Organ-specific metabolism during anoxia and recovery from anoxia in the cherrystone clam, *Mercenaria mercenaria*

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The levels of intermediary metabolites and end products were quantified in the tissues of the cherrystone clam, *Mercenaria mercenaria*, over a time course of 96 h of anoxia followed by 48 h of aerobic recovery. Succinate and alanine accumulated as anaerobic products while glycogen and aspartate were utilized as substrates. Succinate accumulation ranged from 12–14 μmol/g wet weight in muscle (phasic and catch adductor, foot) to 25 μmol/g in gill and mantle with 32 μmol/mL released into the mantle cavity fluid. Lesser amounts of alanine were produced, the ratio succinate:alanine varying from 1.4:1 in phasic adductor to 3.2:1 in mantle at 96 h. Aspartate reserves apparently supply the carbon for succinate synthesis over the first 6–12 h of anoxia; subsequent succinate and alanine production probably results from glycogen fermentation. The imino acids alanopine and strombine were not produced in appreciable amounts (<1 μmol/g) during anoxia. When returned to aerated seawater, control levels of alanine and aspartate were reestablished within 24 h; accumulated succinate was catabolized within 48 h. Glycogen content of all tissues showed a sharp decline after 6 h of recovery, perhaps due to enhanced energy demands, but levels increased later in recovery. Tissue ATP levels, which were depressed during anoxia, were restored by 24 h.

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Les concentrations de métabolites intermédiaires et de produits finals dans les tissus ont été mesurés au cours d'une anoxie de 96 h suivi d'une récupération aérobique de 48 h chez le mollusque *Mercenaria mercenaria*. Durant l'anaérobiose, il y a accumulation de succinate et d'alanine, alors que le glycogène et l'aspartate servent de substrats. L'accumulation de succinate varie de 12–14 μmol/g de masse fraîche dans le muscle (adducteur phasique, muscle d'arrêt et muscle du pied) à 25 μmol/g dans la branchie et le manteau; 32 μmol/g sont largués dans le liquide de la cavité du manteau. La quantité d'alanine produite est moindre, le rapport succinate: alanine allant de 1,4:1 dans le muscle adducteur phasique à 3,2:1 dans le manteau après 96 h. Les réserves d'aspartate constituent la source de carbone pour la synthèse du succinate pendant les premières 6–12 h de l'anoxie; la production subséquente de succinate ou d'alanine provient sans doute de la fermentation du glycogène. Les imino-acides, alanopine et strombine, ne sont produits qu'en faible quantité (<1 μmol/g) durant l'anoxie. De retour en eau de mer aérée, les mollusques retrouvent les niveaux normaux d'alanine et d'aspartate en moins de 24 h et le succinate accumulé est catabolisé en moins de 48 h. La concentration de glycogène dans les tissus subit une baisse rapide après 6 h de récupération, probablement à la suite de besoins énergétiques accrus, mais augmente de nouveau plus tard durant la récupération. Les concentrations d'ATP tissulaire, qui diminuent durant l'anoxie, se rétablissent en moins de 24 h.

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Introduction

Numerous species of marine molluscs show well developed capacities for the survival of environmental anoxia. Anaerobic metabolism has been extensively studied in the sea mussel, *Mytilus edulis* (de Zwaan *et al.* 1976; de Zwaan 1977; Kluytmans *et al.* 1977; Zurburg and Kluytmans 1980; Zurburg and Ebberink 1981), while others have examined aspects of anaerobiosis in various other bivalve species (Stokes and Awapara 1968; Gäde 1975; Collicutt and Hochachka 1977; Meinardus and Gäde 1981). In general succinate and alanine are the major products of anaerobic metabolism while propionate and acetate are also accumulated (de Zwaan 1977). Glycogen and aspartic acid are the substrates fueling anoxic energy production (Collicutt and Hochachka 1977; Zurburg and Ebberink 1981).

The cherrystone clam or quahog, *Mercenaria mercenaria* L., is a major commercial species on the east coast of North America. The present study was designed to examine tissue-specific metabolism in this intertidal burrowing species during anoxia and recovery from anoxia. Particular attention was paid to an assessment of the role(s) of alanopine dehydrogenase (ADH) and strombine dehydrogenase (SDH) in anaerobiosis. These enzyme activities (pyruvate + L-alanine (or glycine) + NADH + $H^+ \rightleftharpoons$ meso-alanopine (or D-strombine) + $NAD^+ + H_2O$) have recently been found in the tissues of many marine bivalves (Fields 1976; Dando *et al.* 1981; de Zwaan and Zurburg 1981). In *M. mercenaria* they occur as the major cytostolic dehydrogenase activities; octopine dehydrogenase is lacking and lactate

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dehydrogenase is present in only low amounts. Thus ADH and SDH are potentially important in the maintenance of cytosolic redox balance during glycolytic function.

Material and methods

Animals and chemicals

Cherrystone clams, *Mercenaria mercenaria*, were purchased from a local seafood retailer and were held, without feeding, in an aerated, recirculating seawater tank at 18°C for at least 1 week before use.

All biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp. Alanopine dehydrogenase was prepared from whelk foot muscle and strombine dehydrogenase from *M. mercenaria* muscle as previously described (Storey *et al.* 1982). Succinyl-CoA synthetase was a gift from Dr. W. Bridger, University of Alberta.

Anoxia and recovery

Control, aerobic animals were sampled directly from the seawater tank. To impose anoxia, animals were removed from the seawater tank and placed in large jars in air which were then flushed with nitrogen gas for 20 min and tightly sealed. Anoxic animals were held at room temperature (20°C). At timed intervals during anoxia groups of animals were sampled. Shells were quickly opened and tissues were rapidly dissected out, blotted, and frozen in liquid nitrogen. For recovery experiments, animals were held for 96 h under anoxic conditions and were then returned to the aerated seawater tank and sampled at timed intervals. Tissues were stored frozen at −80°C until use.

Samples of the fluid trapped between the valves were also taken from the anoxic animals, valves were pried open, the fluid was drained, then adductors were slashed, and the valves opened up. Fluid samples were pooled for each group of clams and were frozen at −80°C until use. Samples were not further processed before analysis.

Sample preparation

Frozen tissues were ground to a powder under liquid nitrogen using a mortar and pestle and were then transferred to preweighed, polypropylene centrifuge tubes stored on dry ice. After a second weighing to determine tissue weight, tubes were transferred one at a time to a dry ice – methanol bath at −8°C. Five volumes (w/v) of 6% perchloric acid containing 1 m*M* EDTA (chilled at −8°C) were quickly added and the tissue was then quickly homogenized using a Polytron PT 10-35 homogenizer. For glycogen determination a 50-μL sample of homogenate was removed at this point. Homogenates were centrifuged at 5900 *g* for 15 min at 4°C to pellet the precipitated protein. The acid supernatant was removed, transferred to a second tube and neutralized by the addition of 3 *N* KOH – 0.3 *M* imidazole – 0.4 *M* KCl. After a second centrifugation to remove precipitated $KClO_4$, neutralized samples were stored at −80°C until analysis.

Metabolite assays

Glycogen was determined by the method of Keppler and Decker (1974). ATP, alanine, aspartate, and glucose were analyzed by the coupled enzyme assays of Lowry and Passonneau (1972). Succinate was determined by the method of Williamson and Corkey (1969) with the substitution of the ATP-dependent succinyl-CoA synthetase from *E. coli*. Alanopine and strombine were determined as outlined by Storey *et al.* (1982).

Measurement of enzyme activities

Fresh tissues were dissected out of aerobic animals, blotted, weighed, and homogenized in five volumes (w/v) 50 m*M* imidazole buffer, pH 7.0 containing 20 m*M* 2-mercaptoethanol. For hepatopancreas, 1 m*M* phenylmethylsulphonyl fluoride was included in the homogenizing buffer. Homogenates were centrifuged at 27 000 × *g* for 20 min at 4°C. Supernatants were collected and dialyzed against homogenizing buffer for 2 h.

Enzymes were assayed in 50 m*M* imidazole buffer, pH 7.0 at 23°C. Optimal substrate conditions were 2 m*M* pyruvate and 0.1 m*M* NADH for lactate dehydrogenase with the addition of 800 m*M* glycine for the determination of strombine dehydrogenase or 100, 200, or 400 m*M* L-alanine for gill, hepatopancreas, or all other tissues, respectively, for alanopine dehydrogenase.

Results

Anoxia in M. mercenaria

Figures 1–5 show the changes in metabolite levels in mantle, gill, foot muscle, and phasic and catch adductor muscles of *M. mercenaria* during 96 h of anoxia stress followed by 48 h of recovery from anoxia. Alanine and succinate were products of anoxia in all tissues. Succinate was the major product, the ratio succinate:alanine produced at 96 h of anoxia ranging from a low of 1.4:1 in phasic adductor to a high of 3.2:1 in mantle tissue. Succinate accumulation was linear in all tissues starting from the earliest (6 h) times of anoxia with the total accumulation of succinate being significantly higher in gill and mantle (25 and 21 μmol/g wet weight, respectively) than in the three muscle tissues (12, 14, and 14 μmol/g in phasic adductor, catch adductor, and foot, respectively). Alanine accumulation was also progressive over the course of the anoxia stress. A significant elevation of alanine levels was first seen in gill tissue after 6 h of anoxia and in mantle after 12 h. Significant elevations of alanine content were seen in muscle tissues only in long-term anoxia. However resting levels of alanine in muscle tissues were quite highly variable between individuals and were much higher in the control situation (20–30 μmol/g) than those in gill and mantle tissues (6.5 μmol/g).

Alanopine and strombine were minor products of anoxia in some tissues of the cherrystone clam. Levels of both imino acids were elevated by 50–60% in adductor muscles within 12 to 24 h of anoxia. However, total accumulation of each imino acid was less than 1 μmol/g. Alanopine content of foot showed a steady decline throughout anoxia but low levels of strombine were accumulated. The three muscle tissues showed similar levels of the two imino acids but in gill and

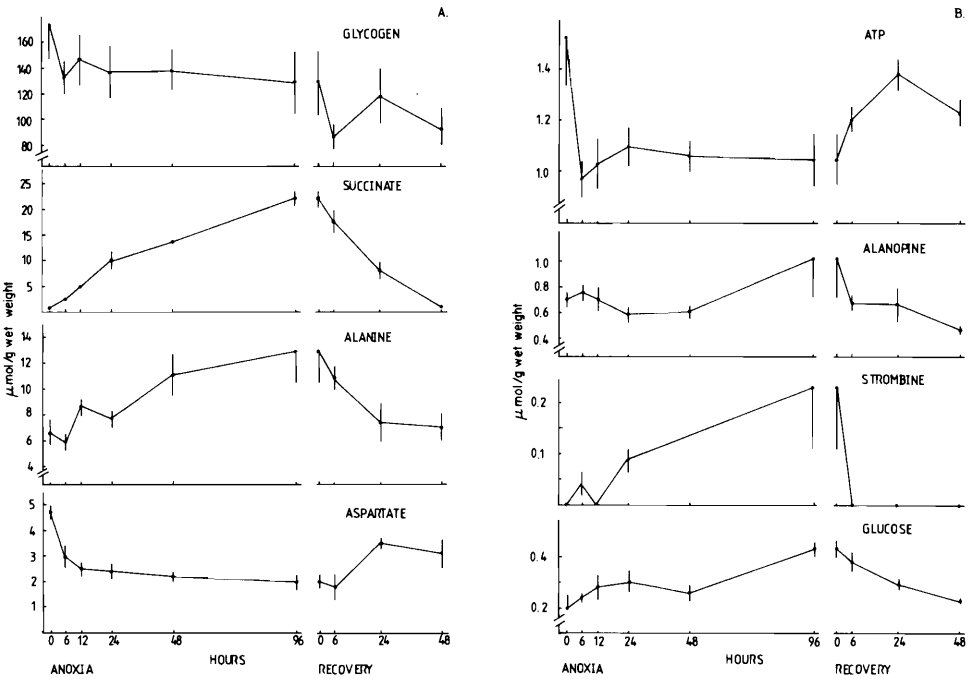

FIG. 1. Levels of some metabolites in the mantle of *M. mercenaria* during anoxia and recovery. Results are expressed as means ± SEM for $n = 7$ animals for time zero and $n = 4$ animals at all other times. Only the inner portion of the mantle was used; the mantle edge was trimmed off.

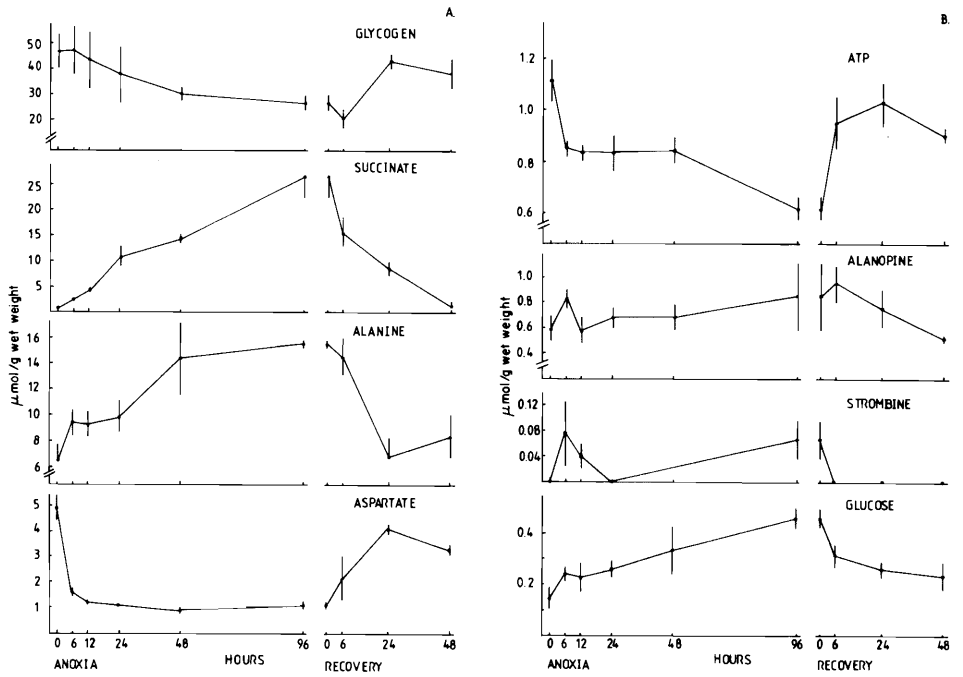

FIG. 2. Levels of some metabolites in the gill of *M. mercenaria* during anoxia and recovery. Results are means ± SEM for $n = 7$ for time zero and $n = 4$ for all others.

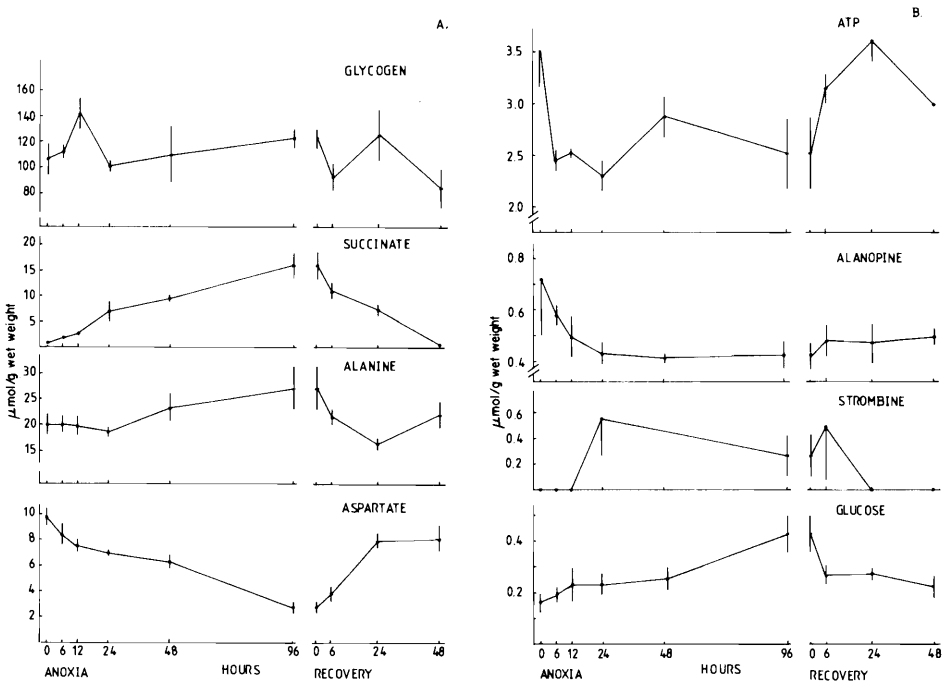

FIG. 3. Levels of some metabolites in the foot muscle of *M. mercenaria* during anoxia and recovery. Results are means ± SEM for $n = 7$ for time zero and $n = 4$ for all others.

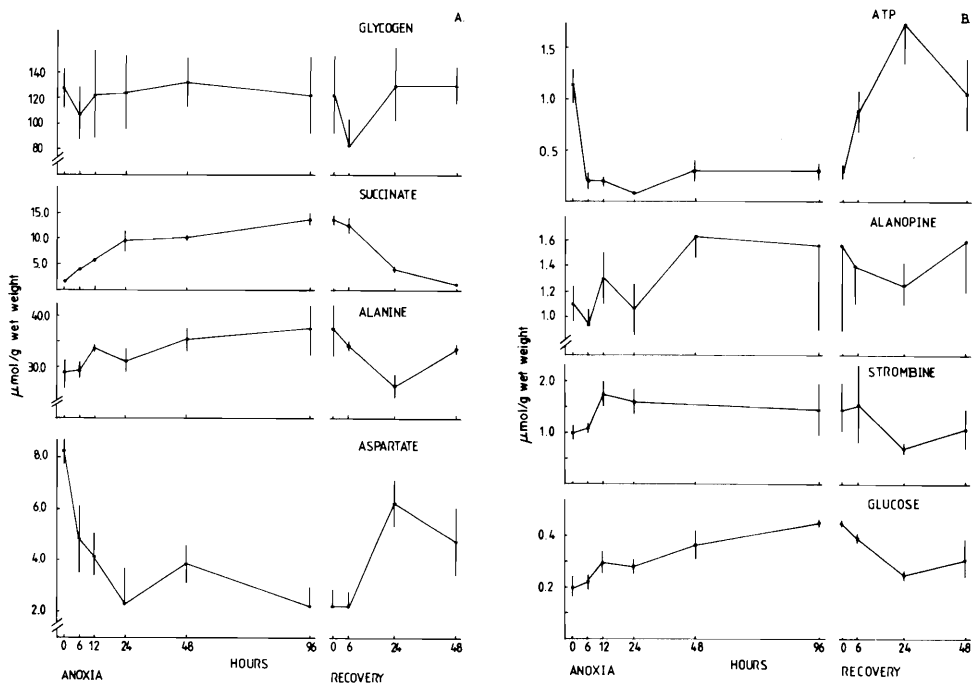

FIG. 4. Levels of some metabolites in the phasic adductor muscle of *M. mercenaria* during anoxia and recovery. Results are means ± SEM for $n = 7$ for time zero and $n = 4$ for all others.

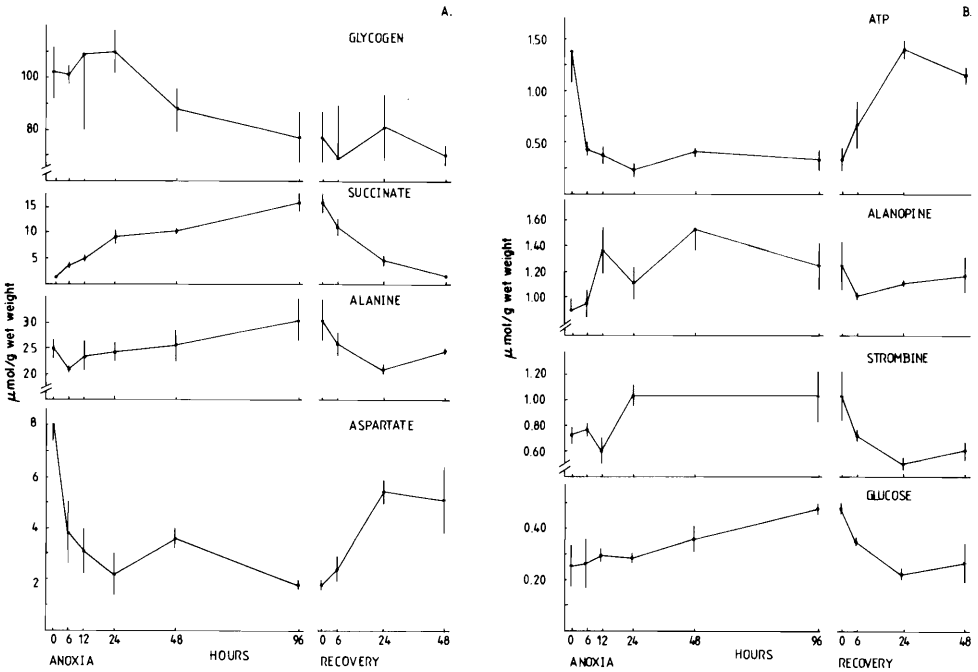

FIG. 5. Levels of some metabolites in the catch adductor muscle of *M. mercenaria* during anoxia and recovery. Results are means ± SEM for $n = 7$ for time zero and $n = 4$ for all others.

mantle alanopine was the predominant imino acid. No significant production of alanopine was found in either gill or mantle during anoxia but a small increase in strombine content was seen (from 0.08 to 0.20 μmol/g). Imino acid levels in hepatopancreas during anoxia were also determined (data not shown). Like gill and mantle, alanopine was the major imino acid at levels of 1.1 to 1.6 μmol/g, whereas strombine content was very low (<0.1 μmol/g). No significant accumulation of alanopine or strombine was found during anoxia.

As anoxia was imposed by exposing animals to a nitrogen gas atmosphere, secretion of end products into the fluid of the mantle cavity during anoxia could also be characterized. Succinate accumulated in a linear fashion during anoxia with concentrations of 3.5, 6.0, 28, and 32 μmol/mL (control levels <0.5 μmol/mL) at 6, 12, 48, and 96 h of anoxia. However only small amounts of alanine were found, alanine content rising from 0.28 to 0.75 μmol/mL after 96 h of anoxia. Strombine was not detected in the mantle cavity fluid but alanopine levels rose from 0 to 0.3 μmol/mL within the first 24 h of anoxia.

Aspartic acid was an important substrate of anaerobic metabolism in *M. mercenaria* during the first 6 to 12 h of anoxia. All tissues showed a rapid decline in aspartate levels during this time and this was matched by an opposite rise in succinate concentrations. Aspartate levels decreased by 4, 2.5, 6, 6, and 7 μmol/g in gill, mantle, phasic adductor, catch adductor, and foot, respectively, and in all cases the carbon from aspartate could account for the accumulated production of succinate. After 12 to 24 h aspartate concentration stabilized at a low level and remained constant throughout the remaining period of anoxia. Foot muscle was the only exception to this as aspartate levels continued to decline throughout anoxia.

All tissues of the clam had high glycogen reserves ranging from about 50 μmol/g in gill and hepatopancreas to 170 μmol/g in mantle (expressed as glucose equivalents). During anaerobiosis the level of tissue glycogen decreased significantly in gill, mantle, and catch adductor muscle but no significant decrease was found in phasic adductor, foot, or hepatopancreas. The decrease in glycogen content in mantle occurred within the first 6 h of anoxia but in gill and catch adductor glycogen content remained constant over the initial hours of anoxia and then declined steadily during long-term anoxia. The decrease in glycogen content in these tissues (20, 30, and 25 μmol/g in gill, mantle, and catch adductor, respectivly) more than accounted for the carbon accumulated in the end products succinate and alanine. All tissues showed a steady rise in glucose concentration during the anoxic period, the levels of glucose rising from about 0.2 to 0.4 μmol/g over the 96 h period. Such a rise in glucose content may suggest an intertissue transport of glucose during anoxia.

ATP content of all tissues of the cherrystone clam decreased rapidly during the first 6 h of anoxia with

levels of 75, 64, 17, 25, and 55% of control values seen at 6 h in gill, mantle, phasic adductor, catch adductor, and foot, respectively. ATP content remained at these low levels throughout the remainder of anoxia.

Recovery

During recovery from anoxia, concentrations of metabolites were rapidly restored to control levels. ATP content of all tissues rose rapidly during the first 6 h of recovery with control levels reestablished by 24 h of recovery in aerated seawater. Accumulated succinate and alanine were rapidly depleted. Control levels of alanine were reestablished within 24 h while succinate showed a linear decline reaching control levels by 48 h. The catabolism of succinate and alanine was met by an opposite rise in aspartate levels which were restored to control concentrations by 24 h of recovery. The nitrogen necessary for aspartate synthesis in all tissues could be accounted for by the depletion of accumulated alanine. Most tissues showed a decline in tissue glycogen content during the first 6 h of recovery but this was restored somewhat in later hours. Glycogen content of tissues did not return to control levels during recovery but this might be expected as animals were starved during the experiment. Tissue glucose content, however, had returned to levels of about 0.2 μmol/g after 24 to 48 h. Alanopine and strombine contents of the tissues were also rapidly restored to control levels in tissues which had accumulated the imino acids. The exception to this was alanopine in the phasic adductor which remained high throughout the recovery period.

Tissue enzyme activities

Table 1 shows the activities of cytosolic dehydrogenases acting at the pyruvate branch point in *M. mercenaria*. Lactate dehydrogenase was a minor activity in all tissues except for substantial amounts in foot muscle. The activity of alanopine dehydrogenase or strombine dehydrogenase (or a single enzyme catalyzing both activities) was highest in foot muscle and phasic adductor. All tissues showed an alanine-dependent activity which was greater than or equal to the glycine-dependent activity. In muscle tissues the ratio alanine/glycine was approximately 1.0 while soft tissues showed a higher ratio suggesting a difference in tissue enzyme complement.

TABLE 1. Activities of cytosolic dehydrogenases acting at the pyruvate branch point in *M. mercenaria*

Tissue	LDH	ADH/SDH		
		Alanine	Glycine	Ala/Gly
Gill	0.83±0.04	6.0±0.5	3.8±0.4	1.6
Mantle	0.75±0.09	8.0±1.0	6.0±1.0	1.3
Phasic adductor	0.34±0.07	41±7	35±7	1.2
Catch adductor	0.70±0.10	32±4	33±5	0.97
Foot	2.50±0.40	77±17	65±16	1.2
Heptopancreas	0.76±0.09	6.5±0.1	4.9±0.5	1.7

NOTE: Enzyme activities of lactate dehydrogenase (LDH) and of alanopine or strombine dehydrogenase (ADH/SDH) are given as means ± SEM for $n = 5$ animals. Activities are expressed as micromoles NADH utilized per minute per gram wet weight.

Discussion

Succinate and alanine were the major products of anaerobic metabolism in *M. mercenaria* as they are in various other marine bivalve species. The further catabolism of succinate to form propionate was not tested in this study but is unlikely to occur, Kluytmans *et al.* (1983) having shown that Mediterranean *Venus* species (*M. mercenaria* was formerly known as *V. mercenaria*) do not produce propionate from accumulated succinate. Succinate was produced in greater amounts than alanine in all tissues and in addition accumulated in high levels in the mantle cavity fluid (alanine did not). This is a major difference compared with the oyster and the mussel which accumulate more alanine than succinate under most circumstances (Collicutt and Hochachka 1977; Eberlee *et al.* 1983; de Zwaan *et al.* 1982) and suggests that the relative importance to total energy production of the pathways producing alanine versus succinate can be modified in different species.

The release of succinate into the mantle cavity fluid has not been documented in other species. *Mytilus edulis* excretes propionate into the hemolymph but not succinate; succinate is retained in the cells (Zurburg 1981). However as succinate appears to be the terminal organic acid produced by *M. mercenaria* tissues it is this acid which is excreted from cells. Succinate in the extracellular fluid can be buffered during anoxia by calcium derived from the shell (Crenshaw and Neff 1969).

Both aspartic acid and glycogen were utilized as anaerobic substrates by *M. mercenaria*. Aspartate was a substrate during short-term anoxia only, all tissues except foot depleting the reserves of this amino acid within the first 12 h of anoxia. During this time, however, the depletion of aspartate carbon could fully account for the accompanying synthesis of succinate in tissues. Metabolism during the early hours of anoxia, therefore, couples the anaerobic fermentation of glycogen to form alanine with the catabolism of aspartate reserves to form succinate. Such a scheme was first postulated for the anoxic oyster heart (Collicutt and Hochachka 1977) and has subsequently been shown to occur in tissues of other species (Zurburg and Ebberink 1981; Meinardus and Gäde 1981). The catabolism of aspartate reserves during short-term anoxia in *M. mercenaria* probably accounts for the full carbon requirements of succinate synthesis. In the natural

environment intertidal animals experience cyclic bouts of anoxia which are unlikely to last more than 12 h. It is probable, therefore, that in the natural environment *M. mercenaria* utilizes aspartate as the sole substrate for succinate synthesis under most anoxic situations. However, *M. mercenaria* must also maintain the capacity for the anaerobic production of succinate from glycogen reserves via the carboxylation of phosphoenolpyruvate to form oxaloacetate. This capacity is evident from the continued synthesis of succinate during long-term anoxia well after aspartate reserves have been depleted. The capacity is apparently present in all tissues of the clam although may be more limited in adductor muscles. Succinate levels increased by only 5–6 μmol/g between 24 and 96 h of anoxia in adductor compared with an increase of 10–15 μmol/g in other tissues (alanine levels rose by 5–6 μmol/g in all tissues). These findings contrast with recent work on *M. edulis*. Adductor muscle in this species appears to be capable of producing succinate from aspartate reserves only while both possible routes of succinate synthesis are present in other tissues (de Zwaan, de Bont, and Hemelraad 1983).

Owing to high tissue glycogen levels and high variability in glycogen content in tissues between individual animals, studies which have attempted to quantitate glycogen depletion during anoxia have met with limited success. The results of a number of studies on different species and tissues (summarized by de Zwaan 1977) indicate, however, an overall depletion of glycogen reserves during anoxia. Similar results were found in the present study for *M. mercenaria*. Glycogen levels were high and variable in all tissues but a significant depletion of glycogen reserves during anoxia was found in mantle, gill, and catch adductor muscle. In these tissues, glycogen depletion more than accounted for the concomitant accumulations of succinate and alanine. Tissue glucose levels were elevated in all tissues during anoxia. Glucose is not an intermediate of intratissue glycogen catabolism but is produced for intertissue transport of carbohydrate. The elevation of glucose in anoxic tissues suggests, therefore, that intertissue transport of substrates (glucose, perhaps others) via the blood may contribute to anoxic energy production. Certainly, externally provided glucose is an excellent substrate for anaerobiosis in the isolated ventricle of the oyster (Collicutt and Hochachka 1977).

Metabolic recovery from anoxia involved the restoration of control levels of succinate, alanine, aspartate, glucose, and ATP in tissues, all occurring within 24 h of the return to aerobic conditions (except for succinate which required 48 h). Glycogen content was not restored to control levels but this is not unexpected as animals were starved throughout the experiment. Changes in aspartate and succinate levels showed an inverse relationship with the depletion of succinate amply accounting for the carbon necessary for the resynthesis of aspartate reserves. Aspartate production may be fueled, therefore, by a direct reconversion of succinate to oxaloacetate with the amino groups necessary transferred from the catabolism of accumulated alanine. This appears to be unlike the situation seen in *M. edulis* (de Zwaan, de Bont, Zurburg *et al.* 1983) and the oyster *Crassostrea virginica* (Eberlee *et al.* 1983). In these species, the time course of succinate degradation is much faster (less than 2 h) than that of aspartate resynthesis (4–10 h); this temporal difference indicates that the succinate pool cannot be directly reconverted to aspartate.

The metabolic responses to anoxia and recovery differed quantitatively between the various tissues of *M. mercenaria* but were not different qualitatively. Thus the amounts of succinate produced during anoxia varied from 12–14 μmol/g in muscles to 21–25 μmol/g in gill and mantle while alanine accumulation ranged from 5 μmol/g in catch adductor to 9 μmol/g in gill. However, the pattern of accumulation of these end products as well as of their loss during recovery was similar between all tissues. Aspartate reserves were higher in mucle tissues providing 6–7 μmol of C_4 for succinate synthesis during the early hours of anoxia versus the 3–4 μmol/g of the amino acid utilized by soft tissues. As a percentage of the total succinate synthesized in each tissue aspartate reserves provided only 12–15% of the C_4 needed in gill and mantle, whereas 40–48% of the succinate produced in muscles could be derived from the catabolism of aspartate reserves. As discussed earlier this could indicate a lower capacity for the production of succinate via the carboxylation of phosphoenolpyruvate in muscles.

Although alanopine and strombine dehydrogenase activities occur in high levels in the cherrystone clam and are the major pyruvate reducing activities in all tissues, the enzymes appear to have no major role in the anaerobic metabolism of the species. Alanopine and strombine accumulated in minor amounts in *M. mercenaria* tissues during anoxia but in all cases this was less than 1 μmol/g each and was relatively insignificant compared with the levels of succinate and alanine produced. Similar results showing imino acids as only minor products of anaerobic metabolism have been found in other species (Zurburg *et al.* 1982; de Zwaan, de Bont, Zurburg *et al.* 1983; Eberlee *et al.* 1983). Alanopine was the major imino acid found in gill and mantle but both imino acids occurred in similar amounts in muscle tissues. This may reflect differences in the amino acid composition of the muscle versus nonmuscular tissues or, perhaps more likely, may result from differences in the amino acid specificities of the tissue alanopine and strombine dehydrogenases. Alanopine and strombine contents of the tissues returned rapidly to control levels during the recovery period except for alanopine in phasic adductor which remained elevated

throughout recovery. However there was no evidence for a specific production of imino acids during the recovery period as has now been shown in *M. edulis* and *C. virginica* (de Zwaan, de Bont, Zurburg *et al.* 1983; Eberlee *et al.* 1983). Both of these species accumulate strombine and (or) alanopine during the first 2 to 6 h of recovery; the elevated metabolic demand of the recovery period appears to necessitate both aerobic and glycolytic energy production to meet the requirements for the restoration of ATP, argininephosphate and aspartate pools, as well as the resumption of normal metabolic activities such as feeding. *Mercenaria mercenaria*, however, does not appear to require an elevated glycolytic energy production as part of its strategy for recovery. Apart from a probable role in supporting muscular work, particularly digging by the foot, the functions of alanopine dehydrogenase and strombine dehydrogenase activities in *M. mercenaria* tissues are still unclear.

Acknowledgements

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