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Metabolic Regulation of Proline, Glycine, and Alanine Accumulation as Intracellular Osmolytes in Ribbed Mussel Gill Tissue

STEPHEN H. BISHOP, DALE E. GREENWALT, MARTIN A. KAPPER, KENNEDY T. PAYNTER, AND LEHMAN L. ELLIS
Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011

Most euryhaline, estuarine bivalve molluscs are osmoconformers, in that the osmotic pressure and ionic composition of the haemolymph closely parallels that of the sea or estuarine water bathing the animals (Burton, '83). The cells in the tissues of these organisms adjust the intracellular osmotic pressure to be more or less equal to that of the haemolymph. In addition to K^+ and other inorganic ions, the cells use highly soluble amino acids such as alanine, glycine and proline along with neutral amines such as taurine and/or betaines as intracellular osmotic buffers (Pierce, '82). The intracellular concentration of these amines rises and falls with the rise and fall in the osmotic pressure of the haemolymph or the media bathing the tissue or the organism.

Our studies have focused on the tissues of the ribbed mussel, *Geukensia (Modiolus) demissa,* because of the excellent data base on the performance of this extremely euryhaline bivalve (Baginski and Pierce, '75, '77, '78; Pierce, '82; Pierce and Greenberg, '76; Shumway and Youngson, '79). With hypotonic stress, internal tissues such as the heart release amino acids to the haemolymph or bathing media as part of the regulatory volume decrease (RVD; Pierce and Greenberg, '76). The whole animal responds by increasing the metabolic rate and releasing ammonia rather than amino acids to the bathing medium (Bartberger and Pierce, '76; Shumway and Youngson, '79; Strange and Crowe, '79a,b). The gills or mantle are thought to be the sites of catabolism of the amino acids released by the internal tissues during hypotonic stress (Baginski and Pierce, '78; Bishop et al., '81). With hypertonic stress, the free amino acids in the tissues rise fairly rapidly as part of the regulatory volume increase (RVI) and come to an unstable equilibrium in about two to four days (Baginski and Pierce, '75).

The response of isolated tissues to hypertonic stress tends to mimic the response of the whole animal (Baginski and Pierce, '75, '77; Greenwalt and Bishop, '80; Bishop et al., '81; Ellis et al., '85). When hearts or gill tissue are removed from animals adapted at low salinity and incubated for a few hours in hypertonic medium (full artificial sea water) with no added organic substrates or ammonium ion, the tissue responds rapidly by decreasing the metabolic rate and increasing the level of free amino acids. The amino acids that increase are mainly alanine, glycine, proline and β-alanine, the same ones that accumulate in the tissues of intact animals. Therefore, the control of the levels of these amino acids is the result of intracellular metabolic processes combined with membrane trapping rather than the result of membrane transport (uptake). Secondly, the carbon and the nitrogen atoms used for the biosynthesis of these amino acids are derived from internal metabolic stores. The levels of taurine change very slowly and because of the slow metabolic turnover of taurine, the levels may be controlled by membrane transport alone (see Bishop et al., '83).

Our experiments have focused on elucidation of the metabolic events regulating the initial increase in the alanine, glycine and proline levels in the gill tissue of the ribbed mussel subjected to hypertonic stress. The objective has been the development of a general theory of metabolic control that might apply to all organic osmolytes in the cells of salt stress-resistant tissues and organisms.

MATERIALS AND METHODS

Mussels were purchased and adapted at 12% as described previously (Greenwalt and Bishop, '80).

Address reprint requests to Stephen H. Bishop, Department of Zoology and Genetics, Iowa State University, Ames, IA 50011.
This paper is dedicated to the memory of James M. Burcham, who died in October 1992.

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Unless stated otherwise all radioisotopically labelled compounds were obtained from New England Nuclear Co. (DuPont, Wilmington, DE) and reagents were purchased from Sigma Chemical Co. (St. Louis), Fisher Scientific Co., Kansas City, or Pierce Chemical Co., Chicago. Radiolabelled chemicals used in studies described here were U-^{14}C-L-glutamate (292 mCi/mmol), U-^{14}C-L-alanine (170 mCi/mmol), U-^{14}C-L-valine (250 mCi/mmol), U-^{14}C-L-aspartate (200 mCi/mmol), U-^{14}C-glycine (90 mCi/mmol), and U-^{14}C-D-glucose (300 mCi/mmol).

The procedures for evaluating changes in the amino acid levels and patterns of radiolabel transfer have been described (Greenwalt and Bishop, '80; Bishop et al., '81; Ellis et al., '85). Tissue extracts were prepared in 80% ethanol. After drying, the extract was dissolved in water and passed through a small Dowex 50 column. The column was washed with water to remove the anionic organic acids and neutral compounds and then with ammonia water to remove the amino acids. After drying, samples were prepared for amino acid analysis on an automated amino acid analyzer using the physiological fluids program (Li-citrate buffer system) or for thin-layer chromatography and radioautography. Radiolabelled compounds were removed from the thin-layer plates and the amount of radioactivity determined using a liquid scintillation counter.

The basic experimental design for the tissue incubation experiments followed that of Baginski and Pierce ('75, '77, '78). Tissues were removed from animals adapted at low salinity (12‰) and incubated in medium at either 12% or the hypertonic stress salinity of 32‰. The experiments involved the use of radiolabelled tracers with and without a variety of metabolic inhibitors. These experiments were complemented by studies of specific enzymes and isolated mitochondria.

RESULTS AND DISCUSSION

Overall amino acid accumulation and metabolism

In the initial experiments of Baginski and Pierce ('75), hypertonic stress of isolated ribbed mussel hearts resulted in a rapid increase in alanine, proline and glycine with no change in taurine. The increase in alanine accounted for 80–90% of the increase in amino acid levels in the first few hours. Because only a few species of amino acids increase, this increase must be due to a reorganization of internal metabolic stores. We reasoned that the carbon skeleton and the amino group of the accumulated amino acids might be derived from protein (Bishop, '76) and shuttled to these few amino acids (i.e., alanine) through a transaminase relay system similar to that seen in skeletal muscle. If this general premise was true, then blockade of amino acid catabolism by transaminase inhibitors should reduce the levels of alanine accumulation and increase the accumulation of the other amino acids.

We exposed *G. demissa* heart tissue to hyperosmotic seawater in the presence and absence of the transaminase inhibitors, aminooxyacetate (AOA) and L-cycloserine (Greenwalt and Bishop, '80). In the control experiments, with no inhibitors, we obtained essentially the same results as Baginski and Pierce ('75). Addition of the transaminase inhibitors reduced the increase in alanine and proline levels, had no effect on the small increase in glycine or β-alanine levels, had no effect on the level of glutamate, and caused an increase in the levels of just about all of the other amino acids, including essential amino acids. Overall, the increase in the variety of amino acids behind the transaminase blockade compensated for the decreased rise in the alanine levels so that the total amino acid accumulation with salt stress did not change with transaminase blockade. This result supported the theory that the carbon and nitrogen in the accumulated amino acids (i.e., alanine, glycine, proline, etc.) were coming from protein, arginine, or phosphoarginine turnover and not from ammonia fixation by glutamate dehydrogenase followed by transamination of glycolytically derived intermediates. The increase in alanine levels was transaminase-dependent. The theory that cellular protein is the source of the nitrogen for the accumulation of amino acids is supported by the studies of Deaton ('87) and Deaton et al. ('85) and Hawkins and Hilbish ('92) with *M. edulis*.

Because the gill is a larger, more manageable tissue and the probable site of amino acid turnover during hypotonic stress, recent studies have focused on the gill. The change in amino acid levels in the gill tissue subjected to hypertonic stress in the presence and absence of AOA (Table 1) was similar to that with heart tissue and to that with gill tissue over an 8 hr period (Deaton, '87). Taurine levels did not change with hypertonic stress or with addition of arsenite or AOA at either salinity. In contrast to its effects on the heart, AOA blocked about 25% of the increase in total amino acid levels in the hypertonically stressed gill, indicating that either some AOA-sensitive process was essential in establishing the increase in the total amino acid pool size or that the higher levels of the L-amino acid oxidase in gill compared to heart tissue (Burcham et al., '80) might have catabolized more of the amino acids as they accumulated behind the transaminase blockade.

TABLE 1. Intracellular free amino acid concentrations in isolated gill tissue (50 mg) of 12‰ ASW adapted G. demissa *exposed to 12% ASW or 32% ASW for four hours with and without aminooxyacetate (1 mM) or arsenite (1 mM)*[1]

Inhibitor	None		Aminooxyacetate		Arsenite	
Salinity	12%	32%	12%	32%	12%	32%
Alanine	4.5 ± 0.7	127.5 ± 12.2	21.4 ± 2.7	31.2 ± 2.3	25.0 ± 9.9	75.2 ± 13.0
Proline	0.1 ± 0.1	12.1 ± 0.9	0.8 ± 0.2	6.8 ± 1.0	0.5 ± 0.5	16.0 ± 1.2
Ornithine	3.0 ± 0.7	7.1 ± 0.8	12.0 ± 1.1	34.5 ± 1.5	10.3 ± 0.7	7.4 ± 0.7
Arginine	4.6 ± 0.4	5.9 ± 0.5	5.7 ± 0.8	5.0 ± 0.3	2.4 ± 0.9	1.6 ± 0.2
Glutamate	13.2 ± 1.5	13.8 ± 1.1	3.9 ± 1.4	9.7 ± 0.8	10.8 ± 3.0	29.9 ± 4.2
Aspartate	41.7 ± 3.0	22.2 ± 1.4	24.1 ± 2.6	27.8 ± 3.8	3.0 ± 0.6	2.5 ± 0.4
Glycine	4.8 ± 1.1	16.7 ± 2.0	9.7 ± 1.4	16.2 ± 2.8	7.7 ± 1.7	10.2 ± 2.2
β-alanine	0.8 ± 0.1	17.4 ± 0.8	3.4 ± 0.1	19.5 ± 0.5	0.2 ± 0.1	19.7 ± 1.6
Serine	2.5 ± 0.4	3.5 ± 0.4	5.1 ± 0.9	5.3 ± 0.5	2.3 ± 0.1	3.8 ± 0.9
Taurine	139.0 ± 7.8	125.6 ± 9.9	103.8 ± 10.7	124.9 ± 11.7	114.1 ± 16.0	121.5 ± 7.7
Other	40.8 ± 6.9	41.8 ± 8.2	55.1 ± 9.3	61.0 ± 5.4	46.0 ± 4.1	38.2 ± 6.0
TOTAL	255.0 ± 8.0	393.6 ± 20.0	245.0 ± 13.8	341.9 ± 7.7	222.3 ± 2.5	326.0 ± 29.2
TOTAL — taurine	116.0 ± 4.5	268.0 ± 11.9	141.2 ± 4.3	217.0 ± 7.2	108.2 ± 20.6	204.5 ± 9.8

[1]Each value (μmoles/g dry wt. ± S.E.M.) represents the mean of tissue samples from at least five animals. ASW, artificial sea water.

With regard to the individual amino acids (Table 1), the increase in alanine levels with hypertonic stress was blocked by AOA; alanine production was transaminase-dependent. Unlike heart tissue, AOA caused a major loss of gill tissue glutamate (Table 1), presumably through the glutamate dehydrogenase reaction. The pattern of the effect of AOA on the other amino acid level increases was similar to that seen with the heart experiments.

Experiments using ^{14}C-labelled amino acids with blue crabs (Pressley and Graves, '83), oysters (Beavers and Hammon, '85) and ascites tumor cells (Lambert and Hoffmann, '82) indicated that hypertonic stress caused a decrease in the catabolism of the amino acids that accumulated during hypertonic stress. To evaluate a change in the catabolism of specific amino acids in ribbed mussel gill tissue during hypertonic stress, CO_2 production (as ^{14}C-CO_2) was measured in mussel gills incubated with small amounts of radiolabelled amino acids at high specific radioactivities that would label this intracellular pool without changing the pool size (Fig. 1). The turnover of aspartate was faster than the other amino acids and was in agreement with the previous work of Baginski and Pierce ('78). Hypertonic stress caused a 95%, 60% and 75% decrease in the rate of glycine, alanine, and aspartate catabolism, respectively. On the other hand, the turnover of glutamate declined only slightly with hypertonic stress. AOA blocked alanine and glycine catabolism, caused a 75% decrease in the rate of aspartate turnover and had only a small effect on glutamate catabolism. The experiment with AOA indicated that the purine nucleotide cycle might be involved to some degree in aspartate metabolism (see Bishop et al., '81) and that direct glutamate catabolism (ammonia production) was mainly through the AOA-insensitive glutamate dehydrogenase (Reiss et al., '77) located in the mitochondria.

From the data in these experiments, it seemed that the accumulation of each amino acid (alanine, glycine and proline) was regulated individually. Therefore, we investigated the metabolism of pro-

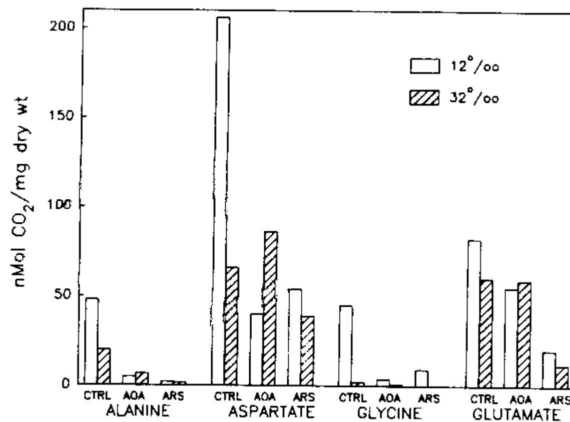

Fig. 1. Effect of hypertonic shift on CO_2 production from intracellular alanine, glycine, glutamate, and aspartate in ribbed mussel gill tissue in the presence and absence of AOA and arsenite. Tissues were incubated at 23°C with 0.5 μCi (undiluted) of uniformly ^{14}C-labelled substrate and the rate of ^{14}C-CO_2 production was determined (Bishop et al., '81). The amount of CO_2 was calculated from the ^{14}C-CO_2 produced and corrected for the isotope dilution due to time course (fairly linear; data not shown) changes in the intracellular pool sizes (Table 1). Values are estimated yields per 4 hr of incubation from 6 to 9 separate determinations on 40 mg of tissue each. Standard errors in the measurements were 10% or less. ARS, arsenite.

line, glycine and alanine as separate entities and looked for common ground.

Proline

The effect of AOA on proline accumulation is most interesting because ornithine levels seemed to rise as proline levels declined in experiments with heart tissue (Greenwalt and Bishop, '80). With the gill, proline levels are essentially nil at 12% and rose considerably with salt stress at 32% (Table 1). AOA causes an approximately 50% decline in the rise in proline levels and a very large increase in the ornithine levels. The ornithine aminotransferase (OAT) is mitochondrial and very sensitive to inhibition by AOA (Bishop et al., '81, '83; Burcham et al., '84). Proline catabolism is not blocked by AOA. It seems that a considerable portion of the accumulating proline may be derived from ornithine and ornithine precursors such as arginine and phosphoarginine.

Initial experiments with U-^{14}C-glucose as a tracer indicated little or no de novo biosynthesis of proline by ribbed mussel tissue (Baginski and Pierce, '78). We repeated the ^{14}C-glucose tracer experiments with isolated gill tissue and found radiolabel transfer from glucose to glutamate, alanine and aspartate, but not to proline or arginine (Bishop et al., '81). Tracer studies with ^{14}C-amino acids such as glycine, alanine, valine, serine, leucine, aspartate, and glutamate indicated no transfer of label to proline, ornithine or arginine.

In another series of experiments, radiocarbon label (Bishop et al., '81) from arginine and ornithine was transferred rapidly to proline and glutamate but label from proline and ornithine was not transferred to arginine. Additionally, isolated gill pieces converted both pyrroline-5-carboxylate (product of the OAT action on L-ornithine) and pyrroline-2-carboxylate (product of L-amino acid oxidase action on L-ornithine; Burcham et al., '80) to proline, glutamate and CO_2 (data not shown). Enzyme studies (Bishop et al., '81) and studies with isolated respiring mitochondria (Burcham et al., '84) indicated a very reactive mitochondrial proline oxidase and P-5-C dehydrogenase and a cytoplasmic P-5-C reductase.

It appears that the transient increase in proline levels during hypertonic stress results from an increase in the catabolism of arginine and ornithine to proline and glutamate plus a release of proline from another source such as protein (Fig. 2). The proline levels are not sustained because, as the proline levels increase, the rate of catabolism by the mitochondrial proline oxidase increases and the proline levels begin to decline as the tissue becomes adapted to the higher salinity, and the flow of carbon from arginine and ornithine to proline and glutamate declines.

To some degree, the rate of proline oxidation depends on the rates of glutamate and ketoglutarate turnover. The rate of glutamate and ketoglutarate oxidation by the gill and by isolated respiring mitochondria is very rapid and apparently not affected by hypertonic stress of the isolated tissue (Bishop et al., '81; Fig. 1). Hypertonic stress (NaCl) causes a reduced glutamate oxidation in isolated respiring mitochondria (Ballantyne and Moyes, '87). The

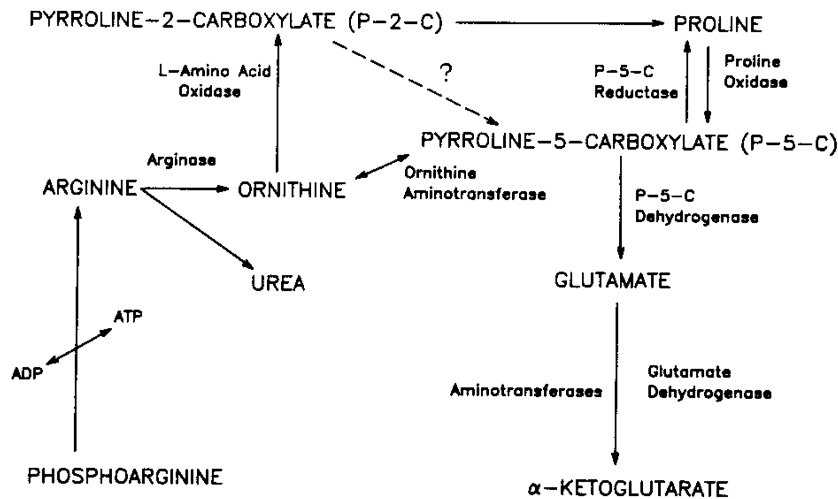

Fig. 2. Outline of arginine, ornithine and proline metabolism in ribbed mussel gills.

activity of the ketoglutarate dehydrogenase in the gill mitochondria seems to depend upon ketoglutarate availability (Km) and upon changes in adenine nucleotide levels but not upon changes in calcium ion levels (Karam et al., '87). The lack of proline and ornithine biosynthesis from glutamate (apparent absence of P-5-C synthase in the mitochondria) indicates that both are essential amino acids. The very low levels of proline in bivalve tissues (see Bishop et al., '83) and the inability to sustain high levels of proline in the tissues suggest that proline is probably not important as an osmotic buffer in most bivalves.

Glycine

Glycine levels accumulated very slowly (weeks) in the tissues of intact ribbed mussels as they adapted to high salinities (Baginski and Pierce, '75, '77). However, in animals that are well adapted to high salinities, glycine comprised a third of the intracellular amino acid pool. The lack of AOA inhibition on the glycine levels in hypertonically stressed gills (Table 1) seemed to disagree with the effect of salt stress and AOA on glycine catabolism (Fig. 1). In a series of tracer experiments, Ellis et al. ('85) found little or no transfer of ^{14}C-label from glucose, aspartate, alanine, or glutamate to glycine or serine, whereas transfer of ^{14}C-label between serine and glycine and from glycine and serine to alanine was quite rapid. Reversal of the glycine oxidase reaction by CO_2 fixation was very slow. Therefore, it appeared that de novo biosynthesis of both glycine and serine were very slow. A metabolic scheme was devised (Fig. 3) and the enzymes in each step evaluated. Although all enzymes were found, some were at very low levels. All of the transaminases, the serine hydroxymethyl transferase and the mitochondrial glycine oxidase were inhibited by AOA and other transaminase inhibitors. A series of experiments with isolated gill mitochondria and whole gill pieces using ^{14}C-glycine labelled in the 1 and 2 positions indicated that the slow rate of glycine accumulation was regulated by the mitochondrial glycine oxidase. Hypertonic stress caused a 95% inhibition of this enzyme. Recently, similar results have been obtained for the regulation of glycine metabolism in another bivalve and some fish (Moyes and Moon, '87) and in rat liver (Jois et al., '92).

It appears that in ribbed mussel gill, the levels of glycine are controlled by regulation of the mitochondrial glycine oxidase complex to adjust the rate of catabolism rather than by control of the rate of glycine biosynthesis. With hypertonic stress, the glycine oxidase complex is inhibited and glycine accumulates slowly behind this metabolic block. Factors regulating this activity are uncertain (see Ellis et al., '85) but recently Jois et al. ('92) have demonstrated activation by increased calcium ion levels in a fashion similar to that seen with the mammalian (Roche and Lawlis, '82) and plant ketoglutarate dehydrogenases (Karam and Bishop, '89).

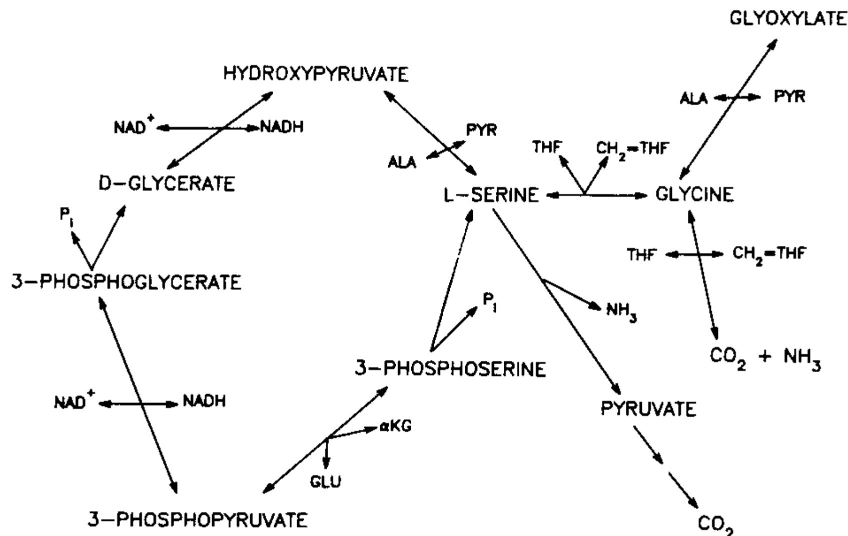

Fig. 3. Outline of serine and glycine metabolism in ribbed mussel gills.

Alanine

The data in Table 1 and Figure 1 indicated that most alanine synthesis and turnover was transaminase-dependent and possibly controlled by an arsenite-dependent reaction. Arsenite, an inhibitor of the keto acid dehydrogenase complexes, including pyruvate dehydrogenase (PDH), caused a large increase in alanine levels at the control salinity and reduced accumulation of alanine at the stress salinity plus an inhibition of alanine catabolism at all salinities.

Two problems were addressed: the source of the carbon skeleton for the alanine that accumulated and the processes regulating alanine accumulation.

In experiments similar to that described for valine (Fig. 4), hypertonic stress causes a 5- to 30-fold increase in the transfer of radiocarbon label from a variety of amino acids (proline, arginine, ornithine, glutamate, serine, glycine, aspartate, leucine, and valine) to alanine. Transfer of label to alanine is blocked by AOA in all cases. It appears that any compound that could provide tricarboxylic acid (TCA) cycle metabolites and pyruvate could serve as a precursor for alanine biosynthesis.

Because both hypertonic stress and anaerobic stress (Baginski and Pierce, '78; Ho and Zubhoff, '82) caused an increase in alanine levels, Baginski and Pierce ('75, '78) suggested that a major source of alanine was glycolytically derived pyruvate and showed that there was a considerable increase in the transfer of radiocarbon label from U-^{14}C-glucose to the accumulating alanine during hypertonic stress. Using the brackish water clam, *Rangia cuneata*, Henry et al. ('80) presented data supporting this idea by showing that iodoacetate (IAA) reduced alanine accumulation by about 50% during hypertonic stress.

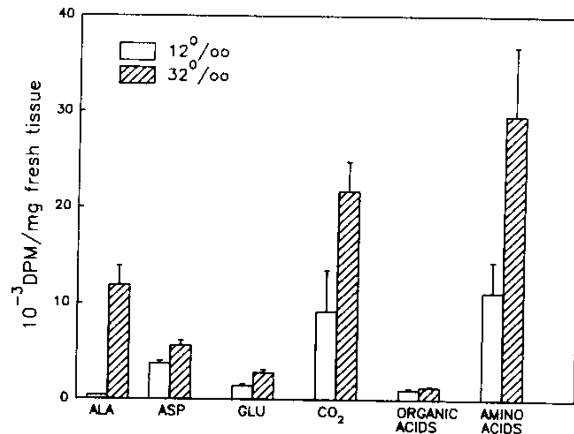

Fig. 4. Effects of hyperosmotic shock (12‰ → 32‰) on the incorporation of ^{14}C-U-valine (3.0 μCi) into metabolic intermediates in isolated gill tissue of from *G. demissa* adapted at 12‰ ASW. Each value represents the mean (± S.E.M.) of tissue samples (75–92 mg) from two mussels. Experimental procedures are described in Bishop et al. ('81).

Table 2 shows the effect of some inhibitors of glycolysis on the rise in alanine levels during hypertonic stress. IAA causes a 30% drop in the rise of alanine levels, whereas deoxyglucose and α-chlorohydrin (α-CH) had a much more modest effect. α-CH inhibits the glyceraldehydephosphate dehydrogenase in much the same fashion as IAA but is more specific and less reactive with other sulfhydryl enzymes (Brown-Woodman et al., '78).

TABLE 2. Intracellular concentrations of selected amino acids in isolated gill tissue (50 mg) of 12‰ ASW adapated M. demissus *exposed to 12‰ → 32‰ ASW with or without inhibitors of glycolysis for 4 hr*[1]

Conditions of incubation	Alanine	Aspartate	Glutamate	Glycine
No inhibitor				
12%	4.7 ± 0.5	39.9 ± 3.8	10.6 ± 1.0	3.4 ± 1.0
32%	124.5 ± 13.5	25.5 ± 2.2	18.8 ± 1.8	15.0 ± 4.9
Iodoacetate[2]				
12%	23.1 ± 2.1	11.2 ± 1.7	8.0 ± 1.1	5.8 ± 1.3
32%	86.2 ± 3.3	8.7 ± 0.7	16.0 ± 0.3	16.8 ± 3.9
2-deoxyglucose[2]				
12%	2.6 ± 0.4	31.8 ± 3.1	6.2 ± 1.0	1.7 ± 0.2
32%	99.9 ± 14.1	29.4 ± 2.9	14.2 ± 2.1	15.1 ± 0.6
α-chlorohydrin[2]				
12%	5.6 ± 2.3	36.5 ± 4.1	11.8 ± 1.9	3.6 ± 0.9
32%	106.0 ± 1.8	28.6 ± 1.0	10.7 ± 0.6	15.5 ± 3.1

[1]Each value represents the mean (± S.E.M.) of four separate determinations. Values are expressed as μmoles/gm dry weight).
[2]Tissue samples preincubated with 0.5 mM iodoacetate, 1 mM deoxyglucose or 2 mM α-chlorohydrin in 12‰ AWW for 15 minutes prior to transfer to 32‰ ASW.

IAA caused about a 50% reduction in transfer of radiolabel from glucose to alanine during hypertonic stress (Fig. 5); therefore, IAA inhibits glycolytic flux to some extent. Other experiments indicated a reduced transfer of radiocarbon label from aspartate and glutamate to alanine, suggesting that the effect of IAA was not specific.

Hypertonic stress causes an 80% reduction in ^{14}C-CO_2 production from U-^{14}C-glucose, 6-^{14}C-glucose and 3-^{14}C-pyruvate. Addition of AOA to the incubations with U-^{14}C-glucose increased ^{14}C-CO_2 production by preventing isotope dilution with the alanine pool. These data support the concept of a metabolic block on pyruvate metabolism during hypertonic stress.

Glycolytic flux, as ^3H incorporation into water from 5-^3H-glucose at the triosephosphate and enolase step in glycolysis (Ashcroft et al., '72), declined about 10–20% with hypertonic stress and averaged 3 μmoles/g wet weight/hr. This method of estimating glucose utilization is troublesome because even modest levels of the transaldolase in the presence of catalytic amounts of erythrose 4-phosphate will result in equilibration of the fructose-6-phosphate and triosephosphate pools in a fashion that bypasses the phosphofructokinase step (Hue and Hers, '74). Therefore, this method may overestimate glycolytic flux because the transaldolase level is about 10% of the phosphofructokinase level (data not shown).

Experiments with glucose labelled with ^{14}C in the 1 and the 6 positions were used to evaluate the contribution of the pentose shunt to glucose oxidation after the method of Katz and Wood ('63). Using the data from the 5-^3H-glucose experiment as a measure of glucose utilization, between 1% and 2% of the glucose appeared to be passing through the pentose shunt so most of the carbon from glucose was proceeding through the Emden-Myrhoff pathway. The results on flux rates and involvement of the pentose shunt are in close agreement with similar studies on sea mussel tissues (Zaba and Davis, '80, '81). Depending upon the method of measurement, glycolytic flux could contribute 10–40% of the carbon in the accumulating alanine.

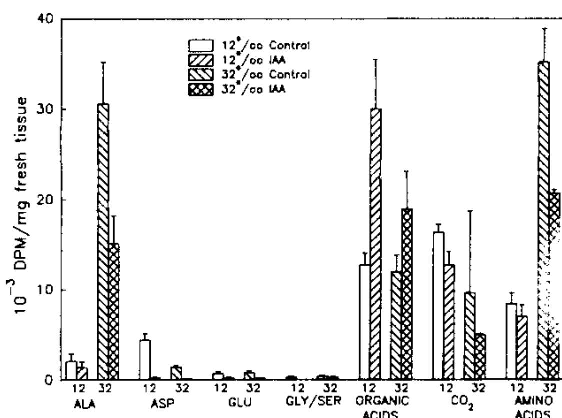

Fig. 5. Effects of 0.5 mM iodoacetic acid (IAA) on the incorporation of ^{14}C-U-glucose (3.0 μCi) into metabolic intermediates in isolated gill tissue of 12‰ ASW adapted *G. demissa* during six hours of hyperosmotic shock (12‰ → 32‰) Each value represents the mean (±S.E.M.) of tissue samples from three mussels. Tissue weights were 65 to 75 mg for each preparation.

Because alanine is a strong inhibitor of pyruvate kinase (K_i = 0.5 mM), accumulation of alanine at 10 to 100 mM should block flux through this step in glycolysis (Kapper and Bishop, ms. in preparation). Fructose 1,6-bisphosphate activates the pyruvate kinase in a fashion that reverses inhibition by alanine. The phosphoenolpyruvate (PEP) branchpoint (deZwaan and Dando, '84) provides an alternative pathway in that carbon flux from PEP might proceed through the phosphoenolpyruvate carboxykinase (PEPCK) reaction to the organic acids (oxaloacetate to malate or aspartate) for eventual conversion to pyruvate and alanine. Harlocker et al. ('91) found that flux through PEPCK was probably minor because of the high Km for bicarbonate and the small (10%) inhibition of alanine accumulation by mercaptopicolinic acid, a specific inhibitor of PEPCK. Either there is a considerable override of the alanine inhibition of PK or glycolytic flux contributes only approximately 10% of the carbon to alanine during alanine accumulation.

Regardless of the source of carbon for alanine, the metabolic control of alanine production seemed to be associated with pyruvate turnover and regulation of the PDH. In mammals (Reed and Yeaman, '87) and helminths (Kominecki and Thissen, '89), pyruvate dehydrogenase is associated with the matrix of the mitochondrion as a multienzyme complex composed of three basic components, E-1 (TPP-dependent decarboxylating dehydrogenase), E-2 (lipoamide-dependent acyl transferase), and E-3 (lipoamide dehydrogenase) plus two regulatory activities (PDH-protein kinase and PDH-protein phosphatase) that phosphorylate and dephosphorylate a subunit of the E-1 component. Phosphorylation causes a loss of activity and dephosphorylation restores activity. The phosphatase is Mg^{2+}-dependent and activated by Ca^{2+} at low Mg^{2+} levels. The kinase and phosphatase are under hormonal control through an as yet undefined second messenger signalling syst[illegible] that involves regulation of the intramitoch[illegible]rial Ca^{++} levels (McCormack et

al., '90). Other factors such as substrate levels, organic acids, nucleotides, anaerobiosis, and salt can inhibit or activate the PDH through a variety of mechanisms (Olson, '89; Patel and Olson, '84).

The PDH in the mitochondria from ribbed mussel gill appears to be very similar to the PDH in the other animal cells (Paynter et al., '85a). Both the kinase and the phosphatase are associated with the gill PDH and cause inactivation and reactivation of the PDH activity. In these studies, no Ca^{2+} activation of the phosphatase was observed, but these experiments were performed at high Mg^{++} levels where the Ca^{++} activating effect might have been overridden.

In experiments with isolated respiring mitochondria showing high coupling ratios with glutamate, α-ketoglutarate, succinate, proline, glycine, and malate, addition of pyruvate caused an initial ADP-dependent stimulation of oxygen consumption which then stopped (Burcham et al., '84). The ATP produced by the initial burst of pyruvate oxidation probably served as substrate for the PDH-kinase which then put the PDH in the inactive, phosphorylated form.

In conjunction with the studies on PDH, Paynter et al. ('84) have shown that the alanine aminotransferase (ALAT) in the ribbed mussel gill tissue is also restricted to the mitochondria. The gills and tissues from some other bivalves that show both cytosolic and mitochondrial isozymes of ALAT (Paynter et al., '85b) do not accumulate as much alanine as ribbed mussel gills.

A general scheme describing our view of alanine production in ribbed mussel gill is shown in Figure 6. It appears that the mitochondrion is the seat of alanine production and turnover and that the regulation of the alanine levels is dependent on pyruvate availability as regulated by the mitochondrial PDH. This compartmentation of alanine biosynthesis in the mitochondrion means that, once synthesized, alanine can be exported to the cytoplasm to act as an osmotic buffer, thereby preventing the ALAT reaction from going to equilibrium and arresting alanine production.

CONCLUSION

Each of the three amino acids under consideration (proline, glycine and alanine) have very different metabolic pathways in terms of biosynthesis. Proline production must depend upon synthesis from essential amino acids such as arginine and ornithine or from protein turnover. Glycine production is very slow and depends upon production from serine or from glycine and serine released during protein turnover. Alanine can be synthesized rapidly

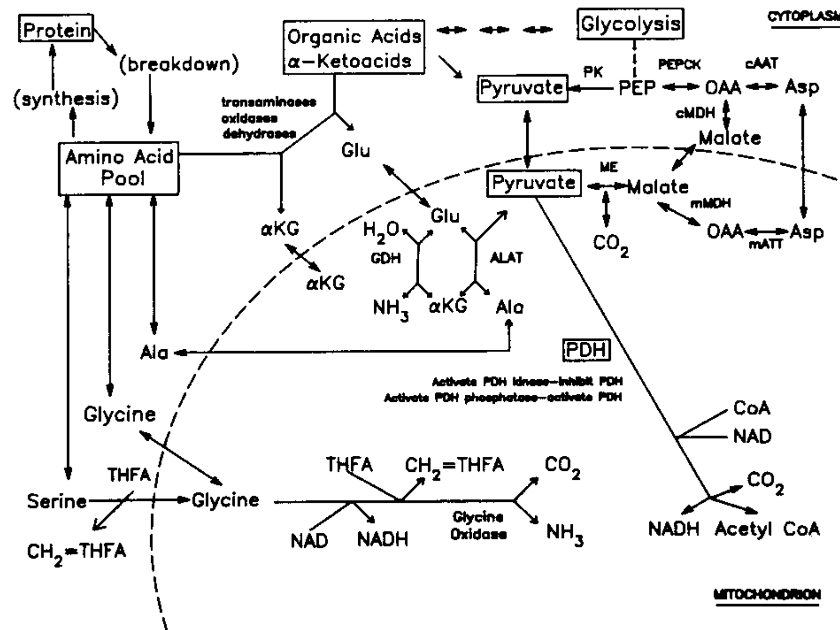

Fig. 6. Outline of the probable metabolic origins and fate of alanine in ribbed mussel gill.

from glucose or any substrate that will produce pyruvate. The nitrogen in the accumulated amino acids appears to be derived from protein or arginine/arginine phosphate turnover. Accumulation may be at the expense of protein turnover (Hawkins and Hilbish, '92).

Because these amino acids do not have a common origin and are synthesized at very different rates, the regulation of catabolism appears to control accumulation during hypertonic stress. Control of turnover seems to be associated with the regulation of three specific flavoprotein-linked, multienzyme complexes within the mitochondrion: the proline oxidase, the glycine oxidase (synthase) and the PDH with associated ALAT.

Although coordinated regulation of these three catabolic enzymes may or may not occur, the packaging of these regulatory elements within the organelle (mitochondrion) may indicate some common control mechanism. At this time, we would predict that both the glycine oxidase and the PDH activities are probably regulated by second messenger(s) generated by a plasma membrane-related tonic or salt receptor activity and that this regulation is probably associated with changes in Ca^{++} ion levels in the mitochondrion (Fig. 7). For instance, if Ca^{++} is involved in the salt stress response, hypotonic stress should raise Ca^{++} levels in the mitochondria and stimulate glycine and pyruvate catabolism, whereas hypertonic stress should not raise Ca^{++} levels and inhibit or not stimulate catabolism of glycine and pyruvate. There are no data on the possible metabolic regulation of proline catabolism through the proline oxidase complex. The cytoplasmic control elements generated by salt stress may also control the changes in plasma and mitochondrial membrane permeability to permit trapping and accumulation of the osmolytes within the cells. The role of Ca^{++} ion in the regulation of membrane activities associated with the control of cell volume during hypotonic stress has been reviewed recently (Pierce and Politis, '90; McCarty and O'Neil, '92). There is no compelling evidence for a change in intracellular Ca^{++} levels during hypertonic stress.

The production and turnover of proline and glycine-betaine (see Dragolovich, '94) in eucaryotic cells that accumulate these compounds as intracellular osmolytes appears to be organelle-based and to occur within the mitochondria or chloroplasts. Table 3 shows a few examples of the localization of key regulatory enzymes that supports a "compartmentation theory" for the metabolic regulation of amine levels during osmotic stress. Possibly, the metabolic control of other organic solutes that are used as osmolytes (sorbitol, inositol, glycerol, glycerol-glycosides, dimethyl-proline, etc.) in eucaryotes is also organelle-based.

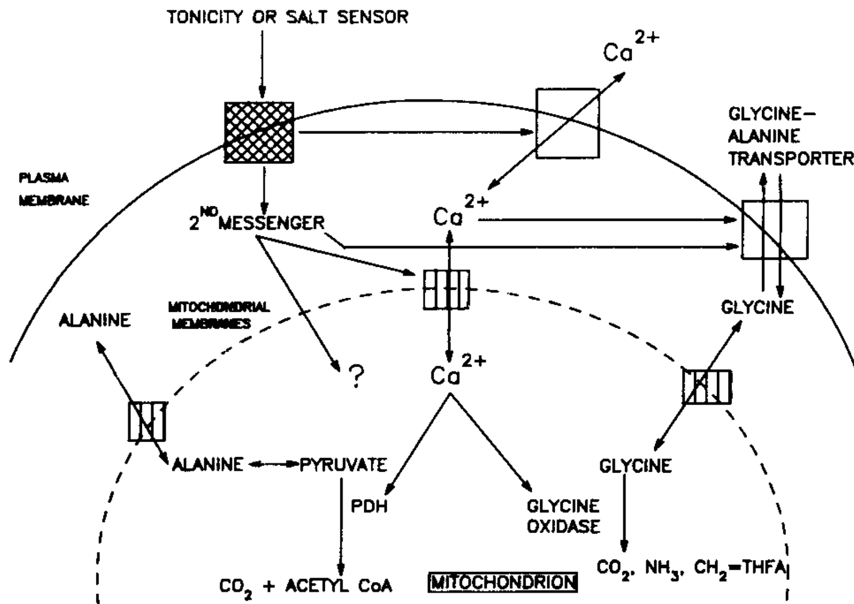

Fig. 7. Scheme outlining probable pathways controlling alanine and glycine levels in ribbed mussel gills.

TABLE 3. Compartmentation of metabolic control points for regulation of intracellular osmolytes

Osmolyte	Regulatory enzyme(s)	Cellular location of enzyme(s)
alanine	pyruvate dehydrogenase, alanine aminotransferase	mitochondria
glycine	glycine synthase	mitochondria
proline	P-5-C synthase, proline oxidase	mitochondria
glutamate	glutamate dehydrogenase, α-ketoglutarate dehydrogenase	mitochondria
glycine betaine	choline oxidase, aldehyde dehydrogenase	mitochondria or chloroplasts

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