Amino Acid Metabolism in Euryhaline Bivalves: Regulation of Glycine Accumulation in Ribbed Mussel Gills

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ABSTRACT Glycine levels in isolated ribbed mussel (Modiolus demissus) gill tissue increased slightly and decreed markedly when incubated at high and low salinities, respectively. Low levels of the enzymes involved in the biosynthesis of serine from triose phosphate intermediates, the serine hydroxymethyltransferase, and serine dehydrase were detected in gill tissue homogenates. Experiments using gill tissue incubated with (U-14C)-glycine and (U-14C)-serine indicated interconversion between serine and glycine and transfer of label to alanine, aspartate, glutamate, CO2, organic acids, and protein. Glyoxylate was metabolized more slowly than glycine and was probably converted to glycine for catabolism. Studies using (1-14C)-glycine and (2-14C)-glycine with isolated gill tissue and mitochondria indicated that the mitochondrial glycine cleavage enzyme was the major route of glycine catabolism. Metabolic controls activating or inhibiting the glycine cleavage enzyme regulate tissue glycine accumulation and catabolism during hypersalinity or hyposalinity stress.

High levels of intracellular amino acids aid in the control of the intracellular osmotic pressure in most marine and estuarine molluscs. The concentrations of free amino acids in the tissues increase and decrease with the increase and decrease of the blood osmotic pressure during environmental salinity changes. With mussels, particularly ribbed mussels (M. demissus), the concentrations of alanine, glycine, and taurine added together comprise about 90% of the total intracellular free amino acid pool of most tissues (Pierce and Greenberg, '72; Baginski and Pierce, '75, '77; Hooykaas et al., '76; Shumway et al., '77; Shumway and Youngson, '79; Livingstone et al., '79; Greenwald and Bishop, '80).

During short-term changes (hours) in environmental salinity, the membrane-controlled processes seem to respond rapidly to adjust the intracellular amino acid concentrations (Pierce and Greenberg, '72; Shumway et al., '77; Shumway and Youngson, '79; Livingstone et al., '79; Strange and Crowe, '79a,b; Crowe, '81). With prolonged hypo-osmotic stress, the amino acids are metabolized within the animals and the nitrogen is released as ammonia (Barbarger and Pierce, '76; Livingstone et al., '79). Transfer of mussels adapted to low salinities to high salinities for extended periods (weeks) results in a rapid increase in tissue alanine levels and a slower, continuous increase in tissue glycine and taurine levels (Baginski and Pierce, '75, '77; Livingstone et al., '79). The metabolic component determines which of the amino acids accumulate. Experiments using ribbed mussel gill and heart tissues with transaminase inhibitors indicate that the metabolic events associated with the adjustment of the alanine levels are transaminase linked. There is no clear indication that the regulation of the glycine levels is entirely transaminase linked (Greenwald and Bishop, '80; Bishop et al., '81) and glycine is not a substrate for the particular L-amino acid oxidase in ribbed mussel tissues (Burcham et al., '82). The metabolic component regulating taurine accumulation is uncertain (see Bishop et al., '83).

Tracer studies using (U-14C)-glucose with a number of molluscs indicate that the labeling of both glycine and serine from intermediates of glycolysis is weak compared to the labeling of the TCA cycle intermediates and the amino acids alanine, aspartate, and glutamate (Chen and Awapara, '69; de Zwaan and van Marrewijk, '73a; Campbell and Bishop, '70; Allen and Kilgore, '75; Baginski and Pierce, '78; Bishop et al., '83). From these limited data, it would appear that the pulmonate gastropods have a

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greater de novo serine and glycine biosynthetic capacity than the bivalves.

In most organisms, glycine metabolism is closely coupled to serine and glyoxylate metabolism and to one carbon transfer (Campbell and Bishop, '70). The transamination of glyoxylic acid to glycine has been demonstrated in tissue homogenates of several bivalves including the ribbed mussel (R. '62; Greenwall and Bishop, '80; Falany and Freidt, '81; Noguchi et al., '82). However, de novo biosynthesis of glycolate by either the dicarboxylic acid cycle (deZwaan and van Marrewijk, '73) or the purine catabolic pathway (Andrews and Reid, '72; Campbell and Bishop, '70) is either unlikely or slow in most molluscs including mussels. The interconversion of serine and glycine by the serine hydroxymethyltransferase (SHMT) and the production of reactive one-carbon derivatives of tetrahydrofolate (THF) have been demonstrated in a number of marine invertebrates including molluscs (Whiteley, '60). The results of tracer studies using (3-14C)-serine and (2,4-14C)-glycine to evaluate urea acid biosynthesis by pulmonate land snails (Lee and Campbell, '65) are suggestive of the presence of both the SHMT and the glycine cleavage (synthase) complex (Kikuchi, '73; Kikuchi and Hiraga, '82). This enzyme complex has multiple subunits with lipoamide and pyridoxal phosphate bound as cofactors and catalyzes the following reaction: H2 + glycine + NAD + THFA + CH2 = THFA + NADH + NH3 + CO2.

Considering that the glycine cleavage enzyme and the SHMT are reversible and that both might be fairly active in some species, the studies using (14CO3) to trace biosynthetic capacities in these molluscs are of interest. Little or no radioactivity from (14CO3) is incorporated into tissue glycine or serine in studies with oysters (Hammen and Wilbur, '59) and sea mussels (Ahmad and Chaplin, '79). The incorporation of radiolabel from (14CO2) into both glycine and serine with the tissues of pulmonate land snails (Campbell and Speeg, '68) supports data from a previous study (Lee and Campbell, '65) on urea acid biosynthesis.

Intracellular glycine can also participate in the biosynthesis of strombine (Fields et al., '80; Dando, '81), a dead-end product of anaerobic glycolysis in many bivalves (Zarburg et al., '82; de Zwaan et al., '83; Eberlee et al., '83; Korycan and Story, '84).

The purpose of this investigation is to define the basic metabolic processes regulating glycine and serine turnover in euryhaline bivalves.

**MATERIALS AND METHODS**

Mussels were purchased from Northeast Marine Environmental Institute (Monument Beach, Mass.) and maintained in the laboratory in 12 000 o/s artificial seawater (ASW) as previously described (Greenwall and Bishop, '80).

Biochemicals and chromatography solvents were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Pittsburgh, Pa.) Aquasol, (U-14C)-glycine, (U-14C)-serine, NaHCO3, (U-14C)-glyoxylate, (U-14C)-NaHCO3, and (U-14C)-thymine were obtained from New England Nuclear (Boston, Mass.); (3-14C)-glycine, (1-14C)-glycine, and (2,3-14C)-glycine were purchased from ICN (Irvine, Cal.). Silicone oil was purchased from Aldrich Chem. Co. (Milwaukee, Wis.). Thin layer chromatographic and radiographic supplies were obtained as described previously (Bishop et al., '81).

**Metabolic studies**

For metabolic studies, gill pieces were incubated at 22°C in 12 000 o/s or 32 000 o/s ASW as described by Bishop et al. ('81) with C14-labeled amino acids or (14CHCO2) in 3.0 ml sterile 12 000 o/s or 32 000 o/s ASW using 25 ml Erlenmeyer flasks fitted with rubber stoppers and plastic center wells (Kontes, Vineyard, N.J.) containing 0.2 ml of 1 M hyamine hydroxide in methanol. Incubations were terminated by the addition of 0.5 ml 2.0 M HClO4. Released (14CO2) was determined by the method of Cooley et al. ('76). Gill pieces and incubation medium were quantitatively transferred to a glass TenBroek homogenizer and then homogenized. The homogenate was centrifuged at 1000 g for 10 min and the supernatant and pellet collected separately. The pellet was dissolved in 0.2 ml of 0.1 M NaOH, neutralized, and transferred to an Aquasol scintillation cocktail for counting. The supernatant was neutralized with 0.3 ml of 2.0 M KOH cooled in an ice-bath for 30 min and KClO4 removed by centrifugation. The supernatant was poured onto a small Dowex 50W-X8 (100 mesh column) in acid form. The columns were washed with five bed volumes of distilled water and the eluate collected. This eluate was lyophilized and the lyophilate redissolved in 1.0 ml distilled water. An aliquot was added to Aquasol and counted (radioactivity). Following the water wash, 3 M NH4OH was applied to the column to elute the amino acid fraction. This eluate was repeatedly taken to dryness in a
rotary evaporator and redissolved in 0.5 ml distilled water. Radioactive amino acids were separated using two-dimensional thin-layer chromatography on 20 x 20 cm silica gel plates with butanol:formic acid:water (5:15:10, v:v:v) and phenol-water (4:1, v:v) as developing solvents. Autoradiography was accomplished using the procedure of Bishop et al. ('81). Unresolved amino acids, usually only serine and glycine, were scraped from the plate and redissolved in 0.1 ml distilled water. The amino acids were danylated by the method of Hartley and Massey ('56) and the danylated derivatives chromatographed on a silica-gel plate with chloroform:dimethylalcohol:formic acid (70:30:3, v:v:v) as the chromatographic solvent. Danylated-amino acid spots were scraped from the plate and the radioactivity counted in Aquasol.

Mitochondrial preparation and metabolism

Gill mitochondria were prepared by homogenizing gills in five volumes of a cold isolation buffer containing 400 mM sucrose, 1.0 mM EGTA, 1.0 mg/ml defatted bovine serum albumin, and 40 mM Tris pH 7.4. Homogenization was performed with an Ultra-Turrax model T45N homogenizer (Ika, Cincinnati, O.) using three passes of 18 seconds each at a setting of 40. The homogenate was mixed with an equal volume of isolation buffer and filtered through cheesecloth. The filtrate was centrifuged at 700 g for 10 min to remove cells and nuclear material. The supernatant was collected and recentrifuged at 10,000 g for 20 min and the resulting supernatant used for some enzyme assays. The pellet was resuspended in isolation buffer and recentrifuged twice at 7000 g for 20 min. The washed pellet (mitochondria) was resuspended in isolation buffer at a concentration of 5 mg mitochondrial protein/ml.

Metabolism of glycine and glyoxylate by gill mitochondria was assayed using 3.0 ml of suspended mitochondria. Radioactive substrates and additives were incubated with the mitochondria for 1 hour. The (14)CO2 evolution was analyzed as in the gill fragment metabolic experiments. In experiments with specifically labeled (14)C glycine, the dimedon-aldehyde trap was employed in the manner described by Motokawa and Kucchini ('74).

Mitochondrial metabolite transport

Mitochondrial uptake of glycine and glyoxylate was assessed using a modification of the silicone oil separation method of LaNeue et al. ('73). The acid layer was 2.4 M HClO4 and the silicone oil layer was a density of 1.050. At timed intervals, a 1.0 ml aliquot of mitochondrial suspension was layered onto the silicone oil layer and centrifuged at 15,600 g for 3 min in an Eppendorf microfuge at 4°C. Extramitochondrial space was estimated from the distribution of 3H-sucrose.

Enzyme assays

All enzyme assays were performed at 22°C. The activity of the following enzymes were measured in tissue extracts which had been desalted on a G-25 column using the following referenced methods: 3-phosphoglycerate dehydrogenase (Pizer, '64), 3-phosphoglycerate (Grillo and Coghe, '66), 3-phosphoglycerate phosphatase in 50 mM Tris HCl pH 9.0 rather than barbital buffer (Fallon et al., '68) using the inorganic phosphate analytical procedure of Dryer et al. ('57), D-glycerate dehydrogenase (Willis and Sallach, '64), hydroxypyruvate-L-alanine aminotransferase (Fukushima et al., '78), L-serine dehydrase (Fallon et al., '68), L-serine hydroxymethyltransferase (SCH) (Taylor and Weissbach, '83; Schur et al., '77), L-threonine aldolase (Schur and Gross, '68), and L-threonine dehydrase (Park and Datta, '79). The products of the hydroxypyruvate-L-alanine aminotransferase and SHMT were confirmed in the following manner. The reactions were allowed to proceed in the absence of NAD and coupling enzymes for 1 hour. The reactions were terminated by the addition of an equal volume of 10 mg/ml bovine serum albumin and immersion in a boiling water bath for 5 minutes. The protein precipitate was removed by centrifugation, the supernatant solution taken to dryness in a rotary evaporator, and the residue redissolved in 0.5 ml of 0.01 N HCl. This solution was subjected to danylation and chromatographed as previously described.

Protein was determined using Miller's ('59) modification of the Lowry procedure. Mitochondrial protein was estimated using the biuret procedure (King, '67). Amino acid levels were determined using the previously described method (Greenwalt and Bishop, '80).

RESULTS

Specific enzymes

As a prelude to the 14C-tracer experiments with glycine and serine, the activities of some
of the enzymes involved in serine and glycine biosynthesis and the effects of aminoxy-acetic acid (AOA) and \( \beta \)-chloro-L-alanine (CA) on the activity of some of these enzymes were evaluated (Table 1). Except for phosphoserine phosphatase, all of the enzymes required for the synthesis of serine by the phosphorylated and nonphosphorylated triose pathways were detected. The levels of most of these enzymes were relatively low. These results were in general agreement with previous tracer studies using \(^{14}\)C-glucose or amino acids indicating only modest labeling of glycine and serine after extended incubation periods (see introduction). Some L-serine dehydrase but no L-threonine dehydrase was detected. Subcellular fractionation showed that the serine dehydrase was cytosolic and that the SHMT and the hydroxypyruvate-L-alanine aminotransferase activities were both cytosolic and mitochondrial. The levels of the glycine:pyruvate aminotransferase have been reported previously (Greenwald and Bishop, '80).

Aminoxyacetic acid inhibited the 3-phospho- 
pyruvate:L-glutamate aminotransferase, the hydroxypyruvate:L-alanine aminotransferase, the SHMT, the serine dehydrase, and the threonine aldolase (Table 1). AOA inhibition of the alanine aminotransferase, the aspartate aminotransferase, and the glycine:pyruvate aminotransferase has been reported previously (Greenwald and Bishop, '80; Paynter et al., '84a,b). Previous work has shown that CA inhibited alanine aminotransferase (Paynter et al., '84b) but not the isoenzymes of aspartate aminotransferase (Paynter et al., '84a). \( \beta \)-Halogen substituted alamines have been shown to inhibit SHMT from mammalian liver (Wang et al., '81). CA inhibited hydroxypyruvate:L-alanine aminotransferase, SHMT, threonine aldolase, and serine dehydrase but not the 3-phospho- 
pyruvate:L-glutamate aminotransferase from the gill tissue (Table 1).

**Tissue experiments**

The rate of oxidation of glycine to \( \text{CO}_2 \) was more than an order of magnitude slower in gills incubated at high salinity compared to gills incubated at low salinity (Table 2). During this time period (1 hr) the free glycine concentrations in the tissue preparations increased from about 10 \( \mu \)mole/g dry wt to 12 \( \mu \)mole/g dry wt with gills at 32 o/oo and decreased from 10 \( \mu \)mole/g dry wt to about 5 \( \mu \)mole/g dry wt with gills held at 12 o/oo. The incorporation of \(^{14}\)C into HClO\(_4\) insoluble components was the same at both salinities. The Dowex column partitioning of the radioactivity from the HClO\(_4\) soluble fractions into the water eluate (acidic and neutral organs) and the ammonia water eluate (free amino acids) indicated considerable metabolism of glycine to other components. AOA inhibited \( \text{CO}_2 \) production from glycine at both high and low salinity with the most pronounced effect at low salinity. Arsenite (As\(_2\)) was more effective at inhibiting \( \text{CO}_2 \) production from glycine at low salinity than at high salinity.

Following thin-layer chromatography of the ammonia water eluate from the Dowex column, it was apparent that in the absence of inhibitors glycine was converted primarily to serine with some label appearing in alanine.

**TABLE 1. Tissue levels of enzymes of serine metabolism in M. demissus gill plus \( I_0 \) levels for aminoxyacetic acid (AOA) and \( \beta \)-chloro-L-alanine (CA)**

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Tissue level (( \mu )M product/g/hr)</th>
<th>AOA ( I_0 )</th>
<th>CA ( I_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Phosphoglycerate dehydrase</td>
<td>0.3</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3-Phosphopyruvate:L-glutamate</td>
<td>0.2</td>
<td>3.6 \times 10^{-5} M</td>
<td>&gt; 1 mM</td>
</tr>
<tr>
<td>aminotransferase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Phosphoglycerate phosphatase</td>
<td>ND(^*)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3-Phosphoglycerate phosphate</td>
<td>0.8</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>transaminase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glycerate dehydrogenase</td>
<td>2.4</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Hydroxypyruvate:L-alanine</td>
<td>2.1</td>
<td>6.8 \times 10^{-5} M</td>
<td>1.7 \times 10^{-5} M</td>
</tr>
<tr>
<td>Serine hydroxymethyltransferase(^2)</td>
<td>6.2</td>
<td>3.2 \times 10^{-5} M</td>
<td>4.9 \times 10^{-5} M</td>
</tr>
<tr>
<td>Threonine aldolase</td>
<td>0.5</td>
<td>3.2 \times 10^{-5} M</td>
<td>4.9 \times 10^{-5} M</td>
</tr>
<tr>
<td>Serine dehydrase</td>
<td>0.8</td>
<td>1.5 \times 10^{-5} M</td>
<td>7.3 \times 10^{-5} M</td>
</tr>
</tbody>
</table>

\(^*\)Below detectable limits (< 0.01 \( \mu \)M product/g/hr).

\(^{1}\)Not determined.

\(^{2}\)Product confirmed using thin-layer chromatography of amino derivatives (see Methods).
### TABLE 2. Effect of salinity and metabolic inhibitors on the distribution of radioactivity from (U-14C) glycine in isolated gill tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Salinity</th>
<th>Inhibitor</th>
<th>CO2</th>
<th>Perchlorate</th>
<th>Water</th>
<th>NH4OH</th>
<th>Glu</th>
<th>Gly</th>
<th>As(A)</th>
<th>Asp</th>
<th>Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>(34.4;33.9)</td>
<td>12</td>
<td>None</td>
<td>7,808 (2.0)</td>
<td>1,488</td>
<td>15.892</td>
<td>4.772</td>
<td>1560</td>
<td>3,440</td>
<td>278</td>
<td>43</td>
<td>61</td>
</tr>
<tr>
<td>(32.7;33.0)</td>
<td>32</td>
<td>None</td>
<td>207 (0.12)</td>
<td>1,054</td>
<td>7,985</td>
<td>19,287</td>
<td>4436</td>
<td>12,925</td>
<td>1,545</td>
<td>87</td>
<td>114</td>
</tr>
<tr>
<td>(30.3;30.0)</td>
<td>12</td>
<td>AOA</td>
<td>385 (0.1)</td>
<td>2214</td>
<td>2,654</td>
<td>18,838</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>(31.4;33.2)</td>
<td>32</td>
<td>AOA</td>
<td>128 (0.09)</td>
<td>8679</td>
<td>2,917</td>
<td>17,718</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>(36.8;35.8)</td>
<td>32</td>
<td>AsO3</td>
<td>2,609 (0.62)</td>
<td>2937</td>
<td>4,719</td>
<td>15,228</td>
<td>3,512</td>
<td>9,594</td>
<td>1,840</td>
<td>45</td>
<td>**</td>
</tr>
<tr>
<td>(30.0;28.9)</td>
<td>32</td>
<td>AsO3</td>
<td>62 (0.06)</td>
<td>4862</td>
<td>2,955</td>
<td>17,301</td>
<td>2,976</td>
<td>13,675</td>
<td>1,659</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

1. Incubations were for 1 hr with the indicated amount of tissue and 0.5 μM of (U-14C) glycine (carrier free, 3A 133 μC/mmol).
2. Values in parentheses are the specific radioactivity in nM/mg wet tissue using the levels of tissue free amino acids determined by Greenwald (1981).
3. Radioactivity below detectable limits.

### TABLE 3. Effect of salinity and metabolic inhibitors on the distribution of radioactivity from (U-14C) serine in isolated gill tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Salinity</th>
<th>Inhibitor</th>
<th>CO2</th>
<th>Perchlorate</th>
<th>Water</th>
<th>NH4OH</th>
<th>Glu</th>
<th>Gly</th>
<th>As(A)</th>
<th>Asp</th>
<th>Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>(31.6;39.9)</td>
<td>12</td>
<td>None</td>
<td>7,048 (4.2)</td>
<td>2,135</td>
<td>3,464</td>
<td>9,047</td>
<td>5,727</td>
<td>637</td>
<td>1,219</td>
<td>538</td>
<td>756</td>
</tr>
<tr>
<td>(32.9;32.0)</td>
<td>32</td>
<td>None</td>
<td>1,571 (1.5)</td>
<td>8,550</td>
<td>2,369</td>
<td>8,668</td>
<td>6,438</td>
<td>521</td>
<td>1,378</td>
<td>187</td>
<td>102</td>
</tr>
<tr>
<td>(32.9;33.6)</td>
<td>12</td>
<td>AOA</td>
<td>49 (0.07)</td>
<td>3,784</td>
<td>1,829</td>
<td>16,583</td>
<td>15,818</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(31.9;25.8)</td>
<td>32</td>
<td>AOA</td>
<td>40 (0.06)</td>
<td>7,589</td>
<td>1,844</td>
<td>11,790</td>
<td>10,415</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(27.9;34.7)</td>
<td>12</td>
<td>AsO3</td>
<td>2,477 (4.6)</td>
<td>3,993</td>
<td>3,947</td>
<td>11,377</td>
<td>7,364</td>
<td>1,297</td>
<td>1,579</td>
<td>497</td>
<td>416</td>
</tr>
<tr>
<td>(28.1;30.6)</td>
<td>32</td>
<td>AsO3</td>
<td>589 (0.8)</td>
<td>9,992</td>
<td>2,680</td>
<td>7,759</td>
<td>4,181</td>
<td>384</td>
<td>1,766</td>
<td>186</td>
<td>168</td>
</tr>
<tr>
<td>(27.9;32.7)</td>
<td>12</td>
<td>CA</td>
<td>46</td>
<td>2,911</td>
<td>1,152</td>
<td>17,069</td>
<td>36,070</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(27.7;30.5)</td>
<td>32</td>
<td>CA</td>
<td>46</td>
<td>2,944</td>
<td>1,385</td>
<td>17,923</td>
<td>15,729</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

1. Incubations were for 1 hr with the indicated amount of tissue and 0.5 μM of (U-14C) serine (carrier free, 3A 133 μC/mmol).
2. Values in parentheses are the specific radioactivity in nM/mg wet tissue using the levels of tissue free amino acids determined by Greenwald (1981).
3. Radioactivity below detectable limits above background (29 dpm).

Glutamate, and aspartate. These results were consistent with the demonstrated presence of SHMT plus the serine dehydrogenase and the serine aminotransferase (Table 1). Addition of AOA to the incubation mixture caused a retention of the 14C-label in glycine. The sensitivity of CO2 production from glycine to AsO3 and AOA inhibition indicated that lipoamide dehydrogenase (decarboxylating) and pyridoxal phosphate linked systems were involved in glycine catabolism.

When gill explants were then incubated with (U-14C)-serine at different salinities (Table 3), (14C)CO2 production was slower at the high salinity compared to the low salinity. However, this reduction in serine metabolism was not as acute as that seen for glycine (Table 2). A significant portion of 14C from serine appeared in alanine, glycine, aspartate, and glutamate with a larger fraction of the label appearing in alanine at the higher salinity. Addition of AOA of CA blocked serine catabolism at both salinities. Although AOA appeared to have no effect on the incorporation of 14C into HClO4 insoluble components, CA depressed incorporation into this fraction. Addition of AsO3 caused a reduction in CO2 production from serine at both low and high salinities and increased incorporation of label into glycine and alanine. These results were consistent with the view that the metabolism of serine was through both glycine (SHMT) and hydroxyproline plus pyruvate (aminotransferase and dehydrogenase).

An examination of threonine metabolism (Table 4) indicated that threonine was catalyzed very slowly by gill tissue at both salinities. When gill fragments were incubated with (14C)-HCO3 (Table 5), radioactivity incorporated into amino acids was primarily in aspartate and alanine. Glutamate was labeled at a much slower rate than aspartate or alanine, indicating CO2 fixation into the TCA cycle intermediates by the organic acid carboxyli-
TABLE 4. Effect of salinity on the distribution of radioactivity in gill tissue incubated with (U-14C)l-threonine

<table>
<thead>
<tr>
<th>Tissue (mg wet)</th>
<th>Salinity (‰)</th>
<th>CO2 Ppt</th>
<th>H2O Cluate</th>
<th>NH4OH Cluate</th>
<th>Thr</th>
<th>Ser</th>
<th>Gly</th>
<th>Ala</th>
<th>Asp</th>
<th>Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>(32.8±31.6)</td>
<td>12</td>
<td>50</td>
<td>1,478</td>
<td>847</td>
<td>15,406</td>
<td>15,047</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(29.4±27.1)</td>
<td>32</td>
<td>45</td>
<td>2,910</td>
<td>470</td>
<td>15,792</td>
<td>14,736</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Incubations were for one hr with the indicated amount of tissue and 0.5 μCi of (U-14C)l-threonine (carrier free. 234 mCi/mmol)
2 Radioactivity below detectable limits above background (20 dpm)

TABLE 5. Fixation of 14CO2 into amino acids and protein of M. demissus gill tissue incubated in 120o/oo ASW; the effect of added amino acids and inhibitors (1 mM)

<table>
<thead>
<tr>
<th>Additions to incubation mixture</th>
<th>Gly</th>
<th>Ser</th>
<th>Ala</th>
<th>Glu</th>
<th>Asp</th>
<th>Perchlorate Ppt</th>
<th>NH4OH Cluate</th>
<th>H2O Cluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>14CO2</td>
<td></td>
<td></td>
<td>844</td>
<td>156</td>
<td>1,313</td>
<td>327</td>
<td>2,431</td>
<td>2,815</td>
</tr>
<tr>
<td>14CO2 + Glu</td>
<td></td>
<td></td>
<td>1,093</td>
<td>37</td>
<td>1,227</td>
<td>450</td>
<td>2,316</td>
<td>3,067</td>
</tr>
<tr>
<td>14CO2 + Gly</td>
<td>76</td>
<td></td>
<td>1,067</td>
<td>232</td>
<td>1,377</td>
<td>436</td>
<td>2,773</td>
<td>2,541</td>
</tr>
<tr>
<td>14CO2 + Ser</td>
<td>187</td>
<td></td>
<td>1,066</td>
<td>256</td>
<td>1,455</td>
<td>350</td>
<td>2,815</td>
<td>3,777</td>
</tr>
<tr>
<td>14CO2 + Gly + AOA</td>
<td></td>
<td></td>
<td>1,288</td>
<td>105</td>
<td>339</td>
<td>309</td>
<td>1,604</td>
<td>2,978</td>
</tr>
<tr>
<td>14CO2 + Ser + AOA</td>
<td>32</td>
<td></td>
<td>1,418</td>
<td>139</td>
<td>216</td>
<td>212</td>
<td>1,796</td>
<td>3,904</td>
</tr>
</tbody>
</table>

1 Each value represents the mean of three determinations. The induced lines indicate radioactivity below detectable limits (20 CPM mg wet weight). Preparations had 40 mg of tissue in 3.0 ml buffered ASW at pH 7.2 (see Materials and Methods containing 10 μCi of 14C CO2 in 2.2 mM of NaHCO3. Incubation were for 1 hour.

Gases (Baginski and Pierce, '78). If glutamate was added to the incubation medium, the amount of label incorporated into glutamate decreased, the labeling of alanine and aspartate increased, and the labeling glycine and serine was unchanged. When cold 13C glycine or serine were added to the incubation medium, 14C was then observed in glycine suggesting the presence of a glycine-CO2 exchange reaction and a trapping of the label by the increased cellular pool size of glycine. Incorporation of 14C into the amino acids was blocked by AOA and partially inhibited by AsO3. The specific incorporation of radiolabel from 14C CO2 into alanine was not appreciably affected by addition of AsO3. The decrease in label in aspartate and glutamate in the presence of AsO3 indicated that CO2 fixation into both aspartate and glutamate was dependent on the cycling of intermediates in the TCA cycle. These results were consistent with other similar CO2 tracer experiments with tissues from various molluscs (see introduction).

The results with (U-14C) glycine and (14C)CO2 tracers were indicative of a glycine cleavage enzyme in gill tissue. To test for the presence of the glycine cleavage enzyme, gill pieces were incubated with specifically labeled (1,14C) glycine and (2,14C) glycine (Table 6). It was apparent that the C-1 of glycine was the principle source of 14CO2 observed in the earlier experiments with (U-14C) glycine (Table 2). The production of CO2 from the C-1 of glycine was sensitive to changes in salinity as shown by the two orders of magnitude decrease in CO2 production at the increased salinity. The slow production of CO2 from the C-2 of glycine was unaffected by salinity. When the dimedon trap for aldehydes was used with specifically labeled glycine, essentially no label was found as the dimedon derivative using (1-14C) glycine. On the other hand, label was found as the dimedon derivative using (2-14C) glycine. This result indicated that the labeled dimedon derivative contained only carbon from C-2 of glycine and that glyoxalate, the product of glycine transamination, was probably not significantly involved in glycine oxidation in the gill tissue. The incorporation of the C-2 of glycine into aldehyde equivalents was influenced by salinity in a fashion parallel to the influence on the production of CO2 from the C-1 of glycine. A much lower level of radioactivity was found in the aldehyde trap with (2-14C) glycine compared the level of radioactivity in CO2 with (1-14C) glycine. This
TABLE 6. Effect of salinity on the distribution of radioactivity from specifically labeled glycine into CO₂ and dimedon-derivative fractions by isolated gill pieces

<table>
<thead>
<tr>
<th>Additions to incubation mixtures</th>
<th>Salinity (12 cec)</th>
<th>Dimedon derivative</th>
<th>Salinity (32 cec)</th>
<th>Dimedon derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO₂</td>
<td>DPD</td>
<td>CO₂</td>
<td>DPD</td>
</tr>
<tr>
<td>(1) 1⁴Cglycine</td>
<td>10,885</td>
<td>33</td>
<td>237</td>
<td>35</td>
</tr>
<tr>
<td>(2) 1⁴Cglycine</td>
<td>164</td>
<td>350</td>
<td>171</td>
<td>91</td>
</tr>
</tbody>
</table>

1The incubations were for 1 hr with 0.5 µg of 1⁴Cglycine carrier free and 35 mg of wet gill tissue. The specific radioactivity of the 1⁴Cglycine was 52.9 µCi/µmol and 51.0 µCi/µmol, respectively.

TABLE 7. Distribution of radioactivity from specifically labeled glycine into CO₂ and dimedon-derivative fractions by gill mitochondria

<table>
<thead>
<tr>
<th>Additions to incubation mixture</th>
<th>CO₂</th>
<th>DPD (X 10⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) ¹⁴Cglycine</td>
<td>2,799</td>
<td>23</td>
</tr>
<tr>
<td>(2) ¹⁴Cglycine</td>
<td>114</td>
<td>480</td>
</tr>
</tbody>
</table>

1The incubations were in triplicate for 1 hr with 0.5 µg of the indicated ¹⁴Cglycine, 20 µg of ¹⁴Cglycine carrier free, and 1 ml of resuspended mitochondria (15 mg mitochondrial protein).

result was probably due to a dynamic turnover of the methylene groups in the THPA one-carbon metabolic pools and accounted for the labeling of serine by reaction of glycine with CH₂=THPA in the SHMT reaction (Tables 1 and 2) (see Sato et al., '69b).

Studies with mitochondria

Subcellular fractionation was employed to determine the cellular location of glycine and glyoxalate metabolism. Only the mitochondrial fractions were found to produce (¹⁴CO₂) from labeled glycine or glyoxalate (data not shown). The fraction of (¹⁴CO₂) from (¹⁴C)glycine was found to be proportional to the amount of mitochondria and to the incubation time (Fig. 1). Mitochondrial preparations (5 mg/mg mitochondrial protein) were then incubated with specifically labeled glycine (Table 7). The results were similar to the results using whole gill pieces (Table 2 and Table 6). The production of (¹⁴CO₂) was primarily from the C-1 of glycine and aldehyde equivalents, trapped as the dimedon derivative, were derived from the C-2 of glycine.

To further evaluate mitochondrial glycine oxidation and the role of glyoxalate in glycine oxidation, a series of competition experiments were performed. To insure that the results were not due to a lack of mitochondrial transport of glyoxalate and glycine, metabolite uptake experiments were performed using the centrifugal silicone oil partitioning.

Fig. 1. a. Time course of (¹⁴CO₂) release per mg mitochondrial protein from (¹⁴C)glycine (specific activity 36,700 dpm/µmol) by gill mitochondria (5 mg mitochondrial protein/mg). b. Effect of mitochondrial concentration of the release of (¹⁴CO₂) from (¹⁴C)glycine (specific activity 36,700 dpm/µmol) after 1 hr.
procedure (Figure 2). With both glycine and glyoxalate, the distribution of $^{14}$C-label was near equilibrium after an hour. Although the rate of glycine uptake was slightly more rapid than the rate of glyoxalate uptake, glyoxalate did access the mitochondrial compartment at a rapid rate. Therefore, both compounds were used as the $^{14}$C-derivatives (cold) to produce isotope dilution (competition) effects during some of the $^{14}$C-tracer experiments.

Glycine metabolism by the mitochondria was more rapid than glyoxalate metabolism (Fig. 3). The metabolism of both was sensitive to inhibition by AOA. Although AsO$_3^-$ inhibited glycine oxidation, it only slightly reduced the slow rate of glyoxalate oxidation. Addition of cold ($^{12}$C) glyoxalate did not reduce the amount of ($^{14}$C)CO$_2$ produced from ($^{14}$C)-glycine. However addition of cold ($^{12}$C)-glycine reduced the amount of ($^{14}$C)CO$_2$ produced from ($^{14}$C)-glyoxalate. Therefore, glycine showed an isotope dilution effect on glyoxalate catabolism but glyoxalate had no effect on glycine catabolism.

Mitochondria were then incubated with ($^{14}$C)-glycine in the presence of added pyruvate or an $\alpha$-ketoglutarate or with ($^{14}$C)-glyoxalate in the presence of added L-alanine or L-glutamate to drive the glycine aminotransferase reactions (Fig. 3). The oxidation of both glycine and glyoxalate was reduced by these treatments. These results indicated that the glycine aminotransferases may be mitochondrial and function mainly in the thermodynamically favored glycine synthesizing direction at high glycine levels (Metzler et al., '53) and/or that the added ketocids and amino acid would compete with them for the oxidative pathway.

![Graph](image)

Fig. 2. Time course of removal of ($^{14}$C)-glycine or ($^{14}$C)-glyoxalate specific activities 36,700 dpm/mmol from incubation medium by gill mitochondria (5 mg mitochondrial protein/ml).

![Graph](image)

Fig. 3. Release of ($^{14}$CO$_2$) from ($^{14}$C)-glycine or ($^{14}$C)-glyoxalate specific activities 36,700 dpm/mmol by gill mitochondria (5 mg mitochondrial protein/ml) with and without added inhibitors or alternate unlabeled substrates. Additives were preincubated with mitochondria for 10 min prior to addition of labeled glycine or glyoxalate. Inhibitor concentrations (AOA, AsO$_3^-$) were 1 mM, and alternate substrates were equimolar to glycine or glyoxalate.
acids interfered with the utilization of glycine and glyoxylate in some other manner (See Hampson et al., '83, '84). Since 2-oxoglutarate failed to simulate glycine decarboxylation in gill mitochondria, it was unlikely that the aminoacetone or succinate-glycine cycles were primary pathways for glycine catabolism (Lewis et al., '67; Yoshida and Kikuchi, '72).

Overall, these studies (Fig. 3) indicated that glycine was oxidized directly and not primarily via glyoxylate and that most of the glyoxylate catabolism occurred after transamination to glycine.

**DISCUSSION**

The results indicate that the major route of glycine catabolism in ribbed mussel gill tissue is through the glycine cleavage enzyme reaction in the mitochondria. The sensitivity of glycine decarboxylation to AOA and AsO₃³⁻, the mitochondrial localization of the glycine catabolic activity, the specific release of CO₂ from the C-1 of glycine, the incorporation of the C-2 of glycine into aldehyde equivalents, and the pattern of CO₂ fixation into glycine are similar to results with other organisms that use the glycine cleavage enzyme as a major route of glycine catabolism (Yoshida and Kikuchi, '71; Kawasaki et al., '66; Sato et al., '69a,b; Nakada et al., '55; Shin and Cassins, '64; Sagers and Gunzalus, '61; Klein and Sagers, '66; Yoshida and Kikuchi, '72, de Boiss and Stoppani, '67; Moore et al., '77; Rathnam, '79; Hampson et al., '83, '84).

From the data in Table 2 and Table 7, one can estimate the relative rates of glycine decarboxylation by isolated gill tissue and by isolated gill mitochondria, respectively. Although the rate of glycine decarboxylation by the isolated tissue at low salinity was fairly rapid (~3 nmoles/g/min), this rate is considerably slower than the maximal rates (125 nmoles/g/min) observed for perfused rat liver preparations (Hampson et al., '84). If one considers the temperature difference of the assays and the low glycine levels in the low salinity adapted gill preparations then corrects for these factors using an apparent Kₘ for glycine of 2 mM (Hampson et al., '84), the calculated maximal glycine decarboxylation rate by the tissue is still five to ten times slower than the maximal rate with rat liver. On the other hand, the calculated rate with isolated gill mitochondria (Table 7) of about 76 nmoles/mg protein/hr is comparable to the rates (60–120 nmoles/mg protein/hr) calculated for isolated rat liver mitochondria (Hampson et al., '83). These results suggest that the glycine cleavage enzyme in the gill mitochondria is just as active as the enzyme in rat liver mitochondria but that the gill tissue must have fewer mitochondria than rat liver resulting in a slower rate of glycine oxidation at the tissue level. The very strong inhibition of glycine oxidation in gills incubated at high salinities indicates an acute regulation of the gill glycine cleavage enzyme that is apparently not found with the rat liver enzyme.

The presence of alanine-glyoxylate aminotransferase suggests that glycine could be transaminated to glyoxylate for oxidation (Bishop et al., '81). However, the slow rate of glyoxylate oxidation compared to glycine oxidation suggests that this is not the case. Further, in competition experiments, glycine inhibited glyoxylate oxidation but glyoxylate had no effect on glycine oxidation. This result suggests that when glyoxylate is available it is primarily converted to glycine by the gill transaminases and agrees with observations on glycine metabolism in the tissues of other organisms (Roswell et al., '72; Yokota et al., '78, Roff and Edwards, '78, Noguchi et al., '82).

Most of the glycine appears to be derived from serine through the SHMT reaction or from protein through the release of preformed glycine during protein turnover (Greenway and Bishop, '80; Bishop et al., '81). At low salinities the relatively large amounts of carbon transferred from serine to glycine compared to carbon transferred to pyruvate or hydroxypyruvate through the dehydrase or transaminase reactions seem similar. The conversion of serine to pyruvate and alanine is favored at high salinity, indicating a high rate of glycine biosynthesis through reversal of the glycine cleavage enzyme seems unlikely considering the tissue levels of glycine and the results of the 13C exchange experiments (Table 5).

The biosynthesis of serine and glycine from glucose by ribbed mussel gill tissue (13C-glucose tracer studies) seems to be slow (Baginski and Pierce, '78; Greenway, '81). All the enzymes of the nonphosphorylated pathway for serine biosynthesis from triose phosphate glycolytic intermediates are present in the gill tissue. Phosphoserine phosphatase was the only enzyme of the phosphorylated pathway that was not detected. The lack of this
phosphatase has also been shown in ammonotile vertebrate livers that produce serine by the phosphorylated pathway (Grillo and Coghe, '68; Grillo et al., '66). The absence of this phosphatase activity may be irrelevant because a variety of alkaline and acid phosphatases are known to occur in mussel tissues (Koehn et al., '73). In any event, it appears that ribbed mussels have the capacity for slow serine biosynthesis from glucose via the glycolytic intermediates.

The catabolism of threonine is slow and a weak threonine aldolase activity (SHMT, data not shown) is present. This result is similar to results obtained with vertebrate liver where threonine aldolase is probably the primary route of threonine catabolism (Schirch and Gross, '68; Bird and Nunn, '79). However, because no label from the (14)C-threonine was detected in glycine, the in vivo role of the threonine aldolase (SHMT) in threonine catabolism in gill tissue is speculative.

In conclusion, it appears that, metabolically, the glycine levels in these tissues are controlled by an acute regulation of the mitochondrial glycine cleavage enzyme, the major route of glycine catabolism. At high salinities, glycine derived from a variety of sources including serine, glyoxylate, or protein appears to accumulate behind a severely blocked, mitochondrial, glycine cleavage enzyme. At low salinities, this blockade is removed and glycine turnover increases about 20-fold. These results account for the rather slow accumulation of tissue glycine as the ribbed mussels are adapted to high salinities (Baginski and Pierce, '75, '77) and for the rapid metabolism of glycine by ribbed mussel tissues at low salinities. Studies on the specific mechanisms regulating the glycine cleavage enzyme in ribbed mussel tissues are in progress.

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NOTE ADDED IN PROOF

Glycine (10 mM) added to coupled, respiring gill mitochondria prepared according to the procedure of Burcham et al. (1984) stimulated ATP-dependent oxygen consumption and showed a P/O ratio of 2.8. This result indicates that the oxidation of NADH produced in the mitochondrial glycine cleavage reaction is coupled through the mitochondrial respiratory chain.