and glutamate by gill tissue. The overall tracer experiments indicated the presence of a mitochondrial P-5-C dehydrogenase (EC 1.5.1.12) converting P-5-C to glutamate although none could be demonstrated in an unambiguous fashion (S. H. Bishop, Dept. of Zoology, Iowa State University, personal communication) in cell free extracts or mitochondrial extracts of gill tissue.

These data from Greenwalt (1981) and the above demonstrated enzyme activities (Tables 1 and 2, Figures 2 and 4) indicate that the gill tissue has the capacity to synthesize proline and glutamate from arginine and ornithine.

Proline catabolism

The data presented above indicated that the processes regulating the metabolism of proline and the metabolities of proline within the mitochondrion held the key to the understanding of the transient increase and decrease in proline accumulation during adjustment to hyperosmotic stress. As previously demonstrated (Bishop et al., 1981; Greenwalt, 1981; Reiss et al., 1977), glutamate can be converted directly to tricarboxylic acid cycle intermediates by the mitochondrial glutamate dehydrogenase and does not require a transaminase step for this conversion. Oxidation of proline to CO_2 also occurred in ribbed mussel gill tissue, and the oxidation was not dependent upon transamination (not blocked by aminooxyacetic acid) while arsenite produced an accumulation of ^{14}C-glutamate from ^{14}C-proline (Greenwalt, 1981). In studies reported

here (section II), proline and glutamate stimulated state 3 respiration of gill nitochondria to the same extent when the mitochondria appeared fully coupled (section II of thesis). Since these results indicated the presence of proline oxidase (converts proline to P-5-C) in the gill mitochondria, initial experiments were performed to further measure the presence of this enzyme. As a comparison, a closely related species, Mytilus edulis, was used which exhibits a similar response (amino acid accumulation) to hyperosmotic stress (Hoyaux et al., 1975; Livingstone et al., 1979). However, mitochondria from the tissues of Mytilus edulis have been shown to lack proline oxidase activity (Zaba et al., 1978).

Table 3 shows a comparison of [^{14}C]-proline and [^{14}C]-ornithine oxidation to ^{14}C-CO_2 by whole gill tissue pieces from Modiolus demissus and Mytilus edulis. It is evident from these results that both species possess ornithine aminotransferase, P-5-C dehydrogenase, and glutamate dehydrogenase, because both animals can oxidize ornithine to CO_2. However, only ribbed mussel gill tissue can oxidize proline to CO_2.

Since Table 3 confirmed the earlier data of proline oxidase in ribbed mussel gill tissue (section II), mitochondrial fractions from gill tissue were examined for P-5-C formation from proline (Table 4).

These results (Table 4) demonstrate the presence of proline oxidase in mitochondrial particles of gill tissue. Because this enzyme has been shown to be coupled to the mitochondrial electron transport chain in vertebrate species (Johnson and Strecker, 1962; Strecker, 1971), the addition of proline to partially disrupted gill mitochondria should then stimulate oxygen consumption if the gill mitochondrial proline oxidase is

Table 3. Comparison of ^{14}C-CO_2 formation from ^{14}C-proline and ^{14}C-ornithine by gill tissue from Modiolus demissus and Mytilus edulis acclimated to 32°/oo ASW. Gill tissue (50 mg) was incubated in 32°/oo ASW with 0.25 µC of tracer for 4 h at 22°C. The specific activities of proline and ornithine added to the media were 290 mCi/mM and 280mCi/mM, respectively

Species/^{14}C-tracer	^{14}C-CO_2 (dpm)	Amino acid pool size
M. demissus		
U-^{14}C-L-proline	48,000	3-5 µmoles
U-^{14}C-L-ornithine	102,000	5
M. edulis		
U-^{14}C-L-proline	0	10.2+
U-^{14}C-L-ornithine	71,000	2.66+

Table 4. Proline oxidase activity in Modiolus demissus gill mitochondria. Reactions were run for 90 min at 22°C

Components of reaction mixture	µmoles P-5-C formed
Less enzyme	0
Less proline	0.12
Complete	0.41

also coupled to cytochrome c oxidase via the mitochondrial electron transport chain.

Table 5 demonstrates proline stimulated oxygen consumption by partially disrupted Modiolus demissus gill mitochondria in the presence and absence of mitochondrial electron transport chain inhibitors. Of the compounds tested, antimycin A, KCN, and sodium azide completely inhibit oxygen consumption in the presence of proline. Antimycin A blocks electron transport from cytochrome b to cytochrome c_1 (phosphorylation coupling site II) while KCN and sodium azide are both inhibitors of cytochrome c oxidase (phosphorylation coupling site III) (Slater, 1967). The lack of inhibition by arsenite (an inhibitor of 2-oxoglutarate dehydrogenase) and ortho-aminobenzaldehyde (combines with P-5-C to prevent oxidation to glutamate) indicates that proline oxidation to P-5-C is the only reaction taking place under these conditions with the mitochondrial particles as enzyme. Rotenone (an inhibitor of phosphorylation coupling site 1) produces only slight inhibition of proline oxidase in the disrupted mitochondria. These results indicate that in disrupted gill mitochondria proline oxidation is coupled to the electron transport chain at the level of coenzyme Q (phosphorylation site II). A comparison of these results (Table 5) to proline stimulated respiration of intact mitochondria (section II) indicated that mechanical disruption of gill mitochondria produced a loss of rotenone inhibition during proline stimulated respiration.

These observations on gill mitochondria were compared to similar studies using rat liver mitochondria (Figures 5A,B). Figure 5A suggested that in intact rat liver mitochondria proline oxidation but not succinate

Table 5. Proline oxidase activity in disrupted gill mitochondria. Maximal activity (100%) represents 4 nmoles O_2 consumed/min

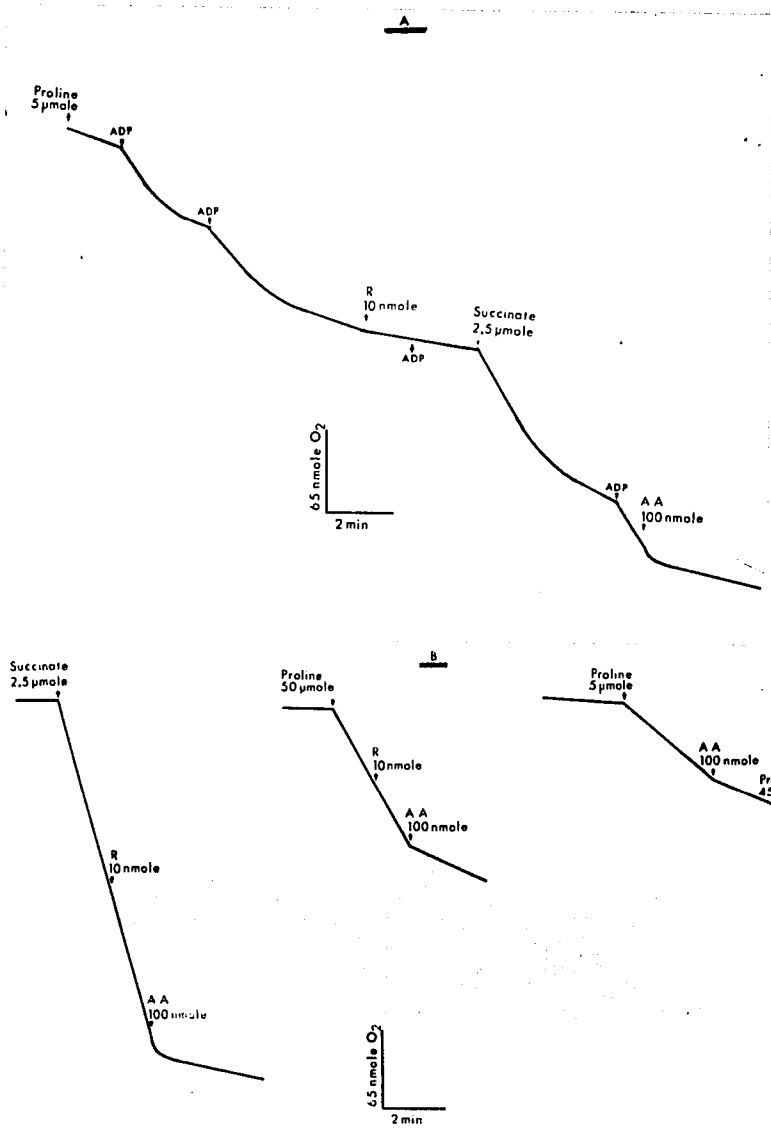
Additions to reaction mixture	Activity
None	0
Proline	100%
Proline + KCN (1 mM)	0
Proline + antimycin A (0.25 mg)	0
Proline + rotenone (1 mM)	80
Proline + arsenite (1 mM)	100
Proline + sodium azide (12 mM)	0
Proline + ortho-aminobenzaldehyde (0.19 mg)	100

oxidation was completely inhibited by rotenone; addition of antimycin A inhibited both succinate and proline oxidation. After this same rat liver mitochondria preparation was disrupted by freeze-thawing (Figure 5B), inhibition by rotenone was completely abolished. Although complete inhibition of oxygen consumption by antimycin A was not observed for freeze-thawed mitochondria in the presence of either proline or succinate, the addition of 45 μmoles proline to an antimycin A inhibited reaction containing 5 μmoles proline did not increase the oxygen consumption rate indicating that the antimycin A insensitive oxygen consumption in the presence of proline was not due to proline oxidation (Figure 5B). Thus, the proline oxidase in rat liver mitochondria disrupted by freeze-thawing is completely inhibited by antimycin A but not rotenone, and in intact

Figure 5. Oxygen consumption by rat liver mitochondria in the presence of proline and succinate. Succinate, proline, antimycin A (AA), and rotenone (R) were added at the arrows in indicated amounts. ADP was added in 200 nmole amounts

A) Oxygen consumption by intact mitochondria in 0.25 M sucrose (pH 7.5)

B) Oxygen consumption by freeze-thawed mitochondria in 0.1 M Tris (pH 7.5)


A
Proline
5 µmole
ADP
ADP
R
10 nmole
ADP
Succinate
2.5 µmole
ADP
A A
100 nmole
6.5 nmole O2
2 min
B
Succinate
2.5 µmole
R
10 nmole
A A
100 nmole
Proline
50 µmole
R
10 nmole
A A
100 nmole
Proline
5 µmole
A A
100 nmole
Proline
45 µmole
6.5 nmole O2
2 min

rat liver mitochondria, proline oxidation is completely inhibited by both rotenone and antimycin A. An analogous experiment on freeze-thawed gill mitochondria indicated that ribbed mussel gill proline oxidase activity was completely inactivated by freezing, therefore, only mechanically disrupted gill mitochondria were used in the above experiments.

These results imply that for both Modiolus demissus gill and rat liver tissue catabolism of proline by intact mitochondria is tightly coupled or modulated through the rotenone sensitive site (phosphorylation site I) of the mitochondrial electron transport chain.

Changes in amino acid levels in isolated mitochondria

Owing to the presence in ribbed mussel gill mitochondria of several enzymes of proline and glutamate metabolism (ornithine aminotransferase, proline oxidase, P-5-C dehydrogenase, and glutamate dehydrogenase), it was of particular interest to investigate changes in the mitochondrial free amino acids during coupled respiration in the presence and absence of added proline and glutamate. The gill mitochondrial preparations for these experiments had respiratory control indices between 5 and 6 and P/O ratios of 3 for proline and glutamate as substrates. In addition, hexokinase and glucose were included in reaction mixtures so as to continuously generate ADP from ATP formed during state 3 respiration, and, thus, continuously maintain state 3 respiration.

Figure 6A describes endogenous free amino acids present in freshly isolated gill mitochondria from ribbed mussels acclimated to low salinity (450 mOsmoles). Taurine, glycine, valine, and a compound tentatively identified as cysteine are present in the highest concentrations. This

Figure 6. Amino acid analysis of gill mitochondria

A) Perchloric acid extract of freshly prepared mitochondria

B) Perchloric acid extract of mitochondria incubated for 15 min with hexokinase, glucose, inorganic phosphate, and ADP

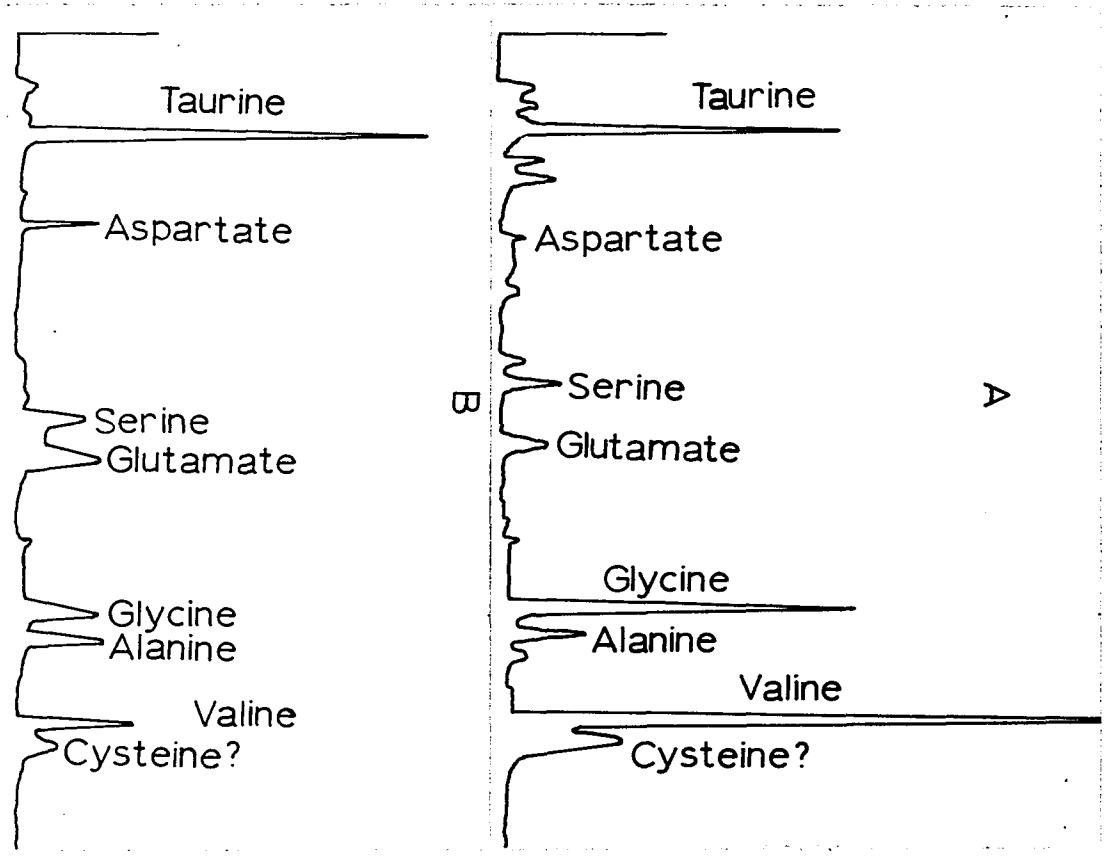

Taurine
Aspartate
Serine
Glutamate
Glycine
Alanine
Valine
Cysteine?
B
Taurine
Aspartate
Serine
Glutamate
Glycine
Alanine
Valine
Cysteine?
A

Table 6. Amino acid analysis of isolated gill mitochondria after sustained state 3 respiration. Each reaction contained 0.75 mg mitochondrial protein. Data represent nmoles amino acid/0.75 mg protein from 4 determinations. All values were scaled to the mean taurine level of 16.8 nmoles (where n = 21). Symbols denote the following: P (proline), A (arsenite), and Py (pyruvate)

Incubation min (additions to reaction)	Taurine	Aspartate	Serine	Glutamate	Glycine	Alanine	Valine
0 (none-fresh mitochondria)	16.8	1.3	4.7	4.0	19.1	4.4	36.8
15 (P)	16.8	2.5	2.7	0.3	4.7	3.1	16.7
45 (P)	16.8	4.1	5.1	0.7	5.2	2.1	16.0
65 (P)	16.8	2.2	4.1	1.2	4.1	3.9	19.8
45 (P+A)	16.8	2.0	6.0	9.3	4.7	5.9	5.6
45 (P+A+Py)	16.8	2.0	2.7	11.5	4.4	6.9	7.5

pattern is quite different from that seen in whole gill tissue where taurine, aspartate, and glutamate represent 75% of the free amino acid pool in animals acclimated to low salinity (Greenwalt, 1981). Sustained state 3 respiration for 15 min by the addition of ADP, potassium phosphate, hexokinase, and glucose results in a rapid decrease of glycine and most notably valine (Figure 6B) while the endogenous levels of taurine, aspartate, serine, glutamate, and alanine remain unchanged during sustained state 3 respiration when compared to the levels found in untreated mitochondria (Figure 6A).

When state 3 respiration is maintained in the presence 0.5 mM proline, no change is observed except for decreases in glycine and valine (Table 6). However, the inclusion of 0.5 mM sodium arsenite (blocks the state 3 respiration of 2-oxoglutarate) in the proline reaction resulted in a small increase in glutamate and alanine levels (Table 6). The inclusion of 0.5 mM pyruvate in the arsenite inhibited proline reaction produced no discernible differences from that seen in the absence of pyruvate (Table 6). These results indicated that in the absence of metabolic inhibitors (e.g. arsenite) the metabolism of proline did not result in the accumulation of an amino acid product. This conclusion was validated by running reactions identicle to those used for the amino acid analysis data, except for the inclusion of [^{14}C-U]-proline in the reactions.

Table 7 shows the radiolabeled components that are produced from 0.5 mM [^{14}C-U]-proline after 45 min of sustained state 3 respiration are primarily $^{14}CO_2$ and ^{14}C organic acids. Thin layer chromatography of the

Table 7. Metabolism of [^{14}C-U]-proline and distribution of radiolabel in the products. Each reaction contained 1.25 μmoles proline (specific activity of 2.2×10^5 dpm/μmole). Data represent total dpm from reactions

Incubation time (min)	$^{14}CO_2$	Organic acid fraction	Amino acid fraction	Proline	Alanine	Glutamate	Aspartate
0	30	10,070	234,360	225,100	5149	4110	
0	29	10,973	227,160	221,770	5573	2655	
45	6387	18,336	236,760	214,500	4206	3208	1529
45	6982	17,415	255,390	232,200	5047	3265	1472

amino acid fraction did not indicate an enrichment of ^{14}C in either glutamate, alanine, or aspartate.

Oxygen consumption measurements on the mitochondria preparations used for the experiments represented in Tables 6 and 7 showed that proline produced a rate of 7.3 nmoles O_2/min/mg mitochondrial protein during sustained state 3 respiration. This value corresponded to 328 nmoles O_2/mg mitochondrial protein for the 45 min incubation time. If it is assumed that 2.0 molecules of O_2 are consumed for each molecule of succinate formed from proline (an 8 electron step oxidation), the formation of 182 nmoles of $^{14}CO_2$ (assuming the specific radioactivity of the proline carboxyl group represents 20% of the radioactive carbons of [^{14}C-U]-proline) arising from the carboxyl group of proline accounted for all of the oxygen consumption by reactions used in Tables 6 and 7.

Since the metabolism of proline did not appear to result in the synthesis of new amino acids, amino acid analysis were run using glutamate as the substrate for sustained state 3 respiration.

Table 8 shows the levels of amino acids after 35 min of state 3 respiration in the presence and absence of 0.5 mM glutamate. In the absence of glutamate, sustained state 3 respiration results in amino acid levels similar to the data of Table 6. However, state 3 respiration in the presence of glutamate causes increases in the levels of aspartate and alanine (Table 8). These results indicate that when excess glutamate is present oxaloacetate (presumably formed from 2-oxoglutarate through the TCA cycle) and pyruvate (presumably by decarboxylation of malate by the action of malic enzyme) undergo transamination with glutamate forming aspartate and alanine from oxaloacetate and pyruvate, respectively.

Table 8. Amino acid analysis of isolated gill mitochondria after sustained state 3 respiration. Each reaction contained 0.75 mg mitochondrial protein. Data represent nmoles amino acid/0.75 mg protein from 4 determinations. All values were scaled to the mean taurine level of 13.6 nmoles (where n = 13). Symbols denote the following: P (proline), and G (glutamate)

Incubation min (additions to reaction)	Taurine	Aspartate	Serine	Glutamate	Glycine	Alanine
0 (none-fresh mitochondria)	13.6	2.2	8.4	6.8	5.3	7.3
45 (none)	13.6	1.65	5.5	3.6	5.1	4.6
45 (P)	13.6	2.0	2.1	3.7	4.5	5.4
45 (G)	13.6	5.2	8.2	not determined	9.5	16.0

Discussion

This study has focused on the metabolic components responsible for the synthesis and degradation of proline in isolated gill tissue of Modiolus demissus. Greenwalt (1981) and Bishop et al. (1981) have previously shown that half of the free proline pool arises directly from protein and half from arginine and ornithine.

Enzyme assays on gill tissue have demonstrated the presence of arginase (Table 1, Figure 2), ornithine aminotransferase (Figures 3 and 4), and P-5-C reductase (Table 2) which together with the data of Bishop et al. (1981) are responsible for the pathway, arginine → ornithine → P-5-C → proline (Figure 1). Of the three enzymes, partially purified arginase and ornithine amino transferase have Km's of 7 mM and 4.8 mM for arginine and ornithine, respectively (Figures 2, 4A). Since the concentrations of free arginine and ornithine is gills of low salinity acclimated mussels (Modiolus demissus) are approximately 1 μmole/g wet tissue and 0.6 μmoles/g wet tissue, respectively (Greenwalt, 1981), the rates of arginine and ornithine conversion to P-5-C by arginase and ornithine aminotransferase are probably regulated by the levels of the substrates.

The presence of P-5-C reductase in the gill tissue (Table 2) indicated that the conversion of arginine and ornithine to proline is indeed possible. The preferred cofactor was found to be NADH rather than NADPH as has been observed for other animal species (Peisach and Strecker, 1962; Wadano et al., 1976; Herzfeld et al., 1977; Matsuzawa and Ishiguro, 1980). Loss of enzyme activity due to extreme temperature lability (both heat and cold labile) has hampered purification from most animal tissues (see

above references), and several preparations from Modiolus demissus gill have rapidly lost activity on standing making Km estimations impractical. However, the best activity measurements from whole gill tissue homogenates have estimated P-5-C reductase activity at 4 to 5 μmoles/g wet tissue/h at 25°C (Bishop et al., 1981). Even if these measurements are underestimates, the measured activity would be sufficient to account for proline increases during hyperosmotic stress.

The presence of proline oxidase in gill mitochondria and isolated gill pieces (Tables 3,4,5, and 7) confirm earlier results (section II; Bishop et al., 1981; Greenwalt, 1981) that proline is catabolized by Modiolus demissus gill tissue. The enzyme from gill mitochondria is similar to rat liver proline oxidase and to the proline oxidase of squid muscle mitochondria (Mommsen and Hochachka, 1981) in being tightly coupled to the rotenone sensitive portion of the electron transport chain in intact mitochondria (section II, Figure 5A). However, the enzyme from both rat liver and mussel gill tissue looses coupling to the rotenone sensitive site after mitochondrial disruption, but the enzymes retain coupling to the electron transport chain as evidenced by antimycin A inhibition (Table 5, Figure 5B). No similar experiments have been performed on the squid proline oxidase. These results demonstrate that proline catabolism in gill tissue requires respiration and is under normal respiratory control.

Although proline oxidase has not been successfully purified from any animal source, the enzyme has been purified to apparent homogeneity from Escherichia coli (Scarpulla and Soffer, 1978). This bacterial enzyme required FAD (flavin adenine dinucleotide) and artificial electron

acceptors for activity, and oxygen did not act as a co-substrate. In addition, the purified enzyme was not inhibited by cyanide. However, in crude bacterial preparations (which also contain P-5-c dehydrogenase), the enzyme was membrane bound with an associated electron transport chain which required oxygen or artificial electron acceptors for activity. Although several attempts to directly assay for the presence of P-5-C dehydrogenase in gill tissue were not successful, several lines of indirect evidence (Table 3 and 7; section II, and Greenwalt, 1981) suggest the presence of this enzyme in ribbed mussel gill tissue. Since the metabolite, P-5-C, is the central or key metabolic product for the catabolism of proline, arginine, and ornithine to glutamate, this enzyme must be active in gill tissue for the formation of CO_2 from proline (Table 3) to occur. In conclusion, the pathway for arginine, proline, and glutamate metabolism is that shown in Figure 1. Recent, studies with excised squid mantle muscle indicate that this pathway probably also functions for arginine catabolism in this tissue (Hochachka et al., 1983).

In addition to the presence of endogenous valine and its rapid disappearance during state 3 respiration, the amino acid analysis data from isolated gill mitochondria indicate that metabolism of proline results in primarily CO_2 and organic acids (Table 7). The increase in alanine levels during metabolism of glutamate (Table 8) and no alanine increase during proline metabolism (Tables 6 and 7) imply that the oxidation of proline to glutamate is slow compared to the deamination of glutamate to 2-oxoglutarate (catalyzed by glutamate dehydrogenase).

The rapid disappearance of the endogenous valine in gill mitochondria subjected to continuous state 3 respiration (Figure 6 and Table 6) was surprising since this amino acid did not act as a substrate for the L-amino acid oxidase (section I). The L-amino acid oxidase was particulate and may be associated with the mitochondria. This experiment (Figure 6A, B) suggests that rapidly respiring mitochondria can somehow selectively block alanine utilization while rapidly metabolizing some of the other endogenous amino acids such as valine, glycine, aspartate, and cysteine(?). Although these experiments do not allow any final conclusions concerning a possible relationship between the aspartate decline and the possible alanine accumulation during anaerobiosis or hyperosmotic stress, they do indicate the possibility for some special regulation of alanine turnover-metabolism by the respiring mitochondria. The processes regulating glycine, aspartate, glutamate, glycine, and serine in respiring and nonrespiring gill mitochondria are the subject of continuing investigations in this laboratory.

GENERAL DISCUSSION

The finding that Modiolus demissus tissues contain an L-amino acid oxidase having a broad substrate specificity implied a possible role for this enzyme in the catabolism of amino acids which do not accumulate during osmotic stress. The enzyme was most active with leucine, the basic, and the aromatic amino acids which do not accumulate to a significant extent in ribbed mussel subjected to salt stress (Greenwalt, 1981). However, its role in ornithine catabolism should be questioned, because ^{14}C-ornithine conversion to $^{14}CO_2$ is blocked by transaminase inhibitors (which have no effect on the L-amino acid oxidase), and the product of the oxidase with ornithine, P-2-C, should be metabolized to CO_2 in the presence of transaminase inhibitors (Greenwalt, 1981). However, the L-amino acid oxidase may account for the nonaccumulation of leucine, histidine, lysine, aromatic amino acids, and some of the other basic amino acids.

Owing to the tight association of this enzyme with a dense particle and its resistance to solubilization by nonionic detergents, the specific intracellular location may only be determined by electron microscopic cytochemistry. Although its physical properties are consistent with those of the dense outer mitochondrial membrane, its acidic pH optimum suggests a lysosomal location. This ambiguity as well as the specific metabolic role of the L-amino acid oxidase will require much additional study before the importance of this enzyme can be realized. The data on gill tissue oxygen consumption showed that ribbed mussel gill mitochondria produced all of the measurable oxygen consumption, and the

mitochondria were tightly coupled in vivo. These findings greatly substantiated a central role for mitochondria in the amino acid metabolism of ribbed mussel gill tissue.

One of the more difficult aspects of the studies reported here was the preparation of ribbed mussel gill mitochondria which exhibited oxidative phosphorylation. Of all the various isolation media, homogenization methods, and centrifugation techniques reported in the literature, only the protocol developed in this laboratory proved successful. The successful demonstration of coupled respiration by isolated gill mitochondria showing predicted P/O ratios and the predicted inhibition patterns by low concentrations of electron transport chain inhibitors has now closed the door on arguments for alternate electron transport chains and assumptions for unusual respiratory coupling in bivalve gill mitochondria (see review in section II) which were based on artifacts caused by improper isolation methods.

In all respects, the ADP stimulated respiration with proline, glutamate, malate, and succinate by ribbed mussel gill mitochondria resembled that found for mammalian mitochondria and provides evidence for a key role of gill mitochondria in the catabolism of proline and glutamate. The lack of pyruvate utilization by isolated gill mitochondria suggests that the mitochondrial pyruvate dehydrogenase or pyruvate transport into gill mitochondria is under strict control and may control the availability of pyruvate for transamination to alanine, thus, regulating the synthesis of alanine during hyperosmotic stress. In this regard, other work from this laboratory has implicated that the accumulation of glycine by ribbed mussel gill tissue may result from

the tight regulation of gill mitochondrial glycine oxidase (Ellis, et al., 1981).

The biosynthesis of proline from arginine and ornithine appears to operate in ribbed mussel gill tissue as judged by the presence of arginase, ornithine aminotransferase, and P-5-C dehydrogenase and complements the radiotracer studies of Greenwalt (1981). It would appear that this pathway is common to many animals. However, some recent findings indicate that some rat tissues are able to catalyze the formation of proline and ornithine from glutamate (Ross et al., 1978) although ribbed mussel gill tissue does not (Greenwalt, 1981; Bishop et al., 1981).

The presence of proline oxidase activity in ribbed mussel gill mitochondria was confirmed by several criteria, and the enzyme appeared to be tightly coupled to the rotenone sensitive site of the electron transport chain as was rat liver proline oxidase. Thus, it appeared that proline levels in gill tissue may be regulated through a respiration linked mechanism. In this regard, proline concentrations in some bivalves, Mytilus edulus and Crassostrea virginica, are not influenced by catabolism since they lack proline oxidase (Zaba et al., 1978; Burcham et al., 1983), and proline concentrations may be controlled by permeability of the cell membrane (Pierce, 1982).

Amino acid analysis of isolated ribbed mussel mitochondria indicate that they contain large amounts of taurine, valine, and in some cases high levels of glycine. If the mitochondria are allowed to maximally respire, the levels of valine and glycine drop rapidly indicating the

presence of possibly a specific deaminating enzyme for valine and the presence of an active glycine oxidase in ribbed mussel gill mitochondria.

In conclusion, the metabolism of proline by sustained state 3 respiration of ribbed mussel mitochondria results in glutamate which is converted into organic acids. However, with glutamate as the substrate, sustained state 3 respiration produces a large alanine increase in ribbed mussel mitochondria and indicates glutamate can be a source alanine accumulation during salt stress.

LITERATURE CITED

Adams, E., and L. Frank. 1980. Metabolism of proline and the hydroxyprolines. Ann. Rev. Biochem. 49:1005-1061.

Addink, A. D. F., and P. R. Veenhof. 1975. Regulation of mitochondrial matrix enzymes in Mytilus edulis (L.). Pages 109-119 in H. Barnes, ed. Proceedings 9th European Marine Biological Symposium. Aberdeen University Press, New York.

Akberali, H. B., and M. J. Earnshaw. 1982. Studies of the effects of zinc on the respiration of mitochondria from different tissues in the bivalve mollusc Mytilus edulis (L.). Comp. Biochem. Physiol. 72C:149-152.

Amoore, J. E., and W. Bartley. 1958. The permeability of isolated rat-liver mitochondria to sucrose, sodium chloride, and potassium chloride at 0°. Biochemical J. 69:223-236.

Azzi, A., and G. F. Azzone. 1967. Ion transport in liver mitochondria II. Metabolism-linked ion extrusion. Biochim. Biophys. Acta 135: 444-453.

Baginski, R. M., and S. K. Pierce. 1975. Anaerobiosis: A possible source of osmotic solute for high-salinity acclimation in marine molluscs. J. Exp. Biol. 62:589-598.

Baginski, R. M., and S. K. Pierce. 1977. The time course of intracellular free amino acid accumulation in tissues of Modiolus demissus during high salinity adaptation. Comp. Biochem. Physiol. 57A:407-412.

Baginski, R. M., and S. K. Pierce. 1978. A comparison of amino acid accumulation during high salinity adaptation with anaerobic metabolism in the ribbed mussel, Modiolus demissus. J. Exp. Zool. 203:419-428.

Ballantyne, J. S., P. W. Hochachka, and T. P. Mommsen. 1981. Studies on the metabolism of the migratory squid, Loligo opalescens: enzymes of tissues and heart mitochondria. Mar. Biol. Lett. 2:75-85.

Barrow, K. D., D. D. Jamieson, and R. S. Norton. 1980. ^{31}P Nuclear magnetic resonance studies of energy metabolism in tissue from the marine invertebrate Tapes watlingi. Eur. J. Biochem. 103:289-297.

Bartberger, C. A., and S. K. Pierce. 1976. Relationship between ammonia excretion rates and haemolymph nitrogenous compounds of a euryhaline bivalve during low salinity acclimation. Biol. Bull. 150:1-14.

Bender, A. E., and H. A. Krebs. 1950. The oxidation of various synthetic α-amino acids by mammalian D-amino acid oxidase, L-amino acid oxidase of cobra venom and the L- and D-amino acid oxidases of Neurospora crassa. Biochem. J. 46:210-219.

van den Bergh, S. G., F. van Vugt, and A. G. M. Tielens. 1980. Anaerobic and aerobic energy metabolism of the common liver fluke, Fasciola hepatica. Pages 231-241 in L. Vitale and V. Simeon, eds. Industrial and Clinical Enzymology. Vol. 61. Pergamon Press, New York.

Bishop, S. H. 1976. Nitrogen metabolism and excretion:regulation of intracellular amino acid concentrations. Pages 414-431 in M. Wiley, ed. Estuarine processes. Vol. 1. Academic Press, Inc., New York, N.Y.

Bishop, S. H., and J. W. Campbell. 1965. Arginine and urea biosynthesis in the earthworm Lumbricus terrestris. Comp. Biochem. Physiol. 15:51-71.

Bishop, S. H., L. L. Ellis, and J. M. Burcham. 1983. Amino acid metabolism in molluscs. Pages 244-327 in P. W. Hochachka, ed. The Mollusca Vol. 1. Academic Press, New York, N.Y.

Bishop, S. H., D. E. Greenwalt, and J. M. Burcham. 1981. Amino acid cycling in ribbed mussel tissues subjected to hyperosmotic shock. J. Exp. Zool. 215:277-287.

Black, R. E. 1962a. Respiration, electron-transport enzymes, and krebs-cycle enzymes in early developmental stages of the oyster Crassostrea virginica. Biol. Bull. 123:58-70.

Black, R. E. 1962b. The concentrations of some enzymes of the citric acid cycle and electron transport system in the large granule fraction of eggs and trochophores of the oyster Crassostrea virginica. Biol. Bull. 123:71-79.

Blaschko, H., and D. B. Hope. 1956. The oxidation of L-amino acids by Mytilus edulis. Biochem. J. 62:335-339.

Boulanger, P., J. Bertrand, and R. Osteux. 1957. Désamination de l'ornithine et de la lysine séhydrogénase du foie de dindon (Meleagris gallopavo L.). Biochim. Biophys. Acta 26:143-145.

Boulanger, P., and R. Osteux. 1956. Action de la L-aminoacide-déshydrogénase du foie de dindon (Meleagris gallopavo L.) sur les acides aminés basiques. Biochim. Biophys. Acta 21:522,561.

Brierley, G. P., M. Jurkowitz, E. Chavez, and D. W. Jung. 1977. Energy dependent contraction of swollen heart mitochondria. J. Biol. Chem. 252:7932-7939.

Burcham, J. M., D. E. Greenwalt, and S. H. Bishop. 1980. Amino acid metabolism in euryhaline bivalves: the L-amino acid oxidase from ribbed mussel gill tissue. Mar. Biol. Lett. 1:329-340.

Burcham, J. M., S. H. Bishop, and L. L. Ellis. 1982. ATP metabolism in mitochondria of Modious demissus gill tissue. Physiologist 25: 326.

Burcham, J. M., K. T. Paynter, and S. H. Bishop. 1983. Coupled mitochondria from oyster gill tissue. Mar. Biol. Lett. (in press).

Bursell, E., and E. Slack. 1976. Oxidation of proline by saccosomes of the tsetse fly, Glossina morsitans. Insect Biochem. 6:159-167.

Chance, B., and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17:65-134.

Chang, Y. F., and E. Adams. 1977. Glutamate semialdehyde dehydrogenase of Pseudomonas. J. Biol. Chem. 252:7979-7986.

Chen, C. H., and R. E. Koeppe. 1970. Amino-acid oxidase action on D- and L-α,γ-diaminobutyric acids. Biochim. Biophys. Acta 215:184-186.

Chen, C. H., and A. L. Lehninger. 1973. Respiration and phosphorylation by mitochondria from the heptopancreas of the blue crab (Callinectes sapidus). Arch. Biochem. Biophys. 154:449-459.

Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. 242:173-181.

Clark, J. B., and W. J. Nicklas. 1970. The metabolism of rat brain mitochondria, preparation and characterization. J. Biol. Chem. 245:4724-4731.

Cooley, L., D. R. Crawford, and S. H. Bishop. 1976. Urease from the lugworm, Arenicola cristata. Biol. Bull. 151:96-107.

Czonka, L. N. 1981. Proline over-production results in enhanced osmotolerance in Salmonella typhimuriuno. Mol. Gen. Genet. 182: 82-86.

Ellis, L. L., D. E. Greenwalt, J. M. Burcham, and S. H. Bishop. 1981. Glycine-secine metabolism in gill tissue of a euryhaline bivalve (Modiolus demissus). Amer. Zool. 21:929.

Estabrook, R. W. 1967. Mitochondrial respiratory control and the polarographic measurement. Pages 41-47 in R. W. Estabrook and M. E. Pullman, eds. Methods in Enzymology Vol. 10. Academic Press, Inc., New York, NY.

Fernandez-Moran, H., T. Oda, P. V. Blair, and D. E. Green. 1964. A macromolecular repeating unit of mitochondrial structure and function. J. Cell Biol. 22:63-100.

Flashner, M. I. S., and V. Massey. 1974a. Purification and properties of L-lysine monooxygenase from Pseudomonas fluorescens. J. Biol. Chem. 249:2579-2586.

Flashner, M. I. S., and V. Massey. 1974b. Regulatory properties of the flavoprotein L-lysine monooxygenase. J. Biol. Chem. 249: 2587-2592.

Gainey, L. F., Jr., and M. J. Greenberg. 1977. Physiological basis of the species abundance-salinity relationship in molluscs: A speculation. Mar. Biol. 40:41-49.

Gamble, J. L., Jr. 1957. Potassium binding and oxidative phosphorylation in mitochondria and mitochondrial fragments. J. Biol. Chem. 228:955-971.

Gaston, S., and J. W. Campbell. 1966. Distribution of arginase activity in molluscs. Comp. Biochem. Physiol. 17:259-270.

Gilles, R. 1979. Intracellular organic osmotic effectors. Pages 111-154 in R. Gilles, ed. Mechanisms of Osmoregulation in Animals. John Wiley and Sons, New York, NY.

Greenawalt, J. W. 1974. The isolation of outer and inner mitochondrial membranes. Pages 310-323 in S. Fleischer and L. Packer, eds. Methods in Enzymology Vol. 31. Academic Press, New York, NY.

Greenwalt, D. E., J. Castiglione, and S. H. Bishop. 1978. Salinity and amino acid levels in mussels. Am. Zool. 18:627.

Greenwalt, D. E., and S. H. Bishop. 1980. Effect of aminotransferase inhibitors on the pattern of free amino acid accumulation in isolated mussel hearts subjected to hyperosmotic stress. Physiol. Zool. 53:262-269.

Greenwalt, D. E. 1981. Role of amino acids in cell volume control in the ribbed mussel:alanine and proline metabolism. Ph.D. Thesis. Iowa State University, Ames, Iowa. 127 pp.

Hammen, C. S. 1976. Respiratory adaptations:invertebrates. Pages 347-355 in M. Wiley, ed. Estuarine Processes Vol. 1. Academic Press, Inc., New York, NY.

Hanes, C. S., and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature 164:1107-1112.

Hanstein, W. G., and Y. Hatefi. 1974. Trinitrophenol: A membrane impermeable uncoupler of oxidative phosphorylation. Proc. Nat. Acad. Sci. (U.S.A.) 71:288-292.

Hendil, K. B., and E. K. Hoffman. 1974. Cell volume regulation in Ehrlick ascites tumro cells. J. Cell Physiol. 84:115-125.

Herzfeld, A., V. A. Mezl, and W. E. Knox. 1977. Enzymes metabolizing Δ^1-pyrroline-5-carboxylate in rat tissues. Biochem. J. 166:95-103.

Hochachka, P. W. 1981. Life without oxygen. Harvard University Press, Cambridge, MA.

Hochachka, P. W., T. P. Mommsen, J. Storey, K. B. Storey, K. Johansen, and C. J. French. 1983. The relationship between arginine and proline metabolism in cepalopods. Mar. Biol. Lett. 4:1-21.

Hogeboom, G. H., W. C. Schneider, and G. E. Pallade. 1948. Cytochemical studies of mammalian tissues I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J. Biol. Chem. 172:619-635.

Holwerda, D. A., and A. de Zwaan. 1979. Fumarate reductase of Mytilus edulis (L). Mar. Biol. Lett. 1:33-40.

Hope, D. B., and K. C. Horncastle. 1967. The oxidation of lysine and oxalysine by Mytilus edulis, identification of the products formed in the presence and the absence of catalase. Biochem. J. 102:910-916.

Hope, D. B., K. C. Horncastle, and R. T. Aplin. 1967. The dimerization of Δ'-piperidine-2-carboxylic acid. Biochem. J. 105:663-667.

Hoyaux, J., R. Gilles, and Ch. Jeuniaux. 1975. Osmoregulation in molluscs of the intertidal zone. Comp. Biochem. Physiol. 53A:361-365.

Humphreys, W. J. 1962. Electron microscope studies on eggs of Mytilus edulis. J. Ultrastruct. Res. 7:467-487.

Jacobus, W. E., R. W. Moreadith, and K. M. Vandegaer. 1982. Mitochondrial respiratory control. J. Biol. Chem. 257:2397-2402.

Jamieson, D. D., and P. de Rome. 1979. Energy metabolism of the heart of the mollusc Tapes watlingi. Comp. Biochem. Physiol. 63B:399-405.

Johnson, A. B., and H. J. Strecker. 1962. The interconversion of glutamic acid and proline. IV. The oxidation of proline by rat liver mitochondria. J. Biol. Chem. 237:1876-1882.

Johnson, D., and H. Lardy. 1967. Isolation of lives or kidney mitochondria. Pages 94-96 in R. W. Estabrook and M. E. Pullman, eds. Methods in Enzymology Vol. 10. Academic Press, Inc., New York, NY.

Kaila, K., and J. Saarikoski. 1981. Membrane-potential changes caused by 2,4-DNP and related phenols in resting crayfish axons are not due to uncoupling of mitochondria. Comp. Biochem. Physiol. 69C: 235-242.

Kernan, R. P. 1980. Potassium fluxes in mitochondria. Pages 120-137 in E. E. Bittav, ed. Cell Potassium. John Wiley and Sons, New York, NY.

King, T. E. 1967. The Keilin-Hartree heart muscle preparation. Pages 202-208 in R. W. Estabrook and M. E. Pullman, eds. Methods in Enzymology Vol. 10. Academic Press, Inc., New York, NY.

Kluytmans, J. H., P. R. Veenhof, and A. de Zwaan. 1975. Anaerobic production of volatile fatty acids in the sea mussel Mytilus edulis (L.). J. Comp. Physiol. 104:71-78.

Kluytmans, J. H., A. M. T. deBont, J. Jones, and T. C. M. Wijsman. 1977. Time dependent changes and tissue specificities in the accumulation of anaerobic fermentation products in the sea mussel Mytilus edulis (L.). Comp. Biochem. Physiol. 58B:81-87.

Kluytmans, J. H., M. van Graft, J. Janus, and H. Pieters. 1978. Production and excretion of volatile fatty acids in the sea mussel Mytilus edulis (L.). J. Comp. Physiol. 123:163-167.

Köhler, P. 1977. The transport of dicarboxylates and some properties of fumarase in the muscle mitochondria of Ascaris swami. Int. J. Biochem. 8:141-147.

Köhler, P., and R. Bachmann. 1980. Mechanisms of respiration and phosphorylation in Ascaris muscle mitochondria. Mol. Biochem. Parasitol. 1:75-90.

LeRudulier, D., and R. C. Valentine. 1982. Genetic engineering in agriculture:osmoregulation. Trend. Biochem. Sci. 7:431-433.

Livingstone, D. R., J. Widdows, and P. Fieth. 1979. Aspects of nitrogen metabolism of the common mussel Mytilus edulis: adaptation to abrupt and fluctuating changes in salinity. Mar. Biol. 53:41-55.

Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randal. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Matsuzawa, T., and I. Ishiguro. 1980. Δ'-pyrroline-5-carboxylate from baker's yeast. Biochim. Biophys. Acta 613:318-323.

Mattisson, A. G. M. 1959. Cytochome c, cytochrome oxidase, and respiratory intensity in some types of invertebrate muscles. Ark. Zool. 12:143-163.

Mattisson, A. G. M. 1961a. The effect of inhibitory and activating substances on the cellular respiration of some types of invertebrate muscles. Ark. Zool. 13:447-474.

Mattisson, A. G. M. 1961b. Flavins in some types of invertebrate muscles. Ark. Zool. 13:545-552.

Mattisson, A. G. M. 1961c. The cytochrome spectra of mitochondria fractions from muscle tissues of some invertebrates. Ark. Zool. 15:65-70.

Mattisson, A. G. M., and A. Birch-Andersen. 1962. On the fine structure of the mitochondria and its relation to oxidative capacity in muscles in various invertebrates. J. Ultrastruct. Res. 6:205-228.

Meister, A. 1954. The α keto analogues of arginine, ornithine, and lysine. J. Biol. Chem. 206:577-585.

Meister, A. 1960. Deamination of amino acids: Specificity and biological significance. Pages 7-26 in J. Roche, ed. Biochimie Comparée des Acid Aminés Basique Vol. 67. Coll. Internat. Centre Nat. de la Res. Sci., Paris.

Meyer, J. 1977. Proline transport in rat liver mitochondria. Arch. Biochem. Biophys. 178:387-395.

Mommsen, T. P., and P. W. Hochachka. 1981. Respiratory and enzymatic properties of squid heart mitochondria. Eur. J. Biochem. 120:345-350.

Moore, M. N. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, Mytilus edulis, and changes induced by thermal stress. Cell Tissue Res. 175:279-287.

Moore, M. N., D. M. Lowe, and S. L. Moore. 1979. Induction of lysosomal destabilization in marine bivalve molluscs exposed to air. Mar. Biol. Lett. 1:47-57.

Murfitt, R. R., K. Vogel, and D. R. Sanadi. 1976. Characterization of the mitochondria of the free-living nematode, Caenorhabditis elegans. Comp. Biochem. Physiol. 53B:423-430.

Nakazawa, T., K. Hori, and O. Hayaishi. 1972. Studies on monooxygenases. V. Manifestation of amino acid oxidase activity by L-lysine monooxygenase. J. Biol. Chem. 247:3439-3444.

Newell, R. C. 1967. Oxidative activity of poikilotherm mitochondria as a function of temperature. J. Zool. (Lond.) 151:299-311.

Olomucki, A., N. V. Thoai, and J. Roche. 1960. Dégradation de l'-ornithine et de la lysine chiz Limnaea stagnalis L. in J. Roche, ed. Biochimie Comparée des Acides Aminés Basques Vol. 67. Coll. Internat. Centre Nat. de la Res. Sci., Paris.

Pablo, I. S., and A. L. Tappel. 1961. Cytochromes of marine invertebrates. J. Cell Comp. Physiol. 58:185-194.

Papa, S., K. S. Cheah, H. N. Rasmussen, I. Y. Lee, and B. Chance. 1970. Mechanism of malate utilization in Ascaris - muscle mitochondria. Eur. J. Biochem. 12:540-543.

Pierce, S. K. 1982. Invertebrate cell volume control mechanisms: A coordinated use of intracellular amino acids and inorganic ions as osmotic solute. Biol. Bull. 163:405-419.

Peisach, J., and H. J. Strecker. 1962. The interconversion of glutamic acid and proline. J. Biol. Chem. 237:2255-2260.

Peraino, C., and H. C. Pitot. 1962. Ornithine-δ-transaminase in the rat. Biochim. Biophys. Acta 73:222-231.

Pho, D. B., A. Olomucki, and J. V. Thoai. 1966. L-arginine decarboxylanle. IV. Incorporation de ^{18}O dans la γ-guanidinobutyramide. Biochim. Biophys. Acta 118:311-315.

Rains, D. W., R. C. Valentine, and A. Holleander. 1980. Genetic engineering of osmoregulation. Plenum Publishing Corp., New York, NY.

Reddy, S. R. R., and J. W. Campbell. 1969. Arginine metabolism in insects. Biochem. J. 115:495-503.

Reiss, P. M., S. K. Pierce, and S. H. Bishop. 1977. Glutamate dehydrogenase from tissues of the ribbed mussel Modiolus demissus: ADP activation and possible physiological significance. J. Exp. Zool. 202:253-258.

Reynoso, G. T., and B. A. De Gamboa. 1982. Salt tolerance in the freshwater algae Chlamydomonas reinhardii: Effect of proline and taurine. Comp. Biochem. Physiol. 73A:95-99.

Roche, J., P. E. Glahn, Ph. Manchon, and N. V. Thoai. 1959. Sur une nouvelle L-aminoacideoxydase, activable par le magnésium. Biochim. Biophys. Acta 35:111-122.

Roche, J., N. V. Thoai, and P. E. Glahn. 1952. Sur la L-amino-acideoxydase de nombreux invertébrés marins. Experientia 8: 428-429.

Rodrick, G. E., S. D. Long, W. A. Sodeman, Jr., and D. L. Smith. 1982. Ascaris suum: Oxidative phosphorylation in mitochondria from developing eggs and adult muscle. Exp. Parasitol. 54:235-242.

Ross, G., D. Dunn, and M. E. Jones. 1978. Ornithine synthesis from glutamate in rat intestinal mucosa homogenates: Evidence for the reduction of glutamate to gamma-glutamyl semialdehyde. Biochem. Biophys. Res. Comm. 85:140-147.

Sactor, B., and C. Childress. 1967. Metabolism of proline in insect flight muscle and its significance in stimulating the oxidation of pyruvate. Arch. Biochem. Biophys. 120:583-588.

Saz, H. J. 1981. Energy metabolism of parasitic helminths: Adaptations to parasitism. Ann. Rev. Physiol. 43:323-341.

Scarpulla, R. C., and R. L. Soffer. 1978. Membrane-bound proline dehydrogenase from Escherichia coli. J. Biol. Chem. 253:5997-6001.

Scher, W. I., and H. J. Vogel. 1957. Occurrence of ornithine δ-transaminase: A dictotomy. Proc. Natn. Acad. Sci. U.S.A. 43: 796-803.

Schneider, W. C., and G. H. Hogeboom. 1950. Intracellular distribution of enzymes V. Further studies on the distribution of cytochrome C in rat liver homogenates. J. Biol. Chem. 183:123-128.

Soper, J. W., G. L. Decker, and P. L. Petersen. 1979. Mitochondrial ATPase complex. J. Biol. Chem. 254:11170-11176.

Slater, E. C. 1967. Application of inhibitors and uncouplers for a study of oxidative phosphorylation. Pages 48-57 in R. E. Estabrook and M. E. Pullman, eds. Methods in Enzymology Vol. 10. Academic Press, Inc., New York, NY.

Strecker, H. J. 1960. The interconversion of glutamic acid and proline. III. Δ'-Pyrroline-5-carboxylic acid dehydrogenase. J. Biol. Chem. 235:3218-3223.

Strecker, H. J. 1971. The preparation of animal proline oxidase (rat liver), and its use for the preparation of Δ^1-pyrroline-5-carboxylate. Pages 251-254 in H. Tabor and C. H. Tabor, eds. Methods in Enzymology Vol. 17B. Academic Press, New York, N.Y.

Strittmatter, P., and C. F. Strittmatter. 1961. Electron transport in eggs, developing embryos and adult tissues of Spisula solidissima. J. Cell Comp. Physiol. 57:87-94.

Struck, J., and I. W. Sizer. 1960. Oxidation of L-α-amino acids by chicken microsomes. Arch. Biochem. Biophys. 90:22-30.

Takeda, H., S. Yamamoto, Y. Kojima, and O. Hayaishi. 1969. Studies on monooxygenases. I. General properties of crystalline L-lysine monooxygenase. J. Biol. Chem. 244:2935-2941.

Tappel, A. L. 1960. Cytochromes of muscles of marine invertebrates. J. Cell Comp. Physiol. 55:111-126.

Thompson, R. J., C. J. Bayne, M. N. Moore, and Carefoot. 1978. Haemolymph volume, changes in the biochemical composition of the blood, and cytological responses of the digestive cells in Mytilus californianus Conrad, induced by nutritional, thermal and exposure stress. J. Comp. Physiol. 127:281-298.

Vogel, H. J., and B. D. Davis. 1952. Glutamic-γ-semialdehyde and Δ'-pyrroline-5-carboxylic acid, intermediates in the biosynthesis of proline. J. Am. Chem. Soc. 94:109-112.

Vorhaben, J. E., J. F. Scott, and J. W. Campbell. 1980. D-Mannitol oxidation in the land snail, Helix aspersa. J. Biol. Chem. 255:1950-1955.

Wadano, A., T. Yamato, K. Yoshida, and K. Miura. 1976. Pyrroline-carboxylate reductase of a blowfly, Aldrichina grahmi. Insect Biochem. 6:657-661.

Weeda, E., C. A. D. de Kort, and A. M. Th. Beenakkers. 1980. Oxidation of proline and pyruvate by flight muscle mitochondria of the Colorado beetle, Lepinotarsa decemlineata saz. Insect Biochem. 10:305-311.

Wekell, M. M. B., and G. W. Brown. 1973. Ornithine aminotransferase of fishes. Comp. Biochem. Physiol. 46B:779-795.

Yamamoto, S., T. Nakazawa, and O. Hayaishi. 1972. Studies on monooxygenases. IV. Anaerobic formation of an α-keto acid by L-lysine monooxygenase. J. Biol. Chem. 247:3434-3438.

Yamauchi, T., S. Yamamoto, and O. Hayaishi. 1975. A possible involvement of sulfhydryl groups in the conversion of lysine monooxygenase to an oxidase. J. Biol. Chem. 250:7123-7133.

Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero. 1982. Living with water stress: Evolution of osmolyte systems. Science 217:1214-1222.

Young, J. P. W., R. R. Koehn, and N. Amheim. 1979. Biochemical characterization of "LAP," a polymorphic aminopeptidase from the blue mussel, Mytilus edulis. Biochem. Genet. 17:305-323.

Zaba, B. N. 1983. On the nature of oxygen uptake in two tissues of Mytilus edulis. Mar. Biol. Lett. 4:59-66.

Zaba, B. N., A. M. T. de Bont, and A. de Zwaan. 1978. Preparation and properties of mitochondria from tissues of the sea mussel Mytilus edulis (L.). Int. J. Biochem. 9:191-197.

Zurburg, W., and J. H. Kluytmans. 1980. Organ specific changes in energy metabolism due to anaerobiosis in the sea mussel Mytilus edulis (L.). Comp. Biochem. Physiol. 67B:317-322.

de Zwaan, A. 1977. Anaerobic energy metabolism in bivalve molluscs. Oceanogr. Mar. Biol. Ann. Rev. 15:103-187.

de Zwaan, A., D. A. Holwerda, and P. R. Veenhof. 1981. Anaerobic malate metabolism in mitochondria of the sea mussel Mytilus edulis (L.). Mar. Biol. Lett. 2:131-140.

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