1980

# Anaerobic metabolism of the ribbed mussel, Geukensia demissa

Ming-Shan Ho
*College of William and Mary - Virginia Institute of Marine Science*

## INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.

University Microfilms International

300 N. ZEEB ROAD, ANN ARBOR, MI 48106
18 BEDFORD ROW, LONDON WC1R 4EJ, ENGLAND

HO, MING-SHAN

# ANAEROBIC METABOLISM OF THE RIBBED MUSSEL, GEUKENSIA DEMISSA

*The University of Wisconsin - Madison* PH.D. 1980

# ANAEROBIC METABOLISM OF THE RIBBED MUSSEL, *GEUKENSIA DEMISSA*

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

by

Ming-Shan Ho

1980

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

Ming-Shan Ho

Approved, April 1980

Paul L. Zubkoff, Ph.D.

Robert E. Black, Ph.D.

John C. Munday Jr., Ph.D.

Craig E. Ruddell, Ph.D.

Carl W. Vermeulen, Ph.D.
Department of Biology
College of William and Mary in Virginia

TABLE OF CONTENTS

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. P.L. Zubkoff for his invaluable advice, training and encouragement throughout the course of this study. His gratitude is also extended to other members of the committee, Drs. R.E. Black, J.C. Munday Jr., C.L. Ruddell and C.W. Vermeulen.

Thanks are also due to Mr. J.E. Warinner, III, Drs. R. Jordan and A.L. Lin for their unforgettable friendship, assistance and encouragement and to Dr. C.P. Mangum for her helpful discussion. The author is also grateful to Mrs. L.L. Jenkins and Mrs. L.T. Marshall for typing this dissertation and to personnel in the library and in the graphic arts and photography section of this Institute for their constant help in preparation of this manuscript.

The author appreciates the research assistantship provided by Dr. M.E. Bender, Head of the Division of Environmental Science and Engineering, Virginia Institute of Marine Science.

It is so difficult to describe the deep appreciation and affection of the author to his parents, wife, brother and sister. Without their help, this work would never be possible.

LIST OF TABLES

LIST OF TABLES (cont'd.)

LIST OF FIGURES

## ABSTRACT

The anaerobic metabolism of the intertidal ribbed mussel, Geukensia demissa (= Modiolus demissus), is reported in this dissertation. This mollusc was selected because anaerobic metabolism is believed to be of vital significance to its survival during the air exposure period of the tidal cycle.

Ribbed mussels, Geukensia demissa, were maintained in either aerated or hypoxic water for up to four days. Major metabolites and polysaccharide of each mussel were then analyzed and compared. Succinate, propionate and alanine were found to accumulate hypoxically, but not malate, α-keto-glutarate, cis-aconitate, citrate, iso-butyrate, butyrate, iso-valerate, lactate and pyruvate. The polysaccharide content of each mussel was quite different and did not show a clear indication of decrease under the hypoxic condition.

Propionate production followed succinate accumulation, which corroborates an earlier report for Mytilus edulis. The linear increase of alanine concentration with hypoxic period observed in this study, has not been reported previously.

The results of this study are consistent with those of Mytilus edulis, Cardium edule, and Anodonta cygnea, bivalves which have recently been studied in Europe. This agreement suggests that bivalves have a similar anaerobic metabolic scheme.

A hypothetical scheme of anaerobic metabolism of bivalves is proposed in which redox balance is achieved in both cytosol and mitochondria. This scheme incorporates parts of the Embden-Meyerhof-Parnas glycolytic pathways, carbon dioxide fixation, the Krebs cycle, transamination, and pathways for propionate formation. To date, this is the only bivalve anaerobic scheme which contains all these essential considerations.

# ANAEROBIC METABOLISM OF THE RIBBED MUSSEL, *GEUKENSIA DEMISSA*

# INTRODUCTION

The ability of molluscs, such as Sydosmya alta, to live in deoxygenated water and survive for more than three days has long been recognized (Moore, 1931). This capability for many molluscs to survive under a condition of low oxygen availability not only allows them to withstand a temporary anoxic condition but also enables them to function in the oxygen deficient habitats for at least part of their life cycle (Theede et al., 1969). It is probably important for the intertidal bivalves because they may not be able to extract enough oxygen from the air to sustain aerobic metabolism. The ribbed mussel, Geukensia demissa (= Modiolus demissus) which dwells in the high intertidal zone and is abundant along the Atlantic coast of the United States is a good example of such an organism. Geukensia demissa are usually found in clumps among roots of cordgrass, Spartina sp., half embedded in the mud of salt marshes (Lent, 1967). During air exposure, their rate of oxygen consumption is reduced (Kuenzler, 1961; Widdows et al., 1979) and the primary pathways of metabolism in the deep tissue are believed to be anaerobic because their oxygen transport system is inefficient (Booth and Mangum, 1978).

The recent progress of understanding molluscan anaerobic metabolism is briefly presented in Table 1. In the freshwater gastropods studied by Von Brand et al. (1950) and Mehlman and Von Brand (1951), lactate and volatile fatty acids were found to accumulate anaerobically. Bivalve anaerobic metabolism was not investigated in detail until De Zwaan and his collaborator

TABLE 1

A CHRONOLOGY OF MOLLUSCAN ANAEROBIC METABOLISM

| | | |
|---|---|---|
| **I. Fresh Water Gastropods** | | |
| **A. Metabolite analysis** | | |
| Anoxic condition<br>Lactate accumulation | *Lymnaea stagnalis*<br>*Lymnaea natalensis* | Von Brand *et al.* 1950 |
| Anoxic condition<br>Propionate and acetate accumulation | *Australorbis glabratus*<br>*Helisoma duryi* | Mehlman and Von Brand 1951 |
| **II. Bivalves** | | |
| **A. Carbon-14 labelling studies** | | |
| Succinate fixes $^{14}C$ at the highest rate among Krebs cycle intermediates | Mantle of oyster *Crassostrea virginica* | Hammen, 1966 |
| Succinate and alanine as end products of $^{14}C$-glucose(U) degradation | Mantle of clam *Rangia cuneata* | Stokes and Awapara 1968 |
| **B. Enzyme activity studies** | | |
| Anoxic condition<br>Fumarate reductase > Succinate dehydrogenase | Oyster *Crassostrea virginica* | Wegener *et al.* 1969 |
| Anoxic condition<br>PEP carboxykinase > Pyruvate kinase | Adductor muscle of oyster *Crassostrea gigas* | Hochachka and Mustafa, 1972 |
| **C. Metabolite analysis** | | |
| Anoxic condition<br>Succinate and alanine accumulation | Mussel *Mytilus edulis* | De Zwaan and Zandee, 1972b |
| Anoxic condition<br>Volatile fatty acids accumulation | Mussel *Mytilus edulis* | Kluytmans *et al.* 1975 |

employed the methods of metabolite analysis. They found that succinate, alanine (De Zwaan and Zandee, 1972b) and volatile fatty acids (Kluytmans et al., 1975) accumulated when sea mussels, Mytilus edulis, were held under a controlled anaerobic condition. Some earlier studies also suggested the accumulation of succinate and alanine, as well as a reversal of part of the Krebs cycle which incorporates carbon dioxide during bivalve anaerobic metabolism:

phosphoenolpyruvate + $CO_2$ → oxaloacetate → malate → fumarate → succinate

(Hammen, 1966; Stokes and Awapara, 1968; Wegener et al., 1969; Hochachka and Mustafa, 1972). The most recent work on anaerobic metabolism utilized Mytilus edulis (De Zwaan and Zandee, 1972b; De Zwaan and Marrewijk, 1973a, b; De Zwaan et al., 1973; Loxton and Chaplin, 1973; Kluytmans et al., 1975; De Zwaan et al., 1975; Kluytmans and De Zwaan, 1976; Kluytmans et al., 1977; Widdows et al., 1979), Cardium edule (Gäde, 1975), and Anodonta cygnea (Gäde et al., 1975). Reactions consistent with a similar anaerobic metabolic scheme have also been found in such other invertebrate organisms as the sea anemone, Diadumene leucolena (Ellington, 1977), the oligochaete, Tubifex sp. (Schöttler, 1975, 1977a, b, 1978; Schöttler and Schroff, 1976), the polychaetes, Arenicola marina (Zebe, 1975; Surholt, 1977) and Lumbricus sp. (Gruner and Zebe, 1978), and the insect, Chironomus sp. (Augenfeld, 1966; Wilps and Zebe, 1976).

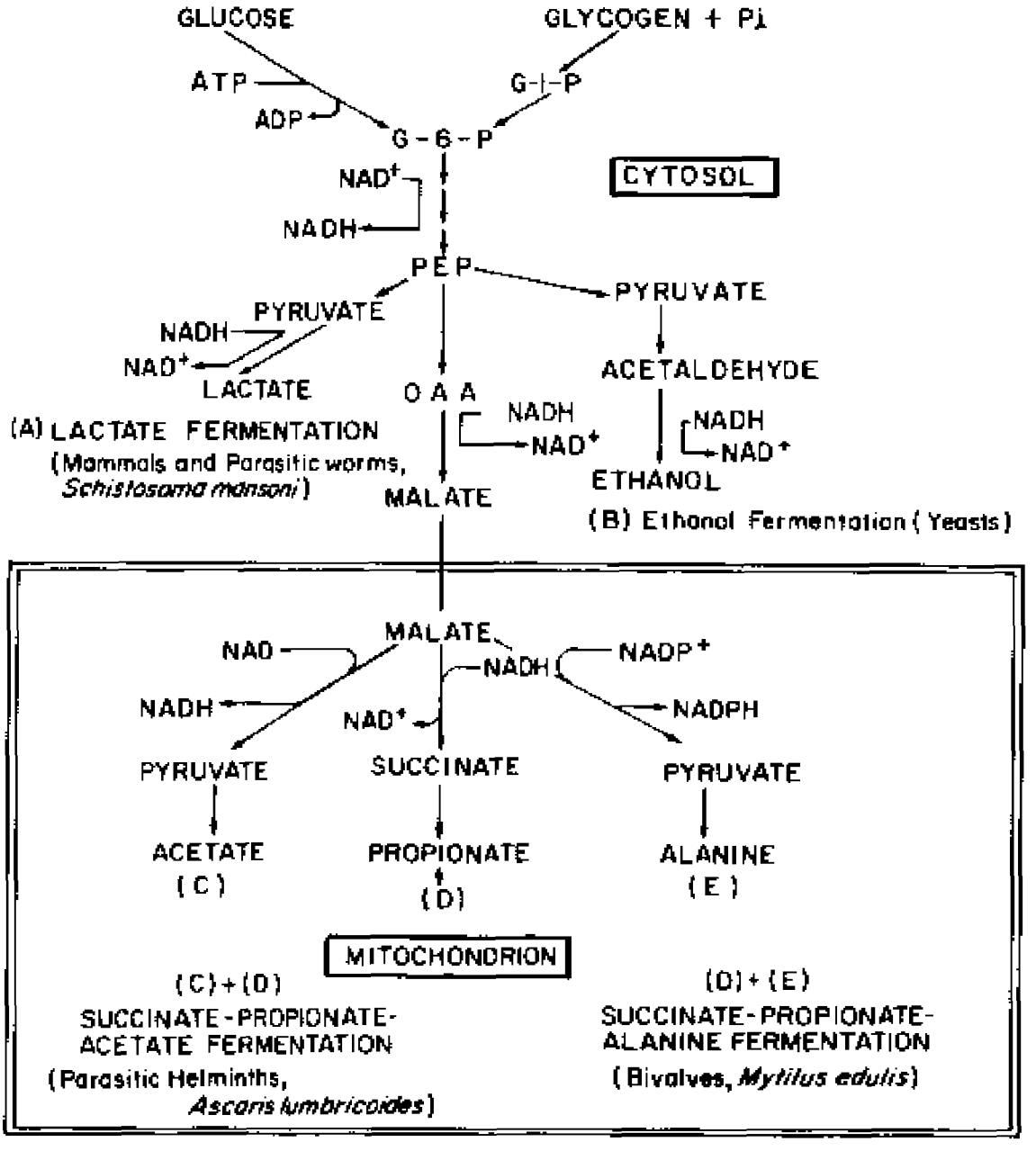
Four major schemes of anaerobic metabolism have been observed and are herein designated (Figure 1) as

(A) lactate fermentation: mammals and some parasitic worms (Schistosoma mansoni);

(B) ethanol fermentation: yeasts;

FIGURE 1. FOUR MAJOR ANAEROBIC METABOLIC SCHEMES.

## FOUR MAJOR ANAEROBIC METABOLIC SCHEMES



(C + D) succinate-propionate-acetate fermentation: parasitic helminths (the pig roundworm, Ascaris lumbricoides [review: Saz, 1969, 1970, 1971]); and

(D + E) succinate-propionate-alanine fermentation: bivalves [review: De Zwaan et al., 1976; De Zwaan and Wijsman, 1976; De Zwaan, 1977].

Of the three major metabolites which accumulate during bivalve anaerobic metabolism (succinate, propionate, and alanine), the formation of succinate through a reversal of part of the Krebs cycle

oxaloacetate → malate → fumarate → succinate

is accepted; propionate is probably generated by the decarboxylation of succinate (Tkachuck et al., 1977; Saz et al., 1978); and the origin of alanine is still controversial. Stokes and Awapara (1968) suggested that in Rangia cuneata, the alanine accumulated during anaerobic metabolism was formed by transamination of pyruvate with amino acids. Such transamination reactions were also shown for Cerastoderma edule and Mytilus edulis by Read (1962). Later, Hochachka and his collaborators further postulated that during molluscan anaerobiosis, amino acids were simultaneously mobilized with carbohydrates in order to generate energy and maintain redox balance (Hochachka and Mustafa, 1972; Hochachka and Somero, 1973; Hochachka et al., 1973; Hochachka, 1976; Collicutt and Hochachka, 1977). However, De Zwaan and his coworkers found that alanine probably only accumulated initially; they disputed both the transamination origin of alanine and the simultaneous mobilization of amino acids during bivalve anaerobiosis (De Zwaan and Van Marrewijk, 1973a; Gäde et al., 1975; De Zwaan et al., 1975; De Zwaan et al., 1976; De Zwaan and Wijsman, 1976; De Zwaan, 1977; Kluytmans et al., 1977). They presented no alternative explanation, and transamination was still proposed as the source of alanine in their metabolic pathways (De Zwaan et al., 1976).

As mentioned previously, the present concept of molluscan anaerobic metabolism was established by De Zwaan and his collaborators with studies predominately undertaken with Mytilus edulis. Although these interpretations are impressive, it is still very useful to have the results confirmed independently by other investigators with different analytical methods and on different bivalve species. Thus, the major objectives of this dissertation are:

(1) To extend the specific observations on the European bivalve, Mytilus edulis, to another species. The ribbed mussel, Geukensia demissa, is chosen for this study because it is ecologically and physiologically interesting and it is taxonomically related to Mytilus edulis;

(2) To compare the results of Geukensia demissa to those derived by De Zwaan and his associates in order to generalize observations on the anaerobic metabolism of bivalves;

(3) To study the functions of the Krebs cycle intermediates (including succinate) in bivalve anaerobic metabolism with the sensitive gas-liquid chromatographic method which analyzes seven intermediates simultaneously; and

(4) To clarify the argument about the role of alanine in bivalve anaerobic metabolism.

To achieve these goals, mussels were maintained under controlled high and low oxygen tensions. Each mussel was then extracted, assayed and compared for concentrations of polysaccharide, Krebs cycle intermediates,

volatile fatty acids, alanine, lactate and pyruvate. To date, this is the first study of bivalve anaerobic metabolism in which all such intermediates were measured in individual organisms instead of pooled tissues. Defined terms and time course studies were conducted using mussels under both winter and summer conditions.

The results of metabolite accumulations determined in this study were integrated with existing knowledge of enzyme localization, kinetics, and regulation and a hypothetical metabolic scheme is proposed. This scheme takes into account cytosol-mitochondrial compartmentation, a modified Embden-Meyerhof-Parnas glycolytic pathway, carbon dioxide fixation, the Krebs cycle, pathways for propionate and alanine formation, and it attains redox balance overall. This scheme of bivalve anaerobic metabolism may assist in the design of further refinements in the understanding of the metabolic control of bivalves.

## MATERIALS AND METHODS

In order to quantify the numerous intermediates potentially involved in intermediary metabolism, several analytical techniques were necessary. These included derivatization, gas-liquid chromatography and ultraviolet spectrophotometry for enzyme analysis. Initial testing of some of these procedures in order to develop technique and verify the method was accomplished using another mollusc, the oyster, as a test organism for extractions.

### Oysters

Oysters (Crassostrea virginica) used in this study were three years old, 11.0 ± 0.5 cm, approximately 20 g (tissue wet weight) and were predetermined to be healthy and superficially free of parasites. They were cultured from larval stage at Virginia Institute of Marine Science, Gloucester Point, Virginia and maintained on a tray in the adjacent York River with salinity from 16-21 o/oo.

### Mussels

Ribbed mussels (Geukensia demissa) with shell lengths of 11.5 ± 1.0 cm were collected on the bank of York River near Mumford Island, Virginia. The average wet weight (including enclosed sea water) was 89.6 g and the dry weight was 2.95 g. Before each experiment was started, the bivalves were placed in glass desiccators with aerated York River water and acclimated at 15°C for two days. The temperature was maintained with circulating water

held constant with a Lo-Temptrol 154 water circulator (Precision Scientific Co., Chicago, Illinois).

Experiments

Two experimental regimes were carried out: (1) Fixed term study: After acclimation, eight mussels were randomly divided into normoxic and anoxic sets and incubated for 51 hours. Incubation was carried out in December 1978. (2) Time course study: 23 mussels were randomly chosen for the following treatments: eight were sacrificed immediately after field collection; three after two days acclimation; and the remaining mussels were incubated anoxically for periods up to four days, with groups of three mussels each sacrificed daily. This experiment was started in June 1979.

Incubation

For the anoxic condition, the river water medium containing mussels was flushed with nitrogen gas (Union Carbide Co., Linde Division, N.Y.). The air trapped under desiccator tops was also replaced with nitrogen gas before sealing. The concentrations of dissolved oxygen for normoxic water was at least 4.2 ppm for the fixed term study, and 7.0 ppm for time course study; dissolved oxygen was below 0.4 ppm for anoxic water as determined by both, polarographic measurement with an oxygen meter (Model 51A, Yellow Springs Instrument Co., Yellow Springs, Ohio) and Winkler titration. The salinity of the river water medium was determined to be 17.1 o/oo with a Beckman RS-7B induction salinometer for the mixed term study and 14.4 o/oo for the time course study. The incubation temperature was 15°C and the organisms were not fed during the experiments. After incubation, the mussels were quickly removed from the desiccator, their sizes and total weights were measured and then the organisms were either frozen (for fixed term

study) or opened under a stream of nitrogen gas and quickly frozen in dry ice-acetone (for time course study) before further treatments.

Preparation of Homogenates

The frozen mussels were opened and their contents collected; the whole process was rapid and the tissues were maintained cold with ice water. Those preparations which were either maintained cold or frozen with dry ice-acetone were minced with scissors and homogenized with a cold buffer of 2-amino-2-methyl-1-propanol (0.1 M, pH 10, Sigma Chemical Co.) using either a hand homogenizer or a VirTis 45 homogenizer (Gardiner, N.Y.). Preparations from each mussel were adjusted to 60 ml with buffer and then divided into sets for various determinations (Figure 2). Homogenized fractions were promptly deproteinized or treated according to the appropriate procedure for each analysis and stored. The analytical methods are summarized in Table 2.

Dry Weight Determination

Aliquots (10 ml) of mussel tissue homogenate were transferred to tared aluminum containers, dried at 60°C for 24 hours and then maintained in vacuum desiccator over phosphorus pentoxide for three days. Samples were weighed with an analytical balance.

Polysaccharide Determination

Polysaccharide was isolated according to the method of De Zwaan and Zandee (1972a) and assayed using the anthrone-$H_2SO_4$ method. One ml of mussel homogenate designated for polysaccharide determination was saponified in one ml of 50% KOH for one hour at 50°C, neutralized with HCl, deproteinized with 0.06 g of trichloroacetic acid and centrifuged for 20 minutes

FIGURE 2. FRACTIONATION OF THE RIBBED MUSSEL, GEUKENSIA DEMISSA.

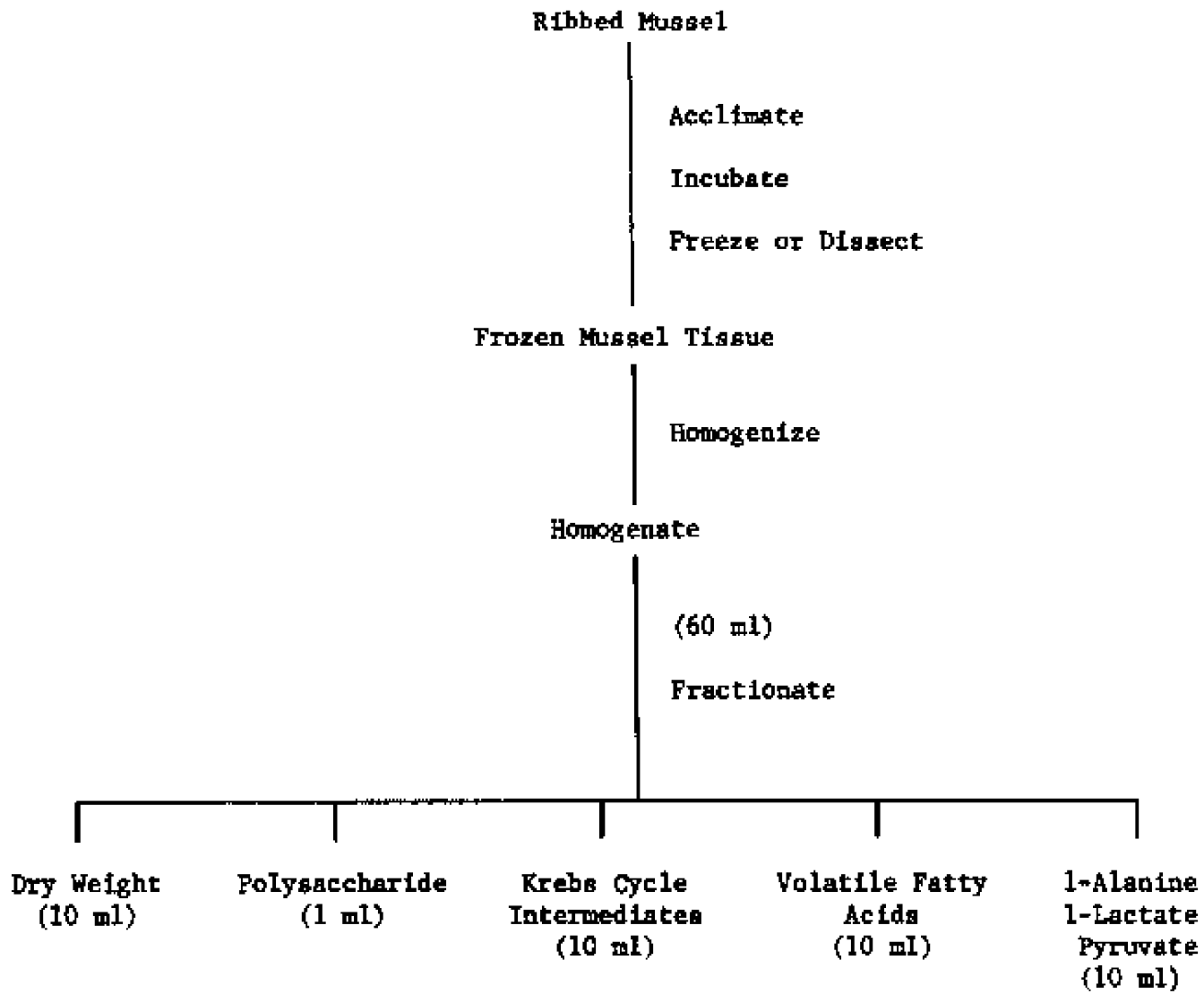


TABLE 2

METHODS FOR THE ANALYSIS OF

KEY METABOLITES AND POLYSACCHARIDE (GLYCOGEN)

| COMPOUNDS | ANALYSIS |
|---|---|
| Kreba Cycle Intermediates<br>De Silva (1971) | Celite$^{R}$ and Basic Alumina<br>Column Purification<br>$BF_3$-Methanol Derivatization<br>Solvent Extraction<br>Gas-liquid Chromatography |
| Volatile Fatty Acids<br>Gibbs *et al.* (1973) | Perchlorate Deproteinization<br>KOH Precipitation<br>Solvent Extraction<br>Gas-liquid Chromatography |
| Alanine<br>Schutgens *et al.* (1977) | Alanine Dehydrogenase<br>$NAD^+ \rightarrow NADH$<br>Spectrophotometry |
| Lactate<br>Sigma 826 UV (1977) | Lactate Dehydrogenase<br>$NAD^+ \rightarrow NADH$<br>Spectrophotometry |
| Pyruvate<br>Sigma 726 UV (1977) | Lactate Dehydrogenase<br>$NADH \rightarrow NAD^+$<br>Spectrophotometry |
| Polysaccharide<br>De Zwaan and Zandee (1972) | Anthrone Reaction<br>Spectrophotometry |

with a clinical centrifuge (International Equipment Co., Mass.). The volume of supernatant was measured and diluted 10 and 100 fold; one ml of each dilution was used for polysaccharide determination.

Preparations from previous treatments were stirred with 0.05 ml of saturated $Na_2SO_4$ solution, followed by three ml of ethanol. Samples were heated in a boiling water bath for three minutes, cooled in an ice bath for at least one hour, and centrifuged; the ethanol was carefully decanted. The polysaccharide pellets obtained after drying with nitrogen gas were dissolved with 0.05 ml of $H_2O$ and 4.0 ml of freshly prepared anthrone reagent was then added; the mixture was vortexed, heated at 90°C for 20 minutes, cooled in ice water and measured at 620 nm with the Cary Model 15 spectrophotometer. Anthrone reagent was prepared by dissolving 0.25 g anthrone in 100 ml of concentrated sulfuric acid.

Krebs Cycle Intermediates Determination

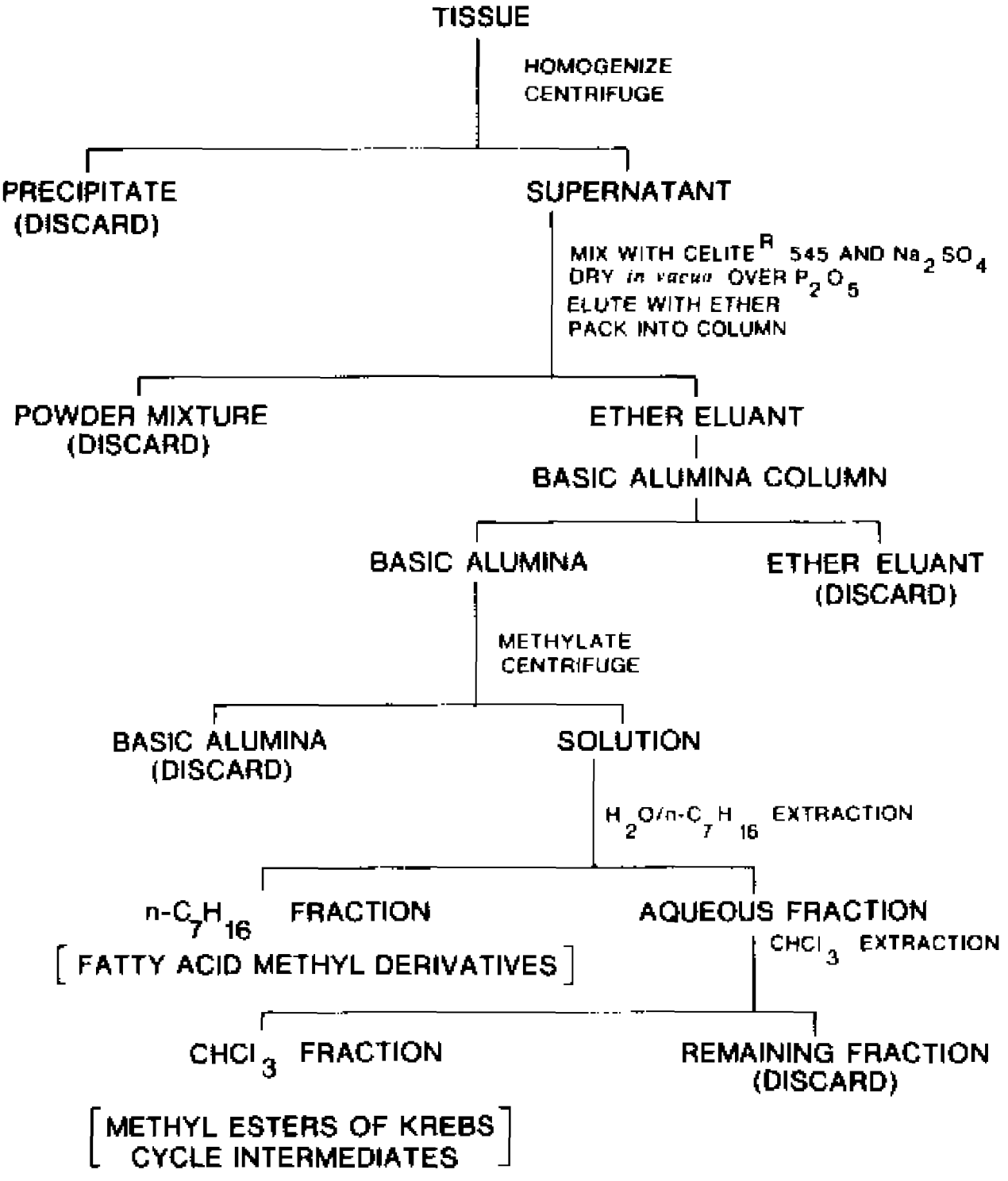
The Krebs cycle intermediates were isolated (Figure 3) and measured according to the method of de Silva (1971).

a. Reagents

The Krebs cycle intermediates, adipic acid and basic alumina (activity grade I) were obtained from Sigma Chemical Co. (St. Louis, Mo.), Celite 545$^R$ from Fisher Scientific Co. and boron trifluoride-methanol ($BF_3$-MeOH, 14% W/V) from Applied Science Laboratories (State College, Pa.). All organic solvents were analytical grade and redistilled in glass before use. $BF_3$-MeOH (7%) was prepared by dilution of a 14% solution with dry methanol. The internal standard, dimethyl adipate, was prepared by reacting 25 mg of adipic acid with 10 ml of 7% $BF_3$-MeOH at 60°C for two hours, cooling to room temperature and then adding an equal volume of water. After extracting the reaction mixture twice

FIGURE 3. PREPARATION OF KREBS CYCLE INTERMEDIATES FOR GAS-LIQUID CHROMATOGRAPHIC ANALYSIS.

## PREPARATION OF KREBS CYCLE INTERMEDIATES FOR GLC ANALYSIS



with 50 ml of chloroform each time, the combined extracts were dried over anhydrous $Na_2SO_4$ and then the volume reconstituted to 100.0 ml with chloroform.

b. Extraction

Ten ml each of mussel tissue homogenate, glass distilled water (blank control), and standard acid mixture were used. The standard acid mixture was prepared by dissolving the weighed compounds (Table 3) in 200 ml of 5N $H_2SO_4$:water:methanol (1:3:16 v/v/v).

c. Purification of Tissue Extracts

The crude acid extract was mixed thoroughly with 10 g of ether-washed Celite 545$^R$. The moist Celite was mixed with 10 g of anhydrous $Na_2SO_4$ and stored overnight in a desiccator containing $P_2O_5$. The resulting powdered mixture was transferred to a 1 cm (i.d.) x 50 cm glass chromatography column in approximately 2 g portions and packed tightly. The organic acids were eluted from the column with ether (approximately 100 ml). The eluant was then passed through a one g column of activated basic alumina oxide (activated by heating in an oven at 100°C for two hours and cooling to room temperature). The alumina was dried under a stream of nitrogen gas, and repeatedly washed with acetone five times and ether twice to completely remove the moisture. The free acids retained by the alumina were converted to their methyl esters by heating with 5 ml of 7% $BF_3$-MeOH to 60°C for two hours in a screw-capped tube.

Following methylation, the mixture was cooled to room temperature, filtered, and an equal volume of water was added to the filtrate. The filtrate contained methyl esters of fatty acids and Krebs cycle intermediates; the fatty acid methyl esters were removed by washing the methanol-$BF_3$-water

TABLE 3

STANDARD MIXTURE OF KREBS CYCLE INTERMEDIATES

| INTERMEDIATES | AMOUNT (mg) |
|---|---|
| Fumarate | 5.0 |
| Succinate | 5.0 |
| Malate | 30.0 |
| α-keto-Glutarate | 15.0 |
| cis-Aconitate | 5.0 |
| Citrate | 20.0 |
| Iso-Citrate (tri-sodium salt) | 50.0 |

All samples were homogenized with 20 ml of 5 N $H_2SO_4$: methanol (1:19 v/v) in an ice bath and then centrifuged. The residue was again homogenized with 10 ml of 5 N $H_2SO_4$: $H_2O$: methanol (1:3:16 v/v/v), centrifuged and then supernatants were combined and reduced by a stream of nitrogen gas to approximately 10 ml and saved for subsequent purification.

solution three times with 1 ml of n-heptane each time; the methyl esters of Krebs cycle intermediates were then extracted with two 10 ml portions of chloroform; the chloroform extract was washed with a 1 M $Na_2CO_3$ solution, followed by water until neutral, and then dried over anhydrous $Na_2SO_4$. One hundred µl of internal standard, dimethyl-adipate, was added and the chloroform extract was reduced to 10-50 µl; a convenient volume (1 µl) was then injected into the column of gas-liquid chromatograph for separation and measurement.

d. Gas-Liquid Chromatography

A gas-liquid chromatograph equipped with a flame ionization detector (Hewlett-Packard 7626A) was used in this study. The column (glass, 1/8" i.d. x 6 ft) was packed with 100-120 mesh GasChrom P coated with 10% EGSS-X (Applied Science Laboratories). Temperature programming was from 100-210°C at 6°C/minute and then maintained isothermally for 15 minutes; the carrier gas flow (Helium, ultra high purity grade, Union Carbide Corp.) was 41 ml/minute.

Before a new column was used, it was conditioned for two days at 220°C and periodically injected with Silyl-8 column conditioner (Pierce Chemical Co.).

e. Quantitation

Peak areas were calculated as peak height x retention time. The volume variations of samples were corrected by adding 25 µg of dimethyl-adipate as internal standard before concentrating the sample. The detector response for dimethyl-adipate was linear up to 2 µg. Retention times and peak areas were then compared with those of both individual pure acids and their standard mixtures which were methylated directly with $BF_3$-methanol and extracted

into chloroform. The recovery from the extraction and purification procedures was calculated and the tissue concentration levels were measured (Table 4).

Volatile Fatty Acids (VFA) Determination

The analysis for volatile fatty acids followed that described by Gibbs et al. (1973).

a. Deproteinization of Tissue Homogenate

Ten µl of diluted n-valeric acid solution (50 µl dissolved in 1 ml of ethyl acetate) was added to 10 ml of mussel tissue homogenate and 10 ml of glass distilled water (blank control) as internal standard (preliminary studies indicated that the ribbed mussel does not contain a measurable amount of n-valeric acid). After one ml of cold 70% perchloric acid was added, the mixture was shaken vigorously for about 30 seconds, maintained cold for an additional 10 minutes to assure complete protein precipitation, and then centrifuged. This deproteinization procedure was repeated with 1 ml of cold 70% perchloric acid. After the supernatant was neutralized with a solution of 50% KOH until cessation of further crystal formation, it was then maintained in a refrigerator overnight. The $KClO_4$ crystals which had formed were removed by centrifugation and the clear supernatant was saved for further preparation.

b. Purification

After deproteinization, the sample was then saturated with NaCl, adjusted to pH 11-12 with 50% KOH, and centrifuged again to remove any remaining $KClO_4$ precipitate; 10.0 ml of ether was then added and the tube capped, shaken on a vortex agitator at high speed, and centrifuged to promote separation; the ether phase was then removed and discarded. The remaining aqueous

TABLE 4

RECOVERY OF A STANDARD MIXTURE OF KREBS CYCLE INTERMEDIATES

(EXTRACTION AND PURIFICATION PROCEDURE)

| | A | | B |
|---|---|---|---|
| Intermediate | Standard Mixture (mg) | Detector Response* | Recovery of Standard Mixture (%) |
| Fumarate | 5.0 | 105.0 | 6.9 |
| Succinate | 5.0 | 135.9 | 87.1 |
| Adipate | 5.0 | 191.5 | - |
| Malate | 30.0 | 61.5 | 31.2 |
| α-keto-Glutarate | 15.0 | 168.6 | 9.8 |
| cis-Aconitate | 5.0 | 69.2 | 59.1 |
| Citrate | 20.0 | 113.3 | 56.3 |

*Detector Response = $\frac{\text{peak height x retention time}}{\mu g \text{ acid}}$

A. Dry standard acid mixture was methylated with 7% $BF_3$-methanol and the methyl esters were extracted directly into chloroform. A one µl aliquot from a total volume of 10 ml was injected into the chromatographic column.

B. Standard acid mixture (Table 3) was taken through the extraction and purification procedure. The recovery (%) was calculated by comparing the detector responses found with the data shown in column A. Internal standard of methyl esters of adipate was used to correct for variation in the final volume of the sample.

phase was brought to pH 2-3 with 4 N HCl, ethyl acetate (10 ml) was added and the tube was vortexed for a few minutes and then centrifuged again. The acidic aqueous phase was extracted twice with 5 ml of ethyl acetate each time; the ethyl acetate fractions were combined and dried over anhydrous sodium sulphate to remove traces of water. The dried extract was then carefully concentrated with a slow stream of dry nitrogen gas to approximately 50-100 µl for gas-liquid chromatographic analysis.

c. Gas-Liquid Chromatography

A convenient aliquot (~1 µl) of concentrated VFA sample was then injected into the gas-liquid chromatograph (Hewlett-Packard 7626A) equipped with a flame ionization detector. The column (glass, 1/8" i.d. x 6 ft) was packed with 80-100 mesh Chromosorb WAW coated with 10% SP 1200 supplemented with 1% phosphoric acid (Supelco, Inc.). Temperature programming was from 70-140°C at 4°C/minute and the carrier gas flow (Helium, ultra high purity grade, Union Carbide Corporation, New York, N.Y.) was 69 ml/minute.

Peak areas were integrated as peak height x retention time and corrected for detector responses. Detector response factors were determined from gas-liquid chromatograms obtained after injection of a standard volatile fatty acid mixture (Table 5). The mixture was prepared by dissolving 10 µl each of propionic, iso-butyric, butyric, iso-valeric and valeric acids (Sigma Chemical Co.) in 500 µl of 1N acetic acid.

4.38 µ mole of n-valeric acid was added to the tissue homogenate as internal standard at the start of sample preparation and the fraction of n-valeric acid was determined from the sample injected; this ratio was used to convert the amount of volatile fatty acid in the sample to that in mussel tissue.

TABLE 5

DETECTOR RESPONSE FACTORS OF FREE VOLATILE ACIDS AS DETERMINED IN A STANDARD MIXTURE

| Volatile Fatty Acid | Detector Response Factor* |
|---|---|
| Propionic ($C_3$) | 3,952 |
| iso-Butyric (iso-$C_4$) | 10,989 |
| Butyric ($C_4$) | 11,905 |
| iso-Valeric (iso-$C_5$) | 21,739 |
| Valeric ($C_5$) | 23,810 |

*Detector Response Factor = $\frac{\text{peak height x retention time}}{\mu \text{ mole of acid}}$

Before a new column could be used successfully, it was stabilized by conditioning for 24 hours at 150°C and then by periodically injecting Silyl-8 column conditioner (Pierce Chemical Co.) to restore column efficiency.

Alanine, Lactate and Pyruvate Determinations

These procedures were modified from Kluytmans et al. (1975) and the Sigma Bulletins 726 UV and 826 UV (Sigma Chemical Co.). The homogenate (10 ml) was deproteinized by addition of 1 ml cold 70% perchloric acid and shaken vigorously for approximately 30 seconds. The mixture was kept cold for an additional 10 minutes to assure complete protein precipitation and then centrifuged for 10 minutes. This procedure was repeated with 1 ml of cold 70% perchloric acid; the supernatant was neutralized with 50% KOH and stored in the refrigerator overnight. The $KClO_4$ crystals formed were then removed by centrifugation and the clear supernatant was used for the determinations of l-alanine, l-lactate and pyruvate.

a. l(+)-Alanine Determination

The rapid alanine dehydrogenase (EC 1.4.1.1) micromethod of Schutgens et al. (1977) was adopted for this determination:

$$\text{l(+)-Alanine} + NAD^+ + H_2O \xrightarrow{\text{Alanine dehydrogenase}} \text{Pyruvate} + NH_3 + NADH + H^+ . \quad (1)$$

(Low $A_{340}$) (High $A_{340}$)

Aliquots of 0.05 ml mussel tissue preparation were pipetted into screw-capped tubes, labelled for reference and experiment, and then 0.05 ml water and 1.0 ml standard incubation mixture were added. The standard incubation mixture was prepared by dissolving 0.5 g hydrazine sulfate into 25 ml of Tris base (200 mM, Sigma Chemical Co.), the solution adjusted to pH 9.0 with 1 M KOH and then diluted to 50 ml with distilled water. 0.1 ml

$NAD^+$ solution was added to the experimental tube (10 mg β-$NAD^+$ dissolved in 0.55 ml standard incubation mixture), while 0.1 ml of the standard incubation mixture was added to the reference mixture. The initial absorbance at 340 nm was measured in the recording spectrophotometer (Cary Model 15) against reference and the enzymic reaction was started by the addition of 10 µl of alanine dehydrogenase (ADH) containing 2.2 units isolated from Bacillus subtilis (Sigma Chemical Co.). The reaction temperature was 37°C and the reaction time was 30 minutes. The standard curve was prepared and the quantity of alanine in the sample was calculated from the increment of absorbance and the standard curve.

b. 1(+)-Lactate Determination

1(+)-Lactate was determined using lactate dehydrogenase (EC 1.3.99.1) according to Sigma Bulletin 826 UV:

$$\text{1(+)-Lactic Acid} + NAD^+ \xrightarrow{\text{Lactate dehydrogenase}} \text{Pyruvic Acid} + NADH + H^+ \quad . \qquad (2)$$

(Low $A_{340}$) (High $A_{340}$)

0.5 ml of tissue preparation was diluted with 0.5 ml of distilled water and then 0.5 ml standard incubation mixture was added. The standard incubation mixture was prepared by gently mixing 2.0 ml glycine buffer (pH 9.2, contains glycine and hydrazine) with 10 mg $NAD^+$ and 0.1 ml lactic dehydrogenase. The reference solution was prepared by deleting $NAD^+$; incubation was at 37°C for 50 minutes, and measured at 340 nm with the recording spectrophotometer (Cary Model 15). The quantity of lactate was interpreted from the standard curve.

c. Pyruvate Determination

Pyruvate was determined with lactate dehydrogenase (EC 1.3.99.1) according to Sigma Bulletin 726 UV using the reverse reaction for the lactate assay:

Lactate dehydrogenase

$$\underset{\text{(High } A_{340})}{\text{Pyruvic Acid + NADH + } H^+} \rightarrow \underset{\text{(Low } A_{340})}{\text{I(+)-Lactic Acid + } NAD^+} \quad . \qquad (3)$$

0.5 ml of Trizma$^R$ base solution (tri-(hydroxymethyl)-aminomethane, 1.5 mol/liter) was added to 2.0 ml of tissue preparation, mixed by gentle inversion, adjusted to pH 9, and 0.5 ml NADH solution was added. The NADH solution was prepared by pipetting 2.2 ml Trizma$^R$ base solution into 1 mg NADH. After two minutes, the absorbance at 340 nm was measured against a reference solution using the Cary Model 15 recording spectrophotometer; the reference was prepared by deleting NADH. The enzymic reaction was started subsequently by the addition of 0.05 ml of lactic dehydrogenase. After two to five minutes, the final absorbance at 340 nm was measured and the quantity of pyruvate in the sample calculated from the increment of absorbance at 340 nm and the standard curve.

# RESULTS

## Preliminary Analysis of the Oyster, Crassostrea virginica

A preliminary analysis of adult oysters was undertaken prior to the more detailed study of the ribbed mussel. In order to investigate anaerobic metabolism, various tissue preparations and procedures were tried. In contrast to the previous reports on bivalve anaerobic metabolism which employed enzymatic methods and analyzed only for succinate, a gas-liquid chromatographic method which analyzes seven Krebs cycle intermediates simultaneously was adopted for this study after modifications (de Silva, 1971).

Satisfactory extraction, purification and quantification of methyl esters of Krebs cycle intermediates from oysters have been obtained and is shown in Figure 4 and Table 6. Single resolved peaks were observed for the dimethyl esters of fumarate, succinate, malate and the trimethyl esters of cis-aconitate, citrate and iso-citrate; two adjacent peaks were observed for trimethyl esters of α-keto-glutarate. The most abundant intermediate in the oyster tissue was malate which was followed by citrate, succinate and then fumarate, α-keto-glutarate; cis-aconitate and iso-citrate were present in trace amounts. The total concentrations in oyster tissue ranged from 752 to 4,092 nmole/g wet weight. These results were reported in part (Ho and Zubkoff, 1978).

## Detailed Study of the Ribbed Mussel, Geukensia demissa

Analysis of the ribbed mussels maintained hypoxically for 51 hours at 15°C revealed that succinate, propionate and alanine accumulated in these

FIGURE 4. GAS-LIQUID CHROMATOGRAPHY OF METHYL ESTERS OF KREBS CYCLE INTERMEDIATES OF OYSTER, CRASSOSTREA VIRGINICA.

(A) Standard intermediate mixture

(B) Standard intermediate mixture + oyster mantle tissue

(C) Oyster mantle tissue

a = Fumarate

b = Succinate

c = Adipate (internal standard)

d = Malate

e, f = α-keto-Glutarate

g = cis-Aconitate

h = Citrate

i = iso-Citrate

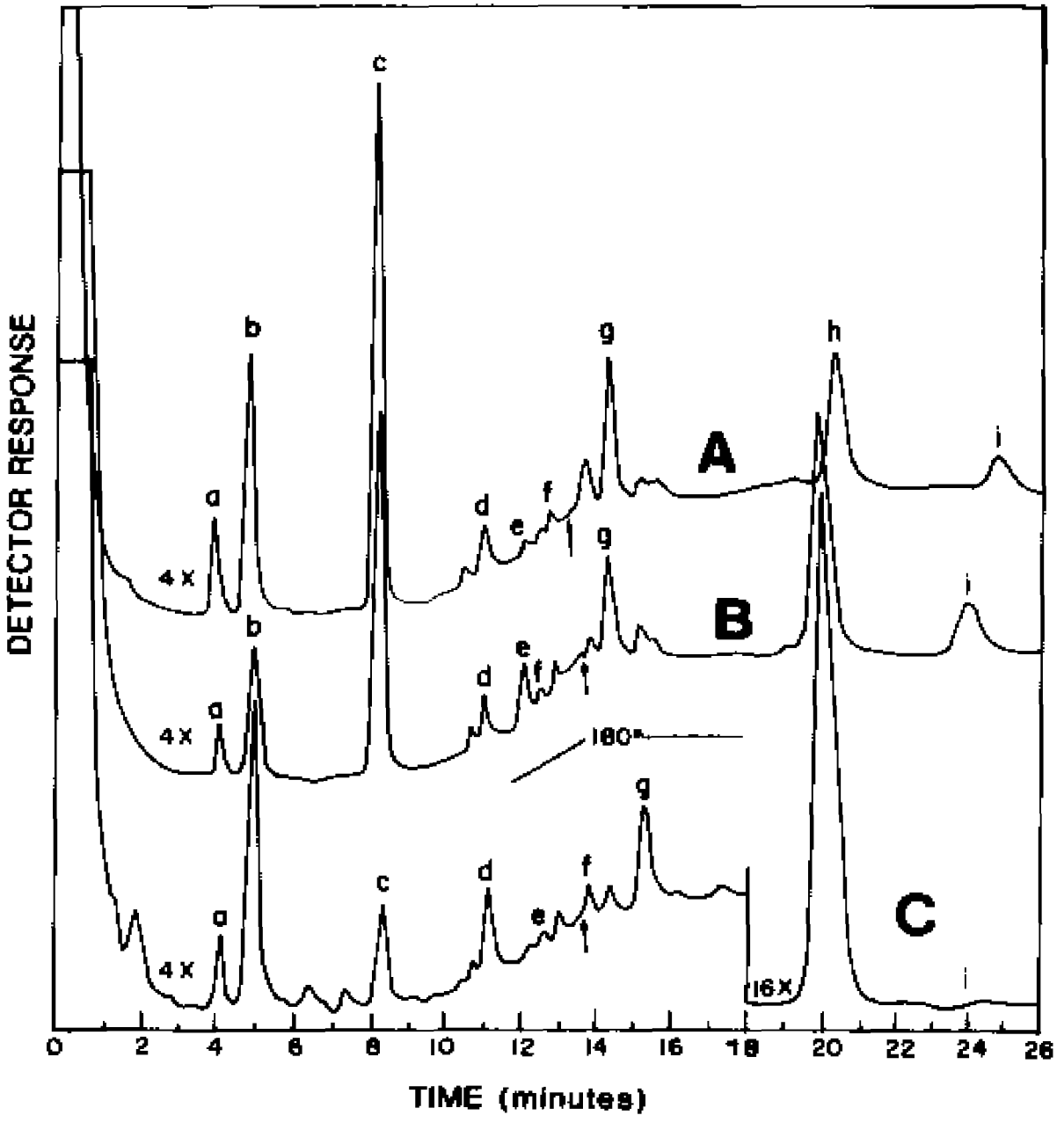

DETECTOR RESPONSE
TIME (minutes)
A
B
C
4X
4X
4X
16X
180°
a
b
c
d
e
f
g
h
i
0
2
4
6
8
10
12
14
16
18
20
22
24
26

TABLE 6

THE CONCENTRATIONS OF KREBS CYCLE INTERMEDIATES OF VARIOUS TISSUES OF THE OYSTER, CRASSOSTREA VIRGINICA AND MUSSEL, GENKENSIA DEMISSA

Data Represent μmoles/g Wet Weight.

| | Crassostrea virginica | | | | | | Genkensia demissa | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mantle* | Mantle | Gill | Hepatopancreas | Adductor Gray | Adductor White | Whole | Whole | Whole | Whole |
| Succinate | 204 | 139 | 417 | 217 | 479 | 302 | 38 | 60 | 132 | 510 |
| Fumarate | 97 | 391 | 145 | 21 | 115 | 179 | n.d. | n.d. | n.d. | n.d. |
| Malate | 810 | 516 | 1118 | 306 | 3146 | 1619 | 35 | 64 | 51 | 118 |
| Citrate | 1801 | 234 | 520 | 156 | 239 | 247 | n.d. | n.d. | 7 | 4 |
| cis-Aconitate | 6 | 7 | 50 | 7 | 11 | n.d. | n.d. | n.d. | n.d. | n.d. |
| iso-Citrate | trace | 79 | 21 | 27 | 7 | 4 | n.d. | n.d. | n.d. | n.d. |
| α-keto-Glutarate | 154 | 55 | 81 | 18 | 95 | 92 | 31 | 21 | 32 | 20 |
| Sum | 3072 | 1421 | 2352 | 752 | 4092 | 2443 | 104 | 145 | 222 | 652 |

* First determination in a single tissue of one organism.

animals. The same experiment showed only small changes which were significant in the quantities of the other metabolites.

A gas-liquid chromatogram of the Krebs cycle intermediates obtained from tissue extracts of control and experimental Geukensia demissa is depicted in Figure 5. The same quantity of dimethyl adipate (peak c) was added as an internal standard to both control and experiment extracts. It is clear that under hypoxic conditions, the succinate peak area (peak b) increased (Figure 5B and 5C). Gas-liquid chromatograms of the volatile fatty acids are shown in Figure 6. Propionate ($C_3$) is clearly demonstrated to accumulate hypoxically. Acetic acid ($C_2$) is also detected but is deleted from further consideration because a similar peak with same retention time appeared in blank controls.

Numerical data depicting and comparing concentrations of polysaccharides and key intermediates in control and experimental mussels is presented in Tables 7, 8, and 9. It can be seen that succinate increased about four-fold from 4.66 μmole/g dry weight in control animals to 19.63 μmole/g dry weight in mussels kept under $N_2$ for 51 hours at 15°C. There is also a concomitant small increase in the level of malate (1.29 μmole/g dry weight), but whether this is significant remains questionable at this time. Concentrations of α-keto-glutarate, cis-aconitate and citrate are low and do not appear to change hypoxically.

The average propionate concentration in the control group is 3.45 μmole/g dry weight, and 14.16 μmole/g dry weight in mussels maintained hypoxically. This represents a four-fold increase. It is also evident that there is virtually no change in iso-butyrate, butyrate and iso-valerate during 51 hours of hypoxia.

FIGURE 5. GAS-LIQUID CHROMATOGRAPHY OF METHYL ESTERS OF KREBS CYCLE INTERMEDIATES OF MUSSEL, GEUKENSIA DEMISSA.

(A) Standard intermediate mixture

(B) Geukensia demissa after maintaining 51 hours with aerated water

(C) Geukensia demissa after 51 hours of hypoxia

a = Fumarate

b = Succinate

c = Adipate (internal standard)

d = Malate

e, f = α-keto-Glutarate

g = cis-Aconitate

h = Citrate

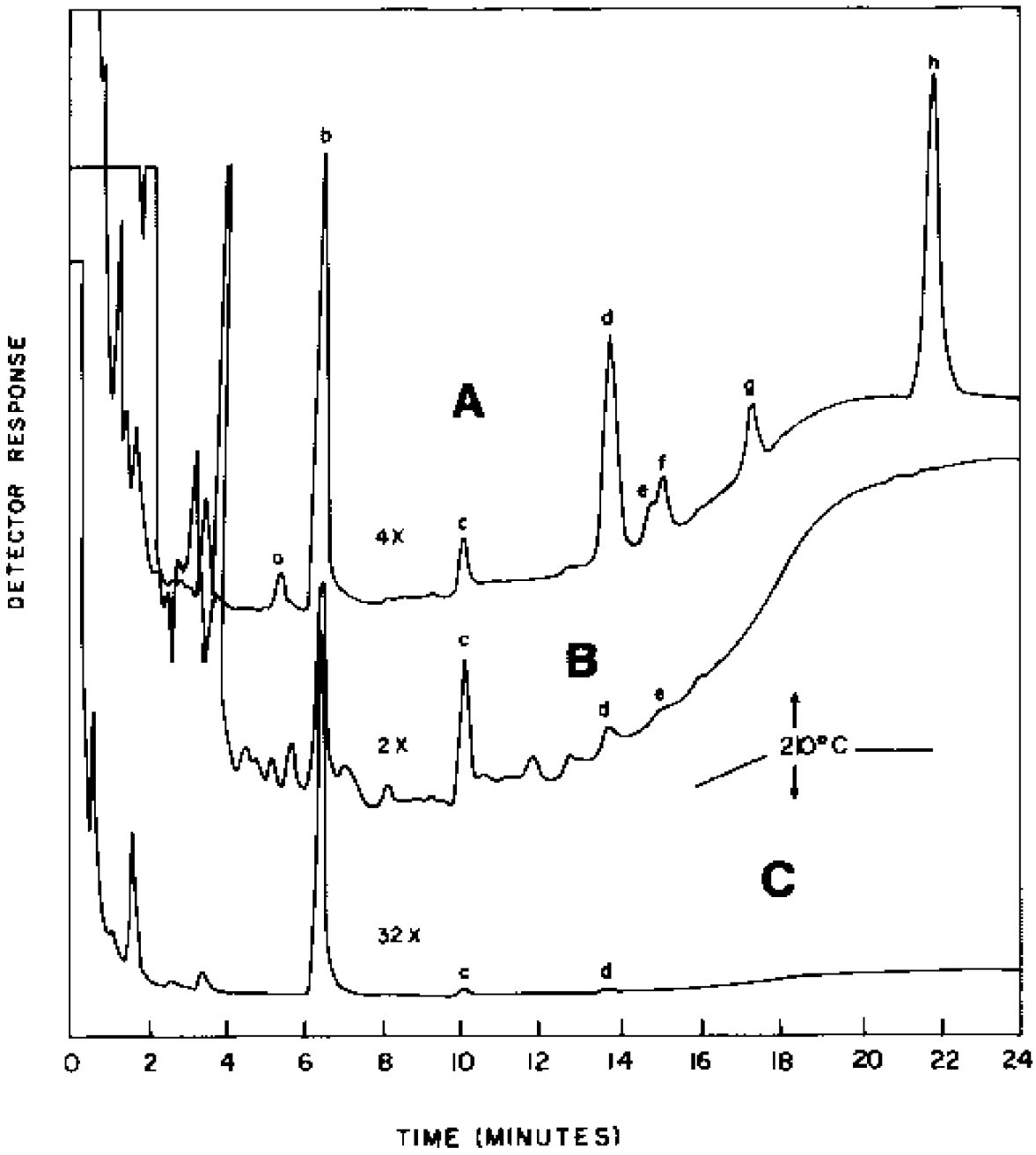

DETECTOR RESPONSE
A
B
C
4X
2X
32X
a
b
c
d
e
f
g
h
210°C
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (MINUTES)

FIGURE 6. GAS-LIQUID CHROMATOGRAPHY OF VOLATILE FATTY ACIDS.

(A) Standard acid mixture

(B) Geukensia demissa after maintaining 51 hours with aerated water

(C) Geukensia demissa after 51 hours of hypoxia

$C_2$ = Acetate

$C_3$ = Propionate

iso-$C_4$ = iso-Butyrate

$C_4$ = Butyrate

iso-$C_5$ = iso-Valerate

$C_5$ = Valerate (internal standard)

(n-Valerate is virtually absent in extracts of mussel tissue)

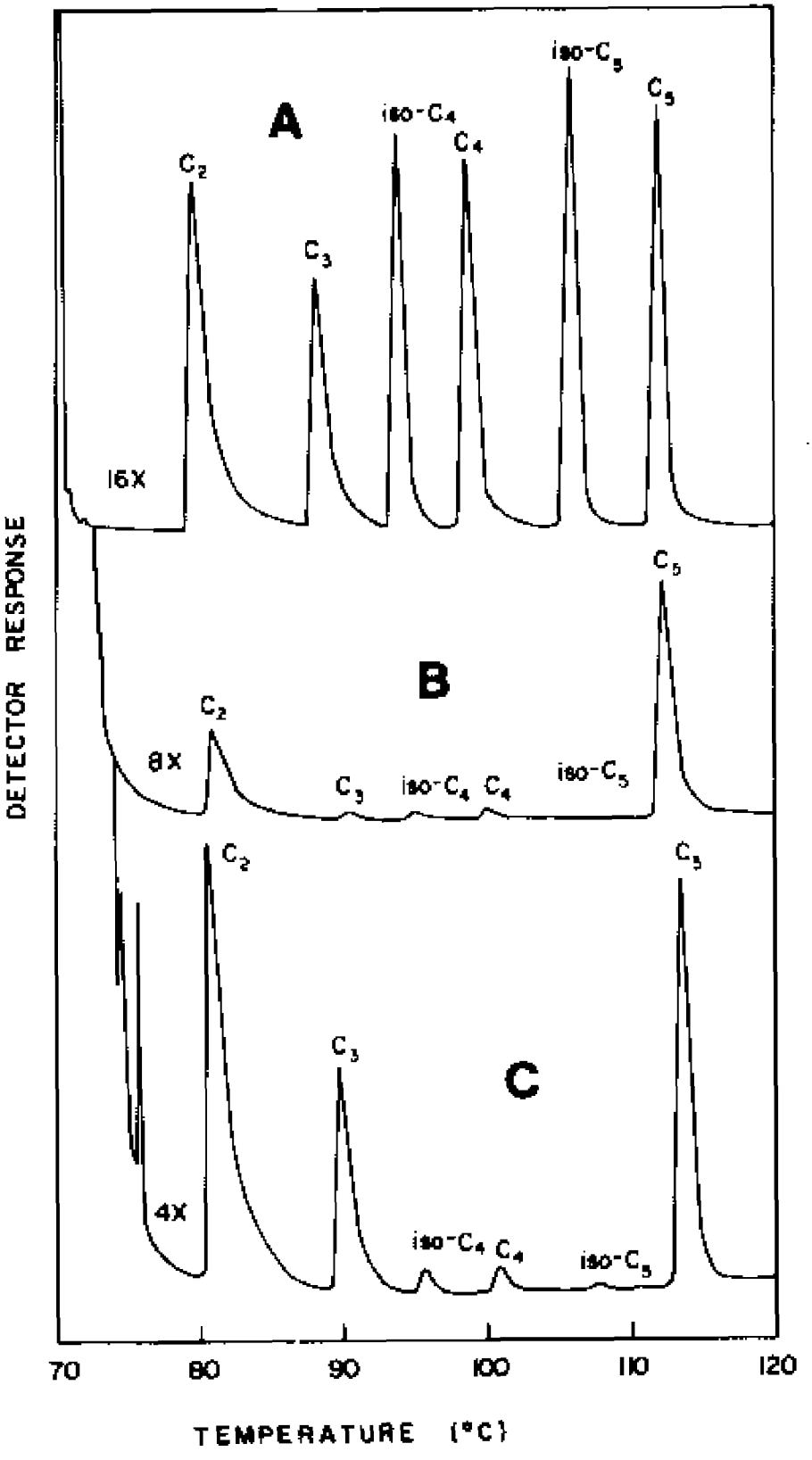

A
B
C
16X
8X
4X
C2
C3
iso-C4
C4
iso-C5
C5
DETECTOR RESPONSE
70
80
90
100
110
120
TEMPERATURE (°C)

TABLE 7

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF

GEUKENSIA DEMISSA AFTER MAINTAINING AT 15°C FOR 51 HOURS

WITH AERATED RIVER WATER (DECEMBER 1978)

| | Mussels | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | $\bar{x} \pm$ S.D. (n = 4) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | | |
| Size (cm) | 12.00 | 11.00 | 10.50 | 11.50 | 11.25 ± 0.65 |
| Dry weight (g) | 2.86 | 4.15 | 2.14 | 2.27 | 2.86 ± 0.92 |
| Polysaccharide | 86.00 | 267.20 | 128.70 | 185.20 | 166.78 ± 78.31 |
| **B. Metabolites (μmole/g dry weight)** | | | | | |
| Succinate | 0.84 | 0.78 | 2.66 | 14.35 | 4.66 ± 6.52 |
| Malate | 0.77 | 0.84 | 1.03 | 3.31 | 1.49 ± 1.22 |
| α-keto-Glutarate | 0.67 | 0.28 | 0.65 | 0.55 | 0.54 ± 0.18 |
| cis-Aconitate | *n.d. | n.d. | n.d. | n.d. | - |
| Citrate | n.d. | n.d. | 0.15 | 0.11 | - |
| Propionate | 2.19 | 1.50 | 4.73 | 5.38 | 3.45 ± 1.89 |
| iso-Butyrate | 0.89 | 0.74 | 1.75 | 1.28 | 1.17 ± 0.28 |
| Butyrate | 0.96 | 0.72 | 1.31 | 1.29 | 1.07 ± 0.28 |
| iso-Valerate | 0.12 | 0.09 | 0.20 | 0.20 | 0.15 ± 0.06 |
| Alanine | 57.58 | 60.73 | 76.78 | 65.66 | 65.19 ± 8.41 |
| Lactate | 5.01 | 3.94 | 6.05 | - | 5.00 ± 1.05 |
| Pyruvate | 0.25 | 0.35 | 0.48 | 0.43 | 0.38 ± 0.10 |
| **C. Sum of metabolites (μmole/g dry weight)** | | | | | |
| | 69.28 | 69.97 | 95.79 | 92.56 | 83.10 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | | | | | |
| | 60.61 | 63.01 | 84.17 | 85.39 | 73.30 |

*n.d. = not detectable

TABLE 8

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF

GEUKENSIA DEMISSA AFTER 51 HOURS OF HYPOXIA (DECEMBER 1978)

| | Mussels | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | $\bar{X} \pm$ S.D. (n = 4) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | | |
| Size (cm) | 11.00 | 11.50 | 11.00 | 11.00 | 11.13 ± 0.25 |
| Dry weight (g) | 2.22 | 2.95 | 2.19 | 2.71 | 2.52 ± 0.37 |
| Polysaccharide | 58.10 | 124.40 | 54.20 | 101.30 | 84.50 ± 34.10 |
| **B. Metabolites (μmole/g dry weight)** | | | | | |
| Succinate | 9.60 | 8.86 | 36.23 | 23.81 | 19.63 ± 13.03 |
| Malate | 2.45 | 2.48 | 4.43 | 1.76 | 2.78 ± 1.15 |
| α-keto-Glutarate | 0.35 | 0.23 | 0.14 | 0.80 | 0.38 ± 0.29 |
| cis-Aconitate | 0.39 | *n.d. | n.d. | n.d. | - |
| Citrate | 0.20 | 0.10 | 0.11 | 0.18 | 0.15 ± 0.05 |
| Propionate | 15.56 | 16.79 | 4.77 | 19.52 | 14.16 ± 6.48 |
| iso-Butyrate | 1.18 | 0.69 | 1.14 | 1.71 | 1.18 ± 0.42 |
| Butyrate | 1.10 | 0.80 | 1.26 | 1.16 | 1.08 ± 0.20 |
| iso-Valerate | 0.19 | 0.11 | 0.22 | 0.24 | 0.19 ± 0.06 |
| Alanine | 61.85 | 79.47 | 71.39 | 71.61 | 71.08 ± 7.21 |
| Lactate | 8.37 | 4.63 | 7.30 | 5.43 | 6.43 ± 1.71 |
| Pyruvate | 1.05 | 0.37 | 0.57 | 0.30 | 0.57 ± 0.34 |
| **C. Sum of metabolites (μmole/g dry weight)** | 102.29 | 114.53 | 127.56 | 126.52 | 117.63 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | 87.01 | 105.12 | 112.39 | 114.94 | 104.87 |

*n.d. = not detectable

TABLE 9

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF

GEUKENSIA DEMISSA AFTER MAINTAINING UNDER OXYGEN AND NITROGEN

AT 15°C FOR 51 HOURS (DECEMBER 1978)

| | Exposed to $O_2$ $\bar{x} \pm$ S.D. (n = 4) | Exposed to $N_2$ $\bar{x} \pm$ S.D. (n = 4) | Difference ($\bar{x}_{N_2} - \bar{x}_{O_2}$) | Accumulation (%) |
|---|---|---|---|---|
| A. Mussels (polysaccharide in mg/g dry weight) | | | | |
| Size (cm) | 11.25 ± 0.65 | 11.13 ± 0.25 | - 0.12 | - |
| Dry weight (g) | 2.86 ± 0.92 | 2.52 ± 0.37 | - 0.34 | - |
| Polysaccharide | 166.78 ± 78.31 | 84.50 ± 34.10 | -82.28 | - |
| B. Metabolites (μmole/g dry weight) | | | | |
| Succinate | 4.66 ± 6.52 | 19.63 ± 13.03 | 14.97 | 43.34 |
| Malate | 1.49 ± 1.22 | 2.78 ± 1.15 | 1.29 | 3.73 |
| α-keto-Glutarate | 0.54 ± 0.18 | 0.38 ± 0.29 | - 0.16 | - |
| Propionate | 3.45 ± 1.89 | 14.16 ± 6.48 | 10.71 | 31.00 |
| Iso-Butyrate | 1.17 ± 0.28 | 1.18 ± 0.42 | 0.01 | 0.03 |
| Butyrate | 1.07 ± 0.28 | 1.08 ± 0.20 | 0.01 | 0.03 |
| Iso-Valerate | 0.15 ± 0.06 | 0.19 ± 0.06 | 0.04 | 0.12 |
| Alanine | 65.19 ± 8.41 | 71.08 ± 7.21 | 5.89 | 17.05 |
| Lactate | 5.00 ± 1.05 | 6.43 ± 1.71 | 1.43 | 4.14 |
| Pyruvate | 0.38 ± 0.10 | 0.57 ± 0.34 | 0.19 | 0.55 |
| C. Sum of Metabolites (μmole/g dry weight) | | | | |
| | 83.10 | 117.48 | 34.54 | 99.99 |
| D. Sum of succinate, propionate and alanine (μmole/g dry weight) | | | | |
| | 73.30 | 104.87 | 31.57 | 91.40 |

Under hypoxic conditions, alanine levels increased from 65.19 to 71.08 μmole/g dry weight. Together, alanine, succinate and propionate account for 91.40% of the accumulation of metabolites (Table 9). There are only small changes of lactate and pyruvate concentrations from 5.00 to 6.43 and from 0.38 to 0.57 μmole/g dry weight, respectively.

Because of the wide range of values measured, the amounts of polysaccharide do not provide a clear indication for changes due to hypoxia (Tables 7 and 8). However, they indicate an increased rate of polysaccharide consumption under the hypoxic condition because the average concentrations dropped from 166.78 to 84.50 mg/g dry weight (Table 9).

The average values and standard deviations during the time course study (-2 to 4 days) are presented in Tables 10 to 15 and summarized in Tables 16 and 17 and Figure 7. The size, dry weight of each mussel, and concentrations of polysaccharides and some metabolites are shown in these tables. These mussels were either assayed immediately after field collection (Table 10), after two days acclimation at 15°C with aerated water (Table 11), or after one to four days of hypoxia (Tables 12-15). The average values of the concentrations of key metabolites (succinate, propionate and alanine) after four days of hypoxia are compared and summarized in Table 17 and plotted in Figure 7.

There is little or no change in concentration of any of the three major metabolites during the two days of acclimation at 15°C. However, succinate increased from 0.95 to 3.47 μmole/g dry weight after the first two days of hypoxia and then leveled off. The concentration of propionate did not change during the first day of hypoxia (2.65 to 2.33 μmole/g dry weight). However, propionate did increase slightly to 7.02 μmole/g dry

TABLE 10

CONCENTRATIONS OF POLYSACCHARIDE AND SOME METABOLITES OF

GEUKENSIA DEMISSA COLLECTED AT LOW TIDE (JUNE 1979)

| | Mussels | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | $\bar{x} \pm$ S.D. (n = 8) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | | | | | | |
| Size (cm) | 11.50 | 12.50 | 12.50 | 11.50 | 12.50 | 11.50 | 10.50 | 11.50 | 11.75 ± 0.71 |
| Dry weight (g) | 3.47 | 3.51 | 3.49 | 2.82 | 2.59 | 3.11 | 3.29 | 2.06 | 3.04 ± 0.52 |
| Polysaccharide | 277.65 | 290.49 | 194.03 | 146.39 | 147.76 | 127.11 | 142.29 | 127.74 | 181.68 ± 66.59 |
| **B. Metabolites (μmole/g dry weight)** | | | | | | | | | |
| Succinate | 3.78 | 3.18 | 0.76 | 0.67 | 0.93 | 1.43 | 1.39 | 0.75 | 1.61 ± 1.20 |
| Malate | 1.41 | 1.01 | 1.93 | 1.80 | 2.40 | 1.22 | 1.87 | 2.45 | 1.76 ± 0.52 |
| α-keto-Glutarate | 1.80 | 0.02 | *n.d. | 0.03 | 0.05 | n.d. | 0.06 | n.d. | - |
| cis-Aconitate | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - |
| Citrate | 0.09 | 0.06 | n.d. | n.d. | 0.07 | n.d. | n.d. | n.d. | - |
| Propionate | 2.52 | 3.14 | 2.62 | 2.45 | 2.97 | 4.40 | 4.75 | 3.87 | 3.34 ± 0.89 |
| iso-Butyrate | 0.62 | 0.44 | 0.61 | 0.62 | 0.61 | 0.47 | 0.46 | 0.84 | 0.58 ± 0.13 |
| Butyrate | 0.91 | 0.61 | 0.88 | 0.79 | 0.83 | 0.69 | 0.47 | 1.15 | 0.79 ± 0.21 |
| iso-Valerate | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - |
| Alanine | 17.46 | 38.33 | 27.14 | 23.99 | 25.63 | 29.89 | 29.89 | 8.56 | 25.11 ± 8.93 |
| Lactate | 4.09 | 2.86 | 4.88 | 5.61 | 4.36 | 5.32 | 4.70 | 9.56 | 5.17 ± 1.96 |
| Pyruvate | 0.02 | 0.02 | 0.54 | 0.56 | 0.39 | 0.09 | 0.24 | 0.13 | 0.25 ± 0.22 |
| **C. Sum of metabolites (μmole/g dry weight)** | 32.70 | 49.67 | 39.36 | 36.52 | 38.24 | 43.51 | 43.83 | 27.31 | 38.61 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | 23.76 | 44.65 | 30.52 | 27.11 | 29.53 | 35.72 | 36.03 | 13.18 | 30.06 |

*n.d. = not detectable

TABLE 11

CONCENTRATIONS OF POLYSACCHARIDE AND SOME METABOLITES OF GEUKENSIA DEMISSA AFTER TWO DAYS ACCLIMATION AT 15°C WITH AERATED RIVER WATER (JUNE 1979)

| | Mussels | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | $\bar{x} \pm$ S.D. (n = 3) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | |
| Size (cm) | 12.00 | 11.50 | 12.00 | 11.83 ± 0.29 |
| Dry weight (g) | 3.70 | 3.58 | 1.40 | 2.89 ± 1.29 |
| Polysaccharide | 63.63 | 190.16 | 37.03 | 96.94 ± 81.82 |
| **B. Metabolites (μmole/g dry weight)** | | | | |
| Succinate | 0.77 | 0.50 | 1.59 | 0.95 ± 0.57 |
| Malate | 2.41 | 1.62 | 1.35 | 1.79 ± 0.55 |
| α-keto-Glutarate | 0.04 | 0.25 | 0.57 | 0.29 ± 0.27 |
| cis-Aconitate | *n.d. | n.d. | n.d. | - |
| Citrate | 0.14 | 0.16 | n.d. | - |
| Propionate | 3.36 | 1.93 | - | 2.65 ± 1.01 |
| iso-Butyrate | 0.56 | 0.48 | - | 0.52 ± 0.06 |
| Butyrate | 0.77 | 0.46 | - | 0.62 ± 0.22 |
| iso-Valerate | n.d. | n.d. | - | - |
| Alanine | 35.39 | 32.04 | 13.20 | 26.88 ± 11.96 |
| Lactate | 5.93 | 3.74 | 10.67 | 6.78 ± 3.54 |
| Pyruvate | 0.01 | 0.62 | 0.24 | 0.29 ± 0.31 |
| **C. Sum of metabolites (μmole/g dry weight)** | | | | |
| | 49.38 | 41.80 | - | 40.77 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | | | | |
| | 39.52 | 34.47 | - | 30.48 |

*n.d. = not detectable

TABLE 12

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF

GEUKENSIA DEMISSA AFTER ONE DAY OF HYPOXIA (JUNE 1979)

| | Mussels | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | $\bar{X} \pm$ S.D. (n = 3) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | |
| Size (cm) | 11.00 | 11.50 | 11.50 | 11.33 ± 0.29 |
| Dry weight (g) | 3.56 | 3.20 | 4.03 | 3.60 ± 0.42 |
| Polysaccharide | 149.18 | 133.09 | 159.48 | 147.25 ± 13.30 |
| **B. Metabolites (μmole/g dry weight)** | | | | |
| Succinate | 3.20 | 3.63 | 1.17 | 2.67 ± 1.31 |
| Malate | 1.50 | 1.26 | 1.21 | 1.32 ± 0.16 |
| α-keto-Glutarate | 0.06 | 0.04 | 0.04 | 0.05 ± 0.01 |
| cis-Aconitate | *n.d. | n.d. | n.d. | - |
| Citrate | 0.03 | 0.03 | 0.03 | 0.03 ± 0.00 |
| Propionate | 1.99 | 2.62 | 2.09 | 2.23 ± 0.34 |
| iso-Butyrate | 0.44 | 0.51 | 0.34 | 0.43 ± 0.09 |
| Butyrate | 0.65 | 0.72 | 0.50 | 0.62 ± 0.11 |
| iso-Valerate | n.d. | n.d. | n.d. | - |
| Alanine | 46.37 | 25.93 | 46.43 | 39.58 ± 11.82 |
| Lactate | 4.33 | 6.70 | 5.34 | 5.46 ± 1.19 |
| Pyruvate | 0.11 | 0.10 | 0.14 | 0.12 ± 0.02 |
| **C. Sum of metabolites (μmole/g dry weight)** | | | | |
| | 58.68 | 41.54 | 57.29 | 52.51 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | | | | |
| | 51.56 | 32.18 | 49.69 | 44.48 |

*n.d. = not detectable

TABLE 13

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF GEUKENSIA DEMISSA AFTER TWO DAYS OF HYPOXIA (JUNE 1979)

| | Mussels | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | $\bar{x} \pm$ S.D. (n = 3) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | |
| Size (cm) | 10.50 | 12.50 | 12.00 | 11.67 ± 1.04 |
| Dry weight (g) | 3.36 | 3.29 | 3.28 | 3.31 ± 0.04 |
| Polysaccharide | 166.10 | 134.12 | 157.72 | 152.65 ± 16.58 |
| **B. Metabolites (μmole/g dry weight)** | | | | |
| Succinate | 1.66 | 2.72 | 6.03 | 3.47 ± 2.28 |
| Malate | 1.33 | 2.31 | 1.47 | 1.70 ± 0.53 |
| α-keto-Glutarate | 0.05 | 0.05 | 0.06 | 0.05 ± 0.01 |
| cis-Aconitate | 0.04 | *n.d. | n.d. | - |
| Citrate | 0.14 | 0.09 | n.d. | - |
| Propionate | 6.54 | 9.91 | 4.62 | 7.02 ± 2.68 |
| iso-Butyrate | 0.33 | 0.53 | 0.64 | 0.50 ± 0.16 |
| Butyrate | 0.51 | 0.80 | 0.81 | 0.71 ± 0.17 |
| iso-Valerate | 0.08 | n.d. | n.d. | - |
| Alanine | 41.72 | 39.49 | 47.53 | 42.91 ± 4.15 |
| Lactate | 3.47 | 5.51 | 5.62 | 4.86 ± 1.21 |
| Pyruvate | 0.52 | 0.06 | n.d. | - |
| **C. Sum of metabolites (μmole/g dry weight)** | 56.39 | 61.47 | 66.78 | 61.22 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | 49.92 | 52.12 | 58.18 | 53.40 |

*n.d. = not detectable

TABLE 14

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF GUKENSIA DEMISSA AFTER THREE DAYS OF HYPOXIA (JUNE 1979)

| | Mussels | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | $\bar{x} \pm$ S.D. (n = 3) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | |
| Size (cm) | 11.50 | 11.50 | 12.00 | 11.67 ± 0.29 |
| Dry weight (g) | 2.50 | 3.84 | 3.60 | 3.31 ± 0.71 |
| Polysaccharide | 86.70 | 267.72 | 207.11 | 187.18 ± 92.14 |
| **B. Metabolites (μmole/g dry weight)** | | | | |
| Succinate | 3.51 | 2.13 | - | 2.82 ± 0.98 |
| Malate | 1.69 | 0.88 | - | 1.28 ± 0.57 |
| α-keto-Glutarate | 0.04 | 0.01 | - | 0.03 ± 0.02 |
| cis-Aconitate | *n.d. | n.d. | - | - |
| Citrate | 0.11 | 0.01 | - | 0.06 ± 0.07 |
| Propionate | 22.35 | 20.69 | 16.44 | 19.83 ± 3.05 |
| iso-Butyrate | 0.73 | 0.57 | 0.52 | 0.61 ± 0.11 |
| Butyrate | 1.10 | 0.74 | 0.71 | 0.85 ± 0.22 |
| iso-Valerate | n.d. | n.d. | n.d. | - |
| Alanine | 53.52 | 53.65 | 34.95 | 47.37 ± 10.76 |
| Lactate | 7.95 | 5.27 | 5.56 | 6.26 ± 1.47 |
| Pyruvate | 0.42 | 0.11 | 0.04 | 0.19 ± 0.20 |
| **C. Sum of metabolites (μmole/g dry weight)** | | | | |
| | 91.42 | 84.06 | - | 79.30 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | | | | |
| | 79.38 | 76.47 | - | 70.02 |

*n.d. = not detectable

TABLE 15

CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF

GEUKENSIA DEMISSA AFTER FOUR DAYS OF HYPOXIA (JUNE 1979)

| | Mussels | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | $\bar{X} \pm$ S.D. ($n = 3$) |
| **A. Mussels (polysaccharide in mg/g dry weight)** | | | | |
| Size (cm) | 12.00 | 11.50 | 11.50 | 11.67 ± 0.29 |
| Dry weight (g) | 4.19 | 3.09 | 2.80 | 3.36 ± 0.73 |
| Polysaccharide | 123.03 | 84.98 | 126.31 | 111.44 ± 22.97 |
| **B. Metabolites (μmole/g dry weight)** | | | | |
| Succinate | 3.95 | 1.65 | 3.75 | 3.12 ± 1.27 |
| Malate | 0.72 | 1.20 | 0.71 | 0.88 ± 0.28 |
| α-keto-Glutarate | *n.d. | 0.03 | 0.05 | 0.04 ± 0.01 |
| cis-Aconitate | n.d. | n.d. | n.d. | - |
| Citrate | 0.07 | 0.03 | 0.06 | 0.05 ± 0.02 |
| Propionate | 21.25 | 50.61 | 58.29 | 33.38 ± 15.33 |
| iso-Butyrate | 0.61 | 0.64 | 0.71 | 0.65 ± 0.05 |
| Butyrate | 0.81 | 1.08 | 0.96 | 0.95 ± 0.14 |
| iso-Valerate | n.d. | n.d. | 0.22 | - |
| Alanine | 45.14 | 51.42 | 61.52 | 52.69 ± 8.26 |
| Lactate | 4.23 | 6.55 | 7.45 | 6.08 ± 1.66 |
| Pyruvate | 0.07 | 0.14 | 0.15 | 0.12 ± 0.04 |
| **C. Sum of metabolites (μmole/g dry weight)** | 76.85 | 113.35 | 103.87 | 97.96 |
| **D. Sum of succinate, propionate and alanine (μmole/g dry weight)** | 70.34 | 103.68 | 93.56 | 89.19 |

*n.d. = not detectable

TABLE 16

AVERAGE CONCENTRATIONS OF POLYSACCHARIDE AND KEY METABOLITES OF GEUKENSIA DEMISSA AFTER FIELD COLLECTION (-2 DAYS), TWO DAYS ACCLIMATION (0 DAY) AND ONE TO FOUR DAYS OF HYPOXIA

| | Days of Hypoxia | | | | | |
|---|---|---|---|---|---|---|
| | -2 | 0 | 1 | 2 | 3 | 4 |
| A. Mussel (polysaccharide in mg/g dry weight) | | | | | | |
| Size (cm) | 11.75 | 11.83 | 11.33 | 11.67 | 11.67 | 11.67 |
| Dry weight (g) | 3.04 | 2.89 | 3.60 | 3.31 | 3.31 | 3.36 |
| Polysaccharide | 181.68 | 96.94 | 147.25 | 152.65 | 187.18 | 111.44 |
| B. Metabolites (μmole/g dry weight) | | | | | | |
| Succinate | 1.61 | 0.95 | 2.67 | 3.47 | 2.82 | 3.12 |
| Malate | 1.76 | 1.79 | 1.32 | 1.70 | 1.28 | 0.88 |
| α-keto-Glutarate | - | 0.29 | 0.05 | 0.05 | 0.03 | 0.04 |
| Propionate | 3.34 | 2.65 | 2.23 | 7.02 | 19.83 | 33.38 |
| iso-Butyrate | 0.58 | 0.52 | 0.43 | 0.50 | 0.61 | 0.65 |
| Butyrate | 0.79 | 0.62 | 0.62 | 0.71 | 0.85 | 0.95 |
| Alanine | 25.11 | 26.88 | 39.58 | 42.91 | 47.37 | 52.69 |
| Lactate | 5.17 | 6.78 | 5.46 | 4.86 | 6.26 | 6.08 |
| Pyruvate | 0.25 | 0.29 | 0.12 | 0.29 | 0.19 | 0.12 |
| C. Sum of metabolites (μmole/g dry weight) | | | | | | |
| | 38.89 | 40.77 | 52.48 | 61.51 | 79.24 | 97.91 |
| D. Sum of succinate, propionate and alanine (μmole/g dry weight) | | | | | | |
| | 30.06 | 30.48 | 44.48 | 53.40 | 70.02 | 89.19 |

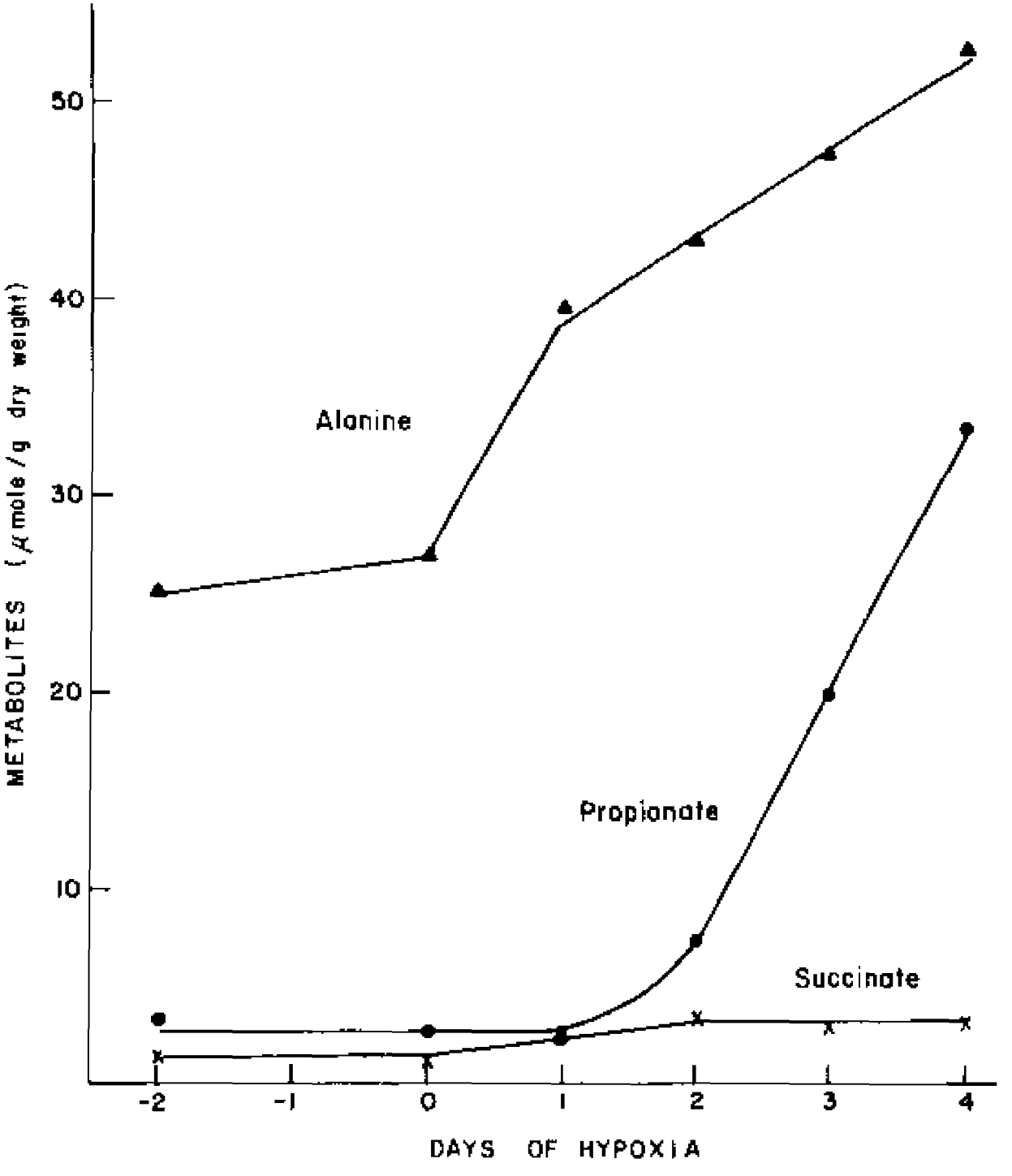
TABLE 17

AVERAGE CONCENTRATIONS OF SUCCINATE, PROPIONATE AND ALANINE OF MUSSELS, GEUKENSIA DEMISSA AFTER FIELD COLLECTION (-2 DAYS), TWO DAYS OF ACCLIMATION (0 DAY) AND ONE TO FOUR DAYS OF HYPOXIA

| | Metabolites (μmole/g dry weight) | | | |
|---|---|---|---|---|
| Days of Hypoxia | Succinate | Propionate | Alanine | Sum |
| -2 | 1.61 | 3.34 | 25.11 | 30.06 |
| 0 | 0.95 | 2.65 | 26.88 | 30.48 |
| 1 | 2.67 | 2.23 | 39.58 | 44.48 |
| 2 | 3.47 | 7.02 | 42.91 | 53.40 |
| 3 | 2.82 | 19.83 | 47.37 | 70.02 |
| 4 | 3.12 | 33.38 | 52.69 | 89.19 |
| Accumulation during four days of hypoxia | | | | |
| | 2.17 | 30.73 | 25.81 | 58.71 |
| % Accumulation | | | | |
| | 3.70 | 52.34 | 43.96 | 100.00 |

FIGURE 7. THE TIME DEPENDENT ACCUMULATION OF SUCCINATE, PROPIONATE AND ALANINE IN GEUKENSIA DEMISSA.

Immediately after field collection (-2 days), two days acclimation with aerated water (0 day), one to four days of hypoxia (1 to 4 days, respectively)


METABOLITES (μ mole / g dry weight)
50
40
30
20
10
Alanine
Propionate
Succinate
-2
-1
0
1
2
3
4
DAYS OF HYPOXIA

weight after the second day and further increased sharply and linearly with time of hypoxia. After four days of hypoxia, a concentration of 33.38 μg mole/g dry weight of propionate was noted. This represents a ten-fold increase in propionate concentration (Table 17, Figure 7).

The initial concentration of alanine was high (26.88 μmole/g dry weight). After the first day under hypoxic conditions, alanine levels increased to 39.58 μmole/g dry weight. From the second day on, a slower but linear accumulation with time was observed (to 52.69 μmole/g dry weight) which was 1.96 times the original concentration (Table 17, Figure 7).

After four days of hypoxia, the μmolar accumulation of propionate and alanine was similar and much higher than succinate, but no increase was observed in malate, α-keto-glutarate, iso-butyrate, butyrate, lactate and pyruvate (Table 16).

The size and dry weight of the mussels used in this study were consistent. However, since the concentrations of polysaccharide were so variable, polysaccharide content was not useful as a further indicator of the effect of hypoxia.

# DISCUSSION

## Accumulation of Intermediates

This study demonstrates that tissue levels of succinate, propionate, and alanine increase in Geukensia demissa held under hypoxic conditions. In contrast to the vertebrates, lactate is found not to increase in Geukensia demissa under hypoxic conditions. These observations extend and confirm the work of other investigators who have found that other lamellibranch molluscs, including Mytilus edulis (Kluytmans et al., 1975), Anodonta cygnea (Gäde et al., 1975), and Cardium edule (Gäde, 1975), respond to hypoxic conditions in much the same manner (Table 18). Therefore, it would seem apparent that the accumulation of succinate, propionate, and alanine under hypoxic conditions is characteristic of many, if not all, lamellibranch molluscs.

With respect to alanine accumulation, a wide range is reported in this study (3.40 to 25.8 μmole/g dry weight) which represents 7.87 to 43.96% of total accumulation (Table 18). Kluytmans et al. (1977) reported that alanine only transiently increased during the first hour of anoxia in Mytilus edulis and then eventually diminished to near its original level. De Zwaan and his associates also suggested that alanine was only the initial end product of bivalve anaerobic metabolism (de Zwaan and van Marrewijk, 1973a; de Zwaan et al., 1975; Gäde et al., 1975; de Zwaan and Wijsman, 1976; de Zwaan et al., 1976; Kluytmans et al., 1977). However, this study shows a substantial increase in the concentration of alanine from 26.88 to 39.58 μmole/g dry weight

during the first day of hypoxia, followed by a linear accumulation with time to 52.69 μmole/g dry weight after four days of hypoxia (Figure 7, Table 17).

Some evidence indicates that the production of succinate and propionate are correlated and that the accumulation of propionate follows that of alanine (Kluytmans et al., 1977). This relationship is clearly illustrated in Figure 7.

Total accumulation of succinate, propionate and alanine ranged from 33.00 to 75.20 μmole/g dry weight in the five studies and is shown in Table 18. Because no significant lactate accumulation was observed in any of these studies, lactate fermentation is believed to have only limited importance (Gäde et al., 1975).

In order to clearly observe these variations in metabolite concentrations, the experimental systems used in this and other studies were somewhat extreme because a submersion of Geukensia demissa in oxygen deficient water for two or more days would probably rarely occur in nature. Since some variability occurred in all studies, there are some likely sources of uncertainty. Among these uncertainties, it is still unknown to what extent animals in aerated water are actually respiring, or whether they have tissues in a hypoxic state. Other possible reasons for the variation of the succinate, propionate and alanine production are temperature (Widdows et al., 1979), tissue (Chaplin and Loxton, 1976; Kluytmans and De Zwaan, 1976; Kluytmans and De Zwaan, 1977; Ahmad and Chaplin, 1977; Livingstone and Bayne, 1977), season (Ahmad and Chaplin, 1977), period of hypoxia, species and habitat.

This study of anaerobic metabolism of Geukensia demissa was conducted during both winter (December 1978) and summer (June 1979), and the results of two days of hypoxia at 15°C are compared in Table 19. Although no clear

TABLE 18

THE ACCUMULATION OF SUCCINATE, PROPIONATE, ALANINE AND LACTATE

DURING ANAEROBIOSIS OF SEVERAL BIVALVE SPECIES

| Metabolites | *Geukensia Demissa* | | | | *Mytilus edulis* | | *Anodonta cygnea* | | *Cardium edule* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | μmole g dry wt. | % | μmole g dry wt. | % | μmole g dry wt. | % | μmole g dry wt. | % | μmole g dry wt. | % |
| Succinate | 14.97 | 45.36 | 2.17 | 3.70 | 29.50 | 39.23 | 13.30 | 30.79 | 20.16 | 43.40 |
| Propionate | 10.71 | 32.45 | 30.73 | 52.34 | 25.70 | 34.18 | 24.80 | 57.41 | 3.14 | 6.76 |
| l-Alanine | 5.89 | 17.73 | 25.81 | 43.96 | 20.00 | 26.60 | 3.40 | 7.87 | 18.90 | 40.69 |
| l-lactate | 1.43 | 4.33 | -0.70 | - | -0.40 | - | 1.70 | 3.94 | 4.25 | 9.15 |
| Total | 33.00 | 99.87 | 58.71 | 100.00 | 75.20 | 100.00 | 43.20 | 100.01 | 46.45 | 100.00 |
| Conditions | 15°C, 51 h | | 15°C, 4 d | | 13°C, 72 h | | 15°C, 15 h | | 15°C, 15 h | |
| Source | Ho 1980 | | | | Kluytmans *et al.* 1975 | | Gäde *et al.* 1975 | | Gäde 1975 | |

TABLE 19

CONCENTRATIONS OF POLYSACCHARIDE AND SOME METABOLITES OF WINTER (DECEMBER 1978) AND SUMMER (JUNE 1979) MUSSELS, GEUKENSIA DEMISSA, AFTER TWO DAYS OF HYPOXIA

| | December 1978 $\bar{x} \pm$ S.D. ($n = 4$) | June 1979 $\bar{x} \pm$ S.D. ($n = 3$) |
|---|---|---|
| **A. Conditions** | | |
| Salinity | 17.1 o/oo | 14.4 o/oo |
| Dissolved oxygen | <0.04 ppm | <0.04 ppm |
| **B. Mussels (polysaccharide in mg/g dry weight)** | | |
| Size (cm) | 11.13 ± 0.25 | 11.67 ± 1.04 |
| Dry weight (g) | 2.52 ± 0.37 | 3.31 ± 0.04 |
| Polysaccharide | 84.50 ± 34.10 | 152.65 ± 16.58 |
| **C. Metabolites (μmole/g dry weight)** | | |
| Succinate | 19.63 ± 13.03 | 3.47 ± 2.28 |
| Malate | 2.78 ± 1.15 | 1.70 ± 0.53 |
| α-keto-Glutarate | 0.38 ± 0.29 | 0.05 ± 0.01 |
| Propionate | 14.16 ± 6.48 | 7.02 ± 2.68 |
| iso-Butyrate | 1.18 ± 0.42 | 0.50 ± 0.16 |
| Butyrate | 1.08 ± 0.20 | 0.71 ± 0.17 |
| iso-Valerate | 0.19 ± 0.06 | - |
| Alanine | 71.08 ± 7.21 | 42.91 ± 4.15 |
| Lactate | 6.43 ± 1.71 | 4.86 ± 1.21 |
| Pyruvate | 0.57 ± 0.34 | - |
| **D. Sum of metabolites (μmole/g dry weight)** | 117.48 | 61.22 |
| **E. Sum of succinate, propionate and alanine (μmole/g dry weight)** | 104.87 | 53.40 |

conclusion can be derived concerning temperature and seasonal effects, concentrations of all metabolites were higher in winter than in summer mussels. This difference may be partially attributed to salinity (17.1 o/oo in December 1978 and 14.4 o/oo in June 1979) or, more likely, the changing physiological state of the organisms during the warmer period.

Concentrations of Krebs Cycle Intermediates

The abundance of Krebs cycle intermediates in various tissues of Crassostrea virginica and whole Geukensia demissa determined in this study is shown in Table 6, those of Crassostrea virginica mantle and other organisms are compiled in Table 20. Although precise comparison may be inappropriate because of variations in experimental conditions and analytical methods, some tendencies are noteworthy. Succinate concentration in nmole/g wet weight varied from 38 to 510 in Geukensia demissa and from 139 to 479 in Crassostrea virginica as determined in this study (Table 6). Succinate was much lower than the 29,661 nmole/g wet weight in oyster mantle reported by Hammen and Wilbur (1959) but was similar to 240 in Mytilus edulis (De Zwaan and Zandee, 1972b). Concentrations of Krebs cycle intermediates in Crassostrea virginica and Mytilus edulis determined from this study are also comparable to those of rat (Kuksis and Prioreschi, 1967) and guinea pig (de Silva, 1971).

Metabolic Scheme for Molluscan Anaerobiosis

As a conceptual tool for focusing on further experimentation in anaerobiosis with lamellibranch molluscs, the following hypothetical scheme (Figure 8) which builds on that of other workers (Gilles, 1972; Hochachka and Mustafa, 1972; Hochachka et al., 1973; De Zwaan et al., 1973; De Zwaan et al., 1976; Hochachka, 1976; Collicutt and Hochachka, 1977) is proposed. The salient

TABLE 20

KREBS CYCLE INTERMEDIATES IN REPRESENTATIVE ORGANISMS

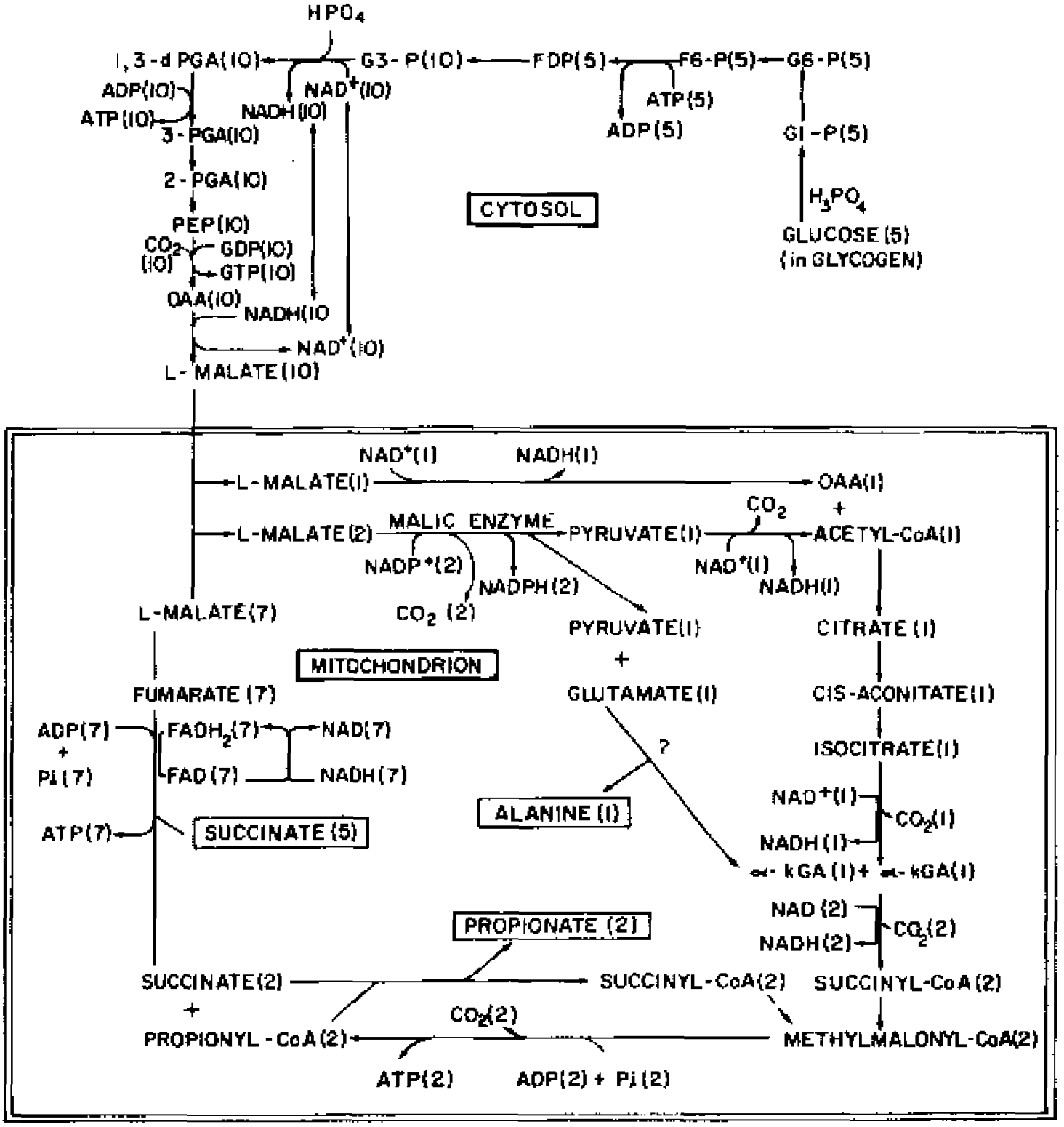
| Acid | Oyster *Crassostrea virginica* Mantle | Oyster *Crassostrea virginica* Mantle | Mussel *Mytilus edulis* Meat | Rat Heart | Rat Muscle | Guinea Pig Heart | Guinea Pig Muscle |
|---|---|---|---|---|---|---|---|
| Citrate | 234 | * | * | 208 | 26 | 157 | 128 |
| cis-Aconitate | 7 | * | * | 57 | 6 | <2 | <2 |
| iso-Citrate | 79 | * | * | 31 | 5 | 43 | 35 |
| α-keto-Glutarate | 55 | * | * | 144 | 7 | 10 | 5 |
| Succinate | 139 | 29661 | 240 | 720 | 8 | 128 | 49 |
| Fumarate | 391 | * | * | 948 | 129 | 15 | 11 |
| Malate | 516 | * | * | 261 | 37 | 502 | 368 |
| Source | Ho & Zubkoff | Hammen & Wilbur 1959 | de Zwaan & Zandee 1972 | Kuksis & Prioreschi 1967 | | De Silva 1971 | |
| Method | GLC | Titration | Enzymatic | GLC | | GLC | |

Units: nmole/g wet weight

*: not reported

FIGURE 8. A HYPOTHETICAL SCHEME OF GEUKENSIA DEMISSA ANAEROBIC METABOLISM.

Double lined box is mitochondrion.




**OVERALL REACTION:**

5 GLUCOSE + 1 GLUTAMATE + 2 $CO_2$ + 24 ADP + 24 Pi ⟶ 5 SUCCINATE + 1 ALANINE + 2 PROPIONATE + 2 SUCCINYL-CoA + 24 ATP

features of this scheme are:

(1) the Embden-Meyerhof-Parnas scheme of glycolysis,

(2) carbon dioxide fixation,

(3) the Krebs cycle,

(4) transamination,

(5) part of the electron transport chain for fumarate reduction, and

(6) methyl malonyl-Co A as an intermediate in the formation of propionate.

This information is advanced one step further with respect to cytosol-mitochodrial compartmentation and redox balance. To date, this is the only bivalve anaerobic metabolic scheme which contains all these essential considerations and differs from others by achieving redox balance.

As a point of reference, this proposed scheme starts from glycogen. Glycogen, known to be the most important carbohydrate reserve for bivalve metabolism, is broken down to pyruvate via the Embden-Meyerhof-Parnas pathways. At this point, the major branchpoint of aerobic and anaerobic metabolism in the cytosol is reached. This branchpoint is controlled by pyruvate kinase for pyruvate formation and by phosphoenolpyruvate carboxykinase for oxaloacetate formation, a component of the Krebs cycle.

The Krebs cycle could also be entered by the formation of malate, catalyzed by the action of malic enzyme on phosphoenolpyruvate and carbon dioxide. Malate may be able to cross the mitochondrial membrane, as in higher organisms (Lehninger, 1970) and thus, the reactions of the cytosol may be coupled through this substrate. Malate dehydrogenase has been localized in both the cytosol and the mitochondrion of the giant scallop, Placapecten magellanicus (O'Doherty and Feltham, 1971).

The further conversion of malate to succinate by reversal of the steps of the Krebs cycle has been extensively studied. These steps are catalyzed by fumarase and fumarate reductase. Both enzymes have been localized in the mitochondrion of the clam, Rangia cuneata (Chen and Awapara, 1969) and the scallop, Placopecten magellanicus (O'Doherty and Peltham, 1971), and Mytilus edulis (Addink and Veenhof, 1975). In this pathway, high energy phosphate is generated and NADPH is produced by several reductive steps in the mitochondrion.

In considering the pivital role that malate may assume in anaerobiosis, the partial sequence of the Krebs cycle
(malate → oxaloacetate → citrate → iso-citrate → α-keto-glutarate)
has been incorporated in this hypothetical anaerobic scheme. This interpretation differs from that of Hochachka and his associates who argued that although citrate synthetase has normal activity in molluscan tissue, these reactions were undesirable under anoxic conditions because an increase in the reductive state of the cell would result (Hochachka et al., 1975; Fields, 1976; Fields et al., 1976; Fields and Hochachka, 1976; Baldwin, 1978). In the scheme proposed here (Figure 8, inside box), redox balance is maintained with these forward reactions and ATP can be generated by reoxidizing NADH. Other enzymes which catalyze these reactions malate dehydrogenase (O'Doherty and Peltham, 1971); malate enzyme (de Zwaan and van Marrewijk, 1973b); isocitrate dehydrogenase (Addink and Veenhof, 1975) have been detected in the molluscan mitochondrion.

In contrast to the central position of malate in anaerobiosis, all studies on bivalve anaerobic metabolism have detected a certain degree of alanine accumulation (Table 18). The source of the alanine is still

questionable but, at present, the transamination of pyruvate with amino acids is the most widely accepted scheme. The enzyme, alanine amino-transferase, localized in molluscan mitochondria (Addink and Veenhof, 1975), is indirect evidence in support of transamination as the source of alanine formation. However, alternative schemes, such as the alanine dehydrogenase reaction, have not been carefully examined. Alanine plays an important role in regulation of aerobic and anaerobic metabolism because it is a strong inhibitor of pyruvate kinase activity and is a positive modulator to phosphoenolpyruvate carboxykinase (Mustafa and Hochachka, 1973a, b).

As in the case of alanine, propionate accumulates but with a different time course. The mechanism of propionate formation in anaerobic tissue has been recently studied in parasitic helminths (Tkachuck et al., 1977; Saz et al., 1978). Available evidence is consistent with the following scheme:

$$\text{Succinyl-CoA} \rightarrow \text{Methylmalonyl-CoA} \quad (4)$$

$$\text{Methylmalonyl-CoA} + E \rightarrow CO_2 - E + \text{Propionyl-CoA} \quad (5)$$

$$\text{Propionyl-CoA} + \text{Succinate} \rightarrow \text{Propionate} + \text{Methylmalonyl-CoA} \quad (6)$$

$$CO_2 - E + ADP + Pi \rightarrow CO_2 + ATP + E \ . \quad (7)$$

---

Sum

$$\text{Succinyl-CoA} + \text{Succinate} + ADP + Pi \rightarrow \text{Propionate} + \text{Methylmalonyl-CoA} + CO_2 + ATP \quad (8)$$

Although the metabolic formation of propionate in molluscs has not been examined, it is not unreasonable to postulate a similar pathway.

In addition to the accumulation of intermediary metabolites and the enzymes associated with them, redox balance is also an important consideration. To facilitate the discussion of redox balance, energy-rich phosphate generation, metabolite accumulation and compartmentation, numbers are assigned to these compounds in the model (Figure 8). In keeping with carbon balance, it is assumed that one mole of glutamate is mobilized simultaneously with every five moles of glucosides consumed. A balance sheet is presented in Table 21 where redox balance is maintained separately in the cytosol and the mitochondrion. The overall reaction may be written as:

5 Glucosides + 1 Glutamate + 2 $CO_2$ + 2 CoASH + 24 ADP + 24 Pi →

5 Succinate + 1 Alanine + 2 Propionate + 2 Succinyl-CoA + 24 ATP , (9)

In the above scheme (9), glutamate may be in an internal pool or derived from protein decomposition. Carbon dioxide may be absorbed from water or generated by decalcification of the shell under the anoxic condition, as proposed by Crenshaw and Neff (1969) and Wijsman (1975) for Mytilus edulis. Of the 24 ATP generated, seven are obtained through oxidative phosphorylation as a result of the flavin-linked fumarate reductase reaction (Saz, 1971), whereas the others are all through substrate phosphorylation. The energy efficiency is 4.8 moles of ATP from the simultaneous consumption of one mole of glucose and one-fifth mole of glutamate; a comparable efficiency was estimated as six moles of ATP from each mole of glucose by De Zwaan et al. (1976).

Furthermore, the two moles of succinyl-CoA may be converted to propionate; thus, the overall reaction for the next five moles of glucosides is:

TABLE 21

BALANCE SHEET OF ANAEROBIC METABOLISM OF

GEUKENSIA DEMISSA ACCORDING TO THE HYPOTHETICAL SCHEME

| Reactions | NADH $FADH_2$ | Energy Rich Phosphate | $CO_2$ Production |
|---|---|---|---|
| A. Cytoplasm | | | |
| Glucoside (5) (in Glycogen) | | | |
| F-6-P(5) → FDP(5) | | -5 | |
| G-3-P(10) → 1,3-dPGA(10) | 10 | | |
| 1,3-dPGA(10) → 3-PGA(10) | | 10 | |
| PEP (10) → OAA (10) | | 10 | -10 |
| OAA (10) → Malate (10) | -10 | | |
| B. Mitochondrion | | | |
| Glutamate (1) mobilized | | | |
| Malate (1) → OAA (1) | 1 | | |
| Malate (2) → Pyruvate (2) | 2 | | 2 |
| Pyruvate (1) → Acetyl-CoA (1) | 1 | | 1 |
| iso-Citrate (1) → α-kGA (1) | 1 | | 1 |
| α-kGA (2) → Succinyl-CoA (2) | 2 | | 2 |
| Methylmalonyl-CoA (2) → Propionyl-CoA (2) | | 2 | 2 |
| Fumarate (7) → Succinate (7) | -7 | 7 | |
| Sum | 0 | 24 | -2 |

5 Glucosides + 1 Glutamate + 2 $CO_2$ + 24 ADP + 24 Pi + 26 CoASH →

5 Succinate + 1 Alanine + 2 Propionate + 2 Succinyl-CoA + 24 ATP

5 Glucosides + 1 Glutamate + 2 Succinyl-CoA + 2 CoASH + 26 ADP + 26 Pi →

3 Succinate + 1 Alanine + 4 Propionate + 4 Succinyl-CoA + 26 ATP . (10)

Propionate formation is coupled with succinate as shown in this model (Figure 8). Thus, this coupling may explain the lag of propionate accumulation and the non-accumulation of succinate under the hypoxic condition (Figure 7). In contrast to anaerobic molluscan metabolism, the pathways of aerobic metabolism in molluscs would be similar to that of higher organisms. The net reaction may be written as:

1 Glucoside + 6 $O_2$ → 6 $CO_2$ + 6 $H_2O$ + 37 ATP . (11)

Of the 37 moles of ATP generated aerobically (Lehninger, 1970), 32 moles are through oxidative phosphorylation with oxygen as final electron acceptor, the primary reason for the greater efficiency of aerobic metabolism. Thus, bivalve anaerobic metabolism is somewhere between the highly efficient aerobic metabolism and the less efficient production of ethanol by fermentation or accumulation of lactate by anaerobic muscle glycolysis.

Although anaerobic metabolism as presented here is only 13% as efficient as aerobic metabolism in energy production, there was no clear indication of increased glucose consumption under the hypoxic condition. A possible explanation is that bivalves may decrease their energy utilization under oxygen deficiency, as suggested by McCarthy (1969), Bayne (1971, 1973, 1975), De Zwaan and Wijsman (1976), De Zwaan et al. (1976) and Philley (1978).

In conclusion, this study determined that succinate, propionate and alanine accumulated when the ribbed mussels, Geukensia demissa were held hypoxically. This result is consistent with those obtained for Mytilus edulis, Anodonta cygnea and Cardium edule by other investigators. Thus, the evidence presented from this study and that of the literature indicates that most bivalve molluscs have similar metabolic pathways.

## SUMMARY

1. Anaerobic metabolism has been investigated in the intertidal bivalve, Geukensia demissa, in order to generalize and elaborate on earlier studies, using other bivalves. Geukensia demissa is taxonomically and ecologically similar to Mytilus edulis and anaerobic metabolism is believed to be essential for survival during air exposure periods.

2. Specimens of Geukensia demissa were maintained in either aerated or hypoxic water for up to four days. Major metabolites and polysaccharide of each mussel were then analyzed and compared.

3. To date, this is the first study of bivalve anaerobic metabolism in which all those compounds were measured in each individual organism instead of pooled tissues.

4. Both defined term and time course studies were conducted using mussels under winter and summer conditions.

5. A sensitive gas-liquid chromatographic method which analyzes seven Krebs cycle intermediates simultaneously was adopted for this investigation after satisfactory tests with oysters, Crassostrea virginica. Within the group of Krebs cycle intermediates, only succinate has been analyzed by other investigators of bivalve anaerobic metabolism.

6. In Geukensia demissa, succinate, propionate and alanine accumulated hypoxically but not malate, α-keto-glutarate, cis-aconitate, citrate, iso-butyrate, butyrate, iso-valerate, lactate or pyruvate. The polysaccharide content of each mussel was quite different and did not show a clear indication of decrease under the hypoxic condition.

7. Propionate production was shown to follow succinate accumulation, which corroborates an earlier report for Mytilus edulis. The linear increase of alanine concentration with increasing duration of the hypoxic period observed in this study has not been reported previously.

8. Results obtained from this study are consistent with those derived for Mytilus edulis, Anodonta cygnea and Cardium edule by other investigators. Thus, these data suggest that similar anaerobic metabolic pathways exist among bivalves.

9. A hypothetical scheme of bivalve anaerobic metabolism is proposed in which redox balance is achieved in both cytosol and mitochondria. This scheme incorporates parts of the Embden-Meyerhof-Parnas glycolytic pathways, carbon dioxide fixation, the Krebs cycle, transamination and pathways for propionate formation. To date, this is the only bivalve anaerobic metabolic scheme which contains all these essential considerations.

# BIBLIOGRAPHY


Addink, A.D.F. and P.R. Veenhof. 1975. Regulation of mitochondrial matrix enzymes in Mytilus edulis L. p. 109-119. In H. Barnes (ed.), Proc. 9th Europ. Mar. Biol. Symp. The Aberdeen University Press, Aberdeen.

Ahmad, T.A. and A.E. Chaplin. 1977. The intermediary metabolism of Mytilus edulis (L.) and Cerastoderma edule (L.) during exposure to the atmosphere. Biochem. Soc. Trans. 5:1320-1323.

Augenfeld, J.M. 1966. Lactic dehydrogenase activities in invertebrates in relation to environment and mode of gas exchange. Comp. Biochem. Physiol. 18:983-985.

Baldwin, J. and A.M. Opie. 1978. On the role of octopine dehydrogenase in the adductor muscles of bivalve molluscs. Comp. Biochem. Physiol. 61B:85-92.

Bayne, B.L. 1971. Ventilation, the heart beat and oxygen uptake by Mytilus edulis in declining oxygen tension. Comp. Biochem. Physiol. 40:1065-1085.

Bayne, B.L. 1973. Aspects of the metabolism of Mytilus edulis L. during starvation. Neth. J. Sea Res. 7:399-410.

Bayne, B.L. 1975. Aspects of physiological condition in Mytilus edulis L., with special reference to the effects of oxygen tension and salinity. p. 213-238. In H. Barnes (ed.), Proc. 9th Europ. Mar. Biol. Symp. The Aberdeen University Press, Aberdeen.

Booth, C.E. and C.P. Mangum. 1978. Oxygen uptake and transport in the lamellibranch mollusc, Modiolus demissus. Physiol. Zool. 51:17-32.

Brand, T. von, H.D. Baernstein, and B. Mehlman. 1950. Studies on the anaerobic metabolism and the aerobic carbohydrate consumption of some fresh water snails. Biol. Bull. Woods Hole 98:266-276.

Chaplin, A.E. and J. Loxton. 1976. Tissue differences in the response of the mussel Mytilus edulis to experimentally induced anaerobiosis. Biochem. Soc. Trans. 4:437-441.

Chen, C. and J. Awapara. 1969. Intracellular distribution of enzymes catalyzing succinate production from glucose in Rangia mantle. Comp. Biochem. Physiol. 30:727-737.

Collicutt, J.M. and P.W. Hochachka. 1977. The anaerobic oyster heart: coupling of glucose and aspartate fermentation. J. Comp. Physiol. B. Metab. Transp. Funct. 115:147-157.

Crenshaw, M.S. and J.M. Neff. 1969. Decalcification and the mantle-shell interface in molluscs. Am. Zool. 9:881-885.

Ellington, W.R. 1977. Aerobic and anaerobic degradation of glucose by the estuarine sea anemone, Diadumene leucolena. Comp. Biochem. Physiol. 58B:173-175.

Fields, J.H.A. 1976. Enzymes of the citrate branch point in the adductor muscle of the oyster. Ph.D. Thesis. University of British Columbia.

Fields, J.H.A., H. Guderley, K.B. Storey, and P.W. Hochachka. 1976. Octopus mantle citrate synthase. Can. J. Zool. 54:886-891.

Fields, J.H.A. and P.W. Hochachka. 1976. Oyster citrate synthase: control of carbon entry into the Krebs cycle of a facultative anaerobe. Can. J. Zool. 54:892-895.

Gäde, G. 1975. Anaerobic metabolism of the common cockle, Cardium edule. I. The utilization of glycogen and accumulation of multiple end products. Arch. Internat. Physiol. Biochem. 83:879-886.

Gäde, G., H. Wilps, J.H.F.M. Kluytmans, and A. de Zwaan. 1975. Glycogen degradation and end products of anaerobic metabolism in the fresh water bivalve Anodonta cygnea. J. Comp. Physiol. 104:79-85.

Gibbs, B.F., K. Itiaba, J.C. Crawhall, B.A. Cooper, and C.A. Mamer. 1973. A rapid gas chromatographic method for the quantitation of volatile fatty acids in urine. Propionic acid excretion in vitamin $B_{12}$ deficiency. J. Chromatogr. 81:65-69.

Gilles, R. 1972. Biochemical ecology of molluscs. Chem. Zool. 7:467-495.

Gruner, B. and E. Zebe. 1978. Studies on the anaerobic metabolism of earthworms. Comp. Biochem. Physiol. 60B:441-445.

Hammen, C.S. 1966. Carbon dioxide fixation in marine invertebrates. V. Rate and pathway in the oyster. Comp. Biochem. Physiol. 17:289-296.

Hammen, C.S. and K.M. Wilbur. 1959. Carbon dioxide fixation in marine invertebrates. I. The main pathway in the oyster. J. Biol. Chem. 234:1268-1271.

Ho, M.-S. and P.L. Zubkoff. 1978. Gas liquid chromatographic quantification of the Krebs cycle intermediates in the mantle tissue of the American oyster, Crassostrea virginica. (abstract). The 144th AAAS National Meeting in Washington, D.C.

Hochachka, P.W. 1976. Design of metabolic and enzymic machinery to fit lifestyle and environment. Biochem. Soc. Symp. 41:3-31.

Hochachka, P.W. and T. Mustafa. 1972. Invertebrate facultative anaerobiosis. Science 178:1056-1060.

Hochachka, P.W. and G.N. Somero. 1973. Strategies of Biochemical Adaptation. 358 p. W.B. Saunders Co., Philadelphia, London, and Toronto.

Hochachka, P.W., J. Fields, and T. Mustafa. 1973. Animal life without oxygen: Basic biochemical metabolisms. Am. Zoologist 13:543-555.

Hochachka, P.W., K.B. Storey, and J. Baldwin. 1975. Squid muscle citrate synthase: control of carbon entry into the Krebs cycle. Comp. Biochem. Physiol. 52B:193-199.

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

Kluytmans, J.H.F.M. and A. de Zwaan. 1976. Production of volatile fatty acids in bivalved molluscs. Biochem. Soc. Trans. 4:475-477.

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

Kuenzler, E.J. 1961. Structure and energy flow of a mussel population in a Georgia salt marsh. Limnol. Oceanogr. 6:191-204.

Kuksis, A. and P. Prioreschi. 1967. Isolation of Krebs cycle acids from tissues for gas chromatography. Anal. Biochem. 19:468-480.

Lehninger, A.L. 1970. Biochemistry. 833 p. Worth Publishers, Inc., New York, N.Y.

Lent, C.M. 1967. Effect of habitat on growth indices in the ribbed mussel, Modiolus (Arcuatula) demissus. Chesapeake Sci. 8:221-227.

Livingstone, D.R. and B.L. Bayne. 1974. Pyruvate kinase from the mantle tissue of Mytilus edulis L. Comp. Biochem. Physiol. 48B:481-497.

Livingstone, D.R. and B.L. Bayne. 1977. Responses of Mytilus edulis L. to low oxygen tension: anaerobic metabolism of the posterior adductor muscle and mantle tissues. J. Comp. Physiol. 114:143-155.

Loxton, J. and A.E. Chaplin. 1973. The metabolism of Mytilus edulis L. during facultative anaerobiosis. Biochem. Soc. Trans. 1:419-421.

McCarthy, R.M. 1969. The carbohydrate metabolism of the clam, Mya arenaria. Ph.D. Thesis. Georgetown University.

Mehlman, B. and T. von Brand. 1951. Further studies on the anaerobic metabolism of some fresh water snails. Biol. Bull. 100:199-205.

Moore, H.B. 1931. The muds of the Clyde sea area. III. Chemical and physical conditions; rate and nature of sedimentation; and fauna. J. Mar. Biol. Ass. U.K. 17:325-358.

Mustafa, T. and P.W. Hochachka. 1973a. Enzymes in facultative anaerobiosis of molluscs. II. Basic catalytic properties of phosphoenolpyruvate carboxykinase in oyster adductor muscle. Comp. Biochem. Physiol. 45B: 639-655.

Mustafa, T. and P.W. Hochachka. 1973b. Enzymes of facultative anaerobiosis in molluscs. III. Phosphoenolypyruvate carboxykinase and its role in aerobic-anaerobic transition. Comp. Biochem. Physiol. 45B:657-667.

O'Doherty, P.J.A. and L.A.W. Feltham. 1971. Glycolysis and gluconeogenesis in the giant scallop, Placopecten magellanicus (Gmelin). Comp. Biochem. Physiol. 38B:543-551.

Philley, J.C. 1978. Anaerobic uptake and utilization of glycine-2-$^{14}$C by the estuarine bivalve Rangia cuneata at three salinities. Comp. Biochem. Physiol. 61:565-569.

Read, K.P.H. 1962. Transamination in certain tissue homogenates of the bivalved molluscs Mytilus edulis L. and Modiolus modiolus L. Comp. Biochem. Physiol. 7:15-22.

Saz, H.J. 1969. Carbohydrate and energy metabolism of nematodes and Acanthocephala. Chem. Zool. 3:329-360.

Saz, H.J. 1970. Comparative energy metabolism of some parasitic helminths. J. Parasit. 56:634-642.

Saz, H.J. 1971. Facultative anaerobiosis in the invertebrates: pathways and control systems. Am. Zoologist 11:125-135.

Saz, H.J., G.A. Dunbar, and A.E. Gardner. 1978. Propionate formation from succinate and possible ATP generation. Abstract. The American Society of Parasitologist Meeting. Chicago, Illinois.

Schöttler, U. 1975. Über den Anaerobiosestoffwechsel von Tubifex. Verh. dt. zool. Ges. 1974:271-274.

Schöttler, U. 1977a. The energy-yielding oxidation of NADH by fumarate in anaerobic mitochondria of Tubifex sp. Comp. Biochem. Physiol. 58B:151-156.

Schöttler, U. 1977b. NADH-generating reactions in anaerobic Tubifex mitochondria. Comp. Biochem. Physiol. 58B:261-265.

Schöttler, U. 1978. Investigations of the anaerobic metabolism of the polychaete worm Nereis diversicolor M. J. Comp. Physiol. 125: 185-189.

Schöttler, U. and G. Schroff. 1976. Untersuchungen zum anaeroben Glykogenabbau bei Tubifex tubifex M. J. Comp. Physiol. 101: 133-145.

Schutgens, R.B.H., C.T. Awie, F.A. Beemer, and W.J.M. Berntsen. 1977. Rapid enzymic micromethod for the quantitative determination of L-(+)-alanine in blood and urine. Clin. Chim. Acta 80:1-5.

Sigma Chemical Company. 1977. The quantitative determination of pyruvic acid and lactic acid in whole blood at 340 nm. Tech. Bull. 726-UV and 826-UV.

Silva, E.M. de. 1971. Analysis of Krebs cycle and related acids in guinea pig tissues by gas-liquid chromatography. Anal. Chem. 43:1031-1035.

Stokes, T.M. and J. Awapara. 1968. Alanine and succinate as end-products of glucose degradation in the clam Rangia cuneata. Comp. Biochem. Physiol. 25:883-892.

Surholt, B. 1977. Production of volatile fatty acids in the anaerobic carbohydrate catabolism of Arenicola marina. Comp. Biochem. Physiol. 58B:147-150.

Theede, H., A. Ponat, K. Hiroki, and C. Schlieper. 1969. Resistance of marine bottom invertebrates to oxygen deficiency and hydrogen sulfide. Mar. Biol. 2:325-337.

Tkachuck, R.D., H.J. Saz, P.P. Weinstein, K. Finnegan, and J.F. Mueller. 1977. The presence and possible function of methylmalonyl CoA mutase and propionyl CoA carboxylase in Spirometra mansonoides. J. Parasitol. 63:769-774.

Wegener, A., A.E. Barnitt, and C.S. Hammen. 1969. Reduction of fumarate and oxidation of succinate in Crassostrea virginica (Gmelin). Life Sci. 8:335-343.

Widdows, J., B.L. Bayne, D.R. Livingstone, R.I.E. Newell, and P. Donkin. 1979. Physiological and biochemical responses of bivalve molluscs to exposure to air. Comp. Biochem. Physiol. 62A:301-308.

Wijsman, T.C.M. 1975. pH fluctuations in Mytilus edulis L. in relation to shell movements under aerobic and anaerobic conditions. p. 139-149. In H. Barnes (ed.), Proc. 9th Europ. Mar. Biol. Symp. The Aberdeen University Press, Aberdeen.

Wilps, H. and E. Zebe. 1976. The end-products of anaerobic carbohydrate metabolism in the larvae of Chironomus thummi thummi. J. Comp. Physiol. 112B:263-272.

Zebe, E. 1975. In vivo-Untersuchungen über den Glukose-Abbau bei Arenicola marina (Annelida, Polychaeta). J. Comp. Physiol. 101:133-145.

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

Zwaan, A. de and D.I. Zandee. 1972a. Body distribution and seasonal changes in the glycogen content of the common sea mussel Mytilus edulis. Comp. Biochem. Physiol. 43A:53-58.

Zwaan, A. de and D.I. Zandee. 1972b. The utilization of glycogen and accumulation of some intermediates during anaerobiosis in Mytilus edulis L. Comp. Biochem. Physiol. 43B:47-54.

Zwaan, A. de, W.J.A. van Marrewijk, and D.A. Holwerda. 1973. Anaerobic carbohydrate metabolism in the sea mussel Mytilus edulis L. Neth. J. Zool. 23:225-228.

Zwaan, A. de and W.J.A. Marrewijk. 1973a. Anaerobic glucose degradation in the sea mussel, Mytilus edulis L. Comp. Biochem. Physiol. 44B: 429-439.

Zwaan, A. de and W.J.A. Marrewijk. 1973b. Intracellular localization of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and "malic enzyme" and the absence of glyoxylate cycle enzyme in the sea mussel, Mytilus edulis L. Comp. Biochem. Physiol. 44B:1057-1066.

Zwaan, A. de, A.M.T. de Bont, and J.H.F.M. Kluytmans. 1975. Metabolic adaptations on the aerobic-anaerobic transition in the sea mussel, Mytilus edulis L. p. 121-138. In H. Barnes (ed.), Proc. 9th Europ. Mar. Biol. Symp. The Aberdeen University Press, Aberdeen.

Zwaan, A. de, J.H.F.M. Kluytmans, and D.I. Zandee. 1976. Facultative anaerobiosis in molluscs. Biochem. Soc. Symp. 41:133-168.

Zwaan, A. de and T.C.M. Wijsman. 1976. Anaerobic metabolism in Bivalvia (Mollusca): characteristics of anaerobic metabolism. Comp. Biochem. Physiol. 54B:313-324.

# APPENDIX

## LIST OF COMMON NAMES AND ABBREVIATIONS

## A. ENZYMES

| Common Name | Enzyme Code (EC) | Systematic Name |
|---|---|---|
| alanine dehydrogenase (ADH) | 1.4.1.1 | L-alanine:$NAD^+$ oxidoreductase (deaminating) |
| citrate synthase | 4.1.3.7 | citrate oxaloacetate-lyase (pro-3S-$CH_2$•$COO^-$ → acetyl-CoA) |
| fumarate reductase | 1.3.1.6 | succinate:$NAD^+$ oxidoreductase |
| glutamate-pyruvate transaminase (alanine aminotransferase) | 2.6.1.2 | L-alanine:2-oxoglutarate aminotransferase |
| iso-citrate dehydrogenase ($NAD^+$) | 1.1.1.41 | threo-$D_s$-iso-citrate:$NAD^+$ oxidoreductase (decarboxylating) |
| lactate dehydrogenase (LDH) | 1.1.1.27 | L-lactate:$NAD^+$ oxidoreductase |
| malate dehydrogenase | 1.1.1.37 | L-malate:$NAD^+$ oxidoreductase |
| malic enzyme | 1.1.1.40 | L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating) |
| octopine dehydrogenase | 1.5.1.11 | $N^2$-(1-carboxyethyl)-L-arginine:$NAD^+$ oxidoreductase (L-arginine forming) |
| phosphoenolpyruvate carboxykinase | 4.1.1.32 | GTP:oxaloacetate carboxy-lyase (transphosphorylating) |
| pyruvate carboxylase | 6.4.1.1 | pyruvate:carbon-dioxide ligase (ADP-forming) |
| pyruvate kinase | 2.7.1.40 | ATP:pyruvate 2-O-phosphotransferase |
| succinate dehydrogenase | 1.3.99.1 | succinate:(acceptor)oxidoreductase |

## B. METABOLITES

| Abbreviations | Full Name |
|---|---|
| α-kGA | α-keto-glutarate |
| 1,3-dPGA | 1,3-diphosphoglycerate |
| FDP | D-fructose-1,6-diphosphate |
| F-6-P | D-fructose-6-phosphate |
| G-1-P | D-glucose-1-phosphate |
| G-3-P | glyceraldehyde-3-phosphate |
| G-6-P | D-glucose-6-phosphate |
| OAA | oxaloacetate |
| PEP | phosphoenolpyruvate |
| 2-PGA | 2-phosphoglycerate |
| 3-PGA | 3-phosphoglycerate |
| VFA | volatile fatty acid |
| $C_2$ | acetic acid=ethanoic acid, acetate |
| $C_3$ | propionic acid=propanoic acid, propionate |
| iso-C4 | iso-butyric acid=2-methyl-propanoic acid, iso-butyrate |
| $C_4$ | n-butyric acid=n-butanoic acid, n-butyrate |
| iso-$C_5$ | iso-valeric acid=3-methyl-butanoic acid, iso-valerate |
| $C_5$ | n-valeric acid=n-pentanoic acid, n-valerate |

## C. COENZYMES

| Abbreviations | Full Name |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| CoASH | coenzyme A |
| FAD | flavine adenine dinucleotide (oxidized) |
| $FADH_2$ | flavine adenine dinucleotide (reduced) |
| GDP | guanosine diphosphate |
| GTP | guanosine triphosphate |
| IDP | inosine diphosphate |
| ITP | inosine triphosphate |
| NAD | nicotinamide adenine dinucleotide (oxidized) |
| NADH | nicotinamide adenine dinucleotide (reduced) |
| NADP | nicotinamide-adenine dinucleotide phosphate (oxidized) |
| NADPH | nicotinamide-adenine dinucleotide phosphate (reduced) |

## D. REFERENCES

Commission on Biochemical Nomenclature. 1972. Enzyme Nomenclature. 443 p. American Elsevier Publishing Co., Inc., New York, N.Y.

Lehninger, A.L. 1970. Biochemistry. 833 p. Worth Publishers, Inc., New York, N.Y.

Morrison, R.T. and R.N. Boyd. Organic Chemistry. 1204 p. Allyn and Bacon, Inc., Boston.

# VITA

## Ming-Shan Ho

Born in Amony, Fu-Chang, China, July 15, 1947. Graduated from the High School of National Taiwan Normal University, Taipei, Taiwan, July 1966; B.S., National Taiwan University, June 1970; M.S., North Carolina State University, December 1973.

Entered the School of Marine Science of the College of William and Mary as a graduate student in the Department of Environmental Physiology in September 1973.

Appointed to the research staff of the School of Pharmacy, University of Colorado in September 1979.